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Distribution and spread of the mobilised RND efflux pump gene cluster *tmexCD-toprJ* in clinical Gram-negative bacteria: a molecular epidemiological study

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Summary

Background TMexCD1-TOprJ1, which is associated with phenotypic resistance to multiple classes of antibiotics, is a transmissible resistance-nodulation-division (RND) family efflux pump. However, the prevalence and genomic and phenotypic characteristics of clinical isolates with this important resistance determinant are poorly understood. In this study, we aimed to survey *tmexCD-toprJ* among clinical Gram-negative isolates collected from hospitals in China between 1991 and 2020 and characterise *tmexCD-toprJ*-positive clinical isolates.

Methods We conducted online data retrieval and active nationwide surveillance in China to screen *tmexCD-toprJ*-positive strains. We characterised *tmexCD-toprJ*-positive clinical strains for their antimicrobial susceptibility, genetic and functional characteristics, and the potential inter-species transmission route of *tmexCD-toprJ* with whole genome sequencing and bioinformatics analyses. The function of *tmexCD-toprJ* in *Pseudomonas* sp and *Proteus* sp was investigated by *tmexD* gene knockdown using an isopropylthio-β-galactoside-inducible CRISPR interference system.

Findings Data retrieval obtained 53 strains carrying *tmexCD-toprJ*, comprising 32 *Pseudomonas* spp, 11 *Klebsiella pneumoniae*, one *Aeromonas* spp, one *Citrobacter freundii*, and one uncultured bacterium from diverse niches. 48 (0·64%) of 7517 clinical isolates from China, including seven *Klebsiella* spp, one *Proteus mirabilis*, and 40 *Pseudomonas* spp, carried *tmexCD-toprJ*. These isolates exhibited multidrug resistance phenotypes and co-harboured resistance genes, such as *mcr* and carbapenemases genes. *tmexCD-toprJ* was encoded on both plasmids and chromosomes in all *Klebsiella* spp that carried plasmid-borne *tmexCD-toprJ* (n=7), *P mirabilis* carried chromosome-borne *tmexCD-toprJ*, and *Pseudomonas* spp carried either plasmid-borne (n=19) or chromosome-borne (n=21) ones. *tmexCD-toprJ* had undergone clonal and horizontal transmission among clinical pathogens. Eight different types of genetic context of *tmexCD-toprJ* were identified, each of which was associated with different mobile elements, including *IntI*, IS*6100*, Tn*As1*-like, IS*Ror5*, IS*Vsa3*, IS*Cfr*-like, Tn*5393*, and IS*222*-like, which might facilitate its transmission. Knockdown of *tmexD* led to a four times decrease in tigecycline minimum inhibitory concentrations in both *Pseudomonas* spp and *Proteus* spp.

Interpretation Our study provides evidence to suggest that *tmexCD-toprJ* contributes to the antimicrobial resistance phenotypes in different bacterial species. *tmexCD-toprJ* has disseminated among diverse species of clinical pathogens, which warrants timely monitoring in clinical pathogens.

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Introduction

Antimicrobial resistance, which leads to longer hospital stays, higher medical costs, and increased mortality, is one of the biggest threats to public health.[1] In addition to intrinsic resistance, bacteria can develop or acquire drug resistance with mechanisms including antibiotic inactivation by enzymatic hydrolysis or modification, overproduction of the antibiotic target through gene amplification, decreased influx or penetration of the drug into the cell wall with changes in cell wall structure, and increased efflux of the antibiotic out of the bacteria cell through porins and efflux pumps.[2] Among these, efflux is the most effective and fastest acting resistance mechanism.[3] Efflux pumps are membrane proteins that are involved in the extrusion of noxious substances, including antibiotics, detergents, toxins, dyes, and waste metabolites from the cell.[4] They are commonly found in all species of bacteria, with genes encoding efflux pumps locating on bacterial chromosomes or mobile genetic elements such as plasmids.[4] Six families of efflux pumps that contribute to multidrug resistance have been identified so far.[5]

The resistance-nodulation-division (RND) family of efflux pumps, which exist as a tripartite system and contain a polyspecific substrate binding pocket that enables them to bind and expel out a wide array of

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Research in context

Evidence before this study

We searched PubMed with no language restrictions for reports that were published up to April 7, 2021, using the terms "multidrug resistance", "mobilized antimicrobial resistance and efflux pumps", "mobilized RND efflux pumps", and "*tmexCD-toprJ*". We found only two publications that described the discovery and functional characterisation of two variants of this mobilised efflux pump determinant, *tmexCD1-toprJ1* and *tmexCD2-toprJ2*, and five publications reporting the characterisation of single (ie, only one isolate was reported in each of these publications) *tmexCD-toprJ*-positive bacteria. However, no study reported the diversity and inter-species transmission route of the *tmexCD-toprJ* gene cluster, as well as the species composition, genetic, and phenotypic characteristics of *tmexCD-toprJ*-positive clinical bacteria in China.

Added value of this study

Our results showed that *tmexCD-toprJ* was distributed in clinically important pathogens including *Pseudomonas* spp, *Klebsiella* spp, *Aeromonas* spp, and *Proteus* spp. *Pseudomonas* spp, as the potential ancestral source of *tmexCD-toprJ*, was the dominant bacterial species carrying this gene cluster. We provide evidence to show that *tmexCD-toprJ* contributes to the antimicrobial resistance phenotypes in different bacterial species, including *Pseudomonas* spp, and *Proteus* spp, which are intrinsically resistant to tigecycline. Inter-species transmission of *tmexCD-toprJ* was achieved by genetic recombination mediated by different mobile genetic elements. Four types of the main functional transporter TMexD, including TMexD1, TMexD2, TMexD3, and TMexD2-like, were identified among the clinical isolates in this study, suggesting the high diversity of the *tmexCD-toprJ* gene cluster. We observed that *tmexCD-toprJ*-positive clinical bacteria carried diverse acquired antimicrobial resistance genes and exhibited multidrug resistance phenotypes. Despite being rare in human clinical isolates, the highly diverse genetic context of *tmexCD-toprJ* and its inter-host transmission potential suggest that its further dissemination among Aeromonadaceae, Enterobacteriaceae, and Pseudomonadaceae is likely, which poses challenges to clinical treatments.

Implications of all the available evidence

The emergence of *tmexCD-toprJ* heralds the breach of the several classes of antibiotics, including the last group of antibiotics, tigecycline, by both chromosome-mediated and plasmid-mediated resistance. Although currently confined to a low number of bacterial species with a low prevalence in clinical settings in China, the prevalence of bacteria with *tmexCD-toprJ* could increase. Measures are needed to monitor and control the further dissemination of *tmexCD-toprJ*.

compounds, are of particular clinical significance.[6] RND efflux pumps are unique to Gram-negative bacteria and have important physiological functions for biofilm formation, modulation of quorum sensing signalling, and virulence, in addition to multidrug resistance among diverse pathogens.[4,7] AcrAB-TolC is the best characterised RND system among Enterobacteriaceae, and homologous pumps have been reported in other Gram-negative bacterial species, including MexAB-OprM, MexCD-OprJ, and MexXY-OprM in *Pseudomonas* spp, CmeABC in *Campylobacter* spp, MtrCDE in *Neisseria* spp, and AdeABC in *Acinetobacter baumannii*.[8]

RND efflux pumps are most frequently chromosomally encoded, with only few studies reporting on plasmid-borne elements.[9] In 2020, a novel plasmid-borne multidrug resistance gene cluster, *tmexCD1-toprJ1*, and its homologues encoding proteins belonging to the MexCD-OprJ efflux family were reported.[10,11] TMexCD-TOprJ confers resistance or reduced susceptibility to several classes of antibiotics, including cephalosporins, phenicols, quinolones, and tetracyclines.[10,11] Of note, TMexCD-TOprJ was the first plasmid-borne RND-type efflux pump that confers resistance to last-line antibiotics tigecycline and eravacycline and can be co-transferred with other mobile resistance genes, such as *mcr-8*, among Enterobacteriaceae.[11,12] Predicted to have primarily originated from a *Pseudomonas* spp and acquired from a chromosome of *Aeromonas* spp through mobile element mediated translocation, plasmid-borne *tmexCD-toprJ* was only reported among particular Enterobacteriaceae, including *Klebsiella* spp and *Raoultella* spp.[11,12] Despite being identified from different sources, including humans, animals, and animal food, *tmexCD1-toprJ1*-positive Enterobacteriaceae are rare among clinical isolates in China.[11–13] Nevertheless, the broader species distribution, the temporal and spatial distribution, and genomic and phenotypic characteristics of the *tmexCD-toprJ*-positive clinical isolates, as well as the types of variants, genetic environments, and the mechanisms underlying the inter-species transmission of *tmexCD-toprJ*, are poorly understood. In this study, we aimed to survey *tmexCD-toprJ* among clinical Gram-negative isolates collected from hospitals in China between 1991 and 2020 and comprehensively characterise *tmexCD-toprJ*-positive clinical isolates.

Methods

Database mining

On Nov 11, 2021, we conducted a BLASTn search in the National Center for Biotechnology Information database and literature retrieval in PubMed. Information on all *tmexCD-toprJ*-positive bacteria retrieved from data mining was recorded (appendix p 33). Multilocus sequence typing (MLST) of isolates with chromosome sequences was determined using mlst (version 2.11; appendix p 2).[14]

See Online for appendix

Retrospective screening of *tmexCD-toprJ*-positive clinical isolates

Strain collection was done as part of an active surveillance process conducted in China. Briefly, Gram-negative bacteria isolated from multiple sources were collected from 77 tertiary-level hospitals across 28 provinces and municipalities in mainland China between 1991 and 2020 (appendix pp 2–3). All isolates were randomly acquired over this period using the Rand function in Microsoft Excel 2021. An accurate number of isolates from each hospital was unavailable because a large number of isolates were from historical collections that were not recorded as being associated with a specific hospital. Species confirmation was conducted using 16S rRNA gene-based sequencing or matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (Bruker Daltonik GmbH, Bremen, Germany; table 1). Ethical approval for this study was given by the Zhejiang University ethics committee (number 2020-319). *tmexCD-toprJ* was screened by PCR and Sanger sequencing according to methods described previously.[11] All *tmexCD-toprJ*-positive isolates from data mining and retrospective screening in this study were classified according to Bergey's Manual of Systematic Bacteriology.[15] Information on all these *tmexCD-toprJ*-positive strains was retrieved to create a classification figure using Adobe Illustrator (version 22.1).

Antimicrobial susceptibility testing, quantitative real-time PCR (rtPCR), and conjugation assay

The minimum inhibitory concentrations (MICs) of *tmexCD-toprJ*-positive strains and 120 randomly selected *tmexCD-toprJ*-negative strains against nine commonly used antibiotics (meropenem, ceftazidime, cefoperazone–sulbactam, ceftazidime–avibactam, tetracycline, tigecycline, colistin, amikacin, and ciprofloxacin) were determined using the broth microdilution method and interpreted according to the Clinical and Laboratory Standards Institute guideline.[16] Expression levels of *tmexCD-toprJ* were tested by quantitative rtPCR analysis. Conjugation between 21 *tmexCD-toprJ*-positive strains and the recipient was conducted using the mixed broth method as previously described (appendix pp 3–4).[17]

Whole genome sequencing and bioinformatics analysis

Genomic DNA of all *tmexCD-toprJ*-positive bacteria was extracted from overnight cultures using the PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA). The procedure for genome sequencing, assembly, annotation, gene environment analysis, and detection of sequence types, plasmid types, and core genome single nucleotide polymorphisms (SNPs) has been described in our previous study (appendix p 5).[18] The procedure for acquiring TMexD homologues, sequence alignment, phylogeny construction, and visualisation has been described in our previous study (appendix p 6).[19]

	Years isolated	Number of isolates	Number of *tmexCD-toprJ*-positive isolates (%)
Enterobacteriaceae			
Enterobacteriaceae (total)	1991–2020	4154	7 (0·2%)
Klebsiella spp	1991–2020	2795	7 (0·3%)*
Escherichia coli	2009–20	984	0
Enterobacter spp	2001–18	169	0
Citrobacter spp	2001–18	124	0
Salmonella spp	2013–16	70	0
Shigella spp	2013–16	12	0
Morganellaceae			
Morganellaceae (total)	1991–2020	489	1 (0·2%)
Morganella morganii	2001–18	38	0
Providencia spp	1991–2020	14	0
Proteus spp†	1991–2020	437	1 (0·2%)†
Yersinia			
Serratia spp	2013–16	244	0
Moraxellaceae			
Acinetobacter spp	2001–20	766	0
Pseudomonadaceae			
Pseudomonas spp	1991–2020	1378	40 (2·9%)‡
Xanthomonadaceae			
Stenotrophomonas maltophilia	2001–16	212	0
Burkholderiaceae			
Burkholderia spp	2007–14	119	0
Flavobacteriaceae			
Flavobacterium spp	2001–18	106	0
Aeromonadaceae			
Aeromonas spp	2008–16	49	0
Total	1991–2020	7517	48 (0·6%)

NA=not applicable. *4 (0·1%) *Klebsiella pneumoniae* and 3 (0·1%) *Klebsiella quasipneumoniae*. †One (0·2%) *Proteus mirabilis*. ‡22 (1·6%) *Pseudomonas aeruginosa*; 10 (0·7%) *Pseudomonas putida*; five (0·4%) *Pseudomonas monteilii*; one (0·1%) *Pseudomonas alcaliphila*; one (0·1%) *Pseudomonas guariconensis* and one (0·1%) *Pseudomonas oleovorans*.

Table 1: **Strain collection and *tmexCD-toprJ*-positive bacteria among clinical Gram-negative isolates**

tmexD gene knockdown

To investigate the contribution of *tmexCD-toprJ* to the resistance phenotype in *Pseudomonas* spp and *Proteus* spp, we used an isopropylthio-β-galactoside (IPTG)-inducible CRISPR interference (CRISPRi) system for tunable repression of gene expression to create *tmexD* knockdown mutants in two representative clinical strains from this study, *P aeruginosa* N18-5 and *P mirabilis* T1010.[20] The detailed protocols for creation of gene knockdown mutants in these strains are shown in the appendix (pp 6–9, 31, 45–47).

Role of the funding source
The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Data mining with sequence query and literature retrieval resulted in 53 strains that carried *tmexCD-toprJ*, including bacteria belonging to Pseudomonadaceae (n=32), Enterobacteriaceae (n=13), and Aeromonadaceae (n=7) families, and one uncultured bacterium. These strains belonged to four genera (*Pseudomonas* spp [n=32], *Klebsiella pneumoniae* [n=12], *Aeromonas* spp [n=7], and *C freundii* [n=1]) from diverse niches, including animals, humans, and the environment (appendix pp 18, 20). These isolates were from regions within Asia (mainland China [n=46], Hong Kong [n=1], India [n=2], Thailand [n=1], and Viet Nam [n=1]) and Europe (Sweden [n=1] and Poland [n=1]), within the period from Jan 1, 1997, to Dec 31, 2019. All strains (except one uncultured bacterium) isolated in the years 1997–2014 belonged to *Pseudomonas* spp, and strains belonging to other genera were isolated between 2015 and 2019 (appendix pp 33, 34). 28 strains could be typed with MLST: four *Aeromonas* spp belonging to ST313 (n=2), ST363 (n=1), and ST645 (n=1); one *C freundii* belonging to ST257; ten *Klebsiella* spp belonging to ST2667 (n=3), ST15 (n=2), and one belonging to each of ST1, ST11, ST273, ST2909, and ST3447; 13 *Pseudomonas* spp belonging to ST235 (n=3), and one belonging to each of ST1284, ST15, ST17, ST234, ST260, ST274, ST292, ST389, ST70, and ST773.

Screening of *tmexCD-toprJ* among clinical isolates in China between 2002 and 2020 showed that a total of 48 isolates (48 [0·64%] of 7517 isolates) were positive, including seven Enterobacteriaceae (four *K pneumoniae* and three *Klebsiella quasipneumoniae*), one Morganellaceae (*Proteus mirabilis*), and 40 *Pseudomonas* spp (22 *P aeruginosa*, ten *Pseudomonas putida*, five *Pseudomonas monteilii*, one *Pseudomonas alcaliphila*, one *Pseudomonas guariconensis*, and one *Pseudomonas oleovorans*; table 1, appendix pp 18, 20). These strains were isolated in five provinces: Zhejiang (n=40), Guizhou (n=2), Henan (n=2), Hunan (n=2), and Tibet (n=2). The strains were isolated from stool (n=21), sputum (n=19), urine (n=3), cerebrospinal fluid (n=1), blood (n=1), secretion (n=1), and unknown samples (n=2; appendix pp 17, 19). Among these, 29 strains could be typed with MLST, including seven *Klebsiella* spp belonging to ST571 (n=3), ST15 (n=2), ST490 (n=1), and ST147 (n=1), and 22 *Pseudomonas* spp belonging to ST1418 (n=15), ST360 (n=2), ST260 (n=1), ST267 (n=1), ST463 (n=1), ST769 (n=1), and ST773 (n=1). ST15 *Klebsiella* spp and ST260 and ST773 *Pseudomonas* spp were identified from both the database and our collection. Apart from three *P aeruginosa* strains isolated before 2010, most (45 [93·8%] of 48) *tmexCD-toprJ*-positive strains in China were isolated during or after 2010. The first *tmexCD-toprJ*-positive clinical *Klebsiella* spp strain was isolated in 2015 in Zhejiang province (appendix pp 35, 36). To investigate the types of *tmexCD-toprJ* among isolates collected in this study, a phylogenetic analysis was conducted on a collection of TMexD proteins homologous to TMexD1 and TMexD2 (appendix pp 39, 40). A previous study[10] classified the TMexD protein into five major clusters, including TMexD1 (GenBank accession number QHW08916), TMexD2 (QQZ45304), TMexD3 (KSR47626), TMexD3-like (AVE20481), and other TMexD-like (AEF23019), each of which included representative proteins. Our result was in line with the previous study[10] in the classification of TMexD except for the addition of one cluster, the TMexD2-like protein. TMexD1, TMexD2, TMexD3, TMexD2-like, and TMexD3-like were closely related, and all TMexD from various bacterial species shared a branching point (figure 1). A large proportion of TMexD belonged to TMexD-like, all of which were *Pseudomonas* spp. Four clusters of TMexD were identified among the clinical isolates in this study, including TMexD1, TMexD2, TMexD3, and TMexD2-like. The TMexD1 cluster includes two *K pneumoniae* isolated from the Henan province encoding protein that were 100% identical to TMexD1. The TMexD2 cluster included three *K quasipneumoniae*, two *K pneumoniae*, and 16 *Pseudomonas* spp producing TMexD protein with 100% similarity to TMexD2. One *P alcaliphila* isolated from Tibet in the year 2020 produced TMexD2-like protein that was 98·9% identical to TMexD2. The TMexD3 cluster included the remaining 24 isolates (one *P mirabilis* and 23 *Pseudomonas* spp), all of which produced proteins that exhibited 98% similarity with TMexD1 (appendix pp 35, 36).

All the 48 clinical *tmexCD-toprJ*-positive bacteria exhibited multidrug resistance phenotypes. In general, the resistance rates of the *tmexCD-toprJ*-positive isolates to different classes of antibiotics were higher than those of the *tmexCD-toprJ*-negative isolates. The *tmexCD-toprJ*-positive Enterobacteriaceae were more commonly resistant to ciprofloxacin (7 [100%] out of 7), meropenem (7 [100%]), ceftazidime (6 [86%]), cefoperazone–sulbactam (5 [71%]), and ceftazidime-avibactam (4 [57%]) than to other antibiotics, including amikacin (3 [43%]), tetracycline (3 [43%]), tigecycline (3 [43%]), and colistin (2 [29%]). The *P mirabilis* isolate was resistant to tetracycline (32 μg/mL), colistin (>8 μg/mL), amikacin (32 μg/mL), and ciprofloxacin (4 μg/mL), intermediated to tigecycline (4 μg/mL), and susceptible to meropenem (≤0·5 μg/mL), ceftazidime (≤2 μg/mL), cefoperazone–sulbactam (≤4–2 μg/mL), and ceftazidime–avibactam (≤1–4 μg/mL). *tmexCD-toprJ*-positive Pseudomonadaceae were commonly resistant to ciprofloxacin (39 [97·5%] out of 40), cefoperazone–sulbactam (34 [85%]), ceftazidime (34 [85%]), ceftazidime–avibactam (26 [65%]), tetracycline (24 [60%]), and amikacin (19 [47·5%]), and only one *tmexCD-toprJ*-positive Pseudomonadaceae was resistant to colistin (1 [2·5%]). Quantitative rtPCR analysis suggested that the sus-

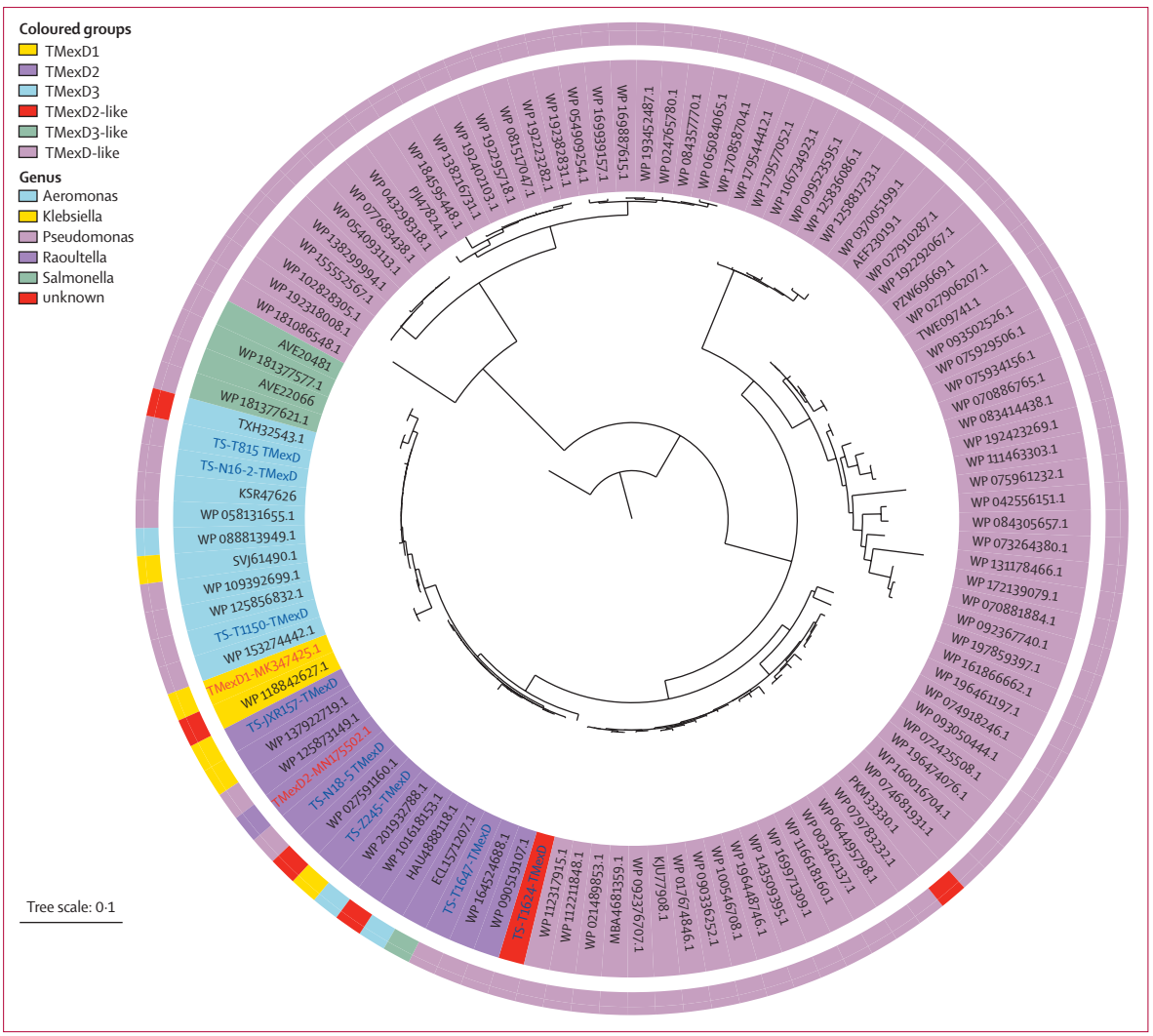

Figure 1: **Phylogenetic tree of TMexD proteins**
The different background colours of the sequence IDs indicate the related variants of TMexD proteins. Colours in the outer circle indicate the genus of *tmexD*-positive bacteria. Previously published sequences TMexD1 (nucleotide acid accession number MK347425) and TMexD2 (MN175502) are shown in red fonts. Sequences from this study are shown in blue fonts and begin with TS-. TMexD sequences of strains from this study were deduplicated to retain only unique and representative sequences. TMexD protein sequence in *K pneumoniae* strains HN6-2 and HN36-2 are identical with that of TMexD1 and were then excluded from the phylogenetic analysis.

ceptibility levels of the isolates were associated with the expression levels and the location (plasmid or chromosome) of *tmexCD-toprJ* (appendix pp 31, 37, 38, 41).

A total of 80 different antibiotic resistance genes (ARGs) were identified among the clinical *tmexCD-toprJ*-positive bacteria, conferring resistance to different classes of antimicrobials, including aminoglycosides, chloramphenicol, colistin, fluoroquinolones, fosfomycin, macrolides, rifampin, sulfonamides, tetracycline, trimethoprim, and β-lactams. A heatmap showing the carriage of ARGs by each strain was constructed (figure 2). The number of acquired ARGs carried by each isolate ranged from 2 to 33. A total of 46 different ARGs were identified among *Klebsiella* spp, 51 different ARGs were identified among *Pseudomonas* spp, and 16 different ARGs were identified among *P mirabilis*. The association between the type of ARG and the family of the host strain was observed as shown in the similarity tree in the heatmap (figure 2), which separated isolates belonging to Pseudomonadaceae and Enterobacteriaceae or Morganellaceae into two different clusters, except for one strain, *P aeruginosa* N16-2, which carried ARGs closely related to Enterobacteriaceae. The coexistence of *tmexCD-toprJ* with both *mcr-1* and *mcr-8* was observed in two *K pneumoniae*. Four *Klebsiella* spp carried both bla_{NDM-1} and *tmexCD-toprJ*. Among these strains, one isolate also carried bla_{IMP-4}. The bla_{KPC-2}-carrying and *tmexCD-toprJ*-positive strains included one *K pneumoniae* and three *P aeruginosa*. A total of three *Pseudomonas* spp carried bla_{IMP} and a total of six *Pseudomonas* spp carried bla_{VIM}. None of

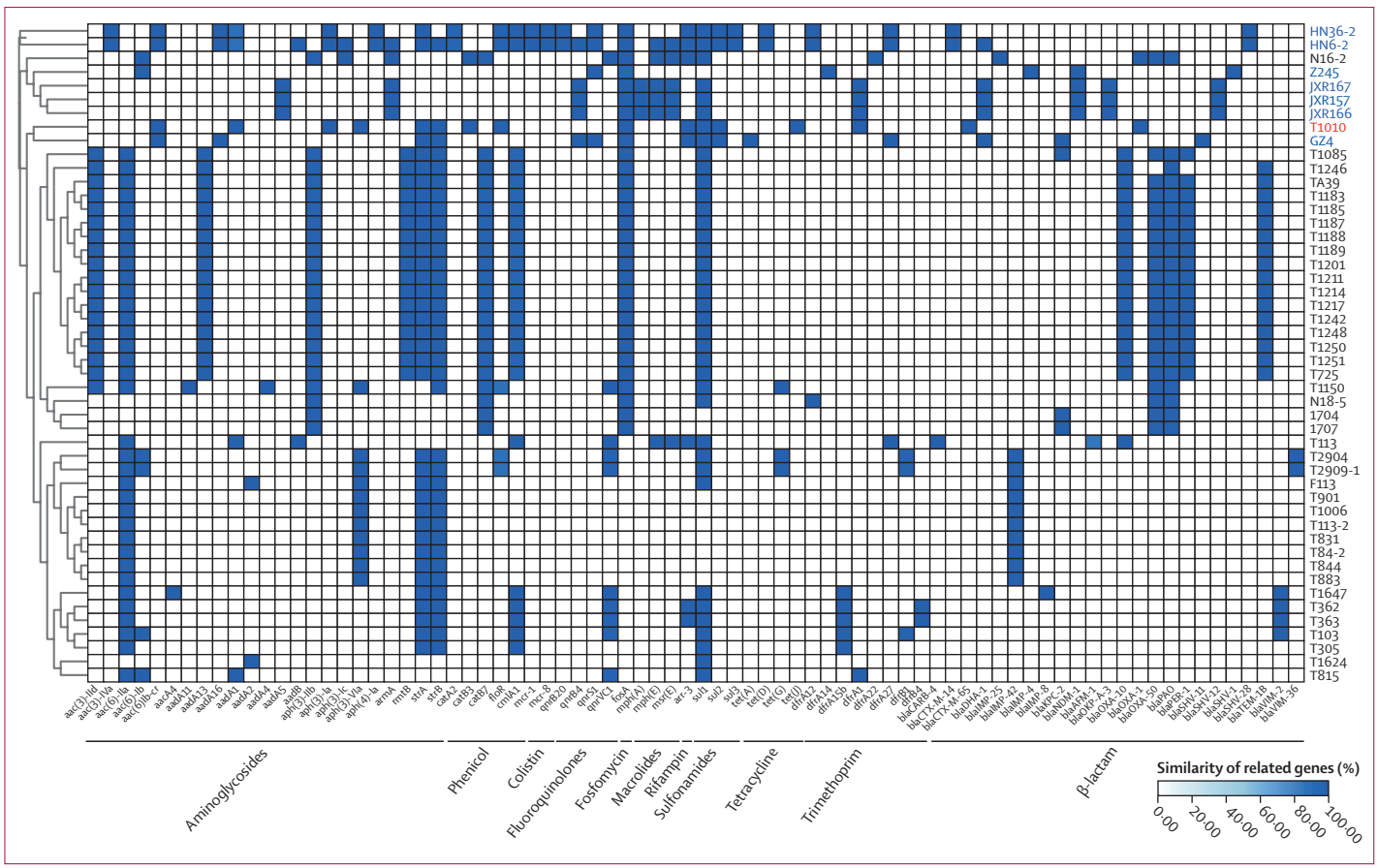

Figure 2: **Heatmap of antimicrobial resistance genes harboured by clinical *tmexCD-toprJ*-positive bacteria in China**
Horizontal axes represent the antimicrobial resistance genes and vertical axes represent the strain IDs. Strains belonging to *Pseudomonas* spp are shown in black, strains belonging to *Klebsiella* spp are shown in blue, and strains belonging to *Proteus mirabilis* are shown in red. The classes of antibiotics to which the genes confer resistance are labelled at the bottom of the figure. Blue boxes represent the presence of the corresponding items among sequenced isolates, and white boxes represent their absence. The gradient identity bar indicates the percentage similarity of related genes. The similarity tree was calculated using agglomerative hierarchical clustering, with the degree of similarity between different clusters calculated by the average linkage method and the degree of similarity of different isolates (calculated using Spearman's rank correlation coefficient).

the *Klebsiella* sp isolates carried mutations that were known to confer tigecycline resistance (appendix pp 35, 36).

Genomic analysis showed that *tmexCD-toprJ* in *Klebsiella* spp (n=7) was located on the plasmids and that, in *P mirabilis* (n=1), were located on the chromosome. *Pseudomonas* spp carried *tmexCD-toprJ* either on the chromosome (n=21) or plasmid (n=19). Chromosome-encoded TMexD proteins included TMexD2-like (n=1), TMexD2 (n=16), and TMexD3 (n=5), and plasmid-encoded TMexD proteins all belonged to TMexD3 in *Pseudomonas* spp.

Clinical *tmexCD-toprJ*-positive *Klebsiella* spp belonged to sequence types ST15 (n=2), ST571 (n=3), ST490 (n=1), and ST147 (n=1). Pairwise SNP analysis suggested the two ST15 *K pneumoniae* strains (HN6-2 and HN36-2) belonged to a single clone (SNP=2). The three ST571 *K quasipneumoniae* strains (JXR157, JXR166, and JXR167) differed by a small number of SNPs (n≤42) and belonged to closely related clones (appendix p 44). Four different types of plasmids, termed pKPGZ4-tmex (n=1), pKPZ245-tmex (n=1), pKPHN6-2-tmex-like plasmid (n=2), and pKQJXR157-tmex-like plasmid (n=3), were identified among *Klebsiella* spp (appendix pp 9, 10, 21–29).

Among the 40 clinical *Pseudomonas* spp, *P aeruginosa* isolates 1704 and 1707 (ST360), *P putida* isolates T883, T1006, T84-2, and T844, and *P monteilii* isolates T362 and T363 each shared a number of pairwise SNPs of ≤10, suggesting that they originated from single clones (appendix pp 35, 43). Plasmid-borne *tmexCD-toprJ* genes clusters were detected among *P aeruginosa* (n=18) and *P putida* (n=1) strains, and *tmexCD-toprJ* were carried by chromosomes in other *Pseudomonas* spp. Three different types of plasmids, termed pPPAN16-2-tmex (n=1), pPTA39-tmex-like plasmid (n=17), and pPPT815-tmex (n=1), were identified among *Pseudomonas* sp (appendix pp 9, 10, 21–29).

Detailed information on *tmexCD-toprJ*-carrying plasmids, including the plasmid length, type of replicons, number of open reading frames, guanine-cytosine content, and antimicrobial resistance genes, are shown in

	tmexCD-toprJ-carrying plasmids												*Non-tmexCD-toprJ*-carrying plasmids
	Plasmid ID	Replicon	Length (bp)	Number of ORFs	G+C content	Homologous plasmid in NCBI (accession number)	Plasmid identity (NCBI)	Plasmid coverage (NCBI)	TMexD type	Antimicrobial resistance genes	*tmexCD-toprJ* gene environment	Strains with similar plasmids	Plasmid size (kb)
K pneumoniae GZ4	pKPGZ4-tmex	IncFIB$_k$/FII$_k$	283 325	319	52·6%	pCAV1193-258 (CP013323)	99·86%	72%	TMexD2	NA	Type III	NA	245, 136, 22
K pneumoniae HN6-2	pKPHN6-2-tmex	IncFIA/FII	134 125	154	52·3%	pHNAH8I-1 (MK347425)	99·62%	90%	TMexD1	*mcr-8*, *qnrS1*, *strAB*	Type VII	*K pneumoniae* HN36-2	253, 185, 69
K pneumoniae Z245	pKPZ245-tmex	IncFIB/HI1B	283 454	318	47·4%	pKOX7525_1 (CP065475)	100%	67%	TMexD2	*bla*$_{IMP-4}$, *catB3*, *qnrS1*, *ant(3″)-Ih-aac(6′)-IId*	Type III	NA	151, 74, 55
K quasipneumoniae JXR157	pKQJXR157-tmex	IncFIB/HI1B	331 364	369	48·0%	pKOX7525_1 (CP065475)	99·90%	65%	TMexD2	*sul1* (2),* *armA*, *msr(E)*, *mph(A)*, *qnrB4*, *bla*$_{DHA-1}$, *bla*$_{SHV-12}$	Type III	*K quasipneumoniae* JXR166 and JXR167	187, 112, 82
P aeruginosa N16-2	pPAN16-2-tmex	NT†	414 321	478	56·3%	pR31014-IMP (MF344571)	99·98%	96%	TMexD3	*msr(E)*, *mph(E)*, *armA*, *sul1*(2), *dfrA22*, *qnrVC1*, *bla*$_{OXA-1}$; *bla*$_{IMP-25}$	Type V	NA	NA
P aeruginosa TA39	pPATA39-tmex	NT	429 633	495	56·7%	pBT2436 (CP039989)	98·88%	95%	TMexD3	*aadA13*, *cmlA1*, *sul1* (n=2), *bla*$_{OXA-10}$, *bla*$_{PER-1}$, *aac(3′)-IId*, *rmtB*, *bla*$_{TEM-1b}$, *strAB*	Type VI	16 *Pseudomonas* spp‡	NA
P putida T815	pPPT815-tmex	NT	473 207	560	55·8%	pVIM-24-ZDHY414 (CP064948)	98·98%	77%	TMexD3	*aacA4*, *aadA1*, *sul1*, *qnrVC1* (2), *dfrA1*, *aac(6′)-IIa*	Type V	NA	NA

BP=base pairs. NA=not applicable. NCBI=National Center for Biotechnology Information. ORF=open reading frame. *Numbers in parentheses indicate the copy number (n) of the related gene if n>1. †NT, plasmid replicons for the *Pseudomonas* spp strains were non-typable by the PCR-based replicon typing. ‡Strain IDs for the 16 *Pseudomonas* spp are T1188, T1085, T1183, T1185, T1187, T1189, T1201, T1211, T1214, T1217, T1242, T1246, T1248, T1250, T1251, and T725.

Table 2: **Profiles of *tmexCD-toprJ*-carrying plasmids harboured by clinical Gram-negative bacteria**

table 2. Our results suggested the clonal spread of *tmexCD-toprJ* in both *Klebsiella* spp and *Pseudomonas* spp. The recovery of pPTA39-tmex-like plasmids from *P aeruginosa* isolates belonging to different clones suggested the potential of *tmexCD-toprJ* to undergo plasmid-mediated horizontal transfer among different *Pseudomonas* spp clones. Although plasmid-mediated horizontal transfer of *tmexCD-toprJ* among *Klebsiella* spp was not observed here, we could not rule out its possibility. All *tmexCD-toprJ*-carrying plasmids in *Klebsiella* spp and *Pseudomonas* spp were non-conjugatively transferable to *Escherichia coli* or *P aeruginosa*.

The lengths of *tmexCD-toprJ*-carrying contigs were all >20 Kb and were associated with eight main genomic backbone profiles, designated types I to VIII (I [n=1]; II [n=1]; III [n=5]; IV [n=1]; V [n=1]; VI [n=20]; VII [n=17]; and VIII [n=1]). In all types, *tmexCD-toprJ* were adjacent to its transcriptional regulator, *tnfxB*. Each type was associated with different mobile elements, including *IntI*, IS*6100*, Tn*As1*-like, IS*Ror5*, IS*Vsa3*, IS*Cfr*-like, Tn*5393*, and IS*222*-like. *xerD* genes, which encode enzyme belonging to the tyrosine recombinase family, were found on genomic backbone types III, IV, V, VI, and VII (appendix pp 10–14, 30). The genetic environment analysis suggested that Tn*As1*-like transposon could have mediated the translocation of *tmexCD-toprJ* from the chromosome to plasmids among *Pseudomonas* spp, and inter-species transmission of *tmexCD*-toprJ was possibly achieved by the capture of Tn*5393*-like transposon by plasmids in *Klebsiella* spp, mediated by XerD through the adjacent conserved XerD binding sites.

An IPTG-inducible CRISPRi system to knock down the main functional transporter gene, *tmexD*, was created to test the role of *tmexD* in *Pseudomonas* sp and *Proteus* sp. As this method has never been used in *Pseudomonas* spp and *Proteus* spp, we introduced a green fluorescent protein (GFP) knockdown system and a *ropBC* knockdown mutant in a reference strain, *P aeruginosa* PA14. Upon addition of IPTG into these mutants, the expression of GFP and the growth of the mutants were lower than that without IPTG, while IPTG showed no effect on the growth of wild-type *P aeruginosa* PA14 (figure 3A–C), suggesting this IPTG-inducible CRISPRi system worked well in *P aeruginosa*. We then created *ropBC* and *tmexD* single gene knockdown mutants in *P aeruginosa* clinical strain N18-5. Upon induction with IPTG, the growth of the *rpoBC* suppressor strain reduced compared with no IPTG induction, and IPTG did not affect the growth of the wild-type strain N18-5, suggesting that this system worked well in this clinical isolate (figure 3D, E). Upon induction with IPTG, tigecycline MICs of the *P aeruginosa* N18-5 *tmexD* knockdown mutant were 1 µg/mL, a four times reduction compared with no IPTG induction, which was 4 µg/mL (figure 3F). We also introduced a GFP knockdown system and created a *tmexD* knockdown mutant in *P mirabilis* T1010. Upon induction with IPTG, the GFP expression levels of *P mirabilis* T1010 were reduced compared with that without IPTG induction, suggesting that this system worked well in this clinical isolate. Upon induction with IPTG, tigecycline MICs of the *P mirabilis* T1010 *tmexD* mutant were 1 µg/mL, a four times reduction compared with no IPTG induction, which was 4 µg/mL (figure 3G–I). These data confirmed that *tmexCD-toprJ* contributed to enhanced tigecycline MICs in *Pseudomonas* sp and *Proteus* sp (appendix pp 14, 15, 31, 32).

Discussion

Our results show that *tmexCD-toprJ*, which contributed to drug resistance phenotypes in different bacteria, including *Pseudomonas* spp and *Proteus* spp, has disseminated worldwide in Aeromonadaceae, Enterobacteriaceae, Morganellaceae, and Pseudomonadaceae, all of which are important clinical pathogens. *tmexCD-toprJ*-positive bacteria are frequently associated with multidrug resistance and carried diverse ARGs, including carbapenemases genes and *mcr*. The main functional transporter, TMexD, has evolved into different subtypes. The genetic environment of *tmexCD-toprJ* is highly diverse and is associated with various mobile elements. Horizontal and vertical gene transfer of *tmexCD-toprJ* could have occurred, which might have led to its clinical dissemination.

Reports on *tmexCD-toprJ*-positive bacteria are mainly from China.[21] However, our data mining results have shown the emergence and global dissemination, and the potential mobilisation of *tmexCD-toprJ*. A clinical *P aeruginosa* strain isolated in 1997 from India was the first strain with *tmexCD-toprJ* on plasmid pMKPA34-1 (accession number MH547560). pMKPA34-1 harboured the *traN* gene, which encodes proteins that are essential for bacterial conjugations and is common among Enterobacteriaceae, suggesting its potential exchangeability with members of the Enterobacteriaceae.[22] Stool (21 [43·8%] of 48 isolates) and sputum (19 [39·6%]) accounted for most samples from which *tmexCD-toprJ*-positive bacteria were isolated, highlighting the diagnosis and clinical significance of these samples.

The positive rate of *tmexCD-toprJ* in clinical *Pseudomonas* spp (40 [2·9%] of 1378) was higher than in *Klebsiella* spp (7 [0·25%] of 2795 isolates), supporting the notion that *Pseudomonas* spp could be the ancestral source of *tmexCD-toprJ*.[12,23,24] Nevertheless, the retrospective sampling method could have affected the observed prevalence of *tmexCD-toprJ*. *tmexCD-toprJ* has been widely reported to be plasmid-borne among Enterobacteriaceae, with evidence of gene acquisition by species of Enterobacteriaceae from chromosomes of Pseudomonadaceae and Aeromonadaceae.[11,12] In line with these findings, we provided evidence to show that horizontal and vertical transmission of *tmexCD-toprJ* could have occurred among clinical strains. In our study,

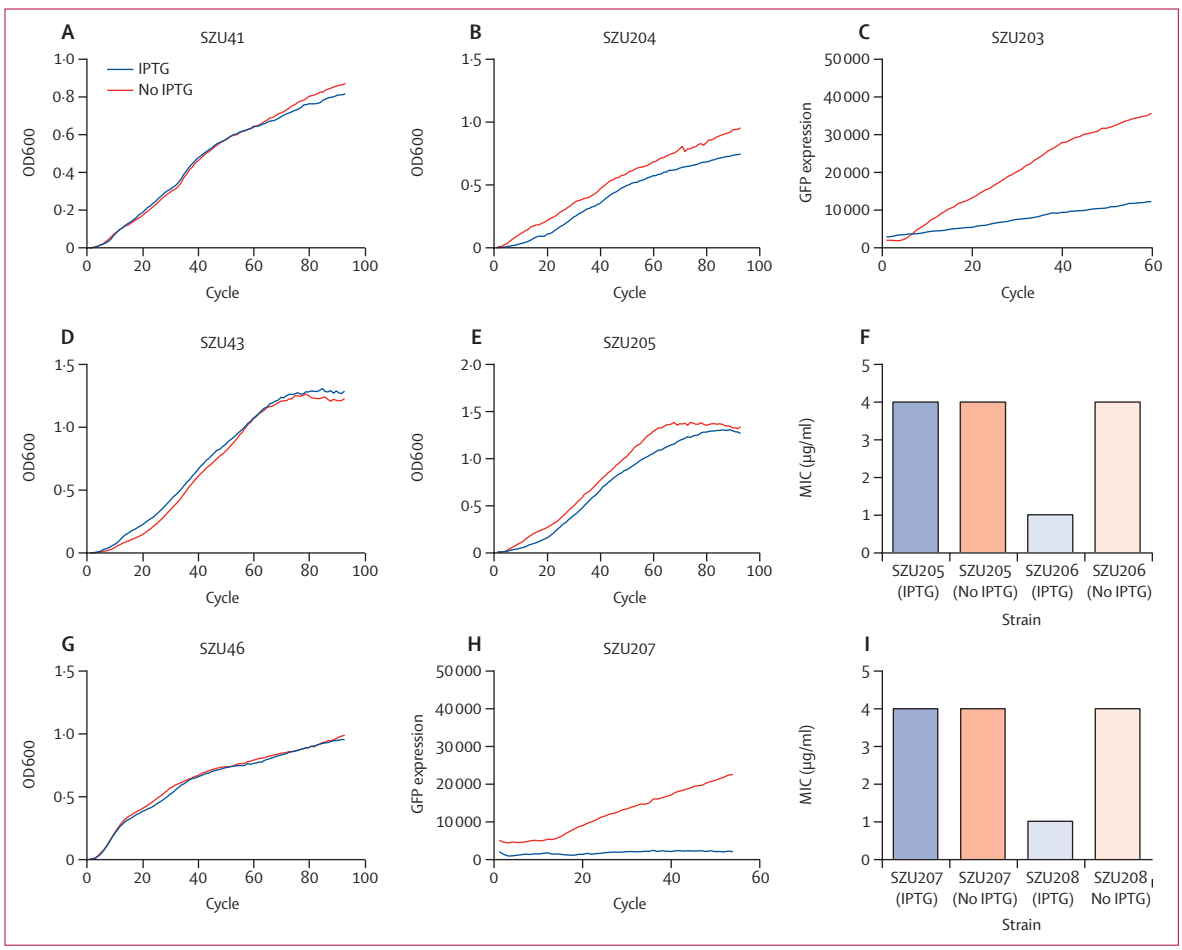

Figure 3: **Contribution of *tmexCD-toprJ* to tigecycline resistance in *Pseudomonas aeruginosa* N18-5 and *Proteus mirabilis* T1010**
Growth curve of SZU41 (*P aeruginosa* PA14; A) and SZU204 (*P aeruginosa* PA14 with *rpoBC* gene knockdown system; B), and green fluorescent protein (GFP) expression in SZU203 (*P aeruginosa* PA14 with *gfp* gene knockdown system; C) with and without isopropyl β-d-1-thiogalactopyranoside (IPTG) induction. Growth curve of SZU43 (*P aeruginosa* clinical isolate N18-5 in this study; D) and SZU205 (*P aeruginosa* N18-5 with *rpoBC* gene knockdown system; E), and tigecycline minimum inhibitory concentrations (MICs) of SZU205 and SZU206 (*P aeruginosa* N18-5 with *tmexD* gene knockdown system) with and without IPTG induction (F). Growth curve of SZU46 (*Proteus mirabilis* clinical isolate T1010 in this study; G), GFP expression in SZU207 (*Proteus mirabilis* T1010 with *gfp* gene knockdown system; H), and tigecycline MICs of SZU207 and SZU208 (*Proteus mirabilis* T1010 with *tmexD* gene knockdown system; I) with and without IPTG induction.
1 cycle=10 min.

the highly diverse genetic context of *tmexCD-toprJ* and its inter-host transmission potential with help of mobile elements and site-specific recombinases suggested its further dissemination among Aeromonadaceae, Morganellaceae, Enterobacteriaceae, Pseudomonadaceae, and even broader hosts (ie, bacterial isolates belonging to other families) is likely, presenting a new and severe challenge for clinical management.

Pseudomonas spp and *Proteus* spp are well known for encoding RND efflux pumps associated with intrinsic resistance phenotypes, but the importance of *tmexCD-toprJ* in these species has not been established in previous studies. By knocking down the *tmexD* gene using an IPTG-inducible CRISPRi system, we provide evidence to show that *tmexCD-toprJ* could increase tigecycline resistance in both species (by four times in this study). TMexCD1-TOprJ1 was reported to increase MICs of tetracyclines, tigecycline, quinolones, and cephalosporins for Enterobacteriaceae by 4–32 times and, for TMexCD2-TOprJ2, the increase in MICs was reported to be by 2–8 times.[10,11,25] Yet antimicrobial susceptibility testing of the *tmexCD-toprJ*-positive isolates in our study suggested that the carriage of *tmexCD-toprJ* does not necessarily render clinical isolates (including *Klebsiella* spp, *Pseudomonas* spp, and *Proteus* spp) resistant to cephalosporin, tetracycline, and tigecycline.[25,26] This finding could be related to the carriage of different variants of *tmexCD-toprJ*, the difference in expression level of *tmexCD-toprJ*, and differences in the host strains. A previous study has shown that the expression of MexCD-OprJ, a homolog of TMexCD-TOprJ, is regulated by its adjacent transcriptional regulator NfxB.[11] We therefore speculate that the different expression levels of *tmexCD-toprJ* could be associated with its upstream

regulator, *tnfxB*. All clinical *tmexCD-toprJ*-positive bacteria (except a *P aeruginosa* strain) were resistant to the fluoroquinolone antibiotic ciprofloxacin, suggesting that *tmexCD-toprJ* tends to be tightly associated with quinolone resistance among clinical hosts. In addition, the coexistence of last-line antibiotic resistance determinants carbapenemases genes and *mcr* with *tmexCD-toprJ* among Enterobacteriaceae and Pseudomonadaceae pose a large public threat.[27–29]

We acknowledge the limitations of the current study. First, the number of species and number of clinical bacteria collected for *tmexCD-toprJ* screening was low. More *tmexCD-toprJ*-positive species could be detected with a larger strain collection. Second, this is a retrospective study and some information on the collected isolates was missing. The clonal spread of *tmexCD-toprJ*-positive strains in local hospitals could not be analysed, which would have been useful for explaining the high carriage rate observed in one province (Zhejiang).

In conclusion, to the best of our knowledge, this study has reported the first nationwide surveillance and molecular characterisation of *tmexCD-toprJ*-positive Gram-negative bacteria, with the largest collection of clinical bacteria to date. *tmexCD-toprJ*-positive bacteria are rare in clinical settings. Nevertheless, given its properties of transmission and the continuous application of antimicrobial agents in clinical settings, the further propagation of *tmexCD-toprJ* among clinical bacteria is inevitable. Control measures should be prioritised and applied to prevent the further transmission of such bacteria.

Contributors

ND, ZS, SC, and RZ designed the project. ND conducted the bioinformatic computation and drafted the manuscript. YZ, CL, JL, CC, YuWu, YFa, YFu, YH, HZ, JC, and GC collected bacterial strains and conducted gene screening. XL and YC conducted the gene knockdown experiments. YaoW, FH, SW, YangW, and YoWu conducted the DNA extraction and whole genome sequencing experiments. ZS, SC, and RZ supervised the project and edited the manuscript. ND, YZ, YaoW, ZS, SC, and RZ verified the data. All authors commented on a draft of the manuscript and approved the final version. All authors had full access to all of the data in the study and had final responsibility for the decision to submit for publication.

Declaration of interests

We declare no competing interests.

Data sharing

All data generated or analysed in this study are available within the main Article and appendix. The newly sequenced 48 *temxCD-toprJ*-positive genomes have been submitted to the GenBank database under BioProject accession number PRJNA674484.

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References

1 WHO. Antibiotic resistance. 2020. https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance (accessed Feb 1, 2021).
2 Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ. Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol* 2015; **13:** 42–51.
3 Du D, Wang-Kan X, Neuberger A, et al. Multidrug efflux pumps: structure, function and regulation. *Nat Rev Microbiol* 2018; **16:** 523–39.
4 Alav I, Sutton JM, Rahman KM. Role of bacterial efflux pumps in biofilm formation. *J Antimicrob Chemother* 2018; **73:** 2003–20.
5 Lowrence RC, Subramaniapillai SG, Ulaganathan V, Nagarajan S. Tackling drug resistance with efflux pump inhibitors: from bacteria to cancerous cells. *Crit Rev Microbiol* 2019; **45:** 334–53.
6 Aron Z, Opperman TJ. The hydrophobic trap—the Achilles heel of RND efflux pumps. *Res Microbiol* 2018; **169:** 393–400.
7 Fernando DM, Kumar A. Resistance-nodulation-division multidrug efflux pumps in gram-negative bacteria: role in virulence. *Antibiotics* 2013; **2:** 163–81.
8 Du D, Wang Z, James NR, et al. Structure of the AcrAB–TolC multidrug efflux pump. *Nature* 2014; **509:** 512–15.
9 Puzari M, Chetia P. RND efflux pump mediated antibiotic resistance in Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*: a major issue worldwide. *World J Microbiol Biotechnol* 2017; **33:** 24.
10 Wang C-Z, Gao X, Yang Q-W, et al. Novel transferable resistance-nodulation-division pump gene cluster *tmexCD2-toprJ2* that confers tigecycline resistance in *Raoultella ornithinolytica*. *Antimicrob Agents Chemother* 2021; **65:** e02229-20.
11 Lv L, Wan M, Wang C, et al. Emergence of a plasmid-encoded resistance-nodulation-division efflux pump conferring resistance to multiple drugs, including tigecycline, in *Klebsiella pneumoniae*. *Mbio* 2020; **11:** e02930-19.
12 Sun S, Gao H, Liu Y, et al. Co-existence of a novel plasmid-mediated efflux pump with colistin resistance gene mcr in one plasmid confers transferable multidrug resistance in *Klebsiella pneumoniae*. *Emerg Microbes Infect* 2020; **9:** 1102–13.
13 He R, Yang Y, Wu Y, et al. Characterization of plasmid-encoded resistance-nodulation-division efflux pump in *Klebsiella pneumoniae* and *Klebsiella quasipneumoniae* from patients in China. *Antimicrob Agents Chemother* 2020; **65:** e02075-20.
14 Seemann T. MLST—Scan contig files against PubMLST typing schemes. 2016. https://github.com/tseemann/mlst (accessed April 28, 2022).
15 Whitman WB, Rainey F, Kämpfer P, et al. Bergey's manual of systematics of archaea and bacteria. East Lansing, MI: Wiley, 2016.
16 Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. Wayne, New Jersey: Clinical and Laboratory Standards Institute, 2021.
17 Magiorakos AP, Srinivasan A, Carey R, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012; **18:** 268–81.
18 Gu D, Dong N, Zheng Z, et al. A fatal outbreak of ST11 carbapenem-resistant hypervirulent *Klebsiella pneumoniae* in a Chinese hospital: a molecular epidemiological study. *Lancet Infect Dis* 2018; **18:** 37–46.
19 Zhang R, Dong N, Shen Z, et al. Epidemiological and phylogenetic analysis reveals Flavobacteriaceae as potential ancestral source of tigecycline resistance gene *tet* (X). *Nat Commun* 2020; **11:** 4648.
20 Liu X, Gallay C, Kjos M, et al. High-throughput CRISPRi phenotyping identifies new essential genes in *Streptococcus pneumoniae*. *Mol Syst Biol* 2017; **13:** 931.
21 Hirabayashi A, Van Nguyen A, Nguyen ST, Shibayama K, Suzuki M. Emergence of a plasmid-borne tigecycline resistance in *Klebsiella pneumoniae* in Vietnam. *J Med Microbiol* 2021; **70:** 001320.
22 Subedi D, Kohli GS, Vijay AK, Willcox M, Rice SA. Accessory genome of the multi-drug resistant ocular isolate of *Pseudomonas aeruginosa* PA34. *PloS one* 2019; **14:** e0215038.
23 Peng K, Wang Q, Yin Y, et al. Plasmids shape the current prevalence of *tmexCD1-toprJ1* among *Klebsiella pneumoniae* in food production chains. *Msystems* 2021; **6:** e00702-21.
24 Wan M, Gao X, Lv L, Cai Z, Liu J-H. IS 26 mediates the acquisition of tigecycline resistance gene cluster *tmexCD1-toprJ1* by IncHI1B-FIB plasmids in *Klebsiella pneumoniae* and *Klebsiella quasipneumoniae* from food market sewage. *Antimicrob Agents Chemother* 2020; **65:** e02178-20.

25 Wang Q, Peng K, Liu Y, Xiao X, Wang Z, Li R. Characterization of TMexCD3-TOprJ3, an RND-type efflux system conferring resistance to tigecycline in *Proteus mirabilis*, and its associated Integrative Conjugative Element. *Antimicrob Agents Chemother* 2021; **65:** e02712-20.

26 Liu C, Wu Y, Fang Y, et al. Emergence of an ST1326 (CG258) MDR *Klebsiella pneumoniae* co-harboring *mcr-8.2*, ESBL genes, and the RND efflux pump gene cluster *tmexCD1-toprJ1* in China. *Front Microbiol* 2022; **17:** 800993.

27 Li R, Peng K, Xiao X, Liu Y, Peng D, Wang Z. Emergence of a multidrug resistance efflux pump with carbapenem resistance gene *bla* VIM-2 in a *Pseudomonas putida* megaplasmid of migratory bird origin. *J Antimicrob Chemother* 2021; **76:** 1455–58.

28 Hirabayashi A, Dao TD, Takemura T, et al. A transferable IncC-IncX3 hybrid plasmid cocarrying *bla* NDM-4, *tet* (X), and *tmexCD3-toprJ3* confers resistance to carbapenem and tigecycline. *Msphere* 2021; **6:** e00592-21.

29 Wang C-Z, Gao X, Lv L-C, Cai Z-P, Yang J, Liu J-H. Novel tigecycline resistance gene cluster *tnfxB3-tmexCD3-toprJ1b* in *Proteus* spp. and *Pseudomonas aeruginosa*, co-existing with *tet* (X6) on an SXT/R391 integrative and conjugative element. *J Antimicrob Chemother* 2021; **76:** 3159–67.