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**Occurrence of *Enterobacter hormaechei* carrying *bla*_{NDM-1} and *bla*_{KPC-2}
in China**

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Running title: Hospital transmission of *E. hormaechei*

Keywords: β -lactamases; carbapenemases; resistance; plasmids; *Enterobacter cloacae*; *Enterobacter hormaechei*.

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Abstract

Three carbapenem-resistant clinical isolates of the *Enterobacter cloacae* complex (ECC) were recovered from different patients in a hospital. All three isolates carried two carbapenemase genes *bla*_{KPC-2} and *bla*_{NDM-1}. A study was performed to characterize their relatedness and to investigate possible links among the patients. Whole genome sequencing revealed that the isolates were *Enterobacter hormaechei* and belonged to ST177 of the ECC. There were 19 to 142 single nucleotide polymorphisms (SNPs) between the isolates, suggesting that the isolates were likely from a central reservoir, which might have existed for some time. *bla*_{KPC-2} and *bla*_{NDM-1} were carried on two different IncF-type plasmids in the isolates. The three *bla*_{NDM-1}-carrying plasmids were almost identical and were self-transmissible, while the *bla*_{KPC-2}-carrying plasmids were only transmissible in the presence of the *bla*_{NDM-1}-carrying plasmid. The source of and direct links among them were not identified, suggesting a hospital transmission of a common multidrug resistant strain.

Introduction

Carbapenem-resistant Enterobacteriaceae (CRE) have emerged as a major challenge to global public health. *bla*_{KPC} and *bla*_{NDM} are the two common gene types encoding carbapenemases able to confer resistance to almost all β -lactam antibiotics, in the Enterobacteriaceae (Temkin, Adler, Lerner, & Carmeli, 2014). Occasionally, the two genes can be found in a single CRE strain. In 2014, we identified a strain of the *Enterobacter cloacae* complex, WCHECI-14653, carrying *bla*_{KPC-2} and *bla*_{NDM-1}, which was recovered from the blood of an ICU patient (Wu, Feng, Carattoli, & Zong, 2015). The *E. cloacae* complex comprises a few closely related species (Mezzatesta, Gona, & Stefani, 2012). During a subsequent screening for CRE in our hospital in 2014, we found two additional non-duplicate isolates of the *E. cloacae* complex carrying *bla*_{KPC-2} and *bla*_{NDM-1}. We therefore investigated the clonal relatedness of the three isolates using whole genome sequencing.

Methods

Strains

The three isolates of the *E. cloacae* complex were recovered from three different patients (Table 1). The initial species identification and *in vitro* antimicrobial susceptibility tests were performed by Vitek II (bioMérieux, Marcy-l'Étoile, France). In addition, MICs of amikacin, ceftazidime, ciprofloxacin, colistin, imipenem, and meropenem against the isolates were determined using the broth dilution method of the Clinical Laboratory Standards Institute (CLSI) (CLSI, 2017).

Whole genome sequencing and phylogenetic analysis

The genome sequence of isolate WCHECI-14653 has been reported before (Wu, et al., 2015). Genomic DNA of the remaining two isolates, WCHECI-Y2402 and WCHECI-Y2403, was prepared using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and was subjected to whole genome sequencing with $150 \times$ coverage using the HiSeq X10 Sequencer (Illumina, San Diego, CA). Reads were trimmed using Trimmomatic (Bolger, Lohse, & Usadel, 2014) and were then *de novo* assembled to contigs using the SPAdes program (Bankevich, et al., 2012). Pair-wise average nucleotide identity (ANI) between the genome sequences of the three isolates and those of the type strain of all *Enterobacter* species were determined using the JSpecies web program (<http://imedea.uib-csic.es/jspecies/>) (Richter & Rossello-Mora, 2009). The $\geq 95\%$ ANI cutoff was used to define bacterial species (Goris, et al., 2007). The sequences of the heat shock protein 60-encoding *hsp60* gene of the three isolates were compared with those of type strains of all *Enterobacter* species, which were retrieved from GenBank, using BLAST. The sequence type (ST) was determined using the genomic sequence to query the multi-locus sequence typing database of *E. cloacae* (<http://pubmlst.org/ecloacae/>). Antimicrobial resistance genes were predicted using ResFinder (<http://genomicpidemiology.org/>).

To determine single nucleotide polymorphisms (SNPs) among the isolates, the trimmed reads of strains WCHECIY2402 and WCHECIY2403 were mapped against the draft genome of WCHECI-14653 using Bowtie2 (Langmead & Salzberg, 2012),

followed by SNP calling using SAMtools (Li, et al., 2009) and VCFtools (Danecek, et al., 2011). Recombination regions were detected using Gubbins (Croucher, et al., 2015). SNPs with a minimum Phred quality of 30 and depth of 10 were included, while SNPs located in recombination regions were discarded. Genomes of isolates of ST93, which has only one allele different from ST177, were retrieved from GenBank and were subjected to phylogenetic analysis together with ST177 genomes based on the core genome using Harvest (Treangen, Ondov, Koren, & Phillippy, 2014). A neighbor-joining phylogenetic tree was constructed using MEGA 7.0 with 1,000 bootstrap iterations (Kumar, Stecher, & Tamura, 2016). Sequences of *bla*_{NDM-1}- and *bla*_{KPC-2}-carrying plasmids were circularized using PCR and Sanger sequencing to close gaps between contigs. Replicon typing for IncF plasmids was performed using the pMLST tool (Villa, Garcia-Fernandez, Fortini, & Carattoli, 2010) at <http://genomicepidemiology.org/>.

Mating

Conjugation experiments were performed using filter- and broth-based methods with the azide-resistant *E. coli* strain J53 as the recipient and 2 µg/ml meropenem plus 150 µg/ml sodium azide for selecting transconjugants. The presence of *bla*_{NDM-1}- and *bla*_{KPC-2} in transconjugants were confirmed by PCR.

Investigation on possible links among the patients

We assembled a team including hospital epidemiologists to retrospectively investigate

the possible source of and direct links among the three patients based on information from their medical records. The investigation has been approved by the Ethical Committee of West China Hospital with consent being waived.

Results and discussion

A total of 4,874,832 to 6,423,425 reads were generated by Illumina whole genome sequencing for strains WCHECIY2402 and WCHECIY2403, which were assembled to 95 and 71 contigs (69 and 56 were $\geq 1,000$ bp in length) with a 55.08 to 55.10% GC content, respectively. The three isolates were all identified as *E. cloacae* by Vitek II. However, pair-wise ANI analysis revealed that the three isolates had $>95\%$ ANI values, which is commonly used to define a bacterial species (Goris, et al., 2007), with *Enterobacter hormaechei* and *Enterobacter xiangfangensis* type strains (Table 3), while they had only 86.89 to 86.95% ANI values with *E. cloacae* ATCC 13047^T (Table 3). Although *E. xiangfangensis* is still considered as a bacterial species, a study has suggested that it may actually be a subspecies of *Enterobacter hormaechei* rather than an independent species (Chavda, et al., 2016). Therefore, the three isolates in the present study belonged to *E. hormaechei*. Among the three currently defined subspecies of *E. hormaechei*, the three isolates had the highest ANI value (98.88 to 98.91%) with *E. hormaechei* subsp. *steigerwaltii* type strain DMS 16691^T but the cutoff of ANI value to define *Enterobacter* subspecies has not been clearly established. Sequences of the heat shock protein 60-encoding *hsp60* gene have also been used for the species identification of the *E. cloacae* complex (Carvalho-Assef, et al., 2014;

Hoffmann & Roggenkamp, 2003). The *hsp60* sequence of the three isolates was 100% identical and was 98.3 to 98.8% identical to those of type strains of *E. hormaechei* and *E. xiangfangensis* while 91.1 to 96.8% to those of types strains of other *Enterobacter* species, suggesting that the three isolates were *E. hormaechei*.

Like isolate WCHECI-14653 (Wu, et al., 2015), WCHECIY2043 and WCHECIY2042 also belonged to the sequence type (ST) 177 of *Enterobacter*. WCHECIY2043 and WCHECIY2042 had 137 and 142 SNPs compared with WCHECI-14653, respectively, while there were only 19 SNPs between WCHECIY2043 and WCHECIY2042. Therefore, it is likely that the three isolates belonged to a common strain, suggesting the transmission of a strain carrying two carbapenemase genes in the hospital.

ST177 has a single allele different from ST93, ST294 and ST828. Eight ST93 isolates have genome sequence available in GenBank, while there are no genome sequences available for isolates belonging to ST294 or ST828. A neighbour-joining phylogenetic tree (Figure 1) revealed that ST93 and ST177 indeed formed two separate groups and the three isolates in this study were clustered together (Figure 1).

*bla*_{NDM-1} and *bla*_{KPC-2} were carried by separate plasmids in the three isolates. The *bla*_{NDM-1}-carrying plasmids (109 kb) contained two IncF replicons, FIB (type 36) and FIIY (type 4), while the *bla*_{KPC-2}-carrying plasmids (88 kb) had a single FIIY replicon (type 5 or 6). The three *bla*_{NDM-1}-carrying plasmids were almost identical in sequence

(99.9% identity and 100% coverage). The *bla*_{KPC-2}-carrying plasmids were also almost identical in sequence (99.9% identity and 100% coverage) but there were four nucleotide mutations in the spacer region between *repA1* and *repA2* genes of the FIIY replicon in WCHECIY2043 and WCHECIY2042 compared with that of WCHECI-14653, resulting in the change of the FIIY type 5 in WCHECI-14653 to 6 in WCHECIY2043 and WCHECIY2042. Transconjugants containing both *bla*_{NDM-1} and *bla*_{KPC-2} or *bla*_{NDM-1} alone were obtained but those containing *bla*_{KPC-2} alone were not detected from the three isolates. These findings suggest that the *bla*_{NDM-1}-carrying plasmids were self-transmissible, while the *bla*_{KPC-2}-carrying plasmids were transmissible only in the presence of the *bla*_{NDM-1}-carrying plasmid in the three isolates. The relaxosome protein-encoding gene *traY* was absent from the conjugative module of the *bla*_{KPC-2}-carrying plasmids, which could explain why these plasmids were not self-transmissible. Nonetheless, in the presence of a similar plasmid, the *bla*_{NDM-1}-carrying plasmid in this case, *bla*_{KPC-2}-carrying plasmids become transmissible, highlighting that the co-existence of two or more plasmids in a single bacterial isolate facilitates the spread of antimicrobial resistance genes. In literature, an *E. hormaechei* (strain CCBH14397) carrying both *bla*_{NDM-1} and *bla*_{KPC-2} has been found in Brazil (Pereira, et al., 2015). However, in strain CCBH14397 *bla*_{NDM-1} and *bla*_{KPC-2} are carried by a 160-kb IncA/C plasmid and a 50-kb IncN plasmid, respectively (Pereira, et al., 2015), which are different from those (IncF) carrying the two genes in the three isolates in this study. This suggests that the co-existence of *bla*_{NDM-1} and *bla*_{KPC-2} in single strains is due to different combinations of plasmids.

The three isolates were resistant to ceftazidime, ciprofloxacin, imipenem, and meropenem, but were susceptible to amikacin and colistin based on MICs (Table 2). They were also resistant to ampicillin-sulbactam, aztreonam, cefepime, ertapenem, gentamicin, levofloxacin, nitrofurantoin, piperacillin-tazobactam, tobramycin, and trimethoprim-sulfamethoxazole, as determined using Vitek II. In addition to *bla*_{NDM-1} and *bla*_{KPC-2}, the three isolates had the same 10 intact antimicrobial resistance genes mediating resistance to aminoglycosides (*aph(3')-Ia*, *aac(3)-IIId*, *aadA2*, *aacA4*), β -lactams (a new variant of *bla*_{ACT}, encoding an AmpC-type cephalosporinase with an amino acid substitution to ACT-7), fosfomycin (*fosA*), macrolides (*mph(A)*), quinolones (*qnrS1*), sulphonamides (*sul1*) and trimethoprim (*dfrA12*) plus a truncated *bla*_{TEM-1} gene.

The three isolates were recovered from clinical samples that were collected at 13 to 63 days after hospitalization when the host patients developed healthcare-associated infections (bacteremia or pneumonia; Table 1). The isolates were considered as pathogens and were hospital acquired. There was a four-month gap between the death of the first patient with the ST177 strain and the recovery of the second and third ST177 isolates from two different patients, suggesting a possible reservoir (environment or human) of the strain. The second and third ST177 isolates were recovered within 11 days of each other, suggesting possible acquisition of the strain by both patients from a common source, a hypothesis supported by the fact that there

were only 19 SNPs between the two isolates. The patients were continuously hospitalized in three different wards during their hospital stay. We were unable to identify any healthcare workers including doctors, nurses, technicians, workers and medical students and medical instruments shared by the three patients or any two of them. The source and direct links of the transmission remain unclear. Nonetheless, as the patients had stayed in the same hospital, it is most likely they have been exposed to a common source such as food, water, medical instruments or staff members.

In conclusion, we identified the in-hospital acquisition of an ST177 strain of *E. hormaechei* carrying both *bla*_{KPC-2} and *bla*_{NDM-1}.

Nucleotide sequence accession numbers. Draft whole-genome sequences of isolates WCHECIY2402 and WCHECIY2403 have been deposited into GenBank under the accession numbers MRAA000000000 and MRAB000000000, respectively. The complete sequences of pKPC2_ECY2402, pKPC2_ECY2403, pNDM1_ECY2402 and pNDM1_ECY2403 have been deposited into GenBank under the accession numbers KY399972, KY399973, KY399974 and KY399975, respectively.

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Potential conflicts of interest. The authors declare no competing financial interests.

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Table 1. Patient demographic data, history and infection control investigation

Isolate	Source	Patient age	Admission time	Collection date	Days between admission and collection ¹	Diagnosis on admission	Diagnoses at the time of collection ¹	Ward
WCHECI-14563	Blood	44	Feb. 2014	Apr. 2014	63	Pancreatitis	Pancreatitis, bacteremia	ICU
WCHECIY2402	Sputum	76	Jul. 2014	Aug. 2014	22	Uremia	Pneumonia, uremia	Nephrology
WCHECIY2403	Sputum	89	Aug. 2014	Aug. 2014	13	Rupture of an occupying hepatic lesion	Pneumonia, rupture of an occupying hepatic lesion	Gerontology

¹collection refers to the collection of samples that grew the three isolates.

Table 2. MICs ($\mu\text{g/ml}$) of antimicrobial agents against the three isolates

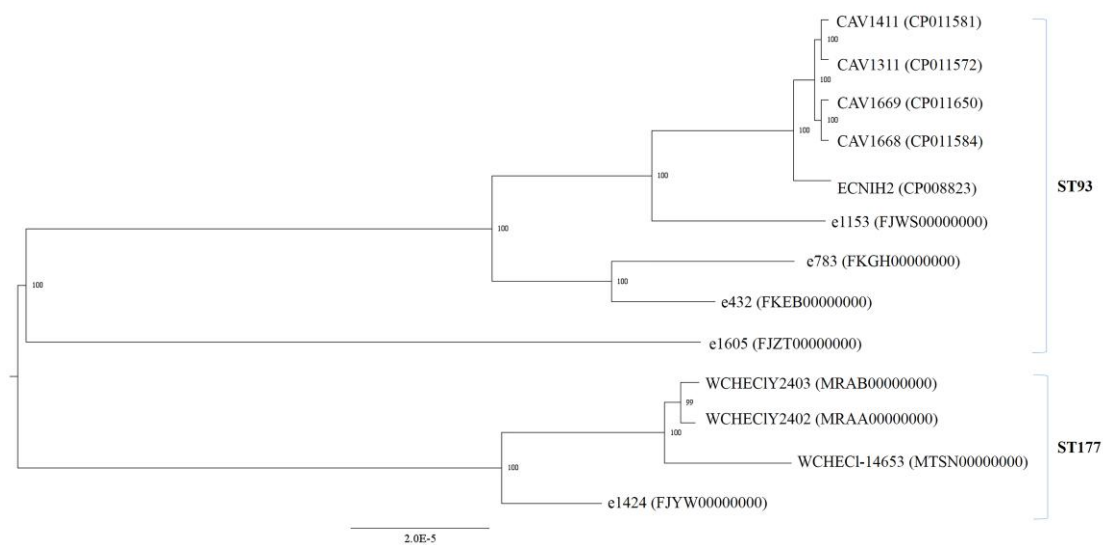
Isolate	Amikacin	Ceftazidime	Ciprofloxacin	Colistin	Imipenem	Meropenem
WCHECI-14563	16	512	64	2	64	64
WCHECIY2402	8	512	8	2	128	128
WCHECIY2403	8	512	8	2	64	64

Table 3. ANI values between the three isolates in this study and the type strains of species belonging to the genus *Enterobacter*.

Strain	Accession no.	ANI [%]		
		WCHECIY2402	WCHECIY2403	WCHECI-14653
<i>Enterobacter hormaechei</i> subsp. <i>steigerwaltii</i> DSM 16691 ^T	NZ_CP017179	98.88	98.91	98.89
<i>Enterobacter hormaechei</i> subsp. <i>oharae</i> DSM 16687 ^T	NZ_CP017180	97.65	97.74	97.75
<i>Enterobacter xiangfangensis</i> LMG2719 ^T	NZ_CP017183	97.04	97.13	97.14
<i>Enterobacter hormaechei</i> subsp. <i>hormaechei</i> ATCC 49162 ^T	AFHR00000000	95.02	95.03	95.02
<i>Enterobacter mori</i> LMG 25706 ^T	AEXB00000000	87.82	87.81	87.81
<i>Enterobacter muelleri</i> JM-458 ^T	ERR1111100	87.79	87.79	87.78
<i>Enterobacter asburiae</i> JCM 6051 ^T	BBED00000000	87.77	87.76	87.76
<i>Enterobacter kobei</i> DSM 13645 ^T	NZ_CP017181	87.46	87.45	87.42
<i>Enterobacter cloacae</i> ATCC 13047 ^T	NC_014121	86.89	86.95	86.95
<i>Enterobacter cancerogenus</i> ATCC 35316 ^T	ABWM00000000	86.52	86.52	86.52
<i>Enterobacter ludwigii</i> EN-119 ^T	NZ_CP017279	86.33	86.33	86.33
<i>Enterobacter soli</i> ATCC BAA-2102 ^T	LXES00000000	85.68	85.67	85.67
<i>Enterobacter lignolyticus</i> SCF1 ^T	NC_014618	80.68	80.68	80.68
<i>Enterobacter massiliensis</i> JC163 ^T	CAEO00000000	79.94	79.94	79.94

Figure legend

Figure 1. Phylogeny of ST93 and ST177 isolates of the *E. cloacae* complex. The neighbor-joining tree was constructed based on the core genome determined using Harvest with bootstrap values being shown. Accession numbers are shown in parentheses. 2.0E-5 refers to 2.0×10^{-5} .



Highlights

- Three nonduplicate clinical isolates of *Enterobacter hormaechei* (ST177) carrying two carbapenemase genes *bla*_{KPC-2} and *bla*_{NDM-1} were identified.
- *bla*_{KPC-2} and *bla*_{NDM-1} were carried on two different IncF-type plasmids in the isolates.
- A hospital transmission of a common multidrug resistant strain from a central reservoir was suggested.