Emergence of *Klebsiella pneumoniae* ST307 Co-Producing CTX-M with SHV and KPC from Paediatric Patients at Shenzhen Children's Hospital, China

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Aim: We investigated the clonal diversity of carbapenemase-producing *Klebsiella pneumoniae* isolates from the Shenzhen Children's Hospital, China, and drew conclusions on the clinical and public health impact of these isolates as multidrug-resistant.

Methods: From January 2014 to December 2018, a total number of 36 unique carbapenemase-producing clinical isolates of *Klebsiella pneumoniae* were collected out of 900 clinical isolates in paediatric patients from the Shenzhen Children's Hospital, China. After carbapenemase production confirmation, antimicrobial susceptibility, resistance determinants and phylogenetic relationship were determined.

Results: The isolates showed resistance to ceftazidime, ertapenem, ampicillin, cefazolin, ceftriaxone, cefotetan, ticarcillin, cefaclor, cefpodoxime, azlocillin, cefcapene, mezlocillin and ampicillin-sulbactam. Of the 36 *Klebsiella pneumoniae* carbapenemase genes coding isolates, *bla*_{NDM} was the mostly detected 50% (n=18) followed by *bla*_{KPC} and *bla*_{IMP} 19% (n=7), *bla*_{VIM} 17% (n=6), *bla*_{OXA-48-like} 8% (n=3) and *bla*_{SME} 5% (n=2), whereas extended-spectrum β -lactamase (*bla*_{SHV}) was predominantly detected 92% (n=33) followed by *bla*_{CTX-M} 53% (n=19) and *bla*_{CMY} 28% (n=10). Pulsed-field gel electrophoresis typing showed eight different patterns, and twenty-five distinct sequences types were observed with ST307 being predominantly identified 11% (n=4), followed by ST2407 8% (n=3). Plasmid replicon typing results indicated that IncFIS, IncHI2, IncFIC and IncFIA plasmids carry *bla*_{CTX-M}, *bla*_{SHV} and *bla*_{NDM} genes.

Conclusion: This study reports on the occurrence and spread of carbapenemase and extended-spectrum β -lactamase encoding genes co-existence in sporadic *Klebsiella pneumoniae* ST307 in paediatric patients from the Shenzhen Children's Hospital, China.

Keywords: *Klebsiella pneumoniae*, carbapenemase, ESBLs, antimicrobial susceptibility, molecular characterization

Introduction

The rise and spread of antimicrobial resistance bacteria are universal symbolic challenges for healthcare due to the restricted treatment choices.¹ In the past year, carbapenem-resistant *Klebsiella pneumoniae* (CRKP) infections have become a growing of global public health concern, particularly in paediatric patients, due to high morbidity and mortality.² Carbapenemase enzymes encoded by alleles of the *bla*_{KPC} gene, depict one of the five substantial carbapenemase families, others being the VIM, IMP and New Delhi metallo- β -lactamase (MBL) (NDM), also, the OXA-48-like oxacillinases have

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been reported worldwide.^{3,4} The commonness of each carbapenemase differs geographically, and its resistance profiles vary.^{5,6} Several *Klebsiella pneumoniae* carbapenemase (KPC) outbreaks were reported in Brazil, Argentina, Poland, Germany, France, Spain and China (Jiangsu),⁷ whereas NDM producing *Klebsiella pneumoniae* is highly prevalent in Canada, Greece, Belgium, Sweden, Norway, India, Pakistan, Bangladesh, Korea and China.⁸ Thus, OXA-48-like oxacillinases producing strains are widely spread in Turkey, Morocco and France, and the VIM is highly spread in Greece.⁹ In China, despite the considerable dissemination of KPC-producing strains, NDM-producing *K. pneumoniae* are the type mainly found in children, and are seldom reported in adults.¹⁰ The CHINET data report of 2005–2014 reported that the detection rate of CRKP isolates in paediatric patients has increased from 5.3% to 15.9%.¹¹ Recently, *K. pneumoniae* isolates producing OXA-232, OXA-163 and OXA-405 carbapenemase were reported in Children's Hospitals globally including China.^{12,13} Carbapenem antibiotics are the foremost alternative to treat conditions caused by multidrug-resistant (MDR) bacteria due to the side effects of other drugs, such as fluoroquinolones and aminoglycosides.¹⁴ This has led to an exponential increase in carbapenem resistance among pathogenic bacteria. Despite the fact that CRKP has spread globally and has serious clinical outcomes in pediatric patients, there are insufficient data available on the susceptibility and genetic platform of carbapenemase production of *K. pneumoniae* in China. CRKP has been significantly increased in China, of particular concerns is the emerging multiple carbapenemase encoding genes in single isolates or co-existence extended spectrum β -lactamase (ESBLs), and the recently discovered colistin resistance plasmid-born *mcr-1*; which results in highly drug-resistant strains (Pan-drug-resistant strains) that are probably untreatable.¹⁵ Carbapenemase encoding genes detection significantly aids in targeted drug use, and helps to prevent further dissemination. Thus, this study aimed to investigate the resistance gene profile, molecular epidemiology, and clinical characteristics of CPKP isolates obtained from paediatric patients in the Shenzhen's Children Hospital in China.

Materials and Methods

Clinical Sample, Bacterial Isolation and Identification

Thirty-six unique (one isolate from one patient) clinical isolates of *K. pneumoniae* were collected from January 2014 to December 2018 from Shenzhen Children's Hospital, Shenzhen, China. Among the 36 CRKP isolates, 61%

(n=22) were from male and 39% (n=14) were from female patients with ages ranging from one month to twelve years. The clinical isolation type of specimens was as follows: urine 44% (n=16), sputum 25% (n=9), blood 12% (n=4) and catheter-associated secretion (CAS) 6% (n=2), while the cerebral-spinal fluid (CSF), throat swab (TS), pus, and abdominal fluid or secretion each had 13% (n=1). This study was conducted in accordance with the declaration of Helsinki.

All isolates were primarily identified by API20E (Biomerieux, Ref. No.27530/275660) automated system and further confirmed by using 16s RNA gene sequence primers.

Phenotypic Detection of CRKP

Carbapenemase production was confirmed by using a newly developed carbapenem inactivation method (CIM) as delineated in the year 2015.¹⁶ To carry out the CIM, an antibiotic susceptibility-testing disc of 10- μ g meropenem (MEM) was incubated for 2 hrs in an aqueous suspension of a *K. pneumoniae*. The disc was removed from the suspension and placed onto a Mueller-Hinton agar (MHA) plate, seeded with an ATCC25922 indicator organism, followed by overnight incubation. The zone of inhibition was measured to determine whether the MEM had been hydrolyzed (growth of the indicator organism close to the disc), or still active (a large zone of inhibition around the disc). The positive control strain was selected from the collected isolates characterized from our laboratory.

Phenotypic Detection of ESBLs Production

The ESBLs production was determined by using VITEK@2 compact system (Biomerieux, Ref. No. 27530/275660). We used a control strain that was characterized from our laboratory, while ATCC25922 was used as a negative control strain. The ESBLs production result was analyzed according to the Clinical and Laboratory Standards Institute (CLSI) guideline.¹⁷

Antimicrobial Susceptibility

Antimicrobial susceptibility was performed by using VITEK@2 compact system (Biomerieux, Ref. No. 27530/275660) for 25 antimicrobial agents, namely ampicillin/sulbactam, piperacillin, ertapenem, amikacin, levofloxacin, nitrofurantoin, ampicillin, cefazolin, ceftazidime, ceftriaxone, cefepime, imipenem, cefotetan, tobramycin, gentamicin, ticarcillin, cefaclor, cefpodoxime, azlocillin, cefcapene,

mezlocillin, trimethoprim, aztreonam, and ciprofloxacin. Colistin susceptibility was performed by the disc diffusion method. To further ascertain the antimicrobial susceptibility, we used CIM disc to detect carbapenemase enzyme, where the disc was removed from the suspension and placed onto a Mueller-Hinton agar (MHA) plate, seeded with an ATCC25922 indicator organism, followed by overnight incubation. The results were construed according to the Clinical and Laboratory Standards Institute (CLSI) guideline (CLSI, 2019)¹⁶ and EUCAST 2016.¹⁸

Detection of Carbapenemase Encoding Genes

The standard PCR was used to detect the presence of carbapenemase encoding genes. Class-A includes *bla*_{IMI}, *bla*_{GES}, *bla*_{SME} and *bla*_{KPC}, while Class-B consists of *bla*_{IMP}, *bla*_{VIM}, *bla*_{GIM}, *bla*_{SIM} and *bla*_{NDM} and Class-D made up of *bla*_{OXA-48} like using specific primers as previously described.^{19–21} In addition, PCR assay was carried out for other β -lactamase encoding genes: *bla*_{CTX-M-(variant)}, *bla*_{SHV}, *bla*_{CMY}, *bla*_{TEM} and *bla*_{VEB} by using specific primers as described earlier.²² Control strain, which was selected from the characterized strain collection of our laboratory. The private company (Sangon Biotech-Shanghai, China) sequenced the purified PCR products. DNA sequences were analyzed using the following URLs <https://blast.ncbi.nlm.nih.gov/Blast.cgi>, <http://www.blddb.eu:4567/> and <https://bigsdgpasteur.fr/klebsiella/klebsiella.html>.

PFGE and Multi-Locus Sequence Typing (MLST)

We performed PFGE to check whether there is a presence of any clonal transmission within the Hospital. Furthermore, we used the MLST to assess the genetic relatedness of the identified isolates.

DNA Extraction Details

Post-extraction DNA was digested with 45U *Xba*I (Takara Biotech) for 2 hours at 37°C. We used CHEFDRIII apparatus (Bio-Rad Laboratories, Hercules, CA, USA) to perform PFGE for *K. pneumoniae* isolates as previously described.²³ PCR assay was performed to amplify internal portions of the seven housekeeping genes of *K. pneumoniae* (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) with specific primers.²⁴ Amplified products were sequenced from private enterprise (Sangon Biotech-Shanghai, China <http://www.pas>

[teur.fr/recherche/genopole/PF8/mlst/Kpneumoniae](http://www.pas-teur.fr/recherche/genopole/PF8/mlst/Kpneumoniae)) and were referred to assign sequence types (STs).

Plasmid Transferability

Streptomycin-resistant *E. coli* C₆₀₀ was used as the recipient strain in conjugation experiments to analyze the horizontal genes transformation of plasmid-borne β -lactamase encoding genes in CRKP isolates, using liquid mating assay as previously described.¹⁴ Transconjugants were selected using Luria Bertani agar containing streptomycin 2000 (μ g/mL), ampicillin (100 μ g/mL) and cefotaxime (32 μ g/mL). Therefore, we further tested them using PCR for β -lactamase encoding genes after performing a phenotypic combination disc test.

PCR-Based Replicon Typing

PCR-based replicon typing was performed for both plasmids from parental and transconjugant isolates. The Inc (incompatibility) groups were determined by using a specific primer as previously described by Carattoli et al 2005.²⁵

Results

Antimicrobial Resistance Profile of *K. pneumoniae*

All the 36 isolates were identified as *K. pneumoniae* using API20E (Biomérieux, Ref. No. 27530/275660) automated system and were further confirmed using 16s RNA gene sequencing. Antimicrobial susceptibility tests were found in all the 36 CRKP isolates (100%) and were all resistant to ceftazidime, ertapenem, ampicillin, cefazolin, ceftriaxone, cefotetan, ticarcillin, cefaclor, cefpodoxime, azlocillin, cefcapene, mezlocillin and ampicillin-sulbactam. However, aztreonam showed 89% (n=32), piperacillin 86% (n=31), cefepime 83% (n=30), imipenem 80% (n=29), nitrofurantoin 47% (n=17), trimethoprim 44% (n=16), ciprofloxacin, gentamicin, levofloxacin 30% (n=11), tobramycin 25% (n=9), amikacin 11% (n=4). All isolates were susceptible to colistin (Figure 1), and the 36 CRKP isolates showed a multi-drug resistant phenotype, hence designated as “superbugs”.

Molecular Analysis of Drug Resistance Genes

All the 36 CRKP isolates were carrying carbapenemase encoding genes, with the most common being *bla*_{NDM} 50% (n=18) which include *bla*_{NDM-1} (n=13), *bla*_{NDM-6} (n=3) and *bla*_{NDM-5} (n=2); followed by *bla*_{IMP} 19%

Figure 1 Antimicrobial resistance patterns of 25 commonly used antibiotics against 36 carbapenemase-producing *Klebsiella pneumoniae* isolates from paediatric clinical cases.

(n=7) including *bla*_{IMP-38} (n=5) and *bla*_{IMP-4} (n=2), *bla*_{KPC-2} 19% (n=7), *bla*_{VIM-2} 17% (n=6), *bla*_{OXA-48} and *bla*_{SME-1} 8% (n=3) each. Additionally, co-existences of other β -lactamases encoding genes were detected in *bla*_{SHV} 92% (n=33), contains *bla*_{SHV-11}

(n=15), *bla*_{SHV-1} (n=13), *bla*_{SHV-27} (n=3), *bla*_{SHV-26} and *bla*_{SHV-33} (n=1), *bla*_{CTX-M} (n=19), *bla*_{CTX-M-3} (n=11), *bla*_{CTX-M-15} (n=5), *bla*_{CTX-M-14}, 26, 40, (n=1 each), *bla*_{CMY-2} 28% (n=10) (Figure 2). The *bla*_{IMI}, *bla*_{GES}, *bla*_{GIM}, *bla*_{SIM} carbapenemase encoding genes

Figure 2 Dendrogram of the 36-PFGE-XbaI identified CRKP isolates collected from paediatric patients showing their genetic relatedness by date of isolation, department of isolation, sources of specimen, sequences types, replicon type, resistant determinants and clonal relatedness.

and ESBLs encoding genes *bla*_{TEM}, *bla*_{VEB} were not observed in this study. It is interesting that approximately 53% (n=19) of CRKP strains co-harboured ESBLs encoding genes *bla*_{CTX-M-variant} were found in *bla*_{SHV-variant}, with this, at least 50% of strains were carrying *bla*_{NDM-variant}.

Multi-Locus Sequences Typing and PFGE

Extensive diversity of MLST was recorded from CRKP isolates. In 25 different STs, we found that ST307 isolates (11%) were recovered from the neonatal intensive care unit (NICU). ST2407 (8%) was found to be highly prevalent and were collected from the general surgery department (GSD). In addition, we found that CRKP ST307 was dominant in NICU and in a reservoir of *bla*_{CTX-M-3} and *bla*_{SHV-11}. However, ST2407 was predominantly in GSD, which is a key transporter for *bla*_{CTX-M-15}, and *bla*_{SHV-11} gene and ST2263 a key transporter for *bla*_{NDM-5}. Nevertheless, *bla*_{KPC-2} was reported in diverse STs (Figure 2). Our particular concern is that the CRKP co-harboured ESBLs encoding genes *bla*_{CTX-M-variant} and *bla*_{SHV-variant} were reported in seventeen different STs in this hospital. This observation suggests that CRKP strains carrying ESBLs encoding genes might have spread to the Shenzhen region and may be widespread in Southern China. These 36 CRKP isolates were allocated to eight distinct PFGE clusters, sharing ≥ 80% of similar bands. The PFGE results showed that the clonal transmission was often observed within different departments of the Hospital (Figure 2).

Plasmid Profiling

In total, 18 out of 36 successful transconjugants were selected using Luria Bertani agar. PCR-based replicon typing assay results showed that Inc plasmids groups lead was IncFIA 55% (n=10), followed by IncFIS 50% (n=9), IncFIC 27% (n=5), IncP 22% (n=4), IncHI2 11% (n=2), while IncK and IncFIB showed 6% (n=1) (Figure 2).

Discussion

CRKP is a serious threat in paediatric patients, particularly newborns, due to limited therapeutic options.^{26,27} This pathogen is now considered as a reservoir for virulent and resistant genes, due to the acquisition of β-lactamase (carbapenemase and ESBLs) and the recently reported colistin resistance *mcr-1* encoding genes; which make it a serious threat to human and

animal.^{28–30} These data revealed for the first time that NDM carbapenemase is widely spread among CRKP, while CTX-M and SHV were the most prevalent β-lactamase in same isolates in Shenzhen's children Hospital, China. So far, there are no data available on molecular analysis of CRKP in paediatric clinical cases, particularly those caused by IMP or NDM co-production of CTX-M and SHV. Here, we first explored the genetic background of CRKP isolates obtained from the paediatric samples. Data showed that CRKP isolates were highly resistant to commonly used antibiotics, except for colistin. We also found that IMP-38 producing *K. pneumoniae* isolates are significantly sensitive to Imipenem, which is in agreement with studies reported in Australia, however further investigation is required.³¹ *bla*_{NDM-1} was found to be the leading genotype of carbapenemase followed by *bla*_{KPC-2}, *bla*_{VIM-2}, *bla*_{IMP-4}, *bla*_{NDM-6}, *bla*_{SME-1}, *bla*_{oxa-48}, *bla*_{IMP-2}, and *bla*_{NDM-5}. A previous study reported the presence of NDM-1 dominant carbapenemase in carbapenem-resistant *K. pneumoniae* over China, which is comparable to our results.³² There were no cases with co-harboured *bla*_{NDM-1} and *bla*_{IMP-4} strain, which is in contrast with Liu et al's finding.³³ Interestingly, nineteen CRKPs were found to contain *bla*_{CTX-M} encoding determinants. To the best of our knowledge, this is the first report on an MDR CRKP isolates co-harboured *bla*_{NDM}, *bla*_{CTX-M} and *bla*_{SHV} has flagged concerns about the spread of such superbugs in the Shenzhen region, China; but also, studies have reported similar results from other provinces of China, Chongqing and Shanghai.^{34,35} The MLST results revealed that *K. pneumoniae* ST307 and ST2407 were highly dominant in Shenzhen area, which encodes carbapenemase and ESBLs genes. Several countries such as Italy, Korea, USA, Mexico, and China have reported CRKP ST307 with ESBLs production.^{36–40} No study has yet reported that the isolate CRKP belonged to ST307 in the Southern region of China. The emergence of this isolate indicates the rapid spread of this isolate. Also, studies have reported the existence of CRKP belonged to ST2407. Data also report the presence of CRKP belonged to ST2407; to the best of our knowledge, no study has yet reported the presence of CRKP ST2407 isolates that exist with ESBLs in Southern China. Our finding of CRKP belonged to ST307 and ST2407 isolates, identify a substantial public health concern.

Clonal dissemination within the hospital corresponded with a previous study by Tian et al 2018 in Shanghai Province, China.⁴¹ We did not analyze isolates from other hospitals to observe whether there was clonal transmission between the hospitals. Plasmid replicon typing and conjugation experiment results conferred that IncFIA, IncFIS, IncFIC, IncHI2, IncFIB, IncK and IncP replicons existed in the transconjugants and carbapenemase coding genes (*bla*_{KPC-2}, and *bla*_{NDM-variants}) co-transferred with ESBLs encoding genes (*bla*_{CTX-M-variant} and *bla*_{SHV-variant}). No apparent relationship between replicon and sequence type was observed in the current isolates. Also, data of Figure 2 show the presence of some previously published isolates with co-production of carbapenemases, which requires further analysis as these isolates could be of clinical concern. Previously published study has done successful clone for KPC such as the clone group GC258,⁴² nevertheless from our 36 KPC isolates reported in our study, none of them corresponded to the clone group GC258. And we did not find the dissemination of any particular clones at the hospital in Shenzhen, China. Our finding stresses out the importance of continuous monitoring to detect multi-drug resistant isolates to promote therapeutic strategies for infections in paediatric patients. Future studies may assess the presence of other resistance-related determinants by using WGS, such as the outer-membrane permeability and to augment sample size from different areas for the molecular study. It is also worth reporting here that although the methods used in our study are adequate, one of the potential limitations of this study is the lack of use of broth micro-dilution susceptibility testing method or the Clinical and Laboratory Standards Institute recommended disk elution method for colistin.

Conclusion

This study reports, for the first time, the emergence of CPKP ST307 co-producing CTX-M with SHV and KPC in paediatric patients from the Shenzhen's Children Hospital in China. Also, our results identify the occurrence of CRKP ST2407 belongs to co-producing CTX-M with SHV and KPC. The emergence of CPKP in our study highlights a substantial global concern, both within hospitals and the wider community. Continued appraisal of clinical experience and monitoring of the spread of CPKP will provide further important information on the emergence of CPKP ST307.

Abbreviations

Res. Dis., respiratory diseases; PICU, paediatric intensive care unit; Gen. Sur., general surgery; NICU, neonatal intensive care unit; CAS, catheter associated secretion; Abd., abdominal; CSF, cerebral spinal fluid; STs, sequences type; In, Incompatibility; N/D, not detected.

Data Sharing Statement

All data files mentioned in this manuscript have been submitted and are available.

Ethics Approval and Consent to Participate

The present study was approved by the Shenzhen Children's Hospital, Research Ethical Committee, reference number: 2018 (013).

Consent for Publication

The clinical isolate samples used in this research were part of the routine Hospital Laboratory procedure. We do not use patients' name or personal information, so in the publication, no written consent was required. Verbal consent was freely taken from the patients' parents after being duly informed of its nature.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and also agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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