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Use of WGS data for investigation of a long-term NDM-1-producing *Citrobacter freundii* outbreak and secondary *in vivo* spread of bla_{NDM-1} to *Escherichia coli*, *Klebsiella pneumoniae* and *Klebsiella oxytoca*

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Objectives: An outbreak of NDM-1-producing *Citrobacter freundii* and possible secondary *in vivo* spread of bla_{NDM-1} to other Enterobacteriaceae were investigated.

Methods: From October 2012 to March 2015, meropenem-resistant Enterobacteriaceae were detected in 45 samples from seven patients at Aalborg University Hospital, Aalborg, Denmark. *In silico* resistance genes, Inc plasmid types and STs (MLST) were obtained from WGS data from 24 meropenem-resistant isolates (13 *C. freundii*, 6 *Klebsiella pneumoniae*, 4 *Escherichia coli* and 1 *Klebsiella oxytoca*) and 1 meropenem-susceptible *K. oxytoca*. The sequences of the meropenem-resistant *C. freundii* isolates were compared by phylogenetic analyses. *In vitro* susceptibility to 21 antimicrobial agents was tested. Furthermore, *in vitro* conjugation and plasmid characterization was performed.

Results: From the seven patients, 13 highly clonal ST18 NDM-1-producing *C. freundii* were isolated. The ST18 NDM-1-producing *C. freundii* isolates were only susceptible to tetracycline, tigecycline, colistin and fosfomycin (except for the *C. freundii* isolates from Patient 2 and Patient 7, which were additionally resistant to tetracycline). The *E. coli* and *K. pneumoniae* from different patients belonged to different STs, indicating *in vivo* transfer of bla_{NDM-1} in the individual patients. This was further supported by *in vitro* conjugation and detection of a 154 kb IncA/C2 plasmid with bla_{NDM-1}. Patient screenings failed to reveal any additional cases. None of the patients had a history of recent travel abroad and the source of the bla_{NDM-1} plasmid was unknown.

Conclusions: To our knowledge, this is the first report of an NDM-1-producing *C. freundii* outbreak and secondary *in vivo* spread of an IncA/C2 plasmid with bla_{NDM-1} to other Enterobacteriaceae.

Introduction

New Delhi metallo-β-lactamase 1 (NDM-1), a metallo-β-lactamase (MBL), was first identified in 2008, in single isolates of *Klebsiella pneumoniae* and *Escherichia coli* from a Swedish patient, previously treated at a hospital in New Delhi, India.[1] The majority of Enterobacteriaceae with bla_{NDM-1} are often extensively co-resistant to multiple antimicrobial agents, but usually remain susceptible to colistin and tigecycline.[1,2]

The occurrence of NDM-producing Enterobacteriaceae is increasing worldwide.[3,4] Several outbreaks of NDM-producing *K. pneumoniae* have been reported, while outbreaks of other species are still rare.[5–8]

Departments of clinical microbiology in Denmark submit suspected carbapenemase-producing Enterobacteriaceae (CPE) for verification and genotyping to the National Antimicrobial Resistance Reference Laboratory at Statens Serum Institut on a voluntary basis. During 2008–13, 37 CPE were detected in Denmark.[9] Until the end of October 2012, all NDM-producing Enterobacteriaceae reported in Denmark were related to travel abroad.[10–13] The spread of NDM-producing Enterobacteriaceae in hospitals has been investigated by several typing methods, including PFGE. For investigation of population structure and global bacterial epidemiology, MLST has been the gold standard. Furthermore, WGS has recently been applied to compare NDM-producing Enterobacteriaceae.[7]

We report a long-term NDM-1-producing *Citrobacter freundii* outbreak and plasmid-borne secondary spread of an NDM-1 producing plasmid *in vivo* to other Enterobacteriaceae investigated by information obtained from WGS data.

Materials and methods

Setting and bacterial isolates

At the end of October 2012, a meropenem-resistant *C. freundii* was detected in a urine sample from a patient hospitalized in the haematology ward at Aalborg University Hospital. Located in the North Denmark Region (580000 inhabitants), this ~830 bed hospital serves all medical specialities. From October 2012 until February 2014, five additional clinical isolates of meropenem-resistant *C. freundii* were detected from the same patient, during his stay in the haematology ward and the infectious disease ward at Aalborg University Hospital (Figure 1). From April 2013 to February 2014, 17 meropenem-resistant Enterobacteriaceae isolates were obtained from clinical samples from three other patients in the haematology ward. Co-detection of meropenem-resistant *C. freundii* and *K. pneumoniae*, in the same sample from Patient 2 (Figure 1 and Table 1), gave early reason to suspect *in vivo* spread of the resistance mechanism.

From March 2014 to April 2014, 14 meropenem-resistant Enterobacteriaceae isolates (5 *C. freundii*, 6 *K. pneumoniae* and 3 *E. coli*) were obtained from a fifth patient during his hospital stay in the infectious disease ward and ICU at Aalborg University Hospital. In November 2014, two meropenem-resistant *C. freundii*, two meropenem-resistant *K. pneumoniae* and one meropenem-resistant *E. coli* were detected from a sixth patient, staying at a minor regional hospital. The sixth patient had previously been hospitalized in the haematology ward, Aalborg University Hospital. In March 2015, three meropenem-resistant isolates (one *C. freundii*, one *E. coli* and one *Klebsiella oxytoca*) were detected from a seventh patient.

In all seven cases, the first isolate of *C. freundii* harbouring NDM was detected in a clinical sample (Table 1). All of the cases were deemed clinically significant and all patients were treated with antibiotics, preferably colistin. It is likely that Patient 4 and Patient 6 died of the NDM-1-producing *C. freundii* infection, regardless of concurrent haematological disease. Patient 1 and Patient 2 died from their end-stage haematological illnesses. Both patients were colonized, but not infected, by the NDM-1-producing *C. freundii* at the time of death. Patient 5 died from complicated intra-abdominal infection.

From October 2012 to March 2015, 45 isolates of meropenem-resistant Enterobacteriaceae from seven patients were detected. Twenty-four of these, along with one meropenem-susceptible *K. oxytoca*, were referred to Statens Serum Institut for further susceptibility testing and WGS.

Infection control

Immediately upon detection of an isolate of meropenem-resistant *C. freundii*, patients were isolated in the ward and contact precautions were initiated. With the manifestation of the fourth case in the haematology ward in October 2013, an ongoing outbreak was suspected. A task force established by the Infection Control Division at Aalborg University Hospital decided to screen all patients in contact with the haematology ward. Hence, in a 14 day period starting 28 October 2013, all haematological patients, including patients admitted to and discharged from the ward, were subjected to anal screening for meropenem-resistant bacteria using eSwabs (Copan, Brescia, Italy). Swabs were plated directly onto ChromID CARBA (bioMérieux, Marcy-l'Étoile, France), then incubated for

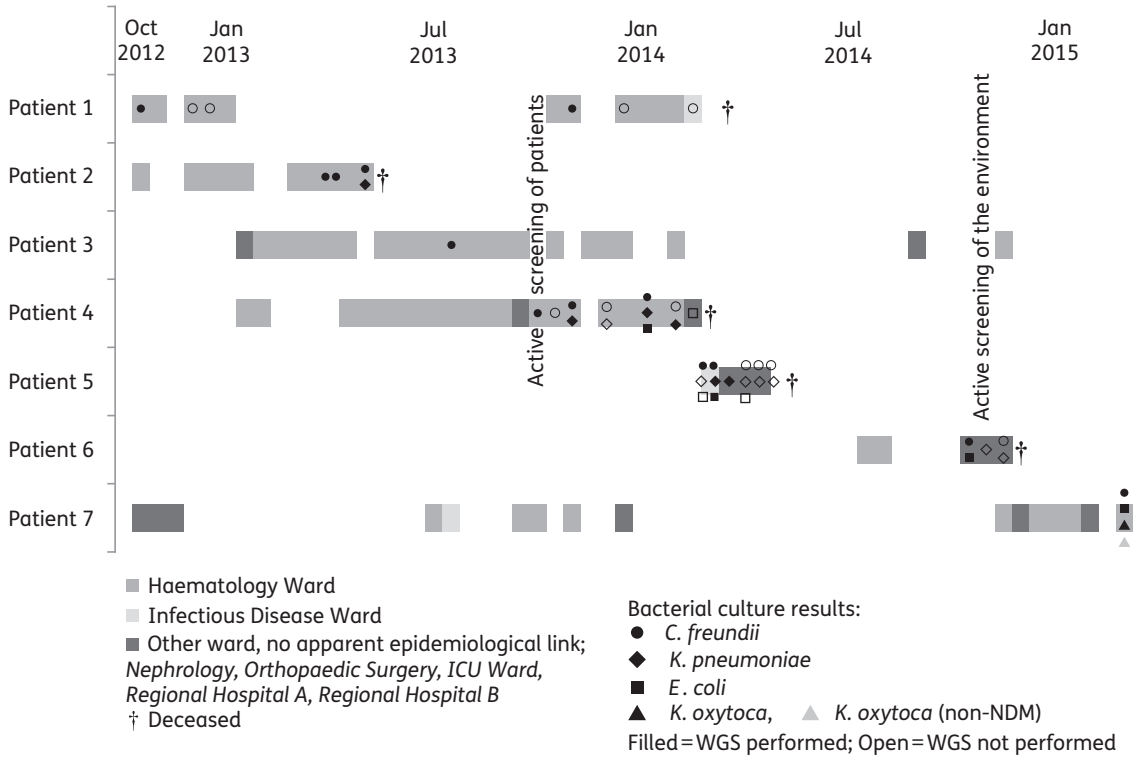

Figure 1. Timeline representing the NDM-1 *C. freundii* outbreak. Ward locations are marked in different shades of grey. Epidemiological links were detected for Patients 1, 2, 3, 4, 6 and 7 in the haematology ward and for Patients 1 and 5 in the infectious disease ward. Screening of all admitted patients and screening of the environment in the haematology ward were performed in October 2013 and November 2014, respectively, without identifying the source of the outbreak.

Table 1. Origin, MLST and resistance gene profiles of the 13 NDM-producing *C. freundii*, the 6 NDM-1-producing *K. pneumoniae*, the 4 NDM-1-producing *E. coli*, the NDM-1-producing *K. oxytoca* and the meropenem-susceptible *K. oxytoca*

							Resistance gene profile[a]								
Isolate name	Patient no.	Sample date	Hospital location[b]	Origin	MLST	SNP profile	other β-lactams	aminoglycoside	fluoroquinolone	fosfomycin	phenicol	rifampicin	sulphonamide	tetracycline	trimethoprim
C. freundii															
AMA 332	1	31-10-2012	H	urine	ST18		**CMY-6**, **OXA-1**, TEM-1b	***strA***, ***strB***, ***aac(6′)-Ib-cr***, ***aacA4***, *aadA5*, ***rmtC***			*catA1*		***sul1***, *sul2*		*dfrA17*
AMA 535		04-11-2013	H	swab, anal screen	ST18	9 SNP differences from AMA332	**CMY-6**, **OXA-1**, TEM-1b	***strA***, ***strB***, ***aac(6′)-Ib-cr***, ***aacA4***, *aadA5*, ***rmtC***			*catA1*		***sul1***, *sul2*		*dfrA17*
AMA 393	2	03-04-2013	H	urine	ST18	11 SNP differences from AMA 332	**CMY-6**, **OXA-1**, OXA-10, TEM-1b, VEB-1	*strA*, *strB*, *aac(6′)-Ia*, ***aac(6′)-Ib-cr***, ***aacA4***, *aadA1*, *aadA5*, *aadB*, *rmtC*			*catA1*, *catB3*, *cmlA1*	*arr-2*	***sul1***, *sul2*	*tet*(D)	*dfrA10*, *dfrA17*
AMA 582		08-05-2013	H	biopsy, anal wound	ST18	12 SNP differences from AMA 332	**CMY-6**, **OXA-1**, OXA-10, TEM-1b, VEB-1	***strA***, ***strB***, *aacA4*, *aac(6′)-Ib-cr aac(6′)-Ia*, *aadA1*, *aadA5*, *aadB*, ***rmtC***			*catA1*, *cmlA1*	*arr-2*	***sul1***, *sul2*	*tet*(D)	*dfrA10*, *drfA17*
AMA 426		21-05-2013	H	swab, anal wound	ST18	9 SNP differences from AMA 332	**CMY-6**, **OXA-1**, TEM-1b	***strA***, ***strB***, ***aacA4***, ***aac(6′)-Ib-cr***, *aadA5*, ***rmtC***			*catA1*		***sul1***	*tet*(D)	*dfrA17*
AMA 463	3	30-07-2013	H	blood	ST18	10 SNP differences from AMA 332	**CMY-6**, **OXA-1**, OXA-10, TEM-1b, VEB-1	***strA***, ***strB***, ***aacA4***, *aac(6′)-Ia*, ***aac(6′)-Ib-cr***, *aadA1*, *aadA5*, *aadB*, ***rmtC***			*catA1*, *cmlA1*	*arr-2*	***sul1***, *sul2*		*dfrA10*, *dfrA17*
AMA 528	4	18-10-2013	H	urine	ST18	1 SNP difference from AMA 332	**CMY-6**, **OXA-1**, TEM-1b	***strA***, ***strB***, ***aacA4***, ***aac(6′)-Ib-cr***, *aadA5*, ***rmtC***	*qnrS1*		*catA1*		***sul1***, *sul2*		*dfrA17*
AMA 533		30-10-2013	H	swab, anal screen	ST18	12 SNP differences from AMA 332	**CMY-6**, **OXA-1**, TEM-1b	***strA***, ***strB***, ***aacA4***, ***aac(6′)-Ib-cr***, *aadA5*, ***rmtC***			*catA1*		***sul1***, *sul2*		*dfrA17*
AMA 570		02-01-2014	H	swab, anal screen	ST18	11 SNP differences from AMA 332	**CMY-6**, **OXA-1**, TEM-1b	***strA***, ***strB***, ***aacA4***, ***aac(6′)-Ib-cr*** *aadA5*, ***rmtC***			*catA1*		***sul1***, *sul2*		*dfrA17*
AMA 639	5	25-03-2014	A	swab, surgical wound	ST18	9 SNP differences from AMA 332	**CMY-6**, **OXA-1**, TEM-1b	***strA***, ***strB***, ***aacA4***, ***aac(6′)-Ib-cr***, *aadA5*, ***rmtC***			*catA1*		***sul1***		*dfrA17*
AMA 653		26-03-2014	A	drainage, peritoneal fluid	ST18	9 SNP differences from AMA 332	**CMY-6**, **OXA-1**, TEM-1b	***strA***, ***strB***, ***aacA4***, ***aac(6′)-Ib-cr***, *aadA5*, ***rmtC***			*catA1*		***sul1***, *sul2*		*dfrA17*

Continued

Table 1. *Continued*

Isolate name	Patient no.	Sample date	Hospital location[b]	Origin	MLST	SNP profile	Resistance gene profile[a]								
							other β-lactams	aminoglycoside	fluoroquinolone	fosfomycin	phenicol	rifampicin	sulphonamide	tetracycline	trimethoprim
AMA 818	6	07-11-2014	GP	urine	ST18	15 SNP differences from AMA 332	**CMY-6- OXA-1**, OXA-10, TEM-1b, VEB-1	***strA***, ***strB***, ***aacA4***, ***aac(6′)-Ib-cr*** *aadA1*, *aadA5*, *aadB*, ***rmtC***			*catA1*, *cmlA1*	*arr-2*	***sul1***, *sul2*		*dfrA10*, *dfrA17*
AMA 941	7	21-03-2015	H	urine	ST18	12 SNP differences from AMA 332	**CMY-6**, **OXA-1**, OXA-10, TEM-1b, VEB-1	***strA***, ***strB***, *aac(6′)-Ia*, ***aac(6′)-Ib-cr***, ***aacA4***, *aadA1*, *aadA5*, ***rmtC***			*catA1*, *catB3*, *cmlA1*		***sul1***, *sul2*	*tet*(D)	*dfrA10*, *dfrA17*
K. pneumoniae															
AMA 425	2	21-05-2013	H	swab, anal wound	ST392		**CMY-6**, CTX-M-15, **OXA-1**, SHV-11-like, TEM-1b	***strA***, ***strB***, *aac(3)-IIa*, ***aac(6′)-Ib-cr***, ***aacA4***, ***rmtC***	*qnrB66*	*fosA*	*catB3*		***sul1***, *sul2*	*tet*(A)	*dfrA14*
AMA 534	4	30-10-2013	H	swab, anal screen	ST17	>24 000 SNP differences from AMA 425	**CMY-6**, **OXA-1**, SHV-11	***strA***, ***strB***, ***aac(6′)-Ib-cr***, ***aacA4***, ***rmtC***	*qnrS1*				***sul1***		
AMA 571		02-01-2014	H	swab, anal screen	ST17	similar SNP profile as AMA 534	**CMY-6**, **OXA-1**, SHV-11	***strA***, ***strB***, ***aac(6′)-Ib-cr***, ***aacA4***, ***rmtC***	*qnrS1*	*fosA*			***sul1***		
AMA 621		17-02-2014	I	tracheal aspirate	ST196	>24 000 SNP differences from AMA 425	**CMY-6**, OKP-A-5-like, **OXA-1**	***strA***, ***strB***, ***aac(6′)-Ib-cr***	*qnrS1*						
AMA 650	5	26-03-2014	A	blood	ST1890	>24 000 SNP differences from AMA 425	**CMY-6**, SHV-11-like, TEM-1B, **OXA-1**	***strA***, ***strB***, ***aac(6′)Ib-cr***, ***aacA4***, ***rmtC***			*catA1*		***sul1***, *sul2*		
AMA 652		26-03-2014	A	drainage, peritoneal fluid	ST1890	3 SNP differences from AMA 650	**CMY-6**, SHV-11-like, **OXA-1**, TEM-1b	***strA***, ***strB***, ***aac(6′)Ib-cr***, ***aacA4***, ***rmtC***			*catA1*		***sul1***, *sul2*		
E. coli															
AMA 569	4	02-01-2014	H	swab, anal screen	ST409		**CMY-6**, **OXA-1**	***strA***, ***strB***, ***aac(6′)-Ib-cr***, ***aacA4***, ***rmtC***					***sul1***		
AMA 648	5	28-03-2014	A	swab, peritoneal	ST5523	>24 000 SNP differences from AMA 569	**CMY-6**, **OXA-1**	***strA***, ***strB***, ***aac(6′)Ib-cr***, ***aacA4***, *rmtC*					***sul1***		
AMA 817	6	07-11-2014	GP	urine	ST131	>24 000 SNP differences from AMA 569	**CMY-6**, **OXA-1**, TEM-1c	***strA***, ***strB***, ***aac(6′)-Ib-cr***, ***aacA4***, *aadA1*, *aadA2*, ***rmtC***			*cmlA1*		***sul1***, *sul3*	*tet*(A)	*dfrA12*

AMA 940	7	21-03-2015	H	urine	ST162	>24 000 SNP differences from AMA 569	**CMY-6**, **OXA-1**, TEM-1b	***strA***, ***strB***, ***aac(6′)-Ib-cr***, ***aacA4***, ***rmtC***			**sul1**, *sul2*	*tet*(A)	*dfrA5*
K. oxytoca													
AMA 942	7	21-03-2015	H	urine	ST2		**CMY-6**, CTX-M-15, **OXA-1**, OXY-2-8-like, TEM-1b	***strA***, ***strB***, *aac(3)-IIa*, ***aac(6′)-Ib-cr***, ***aacA4***, *aadA5*, *aadB*, *aph(3′)-XV*, ***rmtC***	*qnrB1*	*catA1*	**sul1**, *sul2*	*tet*(A)	*dfrA14*, *dfrA17*
AMA 946[c]	7	21-03-2015	H	urine	ST2	7 SNP differences from AMA 942	CTX-M-15, OXA-4, OXY-2-8-like, TEM-1b	*strB*, *aac(3)-IIa*, *aadA5*, *aadB*, *aph(3′)-XV*, *aac(6′)-Ib-cr*	*qnrB1*	*catA1*	*sul1*, *sul2*		*dfrA14*, *dfrA17*

[a]Resistance genes marked in bold were placed on the same plasmid.
[b]H, haematology ward; A, abdominal surgical ward; I, ICU; GP, general practice.
[c]*K. oxytoca* KO-7-2 (AMA 946) was meropenem susceptible.

48 h at 35°C in an ambient atmosphere. Active interventions, such as cleaning and decontamination using the Glosair™ 400 system (Johnson & Johnson AB, Sollentuna, Sweden), were introduced during the screening period in both the haematology ward and associated ambulatory care facilities. In November 2014, the source of the outbreak was still unknown and the accumulating cases of meropenem-resistant *C. freundii* prompted the initiation of further infection control measures. Environmental screenings from the inside of all taps, shower heads and sink holes in the haematology ward were collected and processed similarly to the patient screenings described above.

Antimicrobial susceptibility

At Aalborg University Hospital susceptibility testing was performed using Neo-Sensitabs™ (Rosco Diagnostica, Taastrup, Denmark) according to EUCAST guidelines. Screening for reduced carbapenem susceptibility was done with ertapenem (10 μg), and the primary screening breakpoint applied for Enterobacteriaceae was an inhibition zone <25 mm. All isolates with reduced ertapenem susceptibility were further screened by meropenem and imipenem Etest (bioMérieux) and KPC/MBL and OXA-48 Confirm Kit 98015 (Rosco Diagnostica) before being submitted to Statens Serum Institut.

At Statens Serum Institut, antimicrobial susceptibility testing was performed using Sensititre Trek panels (Thermo Scientific, Waltham, MA, USA) (aztreonam, cefotaxime, ceftazidime, cefepime, piperacillin/tazobactam, chloramphenicol, colistin, ertapenem, imipenem, meropenem, ciprofloxacin, gentamicin, tobramycin, sulfamethoxazole, tetracycline, tigecycline, trimethoprim) according to the manufacturer's instructions. The microbroth dilution method was executed in accordance with the CLSI.[14] Susceptibility to streptomycin, rifampicin, temocillin and fosfomycin was investigated using Etest (bioMérieux). The reference strain *E. coli* ATCC 25922 was used for quality control.

Detection of bla_{NDM}

The bla_{NDM} gene was identified by PCR using primers NDM-F (5′-GAAGCT GAGCACCGCATTAG-3′) and NDM-R (5′-TGCGGGCCGTATGAGTGATT-3′) to amplify an internal fragment of 761 bp. From June 2014, the meropenem-resistant isolates were tested for the presence of carbapenemase genes using the Xpert Carba-R on GeneXpert Infinity System (Cepheid, Sunnyvale, USA).

WGS and assembly

Genomic DNA was extracted from 24 NDM-producing isolates from the seven patients and a meropenem-susceptible *K. oxytoca* isolate from Patient 7 (DNeasy Blood and Tissue Kit, Qiagen, Copenhagen, Denmark). WGS data from the 25 isolates have been deposited at the National Center for Biotechnology Information (NCBI) (BioProject ID PRJEB12145).

Fragment libraries were constructed using the Nextera Kit (Illumina, Little Chesterford, UK) followed by 251 bp paired-end sequencing (MiSeq, Illumina) according to the manufacturer's instructions.

In addition to the 13 genomes of the Danish *C. freundii* isolates, the sequence data were aligned against the chromosome of the MTCC 1658 *C. freundii* reference genome (GenBank accession number ANAV00000000) using the Burrows–Wheeler Aligner (BWA). *K. pneumoniae* ATCC BAA-2146 (GenBank accession number CP006659) was used as the reference genome for comparison of the six *K. pneumoniae* isolates, *K. oxytoca* with GenBank accession number NC_016612 was used as the reference for comparison of the two *K. oxytoca* isolates and *E. coli* JJ1886 (GenBank accession number CP006784) was used as the reference for comparison of the four *E. coli* isolates. Identification of SNP variants was performed using the GATK Unified Genotyper with filtering using NASP (http://tgennorth.github.io/NASP/) to remove positions with less than ×10 coverage and <90% unambiguous variant calls, or within duplicated regions of the reference using NUCmer.[15]

Phylogenetic analyses of the identified SNPs was performed using maximum parsimony implemented in MEGA 6.0.6.[16] The paired-end Illumina data were assembled using CLC Bio Genomic Workbench 8.0 (Qiagen, Aarhus, Denmark). Comparison of the sequences was also performed using CLC Bio Genomic Workbench 8.0.

Identification of resistance genes, plasmid replicon types and MLST

The ResFinder web server, version 2.1, PlasmidFinder (Enterobacteriaceae), version 1.2, and MLST web server, version 1.7 (www.genomicepidemiology.org),[9–11] were used to identify acquired antimicrobial resistance genes, plasmid replicon types *in silico* and MLST profiles from the assembled WGS data, respectively.[17] For resistance genes, a threshold of 100% identity was used for the genes encoding β-lactamases and 98.00% identity for all other genes, and only full-length genes were included. ResFinder detects the presence of resistance genes, but not functional integrity and expression or resistance due to acquired variation in housekeeping genes.

Plasmid transmissibility

C. freundii AMA 332 was used as donor and the plasmid-free recipient was *E. coli* J53azideR (azide resistant). Conjugation was set up as follows. A sterile paper filter (pore diameter 0.2 mm, Advantech) was placed in the centre of a blood agar plate, 1 mL of donor culture and 1 mL of recipient culture in exponential phases of growth were mixed together and 500 μL of the mixture was placed on the paper filter, allowing the liquid to soak into the medium. After overnight incubation at 37°C, filters were washed with 4 mL of 0.9% salt water and 100 μL of the suspension was inoculated onto BHI (Becton, Dickinson & Co., Difco™) agar plates with 1 mg/L meropenem and 100 mg/L sodium azide. After overnight incubation at 37°C the presence of transconjugants was assessed.

Plasmid characterization

Plasmid DNA was extracted from a single transconjugant using the Qiagen Plasmid Midi Kit (catalogue number 12143) and subsequently subjected to Illumina MiSeq paired-end sequencing as described above. Furthermore, the plasmid was sequenced using the Pacific Biosciences platform (Pacific Biosciences, CA, USA). In short, purified plasmid DNA (spiked with chromosomal *Sphingomonas* spp. DNA as carrier) was fragmented using a g-TUBE (Covaris Ltd, Brighton, UK). Five micrograms of DNA in 150 μL was added to the g-TUBE and the tube was spun for 1 min at 4600 rpm, inverted and spun again. Then a size selection was performed on a BluePippin on a 0.75% gel cassette loaded with S1 ladder and a collection range from 10 to 50 kb. Libraries were built following the Pacific Biosciences 10 kb template protocol using the SMRTbell template prep kit 1.0 (Pacific Biosciences). Unligated fragments were removed by exonuclease treatment and the final library was purified twice using AMPure beads from Pacific Biosciences.

Pacific Biosciences BindingCalculator (v2.3.1.1) was used to generate the loading protocol; the sample was loaded accordingly and sequenced on two cells with 360 min movies.

The pT1 plasmid was assembled using a hybrid assembly approach in SPAdes (version 3.7.0) where paired-end Illumina data were used for assembly and Pacific Biosciences reads were used to close gaps. This resulted in a 154437 bp contig that was confirmed to be circular by mapping Pacific Biosciences reads to the contig using BLASTN.

Results and discussion

The epidemiological timeline of the seven patients is shown in Figure 1. Sequence data and antimicrobial susceptibility results were available for 25 isolates, including: (i) 13 meropenem-resistant *C. freundii* isolates from the seven patients; (ii) 6 meropenem-resistant *K. pneumoniae* isolates from three patients; (iii) 4 meropenem-resistant *E. coli* isolates from four patients; and (iv) 1 meropenem-resistant *K. oxytoca* isolate and 1 meropenem-susceptible *K. oxytoca* isolate from Patient 7. All 24 meropenem-resistant isolates were positive for bla_{NDM} by PCR or by GeneXpert. ResFinder analysis of the assembled draft genomes identified bla_{NDM-1} in all 24 meropenem-resistant isolates. Using PlasmidFinder, an IncA/C2 replicon was detected in the 24 NDM-1-producing isolates (data not shown). The 13 NDM-1-producing *C. freundii* isolates from the seven patients all belonged to ST18 using the MLST web server. Phylogenetic analysis of the WGS data showed that the 13 ST18 NDM-1-producing *C. freundii* all belonged to one clade (with 1–15 SNP differences) (Table 1). They had identical antimicrobial susceptibility profiles and were only susceptible to tetracycline, tigecycline, colistin and fosfomycin, except for the *C. freundii* isolates from Patient 2 and Patient 7, which were additionally resistant to tetracycline [encoded by *tet*(D)] (Table 1 and Table S1, available as Supplementary data at *JAC* Online). Even though the *C. freundii* isolates belonged to the same ST and clade, they did not have the same resistance gene profile or Inc plasmid types (except for IncA/C2). This indicates a clonal spread of ST18 NDM-1-producing *C. freundii* between the seven patients during the long-term outbreak, but with additional discursion and acquisition of other plasmids bearing resistance genes. To our knowledge, ST18 *C. freundii* has rarely been detected in hospital settings and has only been reported from Greece (www.pubMLST.org/cfreundii).

NDM-1-producing *K. pneumoniae* isolates were additionally detected in Patient 2, Patient 4 and Patient 5. The *K. pneumoniae* isolate from Patient 2 belonged to ST392, whereas two of the three *K. pneumoniae* isolates from Patient 4 belonged to ST17 and the third isolate from Patient 4 belonged to ST196. The two *K. pneumoniae* from Patient 5 both belonged to ST1890 (Table 1).

The *K. pneumoniae* isolates with the same ST showed no differences in the shared core genome (0–3 SNPs), susceptibility profiles, resistance gene profiles and plasmid replicon profiles (Table 1 and Table S1). NDM-1-producing *E. coli* isolates were additionally detected in Patient 4, Patient 5, Patient 6 and Patient 7. The *E. coli* isolates belonged to different STs (ST409, ST5523, ST162 and ST131) and had different susceptibility profiles, different resistance gene profiles and different plasmid replicon profiles (Table 1 and Table S1).

The occurrence of within-host *in vivo* spread of bla_{NDM-1} from *C. freundii* to *K. pneumoniae* and *E. coli* is likely. The possible *in vivo* transmission was supported by a transfer frequency of 2×10^{-3} transconjugants per donor *in vitro* for *C. freundii* AMA 332 to a plasmid-free *E. coli* J53azideR recipient. A 154 kb plasmid (pT1) was obtained from the *E. coli* transconjugant (GenBank accession number KX147633) and completely sequenced. Analysis of the sequence revealed the presence of bla_{NDM-1}, bla_{CMY-6}, bla_{OXA-1}, *strA*, *strB*, *rmtC*, *aacA4*, *aac(6′)Ib-cr* and *sul1* on pT1. The pT1 complete plasmid sequence was used as the reference template to map the sequencing data from the 23 other isolates in the outbreak. Of these, 22 isolates were found to contain sequencing data covering the whole pT1 sequence. The only exception was isolate AMA 621 *K. pneumoniae*, which was the latest identified isolate from Patient 4. The data did not reveal *aacA4*, *rmtC* or *sul1*, suggesting a single deletion event. Core plasmid genome SNP analysis using pT1 as a reference showed high similarity between the plasmids in the 24 isolates (0–1 SNP differences,

88.5% core genome coverage). Interestingly, a single SNP (A → G) in position 44 571 of pT1 was detected in all isolates originating from Patient 5, but not in any of the other isolates of the study, supporting the (intra-patient) *in vivo* transfer hypothesis.

This hypothesis was further supported by comparison of a meropenem-resistant *K. oxytoca* and a meropenem-susceptible *K. oxytoca*, both isolated from Patient 7. The core genomes of the two isolates were highly similar (difference of 7 SNPs); however, the meropenem-susceptible isolate (AMA 946) differed from the meropenem-resistant isolate (AMA 942) only by these 7 SNPs and by the presence of the pT1 plasmid.

Our analysis supports the hypothesis of multiple transfers of an NDM-1-encoding plasmid *in vivo*. Similarly, Tijet *et al.*[18] recently reported *in vivo* transfer of an NDM-1-producing plasmid in a single patient. This has also been reported for KPC-3-producing plasmids[19,20]

Furthermore, the sequencing data from the NDM-1 plasmid (pT1) was compared by SNP analysis with the other completely sequenced IncA/C2 NDM-1-producing plasmids available at NCBI: plasmids pEC2-NDM-3 (KC999035), pNDM10469 (JN861072), pNDM10505 (JF503991), pNDM102337 (JF714412), pNDM-US (CP006661), pNDM-US-2 (KJ588779), pNDM-KN (NC_019153), pKP1-NDM-1 (NC_023908) and pNDM-PstGN576 (KJ802405). SNP analysis of the core plasmid genomes (covering 83% of the pT1 plasmid genome) suggests that all of these plasmids have highly conserved plasmid backbones (up to 7 SNP differences). Interestingly, an area upstream of bla_{NDM-1} in pT1 containing a complete type I restriction modification system was only found to be present in pT1 and pEC2-NDM-3, but not in the any of the other plasmids.

None of the seven patients had a history of recent travel. The long time span between cases (median of 4 months) indicated that a common external source was unlikely, and the epidemiological links in the haematology ward (Patients 1–4, Patient 6 and Patient 7) and infectious disease ward (Patient 1 and Patient 5), paired with the data obtained from WGS, support the hypothesis that horizontal transmission between patients had taken place. Patient screenings were performed in order to identify possible routes of transmission and to identify patients in whom empirical treatment in case of neutropenic fever needed to be adjusted.

During the 14 day screening period in October–November 2013, 78 anal swab samples from 55 patients were collected. These patient screenings failed to reveal any additional cases. Only Patient 1 and Patient 4, already known to be colonized at the time of the screening, were positive. Patient 1 had been screened negative at admittance, but after 7 days of cefuroxime treatment for suspected pneumonia the patient was found positive in the sample taken at discharge, indicating prolonged gastrointestinal colonization. Since no new cases where identified, and since tracking of patients and beds was incomplete, no conclusions regarding transmission routes could be made and patient screenings were discontinued after the 14 day period.

Among the 33 environmental screening samples from 11 rooms and bathrooms, none yielded any growth of meropenem-resistant Enterobacteriaceae, meropenem-resistant *Pseudomonas aeruginosa* or meropenem-resistant *Acinetobacter* spp.

To date, the origin of the NDM-1-producing *C. freundii* remains unknown. During the preparation of this manuscript, six NDM-1-producing *C. freundii* isolates were detected from six new patients (Patients 8–13), indicating that the outbreak has been ongoing for >3 years.

To our knowledge, clonal NDM-1 outbreaks with secondary horizontal spread have only been described for *K. pneumoniae* and *E. coli* in Canada, previously.[21] Outbreaks with carbapenemase-producing *C. freundii* are rare. Previously, Gaibani *et al.*[22] reported a VIM-producing *C. freundii* outbreak in Italy, but, to our knowledge, our study is the first to disclose an outbreak of NDM-1-producing *C. freundii*.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Yong D, Toleman MA, Giske CG *et al*. Characterization of a new metallo-β-lactamase gene, bla_{NDM-1}, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. *Antimicrob Agents Chemother* 2009; **53**: 5046–54.

2 Kumarasamy KK, Toleman MA, Walsh TR *et al*. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect Dis* 2010; **10**: 597–602.

3 Dortet L, Poirel L, Nordmann P. Worldwide dissemination of the NDM-type carbapenemases in Gram-negative bacteria. *Biomed Res Int* 2014; **2014**: 249856.

4 Struelens M, Monnet D, Magiorakos A *et al*. New Delhi metallo-β-lactamase 1-producing Enterobacteriaceae: emergence and response in Europe. *Euro Surveill* 2010; **15**: pii=19716.

5 Jin Y, Shao C, Li J *et al*. Outbreak of multidrug resistant NDM-1-producing *Klebsiella pneumoniae* from a neonatal unit in Shandong Province, China. *PLoS One* 2015; **10**: e0119571.

6 Chung The H, Karkey A, Pham Thanh D *et al*. A high-resolution genomic analysis of multidrug-resistant hospital outbreaks of *Klebsiella pneumoniae*. *EMBO Mol Med* 2015; **7**: 227–39.

7 Stoesser N, Sheppard AE, Shakya M *et al*. Dynamics of MDR *Enterobacter cloacae* outbreaks in a neonatal unit in Nepal: insights using wider sampling frames and next-generation sequencing. *J Antimicrob Chemother* 2015; **70**: 1008–15.

8 Stoesser N, Giess A, Batty EM *et al*. Genome sequencing of an extended series of NDM-producing *Klebsiella pneumoniae* isolates from neonatal infections in a Nepali hospital characterizes the extent of community- versus hospital-associated transmission in an endemic setting. *Antimicrob Agents Chemother* 2014; **58**: 7347–57.

9 Anon. *DANMAP 2013 - Use of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Bacteria From Food Animals, Food and Humans in Denmark*. 2014. ISSN 1600-2032.

10 Anon. *DANMAP 2012 - Use of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Bacteria From Food Animals, Food and Humans in Denmark*. 2013. ISSN 1600-2032.

11 Nielsen JB, Hansen F, Littauer P *et al*. An NDM-1-producing *Escherichia coli* obtained in Denmark has a genetic profile similar to an NDM-1-producing *E. coli* isolate from the UK. *J Antimicrob Chemother* 2012; **67**: 2049–51.

12 Jakobsen L, Hammerum AM, Hansen F *et al*. An ST405 NDM-4-producing *Escherichia coli* isolated from a Danish patient previously hospitalized in Vietnam. *J Antimicrob Chemother* 2014; **69**: 559–60.

13 Hammerum AM, Toleman MA, Hansen F *et al*. Global spread of New Delhi metallo-β-lactamase 1. *Lancet Infect Dis* 2010; **10**: 829–30.

14 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-second Informational Supplement M100-S22*. CLSI, Wayne, PA, USA, 2012.

15 Delcher AL. Fast algorithms for large-scale genome alignment and comparison. *Nucleic Acids Res* 2002; **30**: 2478–83.

16 Tamura K, Stecher G, Peterson D *et al*. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* 2013; **30**: 2725–9.

17 Zankari E, Hasman H, Cosentino S *et al*. Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother* 2012; **67**: 2640–4.

18 Tijet N, Richardson D, MacMullin G *et al*. Characterization of multiple NDM-1-producing Enterobacteriaceae isolates from the same patient. *Antimicrob Agents Chemother* 2015; **59**: 3648–51.

19 Gona F, Barbera F, Pasquariello AC *et al*. *In vivo* multiclonal transfer of bla_{KPC-3} from *Klebsiella pneumoniae* to *Escherichia coli* in surgery patients. *Clin Microbiol Infect* 2014; **20**: O633–5.

20 Goren MG, Carmeli Y, Schwaber MJ *et al*. Transfer of carbapenem-resistant plasmid from *Klebsiella pneumoniae* ST258 to *Escherichia coli* in patient. *Emerg Infect Dis* 2010; **16**: 1014–7.

21 Borgia S, Lastovetska O, Richardson D *et al*. Outbreak of carbapenem-resistant Enterobacteriaceae containing bla_{NDM-1}, Ontario, Canada. *Clin Infect Dis* 2012; **55**: e109–17.

22 Gaibani P, Ambretti S, Farruggia P *et al*. Outbreak of *Citrobacter freundii* carrying VIM-1 in an Italian hospital, identified during the carbapenemases screening actions, June 2012. *Int J Infect Dis* 2013; **17**: e714–7.