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Original Article

The molecular and epidemiological characteristics of carbapenemase-producing *Enterobacteriaceae* isolated from children in Shanghai, China, 2016—2021

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Abstract *Background:* We isolated the carbapenemase-producing *Enterobacteriaceae* (CPE) strains from children during 2016—2021 in Shanghai, China and investigated the antimicrobial resistance, molecular and epidemiological features of these isolates.
Methods: Antimicrobial susceptibility tests were performed to confirm the carbapenem resistance. Carbapenemase production was assessed by the rapid phenotypic identification of five major carbapenemases (KPC, NDM, VIM, IMP, and OXA-48), which were further confirmed by PCR amplification and sequencing. Multilocus sequence typing (MLST) was conducted for phylogenetic analyses.
Results: A total of 320 CPE strains were collected from 2016 to 2021, consisting of carbapenemase-producing *Klebsiella pneumoniae* (CP-*Kpn*, 55.0%), *Escherichia coli* (CP-*Eco*, 24.5%) and *Enterobacter cloacae* (CP-*Ecl*, 20.4%) and others (2, 0.1%). NDM was the primary

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carbapenemase (67.6%) in children, followed by KPC(26.4%), IMP(5.3%) and OXA-48 (0.6%). The minimum inhibitory concentration (MIC) for imipenem has been increasing from 2016 to 2021. NDM and KPC isolates are high resistant while IMP strains show the lower resistant to imipenem. Invasive infection accounted for 10.7% of CPE-related infections and was mainly caused by CP-*Kpn* (70.6%). NDM-*Kpn* was detected in 51.8% of infants (70.8% of neonates), while KPC-*Kpn* was mainly isolated from non-infants (56.3%～64.3%). ST11 was the primary clone (64.6%) of KPC-*Kpn* and presented an increasing trend from 2016 to 2021.
Conclusion: NDM is widely prevalent and transfers among CPE strains in children. NDM-*Kpn* shows the most serious threat to infants, especially to neonates. High-risk clone of ST11 KPC-*Kpn* should be paid more attention and monitored continuously in children.

Introduction

Infections related to carbapenem-resistant *Enterobacteriaceae* (CRE) are becoming the public concerns in the world.[1] One recent report from the ISPED (Infectious Disease Surveillance of Pediatrics) program has revealed that CRE has been presenting the potential threat to the health of children in China, especially to the poor immunity neonates.[2] Carbapenem resistance due to the emergence of acquired (plasmid-mediated) carbapenemases is of particular concern due to their high ability of transmission within and between bacterial species and genera.[3—5]

Generally, carbapenemases include *Klebsiella pneumoniae* carbapenemase (KPC), imipenemase (IMP), New delhi metallo-β-lactamase (NDM), Verona integron-encoded metallo-β-lactamase (VIM), and oxacillinase (OXA)-48-like enzymes et al.[6,7] Carbapenemase-encoding genes are frequently carried by many mobile genetic elements and easily transmitted among *Enterobacteriaceae*[8] In virtue of the high mortality rates and rapid spread of carbapenemase-producing *Enterobacteriaceae* (CPE), CPE infections are becoming a major threat to children.[9,10]

KPC-producing *K. pneumoniae* (KPC-*Kpn*) is reported as the most common CPE strain, which has been primarily restricted to a clone group CG 258 including ST258, ST11, ST340 and ST512, among which ST11 is the predominant type in China.[11—13] However, a series of studies have shown that KPC-*Kpn* is mainly in adults while NDM-producing *K. pneumoniae* (NDM-*Kpn*) is frequently detected from neonates.[14—16] The bacterial epidemiology and resistance profiles in children are found to be quite different from adult population.[2] Previous study presented the epidemiology features in adult population, data on the clinical and molecular characteristics of CPE strains prevalent in Chinese children are largely lacking or very limited.

In this study, we collected CPE strains including carbapenemase-producing *K. pneumoniae* (CP-*Kpn*), *E. coli* (CP-*Eco*) and *E. cloacae* (CP-*Ecl*) in children from 2016 to 2021 and investigated the antimicrobial resistance, infection and molecular characteristics of those pathogens. This is the first report about the epidemiology of pediatric CPE isolates from 2016 to 2021 in China, our study will be very essential to the control and clinical administration of pediatric CPE infections.

Methods

Bacterial isolates, identification and susceptibility testing

During January 2016 to October 2021, *Enterobacteriaceae* strains from children's hospital (Anonymous) in Shanghai were identified by the MALDI-TOF Mass Spectrometry (Bruker, France). The antimicrobial susceptibility tests were carried out on the Vitek 2 compact system, using AST-GN13 cards (bioMérieux). For the antimicrobials that weren't covered in GN13 card including cefoperazone-sulbactam, cefuroxime, meropenem and fosfomycin, we additionally performed Kirby—Bauer test (KB) to get their susceptibility. The results were interpreted according to the criteria of Clinical and Laboratory Standards Institute (CLSI) breakpoints.[17] MICs = 8 mg/L was defined as intermediate-level to imipenem and MICs≥16 mg/L was defined as high-level resistance to imipenem.

CRE were defined as the unduplicated *Enterobacteriaceae* strains which presented resistance to either of ertapenem, imipenem or meropenem. We chose CRE strains from 2016 to 2021 by random sampling method, and included the strains which caused pediatric infections. All isolates were stored at − 80 °C, sub-cultured aerobically at 35 ± 2 °C and transferred twice prior to testing. Patients were eligible for inclusion if CRE was isolated in a clinical culture from any anatomical site during hospitalization. For the duplicating strains: only the first qualifying culture episode during the first admission for each unique patient enrolled during the study period with CRE infection was included.

Immunochromatographic assay and PCR confirmation for five carbapenemases

CPE strains were confirmed by the immunochromatographic assay for the rapid phenotypic identification of five major carbapenemases (KPC, NDM, VIM, IMP and OXA-48, CARBA-5 assay, Gold Mountainriver, Beijing, China). Confirmation of the initial screening for carbapenemase identification results was performed by PCR amplification and sequencing, according to previously reported primers.[18] The PCR results

served as the gold standard to which the CARBA-5 assay was compared. The strains with Immunochromatographic assay positive but PCR negative were excluded from this study.

Data collection of CPE infections

CPE strains collected from the upper respiratory, oral, skin, or rectal site, etc were considered as colonization and excluded from this study. The diagnosis of clinical CPE infections were based on the electronic medical records, such as clinical manifestation and demographic information, the infection sites, and characteristic laboratory findings which included high level of white blood counts (WBC), C-reactive protein (CRP), procalcitonin (PCT) and interleukin 6 (IL-6), etc. Colonization is defined as CPE strains are found in or on the body but don't cause any symptoms or disease.

An invasive infection was defined as the isolation of a bacterial organism from a normally sterile body fluid, such as blood, cerebrospinal fluid, pleural fluid, pericardial fluid, joint fluid, bone aspirate, or a deep tissue abscess.[19]

Neonates were the children aged between 0 and 28 days (inclusive). Infants were defined as the children aged under 1 year old.

Multilocus sequence typing (MLST)

Total DNA of CPE isolates were prepared by QIA amp DNA mini kit (QIAGEN) according to the manufacturer's instructions. MLST was performed with housekeeping genes of *K. pneumoniae* (*gapA, infB, mdh, pgi, phoE, rpoB,* and *tonB*), *E. coli* (*dinB, icdA, pabB, polB, putB, trpA, trpB* and *uidA*) and *E. cloacae* (*dnaA, fusA, gyrB, leuA, pyrG, rplB* and *rpoB*) according to the protocol described on pasteur MLST database (https://bigsdb.pasteur.fr/index.html).

Statistical analysis

Statistics analyses were performed by using GraphPad Prism 7.0 (GraphPad software, Inc., San Diego, California, USA). Data were analyzed using the $\chi 2$ test or Fisher's exact test, as appropriate. A p-value of less than 0.05 was considered statistically significant.

Results

Epidemiology of CPE strians during 2016—2021

A total of 15,088 *Enterobacteriaceae* strains were isolated from 2016 to 2021, and 7.3% (1098) of them were carbapenem-resistant strains (CRE). The primary CRE were *K. pneumoniae* (56.6%), *E. coli* (20.0%) and *E. cloacae* (12.1%). The ratio of CR-*Kpn* was gradually decreased from 2016 (67.5%) to 2021 (41.9%), while CR-*Eco* and CR-*Ecl* showed the increasing trend in this period (Fig. 1a). A total of 350 CRE isolates responsible for infection were obtained, and 320 (91.4%) of them were CPE strains, consisting of CP-*Kpn* (175, 55.0%), CP-*Eco* (78, 24.5%), CP-*Ecl* (65, 20.4%) and others (2, 0.1%). CPE presented the increasing resistance for antimicrobials including levofloxaxin, gentamicin and trimethoprim/sulfamethoxazole from 2016 to 2021 (Fig. 1b).

CPE strains showed very high level (82.7%～99.3%) resistance to cephalosporin and their enzyme inhibitor combination, and carbapenem antibiotics, moderate resistant (32.6%～37.6%) to gentamicin, ciprofloxacin, levofloxacin and Trimethoprim/Sulfamethoxazole, mild resistant (11.4%～17.7%) to amikacin, tobramycin and fosfomycin, shown in S1.

CPE strains were collected mainly from urine (44.4%) and lower respiratory (35.6%), following by blood (7.2%). CP-*Kpn* was primarily collected from lower respiratory (46.3%), while CP-*Eco* and CP-*Ecl* were mainly isolated from urine (67.9% and 49.2%, Fig. 2a).

The invasive infections took up to 10.7% (34) of CPE-related infections, consisting of septicemia (21), intra-abdominal infections (10), and meningitis (3). Two death cases due to septicemia were caused by one KPC-*Kpn* and one NDM-*Kpn*. CP-*Kpn* was the major pathogen caused pediatric infections, accounting for 70.6% (24) of invasive infection and 52.8% (151) of noninvasive infection (Fig. 2b). The levels of CRP and IL-6 were higher in invasive infection (51.8 ± 9.2 mg/L and 147.4 ± 41.1 pg/mL) than noninvasive infections (15.0 ± 1.7 mg/L and 48.3 ± 9.1 pg/mL) ($p < 0.001$, Table 1).

Carbapenemase, MLST distributions and molecular characteristics of CPE isolates

Among 320 CPE strains, NDM was the primary carbapenemase (67.6%), KPC, IMP-4 and OXA-48 were 26.4%, 5.3% and 0.7% respectively. NDM, KPC and IMP were detected from 52.6%, 45.1% and 2.3% of CP-*Kpn* strains. NDM and KPC took up to 96.2% and 3.8% in CP-*Eco* strains. NDM, IMP and KPC were detected from 73.8%, 23.1% and 3.1% of CP-*Ecl* isolates, respectively (Fig. 3).

There were different clone types in NDM-producing strains (NDM-*Kpn*, 27 MLSTs; NDM-*Eco*, 24 MLSTs; NDM-*Ecl*, 23 MLSTs). Noticeably, ST11 was the dominant type in KPC-*Kpn* (64.6%). Among CP-*Kpn*, ST11 was increasing from 2016 (6.7%) to 2021 (48.6%). ST127 took up to 46.7% of IMP-*Ecl*, but this type was only detected from neonatal intensive care unit (NICU) and caused 41.2% of CP-*Ecl* infection in 2017 (Fig. 4).

Imipenem MICs for different carbapenemase-producing strains during 2016—2020

Fig. 5 depicted that imipenem MICs typically were high for NDM and KPC strains whereas lower for IMP isolates. In this study, 60.5% of NDM strains and 57.1% of KPC strains presented the high-level resistance (MICs$\geq$16 mg/L) for imipenem. There were 22.8% of NDM and 11.9% of KPC strains exhibiting intermediate-level resistance (MICs = 8 mg/L) for imipenem. Moreover, 11.8% and 11.8% of IMP isolates were high-level and intermedaie-level resistant to imipenem, respectively (Shown in Fig. 5a).

CPE strains which showed intermediate or high resistance to imipenem (MICs$\geq$8 mg/L) were increasing from 63.0% in 2016 to 85.5% in 2021. NDM-producing strains were decreasing from 2016 (78.0%) to 2020 (59.7%), whereas KPC-

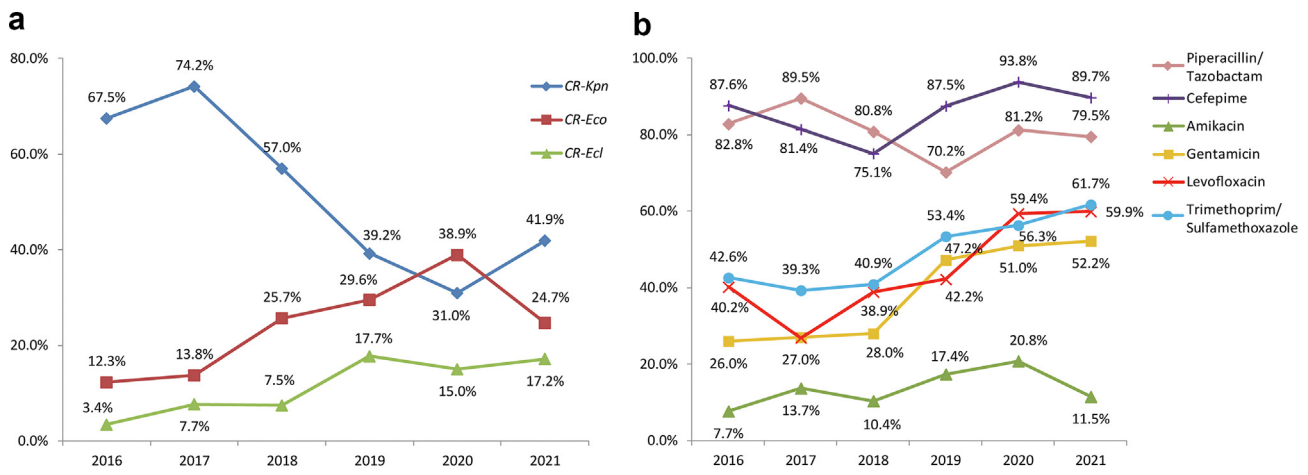

Figure 1. a. The constituent ratios of CR-*Kpn*, CR-*Eco* and CR-*Ecl* in CRE strains during 2016–2021; b. Antimicrobial resistant profiles of CPE strains isolated from 2016 to 2021 in children.

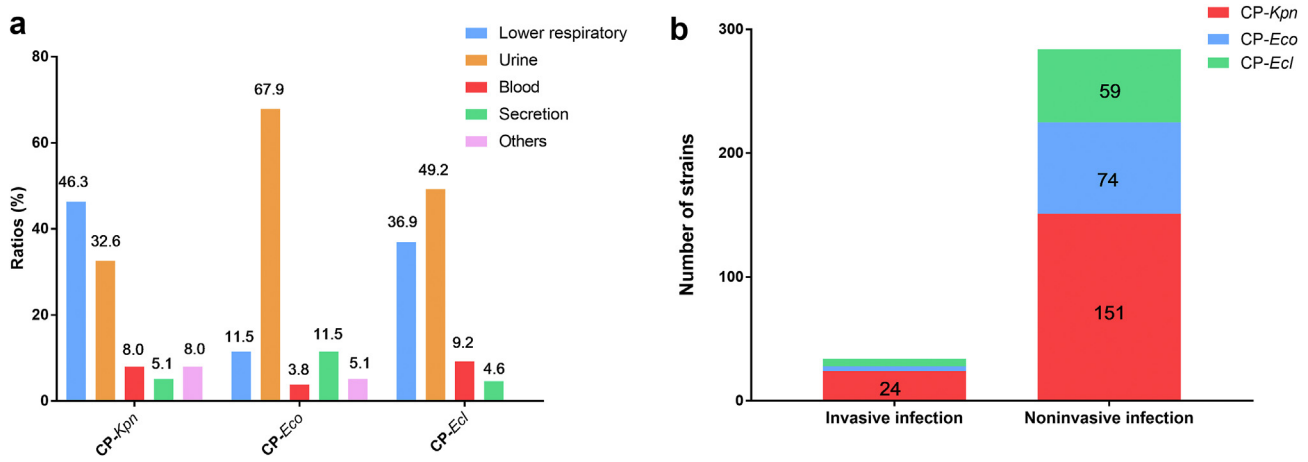

Figure 2. a. The ratios of different specimens of CPE strains; b. The number of CP-*Kpn*, CP-*Eco* and CP-*Ecl* strains between invasive infection and noninvasive infection.

Table 1 Laboratory testing results of noninvasive, invasive, CR-*Kpn*, CR-*Eco* and CR-*Ecl* infection.

Infection types	Laboratoy testing results			
	WBC($\times 10^9$/L)	CRP (mg/L)	PCT (ng/mL)	IL-6 (pg/mL)
Nonivasive infection	11.0 ± 0.9	15.0 ± 1.7	50.6 ± 14.7	48.3 ± 9.1
Invasive infection	10.0 ± 1.1	51.8 ± 9.2****	50.9 ± 23.7	147.4 ± 41.1***
CR-*Kpn* infection	11.3 ± 0.4	21.1 ± 2.8	53.3 ± 18.7	61.0 ± 12.5
CR-*Eco* infection	10.3 ± 0.6	18.8 ± 3.3	30.4 ± 18.3	74.9 ± 34.7
CR-*Ecl* infection	10.3 ± 0.5	14.8 ± 4.4	60.2 ± 22.9	58.5 ± 20.9

Note: ***$p < 0.001$,****$p < 0.001$ compared to nonivasive infection.

producing isolates were increased from 14.0% in 2016 to 33.3% in 2020 (Shown in Fig. 5b).

The clinical characteristics of different carbapenemase-producing strains

NDM-*Kpn* was mostly detected in infants, including 68.0% of neonates and 53.1% of 1 month-1 year children. However, KPC-*Kpn* was mainly isolated from non-infants, the ratios were 64.3%, 57.1% and 56.3% of 1–3 year, 3–5 year and ≥5 year groups, respectively (Fig. 6a).

The invasive infections were caused by KPC-*Kpn* (47.1%), NDM-*Kpn* (23.5%), NDM-*Eco* (11.8%), NDM-*Ecl* (11.8%) and IMP-*Ecl* (5.9%). The noninvasive infections were mainly caused by NDM bacteria (70.1%) including NDM-*Kpn* (29.6%), NDM-*Eco* (25.0%) and NDM-*Ecl* (15.5%), followed by KPC-*Kpn*

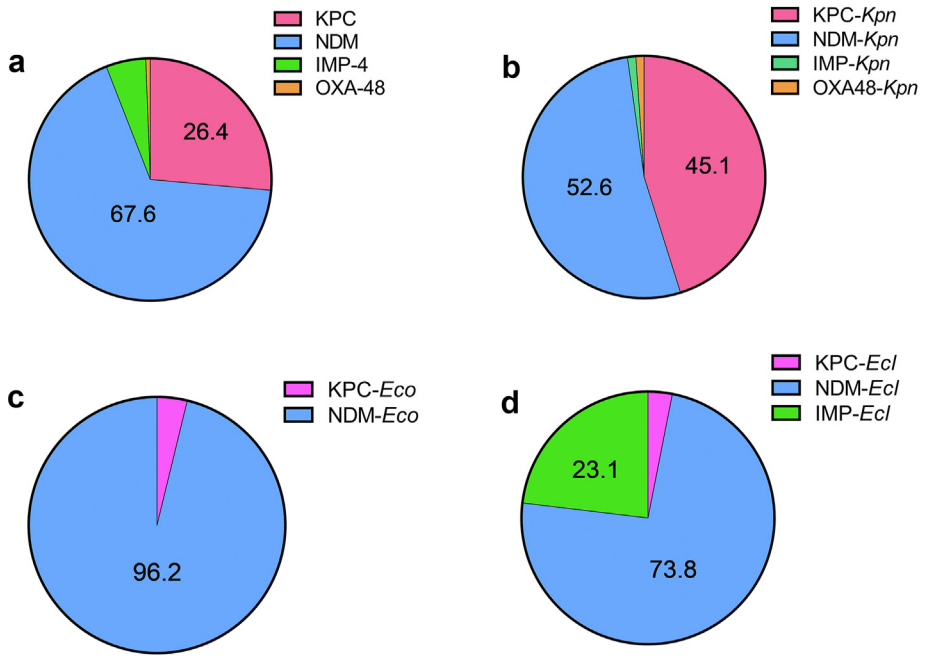

Figure 3. The ratios of different carbapenemases in *Enterobacteriaceae* (a), *K. pneumoniae* (b), *E. coli* (c) and *E. cloacae* (d) strains.

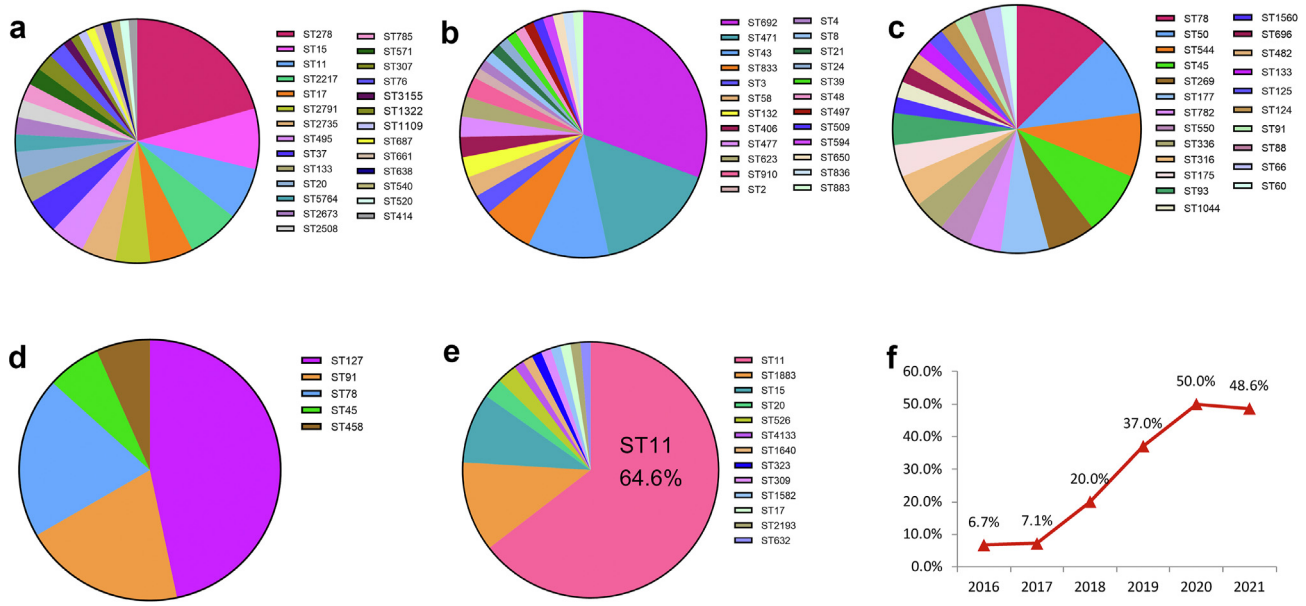

Figure 4. The MLST types of NDM-*Kpn*(a), NDM-*Eco*(b), NDM-*Ecl*(c), IMP-*Ecl*(d), KPC-*Kpn*(e) strains and the ratios of ST11 KPC-*Kpn* in CP-*Kpn* strains (f).

(22.2%), IMP-*Ecl* (4.6%), IMP-*Kpn* (1.4%), KPC-*Eco* (1.1%) and KPC-*Ecl* (0.7%). *K. pneumoniae* was the predominant pathogen which caused pediatric infections, taking up to 70.6% of the invasive infection and 53.2% of the noninvasive infection (Fig. 6b).

Among *K. pneumoniae*-related infections, most of the invasive infection was caused by KPC-*Kpn* (66.7%) while NDM-*Kpn* mainly caused the noninvasive infection (55.6%) ($p < 0.05$, Fig. 6c).

Discussion

The emergence of carbapenemase-producing *Enterobacteriaceae* (CPE) has become a major concern to pediatric infections with relatively few therapeutic options.[20] It's urgent to understand the prevalence of carbapenemase type, the infection and molecular characteristics of CPE strains in Chinese children. In this study, CP-*Kpn*, CP-*Eco* and CP-*Ecl* are the primary CPE strains in children. KPC-*Kpn*

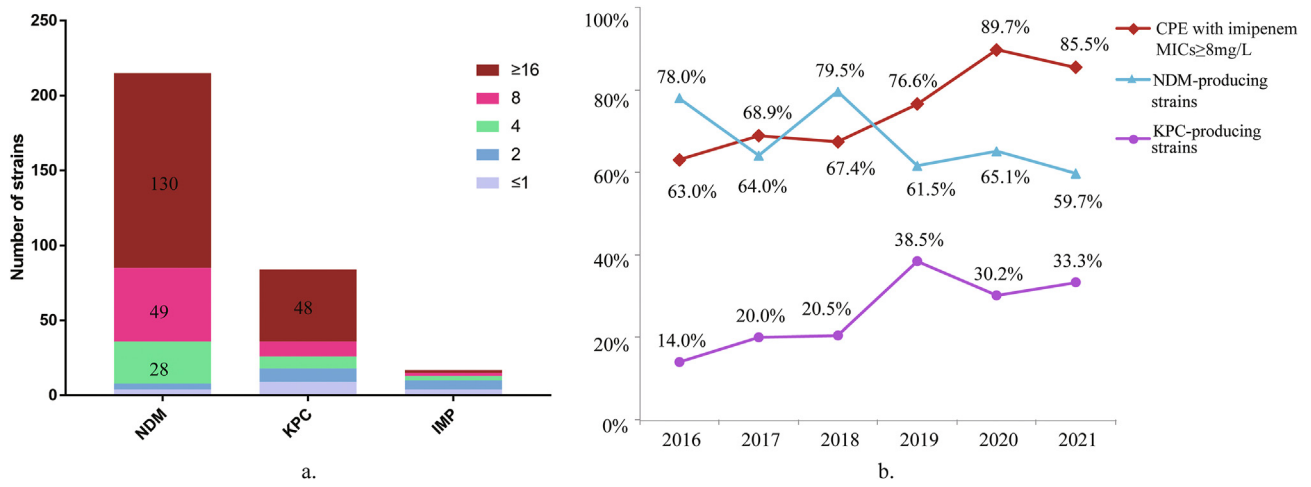

Figure 5. Imipenem MICs for CPE strains during 2016–2020. a. the number of strains with different imipenem MICs; b. The ratios of imipenem intermediate- or high-resistant strains (imipenem MICs≥8 mg/L), NDM-producing strains and KPC-producing strains from 2016 to 2021.

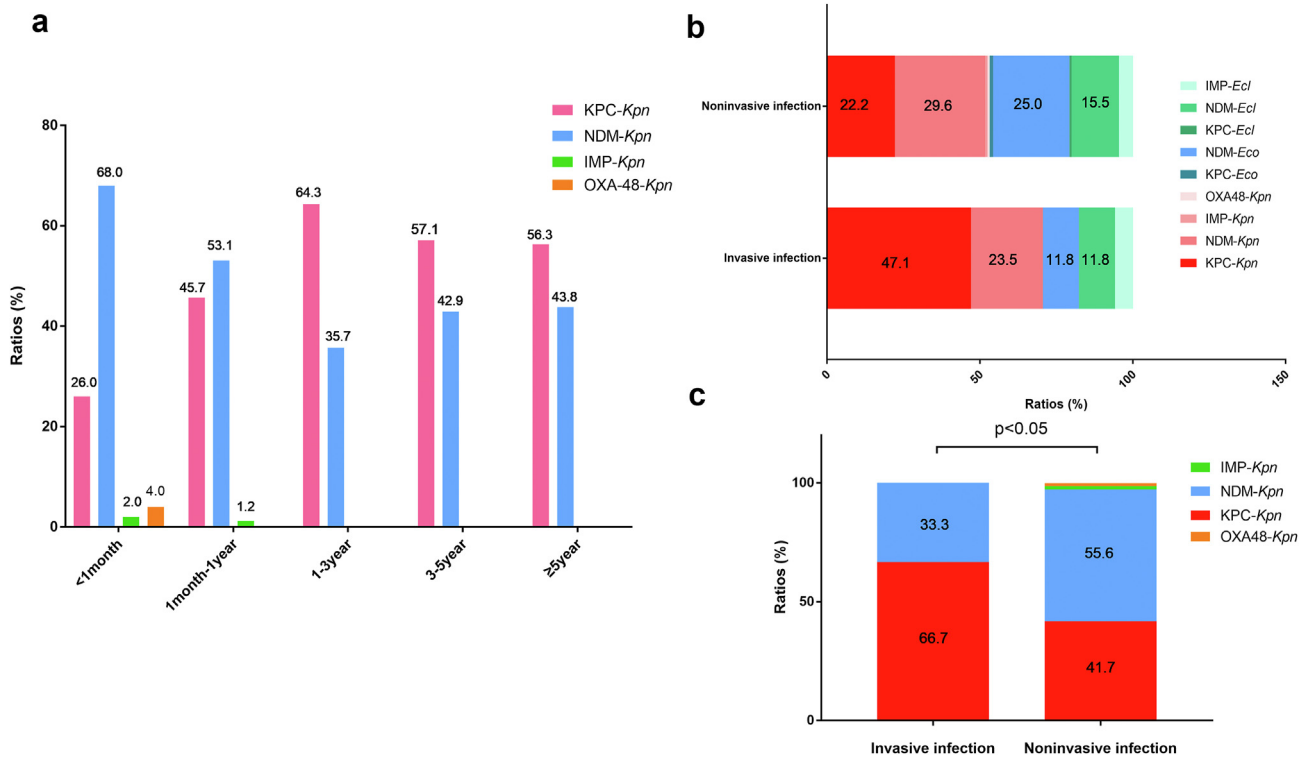

Figure 6. The clinical and molecular characteristics of CPE strains. a. The ratios of different CP-*Kpn* among ages; b. The ratios of different CPE strains between invasive and noninvasive infection; c. The ratios of CP-*Kpn* in invasive and noninvasive infection.

is the major pathogen which causes pediatric invasive infection, and more importantly the high-risk clone of ST11 KPC-*Kpn* is increasing from 2016 to 2021.

CRE isolates include both non-carbapenemase-producing CRE (non-CP-CRE) and carbapenemase-producing CRE (CPE). Actually carbapenemase production is the most prominent mechanism underlying carbapenem resistance. Other mechanisms such as the overproduction of AmpC or production of extended-spectrum β-lactamase can function together with outer membrane protein deficiency and overproduction of certain efflux pumps to confer carbapenem resistance.[21] Previous study indicated that CPE was the primary type of CRE and the ratios varied from 77.3% to 91.3%.[22–25] In this study, 91.4% (n = 320) of clinical CRE were CPE strains, indicating the carbapenemase production is the prominent mechanism of CRE in Shanghai, China. Compared to non-CP-CRE, CPE spread more quickly and became dominant CRE strains because most of the

carbapenemase-encoding genes were carried by mobile transfer elements such as plasmid or transposons, making those genes easily transmitted horizontally among Enterobacter spp.

NDM is accounted for severe infections and morbidities in the pediatric population.[26] We find NDM is the predominant carbapenemase in children and shows the highest resistance to imipenem. In this study, 52.6% of CP-*Kpn*, 96.2% of CP-*Eco* and 73.8% of CP-*Ecl* strains produce NDM, indicating NDM has been successfully transmitting among different *Enterobacteriaceae* species. Polymyxins are used as a last-line therapy against NDM, and combination therapy is recommended widely in clinic.[27–29] Other treatment such as aztreonam in combination with ceftazidime-avibacam is also justified effective to NDM-producing *Enterobacteriaceae*[30] Therefore, we use combination therapy such as polymyxin combined with rifampicin/meropenem or aztreonam combined with ceftazidime-avibactam to treat the NDM infection in clinical practice.

Invasive bacterial infections are the big concern in children because of the related high morbidity and mortality.[19] CPE can cause invasive infections containing septicemia, meningitis, severe intra-abdominal infections and noninvasive infections such as pneumonia and urinary tract infections.[31] In our study, the invasive infection accounts for 10.7% of CPE-related infection and *K. pneumoniae* is the primary pathogen (70.6%). KPC-*Kpn* causes 66.7% of invasive infection whereas NDM-*Kpn* mainly causes noninvasive infection (55.6%). KPC-*Kpn* is prone to cause invasive infections than any other CPE strains.

The international spread of KPC-*Kpn* is primarily due to the expansion of strains belonging to CG258 complex, and more specifically, to ST11 strains in China.[32] Our previous study already showed that ST11 took up to 87.1% of KPC-*Kpn* in Chinese adults group.[15] In this study, ST11 is also the high-risk clone of KPC-*Kpn* among pediatric population (64.6%), and is increasing from 2016 to 2021. The pandemic of ST11 KPC-*Kpn* is associated with the horizontal gene transfer mediated by transposons or plasmids,[33] so we must be concerned about the potential spread of the high-risk clones of ST11 KPC-*Kpn* among children in China. ST127 accounts for 46.7% of IMP-*Ecl* strains but is mainly from NICU ward in 2017, revealing a potential nosocomial infection in that period.

NDM is reported as the most common carbapenemase of CR-*Kpn* in infants and neonates (61%–87.2%).[34,35] In this study, NDM is the primary carbapenemase (54.3%) of CP-*Kpn*, followed by KPC (43.4%). Moreover, NDM-*Kpn* is more prevalent in infants (53.1%～68.0%) but KPC-*Kpn* is mainly detected from non-infants (56.3%～64.3%). One multicenter research shows that KPC is the predominant carbapenemase (81%) of *Klebsiella pneaumoniae* in Chinese adults,[36] which is much higher than our findings, indicating the different molecular and epidemiological characteristics of CP-*Kpn* between children and adults. This study reveals that infants are more sensitive to NDM, or NDM is more easily transmitted among younger children. NDM bacteria are reported mainly spread via the fecal–oral route,[37] so we think infants' poor hygiene awareness makes them prone to get NDM infection.

The first CP-*Ecl* strains in China is a KPC-producing strain isolated from Shanghai in 2010.[38] Later, other carbapenemases including IMP, VIM and NDM have been reported in clinical *E. cloacae* isolates in China.[39] In this study, the metalloenzymes NDM and IMP are the primary carbapenemase in *E. cloacae*, accounting for 73.8% and 23.1% respectively. NDM should be especially worrisome as the gene encoding this enzyme is often located on mobile genetic elements that can be easily transferred between different species.[40]

If newer agents are unavailable or inappropriate, carbapenems are often added to regimens against CPE infection, particularly if their MICs remain low. Certain study suggests that the carbapenem MICs may be as important as the type of carbapenemase.[41] In this study, NDM and KPC isolates are high resistant while IMP strains show the lower resistance for imipenem. Our findings are consistent with previous study which reported NDM and KPC producing strains usually showed high-level imipenem resistance[42,43]. We predicted that carbapenems may remain useful against bacteria with IMP carbapenemases, but not against those with NDM or KPC enzyme Therefore, carbapenem antimicrobials minght have potential therapeutic value in the treatment of IMP infection, especially when we increased the drug dose or combined carbapenem with other antimicrobials such as polymyxin. However, the imipenem MICs are increasing gradually from 2016 to 2021, making the carbapenem less effective to CPE strains. Considering KPC bacteria are increasing during 2016–2020, we think the rising of imipenem MICs for CPE strains may due to the proportion of KPC strains.

In conclusion, NDM is largely prevalent and transmits among CPE strains in children. NDM-*Kpn* showed the most serious threaten to infants, especially to the neonates. The high-risk clone of ST11 KPC-*Kpn* was increasing from 2016 to 2021, must be paid more attention and monitored continuously due to their potential transmission in children. Our study reveal that CPE infections in children are much characteristic and different from adults, effective infection control strategies targeting pediatric CPE infections are thus required in China.

Declaration of competing interest

The authors declare that they have no conflicts of interests, financial or otherwise, related to the publication of this study. Ethical Approval is not required in this study.

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Appendix A. Supplementary data

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