Molecular Epidemiologic Factors Contributing to Guinolone Resistance in Clinical Multidrug-resistant *Klebsiella Pneumoniae* Isolates from Shanghai, China

Yan Wang (✉ wangyan_hf@126.com)

Fudan University <https://orcid.org/0000-0002-8457-5287>

Guoping Cai

Fudan University Jinshan Hospital

Jinan Zhang

Shanghai Jiankang Medical University

Xiaogang Xu

Fudan University Huashan Hospital Institute of Antibiotics

Hongzhou Lu

Fudan University Affiliated Public Health Clinical Center: Shanghai Public Health Clinical Center

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**Molecular epidemiologic factors contributing to quinolone resistance in clinical
multidrug-resistant *Klebsiella pneumoniae* isolates from Shanghai, China**

Yan Wang^{1†*}, Guoping Cai^{2†}, Jinan Zhang³, Xiaogang Xu⁴, Hongzhou Lu^{5*}

[†]Yan Wang and Guoping Cai contributed equally to this work.

*Co-corresponding authors:

Research Center for Clinical Medicine, Jinshan Hospital, Fudan University, 1508 Longhang Road,
Jinshan District, Shanghai 201508, China. wangyan_hf@126.com (YW).

Department of infections and immunity, Shanghai Public Health Clinical Center, Fudan University,
2901 Caolang Road, Jinshan District, Shanghai 201508, China. luhongzhou@fudan.edu.cn (H-ZL).

Full list of author information is available at the end of the article.

Abstract

Background: The soaring quinolone-resistance rate of *Klebsiella pneumoniae*, a common pathogen in immunocompromised individuals, has seriously undermined the wide applications of antimicrobials of this class. This study aimed to investigate the emerging key contributors to quinolone resistance in multidrug resistant *K. pneumoniae* (MDR-KP) isolates from a clinical setting with continuing point-source infection outbreaks.

Methods: A total of 34 *K. pneumoniae* isolates, including 30 carbapenem-resistant *K. pneumoniae* (CRKP) isolates were randomly selected from a teaching hospital participating in an ongoing Bacterial Resistance Surveillance Project in Shanghai, China. Investigations included antimicrobial susceptibility tests, MLST and *wzi*-genotyping, PCR and DNA sequencing for identification of resistance determinants, sequence analyses using MEGA7.0 software, and clinical information retrieving through electronic medical records.

Results: Two predominant high-risk resistant clones, ST11-*wzi*64 and ST15-*wzi*19/*wzi*24, from 30 CRKP isolates caused three point-source nosocomial outbreaks in intensive care unit and neurosurgery department potentially by respiratory-route, promoting the co-selection and evolution of multidrug resistant determinants. Multiple quinolone resistance-determining region (QRDR) mutations occurred in isolates of ST15 (S83F, D87A; S80I), ST11 (S83I, D87G; S80I), and ST218 (D87A; S80I). Plasmid-mediated quinolone resistance determinants, *qnrS1*, *aac(6')-Ib-cr*, *oqxAB*, were detected in 32 (94.1%) isolates alone or in combination, accompanied with β -lactamases, 16S rRNA methylases, and putrescine ABC permeases. AcrR, AcrAB transcriptional repressor, was insertion-inactivated by IS5-transposase in isolates of ST11. Thirteen *ompK36* variants associated with specific ST (n=7) and *wzi*-allele (n=9) clustered into 10 (sub)lineages in the phylogenetic tree possibly affecting the MDR phenotype and infection outcome of isolates. Isolates of ST11, ST15, and ST218 had frameshift disruptions in *OmpK35* coupled with specific GD-insertion at position 134-135 in *OmpK36*, all showing distinct microevolution clusters of *ompK36* genotypes. Seven quinolone-susceptible isolates kept the

porin genes integral, including two each CRKPs of ST13-wzi74 (carbapenemase KPC-2 and NDM-1-coproducers) and ST65-wzi72.

Conclusions: Under multiple selective pressures, accumulation of mutations of three types (QRDR, AcrR, OmpK36/OmpK35) and acquisition of resistance-conferring genes contribute to quinolone-resistance in clinical MDR-KP isolates, reinforcing the importance of continued epidemiologic surveillance on the evolution and transmission of these isolates. Our findings provided detailed mechanistic analyses and epidemiologic implications for further infection control and antibiotic stewardship initiatives.

Introduction

Quinolone antimicrobials are a class of synthetic broad-spectrum agents applicable to a range of gram-negative bacilli (GNB) infections in humans or animals. *Klebsiella pneumoniae*, an important zoonotic pathogen, is naturally susceptible to these agents; however, resistance emerged worldwide with use, which was primarily due to the successive quinolone resistance-determining region (QRDR) mutations on chromosomes^[1] and secondly, facilitated by the wide dissemination of plasmid-mediated quinolone resistance (PMQR) determinants^[2]. Furthermore, chromosomal mutations may also cause decreased outer-membrane permeability^[3,4] and PMQR-encoding plasmids often co-transfer additional resistance determinants, both of which could confer cross-resistance phenotype for antimicrobial agents of multiple families. Thus, *K. pneumoniae* is potentially so important a reservoir for multidrug resistance (MDR) determinants that clinicians were increasingly challenged for effectively treating life-threatening infections caused by those, mainly carbapenem-resistant *K. pneumoniae* (CRKP) isolates that places a significant burden on patient health and healthcare systems^[5,6], particularly in low-middle income countries^[5], a daily issue for the management of antimicrobial therapy for critically ill patients.

Currently, the global spread of CRKP isolates constitutes an urgent threat to public health, making routine phenotypic surveillance combined with comprehensive molecular characterization imperative

for the rapid and accurate diagnosis of epidemic high-risk clones to improve clinical outcome and cut healthcare costs. Data from China Antimicrobial Surveillance Network (CHINET) revealed that *K. pneumoniae* predominated over major GNB increasingly over years, rising to the first-place pathogen in respiratory tract specimens since 2017^[7]. Fluoroquinolones, among the most extensively used antimicrobials in China, have limited application in some clinical settings as the increasing resistance over time, e.g. the ciprofloxacin-resistance rates of *K. pneumoniae* from respiratory tract (31.1%), urine (46.5%), blood (45.9%) and cerebrospinal fluid (59.4%) in 2020^[7]. Furthermore, the aggregated resistance rates to fluoroquinolone in *K. pneumoniae* invasive infections in 2017 worldwide varied geographically, ranging from low- (4%, Australia; 10%, USA) to high- (29%, France; 42%, China) or even extremely high-rate (87%, Russia)^[8]. Herein, we investigated the molecular mechanisms underlying quinolone resistance in MDR *K. pneumoniae* (MDR-KP) isolates from a 700-bed tertiary teaching hospital participating in an ongoing Bacterial Resistance Surveillance Project in Shanghai, an international metropolis in China, to shed light on the new evolution trend of association between resistance to quinolones and to other agents as well the balance between antimicrobial resistance and competitive fitness, for epidemiologic, infection control, and antibiotic stewardship initiatives.

Materials and Methods

Patients, Isolates and Antibigrams

Nosocomial infection was defined as infection occurring > 48 h after admission to the hospital. A total of 34 *K. pneumoniae* isolates from nosocomial infections between January and March 2017 were randomly selected; pertinent information regarding the infections caused by these isolates was retrieved through electronic medical records. The routine antibiograms for all isolates were determined using a disc diffusion assay following CLSI (2017) guidelines^[9], and further confirmed using a BD Phoenix NMIC/ID-4 system according to the manufacturer's instructions when necessary. The

following antimicrobials were tested, including ciprofloxacin, amikacin, gentamicin, ampicillin, ampicillin-sulbactam, piperacillin, piperacillin-tazobactam, cefazolin, cefaclor, cefuroxime, cefotaxime, ceftazidime, cefepime, trimethoprim-sulfamethoxazole, fosfomycin, imipenem, and meropenem. An isolate assigned to the intermediately susceptible, susceptible dose dependent, or resistant category was defined as nonsusceptible to the drug tested in this study; and nonsusceptible to imipenem and/or meropenem was regarded as CRKP.

Homology Analysis of *K. pneumoniae*

MLST targeting seven housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*)^[10], together with *wzi*-genotyping for inference of the capsular (K) types^[11], was performed to investigate the epidemiological associations of the selected isolates. ST and *wzi*-allele were determined by querying the database maintained by the Pasteur Institute.

PCR Detection of Resistance-Related Genes

PCR amplification was performed using the following primers (Table S1): i) QRDR (*gyrA*, *parC*)^[12] and PMQR genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')-Ib-cr*, *qepA*, *oqxAB*)^[13-19]. ii) β -lactamase genes encoding extended-spectrum β -lactamase (ESBL) (*bla*_{CTX-M}, *bla*_{SHV}, *bla*_{TEM}, *bla*_{OXA})^[20], plasmid-borne AmpC β -lactamases (*bla*_{ACC}, *bla*_{CIT}, *bla*_{DHA}, *bla*_{EBC}, *bla*_{FOX}, *bla*_{MOX})^[21], and carbapenemases (*bla*_{KPC}, *bla*_{VIM}, *bla*_{IMP}, *bla*_{NDM}, *bla*_{OXA-48})^[22-23]. iii) 16S rRNA methylase genes (*bla*_{armA}, *bla*_{rmtB})^[24]. iv) The coding sequences of outer membrane (OM) proteins, OmpK35 and OmpK36^[25]. v) The complete sequence of AcrAB efflux pump repressor (AcrR) gene^[26]. Foreign DNA segments inserted in the target genes were identified by primer walking with customized primers.

DNA Sequencing and Phylogenetic Analyses

PCR products were submitted for direct sequencing by 3730xl DNA Analyzer (ABI, USA) and compared with reference sequences available in the GenBank database using BLAST searches. Cloning and sequencing studies were given to samples with weak or bimodal amplification. DNA sequences were analyzed using the EditSeq program in the DNASTAR software package (Madison, WI). Sequence

alignments were performed using the CLUSTAL W algorithm and phylogenetic trees were constructed using the Neighbor-Joining method with 1,000 pseudoreplicate data sets in MEGA 7.0 software^[27].

Results

Specimens and Patients

The specimens of sputum (n=22, 68.8%), urine, blood, and catheter drainage fluid were collected from inpatients in ICU (n=18) and neurosurgery department (ND) (n=14) with a median age of 67 and 52 years, respectively ($P<0.01$). In ICU, the crude mortality rate was 33.3%; two of the six cases died of severe pneumonia, three of ICU-acquired sepsis, and one of intracranial infection. In ND, patients with severe brain injury were mostly subjected to ventilator-associated pneumonia (VAP) after tracheotomy, but all were eventually improved or cured, and discharged. Additionally, two isolates were recovered from infants with urinary tract infection in Pediatric Ward.

Phenotypic and Genotypic Resistance Patterns

All but one isolates were various β -lactamase producers; a total of 30 isolates (88.2%) showed CRKP phenotypes. Isolates susceptible to ciprofloxacin (n=7, 20.6%) included 4 (13.3%) of CRKPs and 3 (75%) of carbapenem-susceptible isolates ($P<0.05$). QRDR-mutations occurred in isolates of ST15 (S83F, D87A; S80I), ST11 (S83I, D87G; S80I) and ST218 (D87A; S80I). PMQR genes, *qnrS1* (n=6, 17.6%), *aac(6')-Ib-cr* (n=10, 29.4%), and *oqxAB* (n=20, 58.8%; n=31, 91.2%), were detected in 32 isolates (94.1%) alone or in combination, accompanied with β -lactamases (CTX-M-14/15, TEM-1, SHV-1; KPC-2, KPC-3, NDM-1; CMY-2), 16S rRNA methylases (ArmA, RmtB), and putrescine ABC transporter permease (PotI) variants (Table 1).

Genetic Analysis of AcrR

Compared to the Spanish isolate K23 (AJ318073), isolates of ST11 (JSK7343) and all non-ST11 in this study harbored the same 7-amino-acid (aa) substitutions (P161R, G164A, F172S, R173G, L195V, F197I, and K201M) at specific positions within the AcrR protein (MK341462), as seen in

fluoroquinolone-resistant strains from Singapore hospitals^[26]. Additionally, one silent change Q127Q (CAG→CAA) was present in isolate JSK7111 (MK341463). Unexpectedly, an abnormal PCR amplicon (1879bp; MK341464) was observed in all ST11 isolates but JSK7343. IS5-like element (981bp) belonging to the ISKpn26 family transposase caused an insertion-inactivation mutation within the AcrR structural gene (651bp) producing an incomplete acrR pseudogene (375bp) with a missing start (+276bp) and a silent change (G441A).

Porin Gene Sequence Analysis

Sequence analysis of *ompK35* genes identified 5 variants that shared 100% aa sequence identity with reference strain JX310555. Disruptions in the OmpK35 coding sequence produced two allelic pseudogenes, namely, O35K7101 (G252-A253 insertion in variant O35K7106) and O35K7118 (A86 deletion in variant O35K7105), with an early frameshift and a series of premature stop codons. Isolate JSK7111 exhibited a panel of heterogeneous *ompK35* variants. The *ompK35* gene was undetected in isolate JSK7109 (Table 1).

Sequence analysis of *ompK36* genes identified 13 variants located in 4 lineages and 6 sublineages in the phylogenetic tree. Except isolates of ST15 and ST218, all had a distinct *ompK36* coding sequence correlated with a specific ST (*wzi*) and grouped into an independent phylogenetic cluster (Figure 1; Table 1). The comparison and alignment of deduced aa sequences of representative *ompK36* genes worldwide revealed that multiple differences in OmpK36 peptides specific for lineages and sublineages mainly occurred in the extracellular Loops (L4-L8); the most common mutation located in the conserved internal loop, L3, which defines the size of the pore, were insertions encoding glycine and aspartic acid at aa positions 134 and 135 (ins aa 134-135 GD) in isolates of ST15, ST11, and ST218 (Figure 2). In all isolates, sequencing revealed no type of disruptions in the OmpK36 coding sequence.

Isolates of ST15 and ST218 each independently harbored a panel of *ompK36* variants as a consequence of microevolution from one common index case of infection. Four *ompK36* variants in isolates of ST15 formed two different evolution directions in sublineage Ia. In ICU, three variants for previously reported isolates ST15 (*wzi*24-K24)^[11] differed by one to two nt displaying an evolutionary

process from prototype, O36K7233, to pre-epidemic intermediate, O36K7340 (CTG→TTG, L9L), and then to a novel variant, O36K7106 (CTG→TTG, L9L; TCC→TTC, S7F). In contrast to ST15 (*wzi*24-K24), emerging isolates ST15 (*wzi*19-K19) mainly epidemic to ND hold different phenotypic and genotypic resistance profiles with specific ins aa134-135 GD in L3 of variant O36K7101. Isolate JSK7111 (ST218) also held a set of heterogeneous *ompK36* variants that belonged to two distinct lineages (Ia/b and IIIa). Nine isolates of ST11 sharing the same *ompK36* sequence (O36K7229) clustered into lineage IV with others in China, USA, etc., despite microevolution of nt sequence existing among which, the *OmpK36* sequences were comparatively conserved showing five sets of identical Loop (L4-L8) insertions and deletions that were predicted to lead to 9 additional aa residues and an increase in mass of approximately 1 kDa^[28], especially a replacement of 4-aa constellation and a insertion of 7-aa string occurring in L6 (Figure 1-3; Table 1).

Discussion

K. pneumoniae has recently achieved notoriety as a common pathogen in immunocompromised individuals due to the increasing number of serious infections and the growing scarcity of effective anti-infective treatments. In the present study, so large a quinolone-resistance armamentarium was accumulated in MDR-KP isolates from nosocomial infections that multiple genetic mechanisms and epidemiologic factors had continuously contributed to the daunting issue.

High-level resistance mutations in either of the two drug target enzymes, DNA gyrase- and topoisomerase IV-encoding genes (*gyrA* and *parC*, respectively) reduced drug binding to the enzyme-DNA complex directly blocking DNA synthesis^[4]; the former and the latter were the primary and the secondary target for many quinolones in clinical use for GNB, respectively. We hereby observed that resistance (n=25; ST15, ST11, ST218) or susceptibility (n=6; ST65, ST13, ST107) to ciprofloxacin in ESBL- and/or KPC-producing *K. pneumoniae* isolates were primarily associated with whether multiple QRDR-mutations occurred or not, indicating the emergence of clinically important resistance would not have been predicted on single-mutation molecular grounds and the additional epidemiologic factors may have come into play. Recently, food, especially fresh raw chicken, was

evidenced as a reservoir of MDR-KP isolates in China^[17,29]. Owing to the role of hospital environment in persistence and onward transmission of MDR-KP^[30], resistant isolates are prone to disseminate among the dense patient population causing foodborne^[31] or airborne^[32] nosocomial outbreaks. Hence, *K. pneumoniae* is increasingly recognized as a foodborne pathogen. Homology analysis revealed that small outbreaks of three *wzi*-allele-K types (*wzi*19-K19, *wzi*24-K24, and *wzi*64-K14.K64) occurred through nosocomial transmission of two epidemic resistant clones, ST15 and ST11, which had different resistance-causing aa changes, S83F-D87A and S83I-D87G, respectively, within the primary target (Figure 3; Table 1). The aqueous environment, as the biggest reservoir for MDR-KP in the hospital environment^[30], might be a source of outbreaks and persistence in the endemic setting. The high frequency (68.8%) of sputum specimens in this study suggested that spread of aerosols containing MDR-KP isolates might sustain these outbreaks as a reservoir, leading to secondary spread, hospital-acquired pneumonia (HAP), among inpatients staying in ICU and ND. Furthermore, the accumulation of QRDR-mutations in MDR-KP clinical isolates had the potential for coselection with several antimicrobial agents and produced highly resistant strains, e.g. one patient died from ICU-acquired septicopyemia caused by a ciprofloxacin-resistant *K. pneumoniae* carbapenemase (KPC)-3 variant (T23N) producing CRKP. Thus, multiresistance was potentially augmented by ongoing selection pressures of different reservoir origins resulted from broad drug use in parallel in animal and human populations coupled with spread of MDR isolates along the food chain and from human-to-human, a fact that posed the noteworthy health risks.

PMQR determinants can confer low-level resistance that facilitates the selection of mutational high-level quinolone resistance through widening the mutation selection window and elevating the mutation prevention concentration^[33], and may promote treatment failure. In this study, a high prevalence ($\geq 10\%$) of QnrS1 occurred in clinical MDR-KP isolates to greatly protect the target enzymes from quinolone action^[2]. AAC(6')-Ib-cr, a cr-variant (W102R, D179Y) of aminoglycoside-modifying enzyme (AME), that acetylates both aminoglycosides and certain quinolones^[16], was more commonly encountered than QnrS1 and particularly associated with ESBL CTX-M-15. No mobile quinolone-pump QepA but the other two multidrug efflux transporters was detected. OqxAB, belonging to the

resistance-nodulation-division (RND) family, firstly identified on olaquinox-resistance plasmids in *E. coli* of veterinary origin^[34], was now widely distributed in diverse clinical MDR-KP genomes from human^[35]. Here, OqxAB existed in almost all isolates and was linked with ESBL CTX-M-14/15 regardless of ciprofloxacin-susceptibility. The grouping of both *oqxA* and *oqxB* sequences was related to specific ST, as a result of the transmission and evolution of progenitor genes from isolates of ST11 (*oqxA1B1*). ATP-binding cassette (ABC) transporters, a ubiquitous superfamily of integral membrane proteins, are responsible for the ATP powered translocation of many substrates across membranes, also involving in a range of other processes including DNA repair and chromosome maintenance^[36]. Putrescine, a product of ornithine decarboxylation, plays multifunctional roles in regulating important cellular processes by polyamine metabolic network^[37]. The putrescine ABC transporter permease variant (R248L) was primarily observed in isolates of ST11 (*potI-v1*) and ST15 (*potI-v2*), suggesting their critical physiologic functions involved in the MDR phenotype. PMQR has been found on heterogeneous plasmids in clinical and environmental isolates worldwide and appears to be spreading independently of QRDR-mutations. High-level quinolone resistance could be seen, in case two or more quinolone resistance determinants were harbored by a plasmid or isolate, e.g. isolate JSK7109 (ST290; *qnrS1*; *oqxAB*) as well as a fluoroquinolone-resistant *E. coli* (overexpressing the AcrAB and other pumps, acquiring both QnrS1 and OqxAB^[38]).

Besides the above-mentioned factors, altered OM permeability reducing cytoplasmic drug concentrations has also been shown to generate a quinolone resistance phenotype in *K. pneumoniae*. AcrAB and OqxAB, two RND efflux pumps, had differential contribution to MDR and virulence^[39]; in the absence of AcrAB, gyrase mutations fail to produce clinically relevant levels of resistance^[40]. The expression of AcrAB operon was down-regulated by a transcriptional repressor, AcrR, whose mutation was related to AcrAB overexpression and fluoroquinolone resistance in *K. pneumoniae*^[26]. Despite no evidence for correlation of low *acrB*-expression with decreased susceptibility to carbapenems^[41] and ceftazidime/avibactam^[42] previously, two types of variations in *acrR*-gene was observed in the present study: one was the genotypic variation in non-ST11 isolates representing geographical difference and the other was *acrR*-mutation, IS5-insertion-inactivation, shown by almost all isolates of ST11, which

was different from frameshift-deletion in genetically unrelated *K. pneumoniae* isolates from Singapore hospitals^[26]. Our finding gave an exciting evidence to decipher the molecular basis of AcrAB overexpression as a resistance mechanism, supporting the ST11 as the predominant clone of MDR-KP infections in China^[43]. Furthermore, OmpK35 and OmpK36, two major co-regulated OM proteins, provided a channel that allows the selective uptake of essential nutrients and multiple antibiotics. Porin deficiency is a widespread phenomenon in *K. pneumoniae*. OmpK36 absence has been related to antimicrobial resistance but also decreased fitness and diminished virulence^[44,45]; OmpK35, unnecessary for pathogenesis and resistance^[44], is commonly absent from ESBL-^[46] or KPC-^[42,47] producers. All seven ciprofloxacin-susceptible isolates in this study kept the coding sequences of both porins integral. However, isolates of ST11 (*wzi*64, *wzi*209) and ST15 (*wzi*19) had frameshift disruptions in OmpK35, accompanied with a specific L3-modification (ins aa134-135 GD) in OmpK36, which has been reported to contribute to the emergence of carbapenem resistance^[28,46,47,48], suggesting as a solution to the fitness cost imposed by OmpK36 loss. The specific OmpK35/OmpK36 profiles formed a trade-off between antimicrobials permeability and nutritional competence, which led high-risk MDR clones to epidemic success. Altogether, reduced influx through porin channels often acted in concert with increased efflux by pumps to confer an antibiotic cross-resistance phenotype in the majority of MDR-KP isolates.

In China, the resistance rates of *K. pneumoniae* to four commonly used antimicrobials, ciprofloxacin (34.0%), ceftazidime (34.7%), piperacillin-tazobactam (26.3%), and amikacin (15.7%), had markedly increased since 2015 to 2020 following a 2005-2014 decline trend, which was consistent with the marked increase in CRKPs from 2005 (3.0%) to 2020 (23.2%), while ESBL production (cefotaxime-resistance) fluctuating at about 30-40% meanwhile^[8]. PMQR (*n*=32, 94.1%) are often associated with co-resistance to other antimicrobials owing to the co-localization of resistance-conferring genes on the same plasmid or isolate^[49], a critical feature that fosters the horizontal dissemination of MDR partly explaining the less optimistic resistance trend since 2015. Thirty CRKP isolates herein carried at least one or more commonly both of the two predominant β -lactamase genotypes, KPC-2-type carbapenemase and CTX-M-type ESBL^[50,51]. Acquisition of class A

(KPC-2, KPC-3) and B (New Delhi metallo- β -lactamase (NDM)-1) carbapenemases^[51] predominantly contribute to carbapenem-resistance mechanisms; production of ESBL (A) and/or AmpC (C) β -lactamases in combination with acquired OM impermeability owing to mutation-derived modifications in major porins^[52] and/or AcrR were also contributors. In view of the extremely high resistance rate of CRKP to ciprofloxacin (93.2%, CHINET, 2020)^[8], co-resistance to quinolones and carbapenems was observed in 26 out of 30 CRKPs, leaving few alternatives to first-line agents for the treatment of serious nosocomial infections. Usually, combination therapy based on tigecycline or polymyxin coupled with carbapenems, aminoglycosides, or fosfomycin was proposed^[53]. The enhanced efficacy of a carbapenem-containing regimen against CPKPs (MICs $\leq$ 4 mg/L) when administered at high-dose/prolonged infusion^[54] kept carbapenems a place in the treatment of CPKP infections. Notably, the occurrence of ArmA and RmtB in isolates of ST15 (*wzi24*) and ST11 (*wzi64/wzi209*), respectively, conferred high level resistance to almost all clinically important aminoglycosides by methylation of ribosomal target^[24] further complicating therapy. Unlike 16S rRNA methylases, AMEs vary in their substrate ranges, e.g. AAC (6')-Ib-cr was active on gentamicin but not amikacin in isolates ST15 (*wzi19*). Fortunately, in the absence of QRDR-mutations, CRKPs might remain susceptible to ciprofloxacin, e.g. isolates (ST13, ST65) from sputum in ND, a phenomenon once underestimated that rendered quinolones a potential complementation to the limited antibiotic options against CRKP infections. Hence, combination or monotherapy based on quinolones was commonly used to manage adult patients with MDR-KP induced HAP or VAP^[55]. Recently, the compound ceftazidime/avibactam was introduced in China to effectively fight against CRKPs carrying all combinations of β -lactamases (class A, C and D)^[42,51,56], with little effect on the emerging KPC-2 and NDM-1-coproducers characterized by high stability, non-inferior fitness and transferability among patients^[57]. Here, such NDM co-producing CRKP of ST13 concomitantly acquired additional β -lactamases of other types, evolving into a super-producer of quadruple enzymes with potentially higher β -lactam hydrolysis activity. Isolates of ST65 (*wzi72-K2*) having unique efflux pumps (*oqxA10B6*, *potI-v3*) shared the same *ompK36* sequence^[28,52] with hypervirulent CRKPs from invasive infections in Southeast Asia that have

become a common pathogen of VAP in mechanically ventilated patients in China^[32,58]. These findings highlighted an emerging and escalating threat of CRKPs to healthcare in the clinical settings.

Nucleotide sequence-based typing methods, MLST^[10] and *wzi*-genotyping^[11], can provide unambiguous and portable data useful for epidemiological studies. We hereby observed a strong correlation of *ompK36* variants with specific lineages of *K. pneumoniae* isolates, as shown by the ST^[59] but also *wzi*-allele, suggesting that *OmpK36* has a higher evolutionary rate. Mutational changes in adaptation to host colonization/infection and antimicrobial pressure are frequently selected, so virulence (e.g. capsule, efflux pump, and porin) and resistance is polymorphic. High-risk epidemic clones employed these mechanisms to change their ability to persist better in the environment and human populations. The *wzi*-encoding surface protein involved in capsule attachment to the OM^[60] was strongly associated with specific K antigens (K1-K78)^[11]. Age- and site-specific distribution of *wzi*-alleles were observed in isolates of ST11 (infant-*wzi*209, adult-*wzi*64) and ST15 (ICU-*wzi*24, ND-*wzi*19), respectively. Besides allelic mutations in genes of *wzi*, *oqxAB*, and *acrR*, multidirectional evolutionary trend of major porin genotypes was presented in isolate JSK7111 (ST218), another trade-off between fitness cost and heterogeneity of antibiotic exclusion by allowing coexistence of mutated and non-mutated versions of porins before reaching a stable state for a new variant. In a fatal nosocomial outbreak, the transmission of isolates ST15 (*wzi*24-K24) among aged inpatients in ICU induced new *ompK36* variants co-circulating, despite all holding single integral *ompK35*. Compared to the epidemic *ompK36*-genotype (O36K7233) in China in the last decade, the sudden predominance of emerging variant O36K7106 was possibly associated with serious infection and poor outcome, accompanied with the emergence of KPC-3 and CTX-M-15 co-producing CRKPs, evidence that the S7F-substitution increased the infectivity and virulence of isolates ST15. Ineffectiveness of QRDR-mutation in a CTX-M-15 producer susceptible to both ciprofloxacin and carbapenem was possibly attributable to a pre-epidemic *ompK36* intermediate (O36K7340) identical to only 3 Lithuania isolates from a global survey^[52]. Furthermore, *OmpK36* phenotype could influence carbapenem susceptibility in KPC-producers, either making them go unrecognized in routine susceptibility testing, e.g. isolate JSK7341 (*potI*-v3), or correlating enhanced resistance to β -lactams with a special *OmpK36*

configuration in ST11 clone^[28,41,42]. Combined, a shift in *ompK36* genotypes seemed to be also involved in the reduction of OM permeability, resulting to changed antibiogram or attenuated virulence. An *ompK36*-targeted PCR method can cost-effectively distinguish major high-risk resistance and virulence clones of *K. pneumoniae*, but lacking the specificity for screening of epidemic ST^[61]. Here, we preliminarily analyzed the exploitability of a genotyping framework based on *ompK36*-alleles of the representative *K. pneumoniae* worldwide. Future work remains to be done to establish a multiple-tiered classification system for the better understanding of how porin heterogeneity and trade-offs might affect the MDR phenotype and infection outcome of isolates.

Conclusions

In conclusion, accumulation of mutations of three categories and acquisition of resistance-conferring genes contribute to quinolone resistance in clinical MDR-KP isolates. Under multiple selective pressures, QRDR-mutations are sufficient but not absolutely necessary for acquiring quinolone resistance, further promoted by spread of PMQR determinants, and frequently co-regulated by mutations in AcrR and/or OmpK36; all closely correlated with combined-resistance to multiple antimicrobials, thereby necessitating and strengthening continuing epidemiologic surveillance, strict infection control measures and restrictive antibiotic stewardship.

Supplementary Information

The online version contains supplementary material

Additional file 1: Table S1. Primer sequences used for PCR amplification of resistant-associated genes for *K. pneumoniae*.

Abbreviations

aa: amino acid; ABC: ATP-binding cassette; AME: aminoglycoside-modifying enzyme; bp: base pair(s); CRKP: carbapenem-resistant *Klebsiella pneumoniae*; Da: dalton(s); ESBL: extended-spectrum β -lactamase; GNB: gram-negative bacilli; HAP: hospital-acquired pneumonia; ICU: intensive care unit;

ins aa 134-135 GD: insertions encoding glycine and aspartic acid at aa positions 134 and 135; IS: insertion sequence; K: capsular; KPC: *Klebsiella pneumoniae* carbapenemase; MDR: multidrug resistant or multidrug resistance; MIC: minimum inhibitory concentration; MLST: multilocus sequence typing; ND: neurosurgery department; NDM, New Delhi metallo- β -lactamase; nt: nucleotide(s); OM: outer membrane; ORF: open reading frame; P: probability; PCR: polymerase chain reaction; PMQR: plasmid-mediated quinolone resistance; QRDR: quinolone resistance-determining region; RND: resistance-nodulation-division; ST: sequence type; VAP: ventilator-associated pneumonia.

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Authors' contributions

YW and H-ZL conceived and designed the study. YW collected the isolates and performed the experiments. G-PC and J-AZ participated in the data collection. YW and G-PC analyzed the data and drafted the manuscript. H-ZL, J-AZ, and X-GX revised the manuscript critically for intellectual content. All authors read and gave final approval of the manuscript.

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Availability of data and materials

All materials and data analyzed during this study are contained within the manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Medical Ethics Committee of Jinshan Hospital, Fudan University. Informed consent was not obtained due to the retrospective and anonymous nature of the study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹Research Center for Clinical Medicine, Jinshan Hospital, Fudan University, Shanghai, China; ²Department of Surgery, Jinshan Hospital, Fudan University, Shanghai, China; ³Department of Internal Medicine, Zhoupu Hospital, Shanghai Jiaotong Medical University, Shanghai, China; ⁴Institute of Antibiotics, Huashan Hospital, Fudan University, Shanghai, China; ⁵Department of infections and immunity, Shanghai Public Health Clinical Center, Fudan University, Shanghai, China

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Figure 1. Phylogenetic analysis based on the coding sequences of *ompK36* genes for *K. pneumoniae* isolates. The dendrogram was constructed using neighbor-joining method by MEGA program version 7.0. Bootstrap resampling (1000 replications) was used, and bootstrap values $\geq 70\%$ were shown. Scale bar represents nt substitutions per site. Reference strains were named in reference to the accession-number, strain name, country of origin, isolation-year, and MLST. ★ denotes *K. pneumoniae* isolates in the present study (GenBank accession number MK341466-MK341478) with its common name and ST shown in bold. The *ompK36* for a *Raoultella planticola* isolate (CP026047) was taken as an out-group. The red box indicates the phylogenetic cluster position of isolates ST11 (Lineage IV).

Figure 1.

Quinolone resistance in MDR *Klebsiella pneumoniae*

Table 1. Characterization of phenotypic and genotypic resistance of 34 *K. pneumoniae* isolates from nosocomial infections

Isolate	Specimen	Susceptibility profiles ^a	PMQR ^b	QRDR mutations		Other resistance genes	ST	<i>wzi</i> -K type ^c	<i>ompK36</i> ^d	<i>ompK35</i> ^d
				GyrA	ParC					
JSK7101	Sputum	AMK, SXT, FOF	<i>aac(6')-Ib-cr, oqxA8B20</i>	S83F; D87A	S80I	CTX-M-15,TEM-1,SHV-1,KPC-2	15	<i>wzi</i> 19-K19	Ia,O36K7101 ^{iGD}	O35K7101 ^{FSI}
JSK7102	Sputum	AMK, SXT, FOF	<i>aac(6')-Ib-cr, oqxA8B20</i>	S83F; D87A	S80I	CTX-M-15,TEM-1,SHV-1	15	<i>wzi</i> 19-K19	O36K7101 ^{iGD}	O35K7101 ^{FSI}
JSK7108	blood	AMK, SXT, FOF	<i>aac(6')-Ib-cr, oqxA8B20</i>	S83F; D87A	S80I	CTX-M-15,TEM-1	15	<i>wzi</i> 19-K19	O36K7101 ^{iGD}	O35K7101 ^{FSI}
JSK7112	Sputum	AMK, SXT, FOF	<i>aac(6')-Ib-cr, oqxA8B20</i>	S83F; D87A	S80I	CTX-M-15,TEM-1,SHV-1	15	<i>wzi</i> 19-K19	O36K7101 ^{iGD}	O35K7101 ^{FSI}
JSK7120	Sputum	AMK, SXT, FOF	<i>aac(6')-Ib-cr, oqxB20</i>	S83F; D87A	S80I	CTX-M-15	15	<i>wzi</i> 19-K19	O36K7101 ^{iGD}	O35K7106
JSK7223	Sputum	AMK, SXT, FOF	<i>aac(6')-Ib-cr, oqxB20, potI-v2</i>	S83F; D87A	S80I	CTX-M-15,KPC-2	15	<i>wzi</i> 19-K19	O36K7101 ^{iGD}	O35K7101 ^{FSI}
JSK7224	Sputum	AMK, SXT	<i>aac(6')-Ib-cr, oqxB20, potI-v2</i>	S83F; D87A	S80I	CTX-M-15,KPC-2	15	<i>wzi</i> 19-K19	O36K7101 ^{iGD}	O35K7101 ^{FSI}
JSK7233 [×]	Urine	NTA	<i>oqxA8B20, potI-v2</i>	S83F; D87A	S80I	CTX-M-15,TEM-1,SHV-1,KPC-2,armA	15	<i>wzi</i> 24-K24	Ia, O36K7233	O35K7106
JSK7338	Sputum	FOF	<i>oqxA8B20</i>	S83F; D87A	S80I	CTX-M-15,TEM-1,SHV-1,KPC-2,armA	15	<i>wzi</i> 24-K24	O36K7233	O35K7106
JSK7340	Sputum	CIP, TZP, IPM, MEM, FOF	<i>oqxA8B20, potI-v2</i>	S83F; D87A	S80I	CTX-M-15,TEM-1,SHV-1,armA	15	<i>wzi</i> 24-K24	Ia, O36K7340	O35K7106
JSK7106*	Sputum	FOF	<i>oqxB20</i>	S83F; D87A	S80I	CTX-M-15,armA	15	<i>wzi</i> 24-K24	Ia, O36K7106	O35K7106
JSK7107*	Sputum	FOF	NPA	S83F; D87A	S80I	CTX-M-15,TEM-1,armA	15	<i>wzi</i> 24-K24	O36K7106	O35K7106
JSK7110 [×]	Sputum	FOF	<i>oqxA8B20</i>	S83F; D87A	S80I	CTX-M-15,TEM-1,SHV-1,armA	15	<i>wzi</i> 24-K24	O36K7106	O35K7106
JSK7119* [×]	Sputum	FOF	<i>oqxB20</i>	S83F; D87A	S80I	CTX-M-15	15	<i>wzi</i> 24-K24	O36K7106	O35K7106
JSK7225	Sputum	FOF	<i>oqxB20, potI-v2</i>	S83F; D87A	S80I	CTX-M-15,KPC-3,armA	15	<i>wzi</i> 24-K24	O36K7106	O35K7106
JSK7345 ^{^×}	Urine	FOF	<i>oqxA8B20</i>	S83F; D87A	S80I	CTX-M-15, KPC-3 variant (C68A→T23N)	15	<i>wzi</i> 24-K24	O36K7106	O35K7106

(Continued)

Quinolone resistance in MDR *Klebsiella pneumonia*

Table 1. (Continued)

Isolate	Specimen	Susceptibility profiles ^a	PMQR genes ^b	QRDR mutations		Other resistance genes	ST	<i>wzi</i> -K type ^c	<i>ompK36</i> ^d	<i>ompK35</i> ^d
				GyrA	ParC					
JSK7111	Sputum	AMK, SXT, FOF	<i>aac(6')-Ib-cr</i> , <i>oqx</i> A8B20(G138A)	S83; D87A	S80I	CTX-M-15,TEM-1,SHV-1	218	<i>wzi</i> 77-K57 (T56C)	Ia, O36K7101 ^{iGD} ; Ib, O36K7111-3 ^{iGD} ; IIIa,O36K7342, O36K7111-1, O36K7111-2, O36K7111-A1	O35K7101 ^{FSI} ; O35K7111-S1 ^{FSI} ; O35K7111-2 ^{FSI} ; O35K7111-S3
JSK7109* ^x	Sputum	AMK, GEN, SXT, FOF	<i>qnrS1</i> , <i>oqx</i> A1(C369G)B5	S83; D87	S80	CTX-M-14	290	<i>wzi</i> 21-K21	II, O36K7109	NPA
JSK7105	Sputum	NTA	NPA	S83; D87	S80	NPA	65	<i>wzi</i> 72-K2	IIIb, O36K7105	O35K7105
JSK7114	Sputum	CIP,AMK,GEN,SXT	<i>oqx</i> A10B6	S83; D87	S80	SHV-1	65	<i>wzi</i> 72-K2	O36K7105	O35K7105
JSK7341	Sputum	CIP,AMK,GEN,SXT,FOF, IPM,MEM	<i>oqx</i> A10B6, <i>potI</i> -v3	S83; D87	S80	KPC-2	65	<i>wzi</i> 72-K2	O36K7105	O35K7105
JSK7347	Sputum	CIP,AMK,GEN,SXT,FOF	<i>oqx</i> B6	S83; D87	S80	KPC-2	65	<i>wzi</i> 72-K2	O36K7105	O35K7105
JSK7116	Sputum	CIP, FOF	<i>aac(6')-Ib</i> , <i>qnrS1</i> , <i>oqx</i> A8B20	S83; D87	S80	CTX-M-14, SHV-1, KPC-2, NDM-1	13	<i>wzi</i> 74-K47	O36K7231	O35K7118 ^{FSD}
JSK7231	Sputum	CIP, FOF	<i>aac(6')-Ib</i> , <i>qnrS1</i> , <i>oqx</i> B20	S83; D87	S80	CTX-M-15, CMY-2, KPC-2, NDM-1	13	<i>wzi</i> 74-K47	IIIa, O36K7231	O35K7231
JSK7342	Urine	CIP,AMK,GEN,TZP,IPM ,MEM,FOF	<i>aac(6')-Ib-cr</i> , <i>qnrS1</i> , <i>oqx</i> B19(T447G)	S83; D87	S80	CTX-M-15, TEM-1	107	<i>wzi</i> 173	IIIa, O36K7342	O35K7342
JSK7113	Urine Catheter	SXT	<i>oqx</i> A1B1, <i>potI</i> -v1	S83I; D87G	S80I	TEM-1, SHV-1, KPC-2, rmtB	11	<i>wzi</i> 209	O36K7229 ^{iGD}	O35K7118 ^{FSD}
JSK7115*	drainage fluid	NTA	<i>oqx</i> A1B1, <i>potI</i> -v1	S83I; D87G	S80I	CTX-M-14,TEM-1, SHV-1, rmtB	11	<i>wzi</i> 64-K14.K64	O36K7229 ^{iGD}	O35K7118 ^{FSD}

(Continued)

Quinolone resistance in MDR *Klebsiella pneumoniae*

Table 1. (Continued)

Isolate	Specimen	Susceptibility profiles ^a	PMQR genes ^b	QRDR mutations		Other resistance genes	ST	<i>wzi</i> -K type ^c	<i>ompK36</i> ^d	<i>ompK35</i> ^d
				GyrA	ParC					
JSK7117*	blood	SXT	<i>oqxA1B1</i>	S83I; D87G	S80I	CTX-M-14,TEM-1, KPC-2, rmtB	11	<i>wzi64</i> -K14.K64	O36K7229 ^{iGD}	O35K7118 ^{FSD}
JSK7118	Urine	SXT	<i>oqxA1B1, potI-v1</i>	S83I; D87G	S80I	CTX-M-14,TEM-1, SHV-1, KPC-2, rmtB	11	<i>wzi64</i> -K14.K64	O36K7229 ^{iGD}	O35K7118 ^{FSD}
JSK7221	Urine	AMK, GEN, FOF	<i>oqxB1, potI-v1</i>	S83I; D87G	S80I	CTX-M-15, SHV-1, KPC-2	11	<i>wzi64</i> -K14.K64	O36K7229 ^{iGD}	O35K7118 ^{FSD}
JSK7227 [^] [×]	Urine	SXT	<i>oqxB1, potI-v1</i>	S83I; D87G	S80I	CTX-M-14, KPC-2, rmtB	11	<i>wzi64</i> -K14.K64	O36K7229 ^{iGD}	O35K7118 ^{FSD}
JSK7229	Sputum	NTA	<i>qnrS1, potI-v1</i>	S83I; D87G	S80I	CTX-M-14, KPC-2, rmtB	11	<i>wzi64</i> -K14.K64	IV,O36K7229 ^{iGD}	O35K7118 ^{FSD}
JSK7343	Urine	FOF	<i>aac(6')-Ib-cr,qnrS1, oqxA8B20</i>	S83I; D87G	S80I	CTX-M-14, KPC-2, rmtB	11	<i>wzi64</i> -K14.K64	O36K7229 ^{iGD}	O35K7118 ^{FSD}
JSK7339	Urine	AMK,GEN,PIP,CEC,CTX,CAZ,TZP,FEP,IPM,MEM,FOF	<i>oqxA1B1, potI-v1</i>	S83I; D87G	S80I	SHV-1	11	<i>wzi64</i> -K14.K64	O36K7229 ^{iGD}	O35K7118 ^{FSD}

*, severe pneumonia; ^, septicopyemia; [×], death; iGD, ins aa134-135 GD; FSD, frameshift deletion; FSI, frameshift insertion; I-IV, a-c, lineage and sublineage of *ompK36* ORFs; NPA, no PCR amplification; NTA, nonsusceptible to all agents tested in this study.

a. all antimicrobial agents tested, including CIP, ciprofloxacin; AMK, amikacin; GEN, gentamicin; AMP, ampicillin; SAM, ampicillin-sulbactam; PIP, piperacillin; TZP, piperacillin-tazobactam; CFZ, cefazolin; CEC, cefaclor; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; SXT, trimethoprim-sulfamethoxazole; FOF, fosfomycin; IMP, imipenem; MEM, meropenem.

b. *potI*-v1, v2, v3 denoted three types of Putrescine ABC transporter permease (*PotI*) variant (T743G→R248L). Isolates of ST11 except JSK7343 hold 100% similarity to the original *oqxAB* sequence of plasmid pOLA52 (EU370913), with a G148N-substitution identified in *oqxB* of all the other isolates.

c. Isolates of JSK7113 and JSK7342 had no associated K types.

d. Sequences of *ompK36* and *ompK35* have been submitted to GenBank under accession numbers MK341466–MK341478 and MK341479– MK341487, respectively.

Figure 3. MLST and *wzi*-genotyping of 34 *K. pneumonia* clinical isolates. The dendrogram of *wzi*-alleles (447bp) was built using neighbor-joining method by MEGA program version 7.0. Bootstrap resampling (1000 replications) was used, and bootstrap values $\geq 70\%$ were shown. Scale bar represents nt substitutions per site. Each known *wzi*-allele number is followed by the corresponding capsular (K) type, with dots separating several K types indicating cross-reactions. The three main branches (A, B, and C) are labeled. The representative *wzi* sequences (GenBank accession number MK341453-MK341461) of sample isolates from the present study and their identical *wzi*-alleles in the same cluster were shown in bold. ○ denotes the new *wzi* variants without known allele-number. The common names of isolates were followed by ward of origin, isolation-date, and MLST. ND, Neurosurgery Department; PW, Pediatric Ward.

Figure 3.

Figures

Figure 1.

Figure 1

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region	L1	B2	L3	L4-H	B8			T5	B9	H-L5			B11 L6			B12 B13 L7			B14			H-L8																																
position	7	49	59	136/7	180/184-188			194-196	203	207	212	222	223	227-229 232-236			260	271-274	276-283			293	309	315-318 325 330 336			359	361	364	365																								
consensus	S	S	V	-	V	G	A	L	S	P	G	A	L	F	L	Y	S	N	L	G	D	S	K	L	A	L	T	A	G	S	L	-	-	-	-	-	F	V	L	D	L	E	-	Y	I	V	D	S	R	H	N			
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Figure 2

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Figure 3.

Figure 3

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