RESEARCH ARTICLE

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ST37 *Klebsiella pneumoniae*: development of carbapenem resistance *in vivo* during antimicrobial therapy in neonates

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Aim: To investigate the mechanism leading to *in vivo* carbapenem resistance development in *Klebsiella pneumoniae*. **Methods:** Carbapenemase was detected using the modified carbapenem inactivation method. β -lactamases resistant genes were identified by PCR and sequencing. Clonal relatedness was evaluated by random amplified polymorphic DNA and multiple locus sequence typing. The relationship between sequence typing and resistant genes was analyzed by using the chi-squared test. **Results:** All ST37 carbapenem-resistant isolates were *bla*_{OXA-1} positive and all ST37 carbapenem-sensitive isolates were *bla*_{OXA-1} negative at Stage I. A significant relationship between carbapenem resistance and *bla*_{OXA-1} was observed. The *bla*_{OXA-1}-positive rate was significantly higher in ST37 *K. pneumoniae* than others. **Conclusion:** This is the first study about the development of carbapenem resistance *in vivo* potentially mediated by *bla*_{OXA-1} in ST37 *K. pneumoniae* among neonates.

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Klebsiella pneumoniae (*K. pneumoniae*) is an opportunistic pathogen and a notable cause of clinical diseases including respiratory, urinary tract infections, septicemia and other related diseases. However, antimicrobials (such as carbapenems, cephalosporin), which are widely used in the treatment of *K. pneumoniae* infection, have been proven to be a major factor to mortality and morbidity in settings with limited diagnostic facilities and treatment options [1], especially in increasing emergent multidrug resistant (MDR) *K. pneumoniae*. MDR *K. pneumoniae*, internationally defined as having nonsusceptibility to at least three classes of antibiotics or more [2], poses tremendous threat to clinical setups in hospitals. Currently, carbapenems are an antibiotic option against MDR *K. pneumoniae* worldwide. However, carbapenem resistance is being reported on a large scale, leaving limited therapeutic options and creating a significant health concern for the public [3–5]. Production of *K. pneumoniae* carbapenemase (KPC) or metallo- β -lactamase is postulated as the main mechanism of carbapenem resistance in *K. pneumoniae*. Based on β -lactamase protein sequence similarities, they are divided into four major classes (Classes A–D) [6]. Currently, the most prevalent carbapenemases among these bacteria include class A *K. pneumoniae* carbapenemase, class B New Delhi metallo- β -lactamase (NDM) and class D oxacillinase (OXA) [7].

KEYWORDS

- *bla*_{OXA-1}
- development of carbapenem resistance
- *Klebsiella pneumoniae*
- modified carbapenem inactivation method

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Table 1. Antimicrobial susceptibilities and clinical characteristics of 39 *Klebsiella pneumoniae* strains.

Cases	Isolates and mCIM	MIC (mg/l), Drug resistance rate (%)										β-lactamase genes				ST	Date isolate Identified [#]	Source	Antimicrobial therapy	Hospital stay (days)	Outcome
		IPM	MEM	TZP	LEV	CIP	GEN	SXT	CAP	AZT	TET	MXF [†]	PMB [†]	OXA-1	Others						
Patient 1	KP1 +	>8R	>8R	>64/4R	8R	>2R	>8R	>2/38R	>16R	>16R	>8R	≤1	1	+	CMY-8, CTX-M-1, CTX-M-14, CTX-M-15, SHV	37	20150729	Sputum	MSU, CAZ, TZP, MOX, MEM	46	Improvement
Patient 2	KP2 -	≤1S	≤1S	>64/4R	8R	>2R	>8R	>2/38R	>16R	>16R	>8R	>4	1	-	CTX-M-1, CTX-M-15, SHV	37	20150817	Sputum	CAZ, MSU, MOX, MEM	69	Improvement
Patient 3	KP3 -	≤1S	≤1S	≤4/4S	≤1S	≤0.5S	≤2S	≤0.5/9.5S	≤4S	>16R	≤2S	≤1	1	-	CTX-M-1, CTX-M-15, SHV	37	20150913	Sputum	MSU, CAZ, MOX, MEM	48	Improvement
	KP4 +	>8R	>8R	32/4I	≤1S	≤0.5S	≤2S	≤0.5/9.5S	≤4S	≤2S	≤2S	≤1	≤0.5	+	CTX-M-1, CTX-M-15, SHV	70	20150918	Feces			
Patient 4	KP5 +	>8R	>8R	>64/4R	8R	>2R	>8R	>2/38R	>16R	>16R	>8R	>4	1	+	CTX-M-1, CTX-M-14, CTX-M-15, SHV	37	20151013	Sputum	MSU, CAZ	46	Improvement
Patient 5	KP6 -	≤1S	≤1S	>64/4R	≤1S	≤0.5S	≤2S	1/19S	>16R	>16R	>8R	≤1	≤0.5	-	CTX-M-1, CTX-M-15, SHV	37	20151016	Sputum	MSU, MOX, MEM	70	Improvement
	KP7 +	>8R	>8R	>64/4R	8R	>2R	>8R	>2/38R	>16R	>16R	>8R	>4	≤0.5	+	CTX-M-1, CTX-M-14, CTX-M-15, SHV	37	20151023	Feces			
Patient 6	KP8 -	≤1S	≤1S	≤4/4S	≤1S	≤0.5S	≤2S	≤0.5/9.5S	≤4S	>16R	≤2S	≤1	1	-	CTX-M-1, CTX-M-15, SHV	1083	20151109	Sputum	MSU, TZP, MEM, MOX	66	Improvement
Patient 7	KP9 +	8R	8R	>64/4R	≤1S	≤0.5S	≤2S	≤0.5/9.5S	>16R	>16R	>8R	≤1	1	+	CTX-M-1, CTX-M-15, SHV	70	20151129	Sputum	MSU, CAZ, MEM, TZP	94	Improvement
	KP10 +	8R	>8R	>64/4R	≤1S	≤0.5S	≤2S	>2/38R	>16R	>16R	>8R	≤1	≤0.5	+	CTX-M-1, CTX-M-15, SHV	37	20151211	Feces			
Patient 8	KP11 +	>8R	>8R	>64/4R	≤1S	2I	>8R	>2/38R	>16R	>16R	>8R	2	≤0.5	+	CMY-8, CTX-M-1, CTX-M-14, CTX-M-15, SHV	37	20151208	Sputum	MSU, MEM, CAZ	74	Poor prognosis
	KP12 +	>8R	>8R	>64/4R	≤1S	≤0.5S	≤2S	≤0.5/9.5S	≤4S	>16R	≤2S	≤1	1	-	CTX-M-1, CTX-M-15, SHV	70	20151211	Feces			
Patient 9	KP13 +	>8R	>8R	>64/4R	8R	>2R	>8R	>2/38R	>16R	>16R	>8R	>4	1	+	CTX-M-14, CTX-M-15, SHV	37	20151209	Sputum	MSU, MEM, CTF	65	Poor prognosis
	KP14 +	>8R	>8R	64/4I	≤1S	≤0.5S	≤2S	≤0.5/9.5S	≤4S	>16R	≤2S	≤1	≤0.5	-	CTX-M-1, CTX-M-15, SHV	70	20151216	Feces			

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[†]Stage I: from 20150729 to 20160402; Stage II: from 20140731 to 20150401.

AZT: Aztreonam; CAP: Chloramycetin; CAZ: Ceftazidime; CIP: Ciprofloxacin; CTF: Cefotiam; GEN: Gentamicin; I: Intermediate; IPM: Imipenem; LEV: Levofloxacin; mCIM: Modified carbapenem-activation method; MEM: Meropenem; MOX: Moxalactam; MSU: Mezlocillin-sulbactam sodium; MXF: Moxifloxacin; PMB: Polymyxin-B; R: Resistant; S: Susceptible; SXT: Sequence type; ST: Tetracycline; TZP: Piperacillin/tazobactam.

Table 1. Antimicrobial susceptibilities and clinical characteristics of 39 *Klebsiella pneumoniae* strains.

Cases	Isolates and mCIM	MIC (mg/l), Drug resistance rate (%)										β-lactamase genes			ST	Date isolate Identified [†]	Source	Antimicrobial therapy	Hospital stay (days)	Outcome
		IPM	MEM	TZP	LEV	CIP	GEN	SXT	CAP	AZT	TET	MXF [†]	PMB [†]	OXA-1						
Patient 10	KP15 +	>8R	>8R	>64/4R	8R	>2R	>8R	>2/38R	>16R	>16R	>8R	>4	≤0.5	+	37	20160111	Sputum	MSU, MEM, TZP	27	Improvement
Patient 11	KP16 -	≤1S	≤1S	>64/4R	≤1S	≤0.5S	≤2S	≤0.5/9.5S	>16R	>16R	>8R	≤1	1	-	37	20160112	Blood	MSU, MEM, TZP	23	Improvement
	KP17 +	>8R	>8R	>64/4R	8R	>2R	>8R	>2/38R	>16R	>16R	>8R	>4	≤0.5	+	37	20160124	Feces			
Patient 12	KP18 +	>8R	>8R	>64/4R	2S	>2R	>8R	>2/38R	>16R	>16R	>8R	4	≤0.5	+	37	20160215	Sputum	MSU, MEM, TZP	59	Improvement
	KP19 +	>8R	>8R	>64/4R	2S	>2R	>8R	>2/38R	>16R	>16R	>8R	>4	≤0.5	+	37	20160303	Feces			
Patient 13	KP20 -	≤1S	≤1S	>64/4R	≤1S	≤0.5S	≤2S	≤0.5/9.5S	>16R	>16R	>8R	≤1	≤0.5	-	37	20160221	Sputum	MSU, MEM, CTF	88	Improvement
Patient 14	KP21 -	≤1S	≤1S	≤4/4S	≤1S	≤0.5S	≤2S	≤0.5/9.5S	≤4S	>16R	≤2S	≤1	1	-	1083	20160223	Sputum	MSU, CTF, TZP	41	Poor prognosis
	KP22 +	4R	4R	>64/4R	8R	>2R	>8R	>2/38R	>16R	>16R	>64R	>4	≤0.5	+	37	20160303	Feces			
Patient 15	KP23 +	>8R	>8R	>64/4R	≤1S	>2R	>8R	>2/38R	>16R	>16R	>64R	4	1	+	37	20160223	Sputum	MSU, MEM, CTF, CAZ, TZP	80	Improvement
	KP24 -	≤1S	≤1S	>64/4R	≤1S	≤0.5S	≤2S	≤0.5/9.5S	>16R	>16R	>64R	≤1	≤0.5	-	37	20160303	Feces			
Patient 16	KP25 +	>8R	>8R	>64/4R	≤1S	>2R	>8R	>2/38R	>16R	>16R	>64R	4	1	+	37	20160224	Sputum	MSU, MEM, CTF, TZP	71	Improvement
Patient 17	KP26 +	>8R	>8R	>64/4R	2S	>2R	>8R	>2/38R	>16R	>16R	>64R	4	≤0.5	+	37	20160323	Sputum	MSU, TZP	31	Improvement
Patient 18	KP27 +	>8R	>8R	>64/4R	≤1S	>2R	>8R	>2/38R	>16R	>16R	>64R	4	≤0.5	+	37	20160402	Blood	MSU, MEM, CAZ, TZP	52	Improvement
Patient 19	KP28 +	8R	8R	>64/4R	≤1S	≤0.5S	≤2S	≤0.5/9.5S	≤4S	≤2S	≤2S	≤1	≤0.5	-	54	20140731	Blood	MSU, MOX, MEM	15	Poor prognosis

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		IPM	MEM	TZP	LEV	CIP	GEN	SXT	CAP	AZT	TET	MXF [†]	PMB [†]	OXA-1	Others						
Patient 20	KP29 +	8R	8R	>64/4R	≤1S	≤0.5S	≤2S	>2/38R	>16R	>16R	4S	2	≤0.5	-	CTX-M-1, CTX-M-15, SHV, TEM	54	20140810	Sputum	MSU, CAZ, TZP, MOX	47	Improvement
Patient 21	KP30 +	8R	8R	>64/4R	8R	>2R	>8R	>2/38R	>16R	>16R	>8R	>4	1	-	CTX-M-1, CTX-M-15, SHV	54	20141201	Sputum	CAZ, MEM, TZP	93	Improvement
Patient 22	KP31 -	≤1S	≤1S	≤4/4S	≤1S	1S	≤2S	≤0.5/9.5S	≤4S	≤2S	≤2S	≤1	≤0.5	-	CTX-M-1, CTX-M-14, CTX-M-15, SHV, TEM	556	20141201	Feces	MSU	17	Improvement
Patient 23	KP32 +	>8R	8R	>64/4R	2S	≤0.5S	≤2S	>2/38R	≤4S	>16R	>8R	≤1	1	-	CTX-M-1, CTX-M-14, CTX-M-15, SHV, TEM, NDM-1	20	20141214	Feces	MSU, CAZ, MEM	84	Improvement
Patient 24	KP33 -	≤1S	≤1S	≤4/4S	≤1S	1S	>8R	>2/38R	≤4S	≤2S	>8R	2	1	-	CTX-M-1, CTX-M-14, CTX-M-15, SHV, TEM	556	20141225	Feces	MEZ, MOX	35	Improvement
Patient 25	KP34 +	8R	8R	>64/4R	8R	>2R	>8R	>2/38R	>16R	>16R	>8R	>4	1	-	CTX-M-1, CTX-M-14, CTX-M-15, SHV	37	20141225	Sputum	MSU, CAZ, MEM, MOX	74	Improvement
Patient 26	KP35 +	8R	8R	>64/4R	2S	>2R	>8R	≤0.5/9.5S	≤4S	>16R	≤2S	4	≤0.5	-	SHV, CTX-M-15	54	20141227	Sputum	MSU, MOX, MEM	48	Improvement
Patient 27	KP36 +	8R	8R	>64/4R	≤1S	≤0.5S	≤2S	>2/38R	>16R	>16R	4S	≤1	1	-	SHV, CTX-M-15	54	20150117	Gastric juice	MSU, MOX, MEM	59	Improvement
Patient 28	KP37 +	8R	8R	>64/4R	≤1S	≤0.5S	≤2S	>2/38R	>16R	>16R	4S	≤1	≤0.5	+	CTX-M-1, CTX-M-14, CTX-M-15, SHV	54	20150324	Sputum	MSU	42	Improvement
Patient 29	KP38 +	8R	8R	>64/4R	≤1S	≤0.5S	≤2S	>2/38R	>16R	>16R	4S	≤1	≤0.5	-	SHV	54	20150329	Sputum	MSU, MEM, TZP	60	Improvement
Patient 30	KP39 +	8R	8R	>64/4R	≤1S	≤0.5S	≤2S	>2/38R	>16R	>16R	4S	2	≤0.5	-	CTX-M-1, CTX-M-15, SHV	54	20150401	Sputum	MSU, MEN	59	Improvement

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The class D β -lactamases are called oxacillinases because of their ability to hydrolyze oxacillin and cloxacillin, two- to four-times faster than classical penicillins such as penicillin G [8]. Oxacillinases have been classified into five distinct groups, of which *bla*_{OXA-1} belongs to OXA group III [9]. The *bla*_{OXA-1} β -lactamase gene is a part of Tn2603, which is borne on a highly conjugative resistance plasmid RGN238 that was initially identified in *Escherichia coli* in 1974 [10,11]. Unlike most class A enzymes, which have been extensively studied, class D β -lactamases confer higher-level resistance to a broad spectrum of β -lactam inhibitors and are the least understood class [12,13], its resistance mechanism to carbapenems in *K. pneumoniae* are especially unclear.

Here, we report on the alternate resistance isolates, seen in **Table 1**, of carbapenem-resistant *bla*_{OXA-1}-producing and carbapenem-susceptible *K. pneumoniae* from 30 neonates in the neonatal department of a teaching hospital. To our knowledge, this is also the first case of the development of carbapenem resistance *in vivo* and potential mediation by the *bla*_{OXA-1} gene in *K. pneumoniae*.

Materials & methods

• Bacterial strains & clinical characteristic

From 2014 to 2016, a total of 39 *K. pneumoniae* isolates were collected from 30 newborn infants (in the Second Xiangya Hospital, Central South University), and all isolates were frozen at -80°C. The clinical information for each patient was collected via chart review from the hospital's unified electronic database (**Table 1**). The 39 isolates were from different specimens: sputum (n = 23, 59.0%), feces (n = 12, 30.8%), blood (n = 3, 7.7%) and gastric juice (n = 1, 2.5%). Identification and initial antimicrobial susceptibility were determined using the BD Phoenix 100 Automated Microbiology System (BD Diagnostic Systems, MD, USA). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as reference controls during the identification of isolated bacteria.

• Antimicrobial susceptibility test

An antimicrobial susceptibility test for the *K. pneumoniae* strains was initially performed using a BD Phoenix 100 Automated Microbiology System according to the manufacturer's instructions and further confirmed by the Kirby–Bauer test. The results were interpreted using the 2017 standards of Clinical and Laboratory Studies Institute guidelines [14].

• Modified carbapenem-inactivation method

The modified carbapenem-inactivation method (mCIM) was performed according to Clinical and Laboratory Studies Institute guidelines to detect carbapenemase-producing *K. pneumoniae* [14]. A 1 μ l loopful of each isolate was suspended in 2 ml tryptic soy broth. The suspension was vortex-mixed for 10–15 s and a 10 μ g meropenem disk was added to each tube. Tubes were incubated at 35°C in air for 4 h. After incubation, each disk was placed onto a Mueller–Hinton agar plate inoculated with a 0.5 McF standard of *E. coli* ATCC 29522 (a meropenem-susceptible strain). Plates were incubated at 35°C in air for 18–24 h. *K. pneumoniae* ATCC BAA-1705 and *K. pneumoniae* ATCC BAA-1706 were used as positive and negative controls. Measure the zones of inhibition to judging results positive: 6–15 mm or presence of colonies with a 16–18 mm; negative: ≥ 19 mm; indeterminate: 16–18 mm.

• Detection of various resistance determinants

β -lactamases genes (e.g., NDM-1, SHV, TEM, KPC-2, OXA-1, OXA-2, OXA-9, OXA-48, OXA-181, CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-14, CTX-M-15, CMY-4, CMY-8, IMP-8, DHA, ACT and SME; **Table 2**) were detected by PCR amplification. The positive and negative isolates confirmed by DNA sequencing were used as positive and negative controls. The amplicons were sequenced, and the entire sequence of each gene was compared with the sequences in the Gen-Bank nucleotide database [15].

• Clonal relatedness

The clonal relationship of all *K. pneumoniae* strains were assessed by random amplified polymorphic DNA (RAPD) analysis and further confirmed by multiple locus sequence typing (MLST) according to protocols provided on the MLST websites [15,22]. eBURST was performed for MLST analysis. The eBURST algorithm groups strains by their allelic profiles employing a user-specified group definition and draws a rough sketch to show the genetic relationship. RAPD experimental steps: briefly, DNA was amplified by adding the random primer OPA-2 (5'-TGCCGAGCTG-3'). PCR amplification was performed in a total volume of 20 μ l, comprising 2 μ l DNA template, 2 μ l primer, 7 μ l

Table 2. Primers used in this study.

Primers	Primer sequence (5'-3')	Annealing temperature (°C)	Length of products (bp)	Ref.
NDM-1	Sense: 5'-CCGCAACCATCCCCTCTT-3' Anti: 5'-CAGCACACTTCCTATCTC-3'	53	888	This study
KPC-2	Sense: 5'-GGCACTTTTCGTCCA-3' Anti: 5'-ATGATTTTCAGAGCCTTACT-3'	52	1003	This study
SHV	Sense: 5'-GCCTTTATCGCCTTCACTCAAG-3' Anti: 5'-TTAGCGTTGCCAGTGTCTGATCA-3'	60	898	[17]
TEM	Sense: 5'-TTCGTGTCGCCCTTATTC-3' Anti: 5'-ACGCTCGTCGTTTGGTAT-3'	55	512	This study
OXA-1	Sense: 5'-CTGTTGTTTGGGTTTCGCAAG-3' Anti: 5'-CTTGGCTTTTATGCTTGATG-3'	55	440	This study
OXA-2	Sense: 5'-TAAGCAACACCGACAGG-3' Anti: 5'-TCGTGATGAGTTCCAGAT-3'	51.2	879	This study
OXA-9	Sense: 5'-ACAGCGGAGCAATGAAG-3' Anti: 5'-CGACAAAGCGTAGAAGAAAC-3'	52.6	549	This study
OXA-48	Sense: 5'-TTTTCTGTGTTGAGCACT-3' Anti: 5'-TACCCGCATCTACCTTT-3'	50	586	This study
OXA-181	Sense: 5'-5CGTTATGCGTGTATTAGC-3' Anti: 5'-CACTTCTTTTGTGATGGC-3'	51	775	This study
CTX-M-1	Sense: 5'-CAGCGCTTTTGCCGTCTAAG-3' Anti: 5'-GGCCCATGGTTAAAAAATCACTGC-3'	60	945	[17]
CTX-M-2	Sense: 5'-CTCAGAGCATTCGCCGCTCA-3' Anti: 5'-CCGCCGCAGCGAGAATATCC-3'	61.5	843	[17]
CTX-M-8	Sense: 5'-ACTTCAGCCACACGGATTCA-3' Anti: 5'-CGAGTACGTACGACGACTT-3'	52.5	1024	[17]
CTX-M-14	Sense: 5'-GCAGATAATACGCAGGTG-3' Anti: 5'-GCTGGGTAAAAATAGGTCAC-3'	55.1	640	This study
CTX-M-15	Sense: 5'-ATTAGAGCGGCAGTCGG-3' Anti: 5'-AAGGAGAACCAGGAACCAC-3'	55.1	883	This study
CMY-4	Sense: 5'-GCCGTTGCCGTATCTAC-3' Anti: 5'-CCAATGCCACTTTGCTGT-3'	55.2	796	[18]
CMY-8	Sense: 5'-AGCGGTAAACGAGTGAG-3' Anti: 5'-AGTAATGCCCTTTGTGG-3'	52	1042	[18]
IMP-8	Sense: 5'-GTTTTATGTGTATGCTTCC-3' Anti: 5'-AGCCTGTTCCCATGTAC-3'	50	678	[19]
DHA	Sense: 5'-GCAGGATATCCCGGGATGG-3' Anti: 5'-CGTACGCATACTGGCTTGC-3'	59	405	[20]
ACT	Sense: 5'-TCGGTAA AGCCGATGTTGCGG-3' Anti: 5'-CTTCCACTGCGGCTGCCAGTT-3'	59	302	[20]
SME	Sense: 5'-A ACGGCTTCATTTTGTTTAG-3' Anti: 5'-GCTTCCGCAATAGTTTATCA-3'	48	831	[21]

deionized water and 9 µl of 2× PCR mix. The mixture was subjected to 45 cycles of amplification (92°C for 60 s, 32°C for 120 s, 72°C for 120 s for each cycle) with an initial incubation step at 95°C for 5 min and a final extension at 72°C for 15 min. The amplified fragments were separated using 1.5% agarose gel electrophoresis at 150 V/cm for 20 min. Gels were photographed under ultraviolet illumination by the FluorChem

system and Image J software, and the comparison was performed for the distribution and number of the DNA bands.

• Statistical analysis

All data were analyzed using SPSS18.0 statistical software. The relationship between ST37 *K. pneumoniae* and non-ST37 *K. pneumoniae* in the *bla*_{OXA-1} gene and the relationship between

carbapenem resistance and resistant genes were analyzed by the chi-square test. P values smaller than 0.05 were considered statistically significant.

Results

• Antimicrobial susceptibility test

All 39 isolates were sensitive to amikacin and resistant to ampicillin/sulbactam, amoxicillin/clavulanic, cefotaxime, ceftazidime, cefazolin, cefepime and piperacillin, which were not shown in **Table 1**. The MIC results of the 39 *K. pneumoniae* isolates revealed that most isolates were resistant to most antibacterial agents of the following: penicillins, cephalosporins, β -lactamase inhibitors and carbapenems. Most isolates were resistant to the vast majority of antibiotics tested: ampicillin/sulbactam (100%), amoxicillin/clavulanic (100%), cefotaxime (100%), ceftazidime (100%), cefazolin (100%), cefepime (100%), piperacillin (100%), aztreonam (89.7%), piperacillin/tazobactam (82.1%), imipenem (73.4%), meropenem (73.4%), chloramycetin (71.8%), sulfamethoxazole (64.1%) and tetracycline (64.1%). Medium susceptibility to ciprofloxacin (43.6%) and gentamicin (48.7%) were identified. Susceptibility was noted only to amikacin (100%) and levofloxacin (74.4%).

• Modified carbapenem inactivation method

29 of the 39 isolates (73.4%) were positive for mCIM. All carbapenem resistance isolates were positive for mCIM and all carbapenem-sensitive isolates were negative for mCIM. See **Figure 1** and **Table 1**.

• Detection of various resistance determinants

18 of the 39 isolates (46.2%) were positive for the *bla*_{OXA-1} gene. The *bla*_{NDM-1} and *bla*_{CMY-8} gene were detected in only two strains (5.1%). The positive rates of the *bla*_{CTX-M-15}, *bla*_{CTX-M-1} and *bla*_{CTX-M-14} genes were 94.9% (n = 37), 84.6% (n = 33) and 43.6% (n = 17) respectively. The *bla*_{SHV} gene was positive in nearly all isolates, except for the KP18 isolate. Lower levels of resistance genes were observed for *bla*_{NDM-1} (n = 2, 5.1%), *bla*_{CMY-8} (n = 2, 5.1%) and *bla*_{TEM} (n = 5, 12.8%). Other β -lactamase genes, including *bla*_{CMY-4}, *bla*_{CTX-M-2}, *bla*_{CTX-M-8}, *bla*_{OXA-48}, *bla*_{OXA-181}, *bla*_{OXA-9}, *bla*_{OXA-2}, *bla*_{IMP-8}, *bla*_{DHA}, *bla*_{ACT}, *bla*_{SME}, *bla*_{KPC} were not detected in any isolates. The final results are shown in **Table 1**.

Figure 1. Partial results of modified carbapenem inactivation method.

1: Negative control; 2: Positive control; 3: Negative strain (20 mm); 4: Positive strain (6 mm).

• Clonal relatedness

Six distinct RAPD patterns (RAPD types A–F) were observed by the FluorChem system and Image J software among the 39 isolates when differences in one or two bands were relevant: type A (22/39), type B (8/39), type C (4/39), type F (1/39) and two isolates each for type D and type E (**Figure 2**). Six distinct MLST sequence types (STs) were observed among the 39 isolates, including ST37 (22/39, type A), ST54 (8/39, type B), ST70 (4/39, type C), ST556 (2/39, type D), ST1083 (2/39, type E) and ST20 (1/39, type F). The 18 *bla*_{OXA-1}-positive *K. pneumoniae* isolates were identified as ST37 (n = 15), ST70 (n = 2) and ST54 (n = 1). Two *bla*_{NDM-1}-positive *K. pneumoniae* belonged to ST54 and ST20 (**Table 1**). The eBURST result showed that these STs were clustered into one clonal complex (ST37 and ST1083) and four singletons (ST54, ST70, ST556, ST20; **Figure 3**).

• Clinical history information

The clinical data from patients were retrospectively reviewed and clinical details of the patients are partially shown in **Table 1**. The most common underlying conditions were low-birth weight premature infants, neonatal respiratory distress syndrome, neonatal pneumonia, neonatal asphyxia and anemia. Indwelling devices were used in most patients, including mechanical ventilation, tracheal intubation, peripherally inserted central catheter and gastric tube. All patients were treated with one or more

Figure 2. Partial results of RAPD-PCR analysis of *Klebsiella pneumoniae* strains. Six distinct RAPD patterns (RAPD types A–F) were observed.

RAPD: Random amplified polymorphic DNA.

antimicrobial agents for a long time, including mezlocillin–sulbactam sodium, piperacillin–sulbactam sodium, ciprofloxacin, ceftazidime, cefotiam and meropenem. Although, almost all isolates were susceptible to amikacin and levofloxacin, and of the half isolates were susceptible to ciprofloxacin (56.4%) and gentamicin (51.3%), these antibiotics are not used in newborn infants to avoid their side effects, if other antibiotics are available. Most patients were improved and discharged after treatment for a long stay (Table 1).

• Relationship between ST37 *K. pneumoniae* & *bla*_{OXA-1} gene

All *K. pneumoniae* isolates were divided into two subgroups according to *bla*_{OXA-1} gene expression (Table 3). The ST37 *K. pneumoniae* *bla*_{OXA-1}-positive rate was significantly higher than the others as analyzed by the chi-squared test ($\chi^2 = 15.63$, $p < 0.05$), suggesting that the *bla*_{OXA-1} gene was more easily expressed in ST37 *K. pneumoniae* in the hospital neonatal department during the term. A significant relationship between carbapenem resistance and the *bla*_{OXA-1} gene was also observed ($p < 0.05$; Table 4). There was no significant correlation between carbapenem resistance and other resistant genes in our study, which were not shown in Table 4.

Discussion

The spread of carbapenem-resistant *K. pneumoniae* has been a global concern. In this study, 100% of carbapenem-resistant strains were confirmed as KPC-producers. The presence of carbapenemase rate among infants detected here is higher than in other studies [23]. The most common carbapenem-resistant mechanism is the acquisition of plasmid-borne β -lactamases that can hydrolyse carbapenems, such as NDM-1, KPC-2, OXA. NDM-1 was first detected in *Escherichia coli* and *K. pneumoniae* isolated from a Swedish patient of Indian origin in 2008 [3]. NDM-1 carbapenemases are highly resistant to many antibiotic classes, including those of the carbapenem family, and potentially herald the end of treatment [24]. Pathogens harboring KPC-2 are resistant to all β -lactam and β -lactamase inhibitors, severely limiting treatment options and thus leading to high fatality rates [25]. However, all carbapenem-resistant isolates were KPC-2 negative, and only two isolates harbored NDM-1 in our study. This outcome leads to a lower positive rate compared with previous studies in our hospital neonatal department [16]. We speculate that different genotypes of *K. pneumoniae* may be expressing different resistant genes at different time. Notably, SHV- and CTX-M-resistant genes, which are the main

Figure 3. Population ‘snapshot’ determined by eBURST analysis showing the cluster of all sequence types in this study and some linked sequence types of *Klebsiella pneumoniae* in Pub Multiple Locus Sequence Typing. The epidemic sequence types in our study are indicated by red squares: Four singletons and one group (ST37 and ST1083). The blue dot indicates a predicted founder; line distances have no significance. The area of each circle corresponds to prevalence of sequence type in the multiple locus sequence typing data of this study.

Table 3. Relationship between sequence type 37 *Klebsiella pneumoniae* and nonsequence type 37 *K. pneumoniae* in the *bla*_{OXA-1} gene.

	Positive	Negative	Total	χ^2	p-value
ST37	15	7	22	15.63	<0.05
Others	3	22 [†]	25		
Total	18	29	47		

[†]In this study, there are 15 non-ST37 *bla*_{OXA-1} negative *K. pneumoniae* isolates, the other eight isolates which were *bla*_{OXA-1} negative and resistant to carbapenem were presented in our previous study [16].
ST: Sequence type.

epidemic-resistant genes on a global scale, were present in almost all isolates in our study.

In our study, the most types of *K. pneumoniae* were observed for ST37 (22/39), ST54 (8/39) and ST70 (4/39), followed by ST556 (2/39), ST1083 (2/39) and ST20 (1/39), which were clustered into one clonal complex and four singletons (Figure 3). Therefore, we should also pay close attention to the sporadic ST in addition to the major epidemic clone complex. It was discovered that ST37 are international clones associated with multidrug-resistant *K. pneumoniae* such as extended-spectrum β -lactamase, AmpC or carbapenemase producers [26]. Recently, NDM-1-producing *K. pneumoniae* belonging to ST37 were reported in China [27], which indicated that multidrug-resistant *K. pneumoniae* ST37 have been identified in our country. The *bla*_{OXA-1}-producing *K. pneumoniae* has been reported in some sequencing types, such as ST48, ST15 and ST23 [28]. However, ST37 has never been reported before. To the best of our knowledge, this is the first report of *bla*_{OXA-1}-producing ST37 *K. pneumoniae* in our country.

According to other authors, the *bla*_{OXA-1} gene is a part of a class I integron [29] that has been found in plasmid and integron locations in a wide variety of Gram-negative organisms, mainly *E. coli*. The *bla*_{OXA-1} gene was frequently found to be associated with genes encoding extended-spectrum β -lactamases (ESBLs), which can hydrolyze both amino and ureidopenicillins (piperacillin) significantly and narrow-spectrum cephalosporins weakly [30].

Rasmussen *et al.* and Andres Opazo *et al.* reported that most OXA-type carbapenemases show weak carbapenemase activity [31,32]. Madhan Sugumar [30] found that *bla*_{OXA-1} β -lactamase-producing strains were resistant to piperacillin/tazobactam (100%), levofloxacin (91.6%), amikacin (75%), cefoxitin (50%), ertapenem (25%), imipenem (16.6%) and meropenem (16.6%). Eva Torres [33] reported that reduced susceptibility to cefepime was mostly due to *bla*_{OXA-1} β -lactamase in *E. coli*. However, the relationship between the *bla*_{OXA-1} gene and carbapenems has never been reported. Only Madhan Sugumar, Rasmussen *et al.* and Andres Opazo *et al.* have reported that OXA carbapenemases show weak carbapenemase activity [30–32]. In our study, a significant relationship between carbapenem resistance and the *bla*_{OXA-1} gene was observed ($p < 0.05$; Table 4). Through point mutations and plasmid transfer, natural OXA variants (e.g., OXA-15, OXA-18 and OXA-19) have arisen with an expanded substrate spectrum, including imipenem and third-generation cephalosporins such as cefotaxime, ceftriaxone and aztreonam [34]. However, other *bla*_{OXA} genes, such as OXA-48, OXA-181, OXA-9 and OXA-2, were negative in our study.

Interestingly, among 22 *K. pneumoniae* ST37 isolates, drug susceptibility to carbapenem varied. 16 isolates were carbapenem resistant and six were carbapenem sensitive. We found the same ST in patient 11 and patient 15, but reversed drug susceptibility to carbapenem in isolates

Table 4. Relationship between susceptibility to carbapenem and the *bla*_{OXA-1} gene.

Carbapenem susceptibility	<i>bla</i> _{OXA-1} positive	<i>bla</i> _{OXA-1} negative	Total	χ^2	p-value
R	18	19 [†]	37	7.88	<0.05
S	0	10	10		
Total	18	29	47		

[†]In this study, there are 15 non-ST37 *bla*_{OXA-1} negative *K. pneumoniae* isolates, the other eight isolates which were *bla*_{OXA-1} negative and resistant to carbapenem were presented in our previous study [16].
R: Resistant; S: Susceptible.

from different parts of the body. On day 1, blood cultures identified ST37 carbapenem-sensitive *K. pneumoniae* (K16) from patient 11. After 12 days, ST37 carbapenem-resistant *K. pneumoniae* (K17) was found in feces. Analogously, ST37 carbapenem-resistant *K. pneumoniae* (K23) was found in sputum from patient 15 on day 5, but ST37 carbapenem-sensitive *K. pneumoniae* (K24) was found in feces after 9 days. Curiously, the carbapenem-susceptible and carbapenem-resistant isolates were isolated alternately from different patients or the same patient. Thus, we speculate that the mechanism might be connected to acquisition or loss of a drug resistance gene or the long-term concurrence of carbapenem-susceptible and carbapenem-resistant ST37 *K. pneumoniae* in our hospital neonatal department. *In vivo* evolution of resistance has been reported previously by Ana Mena in 2006 [35], and Ju-Hsin in 2010 [5] reported inactivation of OmpK36 of *K. pneumoniae* and loss leads to *in vivo* carbapenem resistance development. In 2005, Tao Hong reported that the mechanism was *bla*_{KPC} gene acquisition [36]. In 2010, Rodríguez-Avial reported that the mechanism was *bla*_{VIM-1} gene acquisition [37]. In 2015, Junying Zhu reported that the mechanism was *bla*_{NDM-1} gene acquisition [27]. In our study, we found that all ST37 carbapenem-resistant isolates were *bla*_{OXA-1} positive and all ST37 carbapenem-sensitive isolates were *bla*_{OXA-1} negative in Stage I (from 20150729 to 20160402). To determine whether ST 37 was significantly correlated with the *bla*_{OXA-1} resistant gene, we conducted further statistical analysis. We found that the *bla*_{OXA-1}-positive rate of ST37 *K. pneumoniae* was significantly higher than that in others as analyzed by a chi-squared test ($\chi^2 = 15.63$, $p < 0.05$), suggesting that the *bla*_{OXA-1} gene was significantly more prevalent in ST37 *K. pneumoniae* in our hospital neonatal department during Stage I. However, this relationship did not exist in other β -lactamase genes in our study, such as *bla*_{NDM-1}, *bla*_{KPC-2}, *bla*_{CTX-M}, *bla*_{SHV} and *bla*_{CMY}. These findings indirectly demonstrate that acquisition of the drug resistance gene *bla*_{OXA-1} acquisition is the mechanism leading to the development of *in vivo* carbapenem resistance in ST37 *K. pneumoniae* in our neonatal department. In addition, patient 11 and patient 15 revealed that a mixed population of *bla*_{OXA-1}-positive/negative isolates coexisted in one patient. However only the dominant isolate, adapting to the antimicrobial pressure at the time, was obtained in every instance, giving essentially the same result as that reported by Rodríguez-Avial [37].

From our study data (Table 1), we found that the major epidemic strain was ST54 at Stage II (from 20140731 to 20150401), and only one ST54 isolate was *bla*_{OXA-1} positive (KP37). The major epidemic strain was ST37 at Stage I (from 20150729 to 20160402). However, the strain KP34 isolated at Stage II in 2014 is also ST37 and is resistant to carbapenem, but is *bla*_{OXA-1} gene negative. We speculate that the presence of other new metallo- β -lactamases or variants of certain carbapenemases might contribute to resistance to carbapenem at that time.

However, the possible mechanism of resistance gene acquisition or loss is not understood. The clinical historical information in our study indicated that immunosuppression, invasive procedures (especially tracheal intubation and peripherally inserted central catheter), long hospital stays and prior or long-term use of broad-spectrum antibiotics might increase the chance of infection or colonization of MDR isolates. According to the historical clinical information, we speculate that the possible mechanisms of resistance gene acquisition or loss could include: (i) long-term use of broad-spectrum antibiotics; (ii) a change in the host-immune system; and (iii) competition between resistance genes. Meanwhile, recent research found antibiotic resistance genes, such as the *K. pneumoniae* β -lactamase resistance gene carried on microbial chromosomes showed a peak in abundance after antibiotic treatment followed by a sharp decline [38], which further demonstrated our speculation (i) indirectly. Certainly, these speculations need further confirmation in our future studies.

There are some limitations to this study. Some studies have reported that most OXA-type carbapenemases show weak carbapenemase activity [31,32]. There may be other cooperative mechanisms. Other studies [32] have reported that OXA-type carbapenemases have lower efficiency to hydrolyze carbapenems, but it is important to consider them as potentially dangerous because their expression can be regulated by the upstream insertion of insertion sequence (IS) elements such as ISAbal, which can be intensified when other mechanisms of resistance are present, such as increased expression of efflux pumps and loss of porins [39,40]. The presence of new metallo- β -lactamases, variants of certain carbapenemases or some resistant genes that we did not test could also be possible cooperative mechanisms. These will all require further study in the future.

Conclusion

In summary, we describe here for the first time the *in vivo* development of carbapenem resistance in ST37 *K. pneumoniae* under antibiotic selective pressure by acquisition of the *bla*_{OXA-1} gene among neonates. However, we cannot exclude other possible cooperative mechanisms not captured by our measures, such as the presence of new metallo-β-lactamases, variants of certain carbapenemases, loss of outer membrane proteins or untested resistant genes, which will require further study and investigation. At present, most mobile carbapenem-hydrolyzing OXA β-lactamases are unknown and merit further study. Meanwhile, we must avoid indiscriminate and long-term antibiotic use, which may result in increasing emergent MDR *K. pneumoniae*. In addition, effective measures for prevention and control of the spread of these resistance determinants should be implemented immediately, such as frequent hand-washing, limitation of antibiotic use and isolation of infected patients. Curiously, the results of other antimicrobial categories also appear different for the same ST (e.g., levofloxacin) and deserves further exploration.

Future perspective

MDR *K. pneumoniae* has been recognized as one of the most troublesome pathogens and poses increasing threat to clinical areas in hospitals. The common underlying conditions or indwelling devices in newborn infants may be promoting factors in the emergence of carbapenem resistance. Effective measures for prevention and control of the spread of these resistance determinants should be implemented immediately to prevent increased mortality of *K. pneumoniae* infection in the future. The *bla*_{OXA-1} gene may be a new carbapenem resistance gene and may promote

the development of new antibiotics for MDR *K. pneumoniae*. Whether efflux pump overexpression or outer membrane protein loss cooperatively contributes to carbapenem-resistance acquisition will require further study.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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SUMMARY POINTS

- To our knowledge, this is the first study of the development of carbapenem resistance *in vivo*, potentially mediated by the *bla*_{OXA-1} gene in ST37 *Klebsiella pneumoniae* among neonates.
- Long-term use of antibiotics can lead to the *in vivo* development of carbapenem resistance in ST37 *K. pneumoniae* under antibiotic selective pressure.
- This is the first report of *bla*_{OXA-1}-producing ST37 *K. pneumoniae* in China.

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