Emergence of the resistance-nodulation-division efflux pump tmexCD3-toprJ3 gene confers resistance to tigecycline in Pseudomonas juntendi and Proteus terrae isolated from a pig farm in China

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Abstract

Background: Tigecycline is regarded as a last-resort antimicrobial agent against multidrug resistance (MDR). However, resistance-nodulation-division efflux pump resistance genes, such as tmexCD3-toprJ3, that confer resistance to tigecycline have emerged.

Objectives: This study aimed to characterise the tmexCD3-toprJ3 gene cluster in Pseudomonas juntendi and Proteus terrae isolated from a pig farm.

Methods: Samples were obtained from a Chinese piggery and included 92 pig faecal samples, 56 wastewater samples, 23 drinking water samples, 28 sow vagina samples and nine sow uterus swabs. The presence of tmexCD3-toprJ3 was detected using a polymerase chain reaction assay, and the antimicrobial susceptibility profile of the tmexCD3-toprJ3-positive isolates was determined using the broth dilution method. Genomic locations were identified using whole-genome sequencing and bioinformatics.

Results: We identified two tmexCD3-toprJ3-positive isolates, and both isolates were highly resistant to antibiotics such as tigecycline. In addition, we identified several mobile elements (ISPa7, ISCfr1, ISVsa3) and insertion sequences (TnAs2, TnAs3) in tmexCD3-toprJ3-positive isolates that could increase the potential for the spread of MDR.

Conclusions: To the best of our knowledge, this study is the first to report the detection of tmexCD3-toprJ3 in P. juntendi and P. terrae isolated from a pig farm.

KEYWORDS
Proteus terrae, Pseudomonas juntendi, resistance-nodulation-division, tigecycline, tmexCD3-toprJ3
1 INTRODUCTION

In China, tetracyclines such as chlortetraycline and oxytetracycline are widely used in livestock farming owing to their low price and broad antibacterial spectrum (Felmingham, 2005). Accordingly, various resistance mechanisms against tetracyclines have emerged, such as the tetracycline-specific efflux pump acquisition and ribosomal protection (Grossman, 2016). Tigecycline, a new type of glycycline antibiotic, can effectively avoid the above resistance mechanisms and is considered the last line of defence against multidrug resistance (MDR; Zhai et al., 2022). Recently, the emergence of two novel tet(X) variants, tet(X3) and tet(X4), poses a serious threat to human health (He et al., 2021; Sun et al., 2019). Moreover, a novel plasmid-mediated resistance-nodulation-division (RND) superfamily efflux pump gene cluster tmexCD1-toprJ1 was detected in Klebsiella pneumonia, conferring high resistance to tigecycline (Lv et al., 2020).

RND superfamily efflux pumps play a significant role in the intrinsic and acquired resistance of gram-negative pathogens to antibiotics (Ruggerone et al., 2013), and MexCD-OprJ efflux pump is one example of such pumps (Masuda et al., 2000). Subsequently, based on the tmexCD1-toprJ1 study, mutants tmexCD2-toprJ2 and tmexCD3-toprJ3 were identified in Raoultella ornithinolytica and Proteus mirabilis, respectively (Wang, Gao, Lv et al., 2021; Wang, Gao, Yang et al., 2021). Here, we report the emergence of the tmexCD3-toprJ3 gene in Pseudomonas juntendi and P. terrae isolated from a pig farm in China. We identified several mobile elements and insertion sequences (IS) in tmexCD3-toprJ3-positive isolates that could increase the potential for the spread of MDR.

2 MATERIALS AND METHODS

2.1 Sample collection, isolation and identification

In July 2020, 208 samples (92 pig faecal samples, 56 wastewater samples, 23 drinking water samples, 28 sow vagina samples and nine sow uterus swabs) were collected from a pig farm (comprising 3800 sows, 6000 piglets and 1000 fattening pigs) in Hunan Province, China, by ESwab Collection and Transport System (Copan). All samples are stored in ice packs after collection and delivered to the laboratory within 3–4 h. All samples were enriched in 1 ml of Luria broth containing 4 mg/L tetracycline and 30 mg/L vancomycin at 37°C overnight. Enrichment liquid was inoculated onto China-blue agar with 2 mg/L tigecycline at 37°C overnight. One random colony was selected for tigecycline-resistant isolates, and tmexCD3-toprJ3 gene was detected as previously described (Wang, Peng et al., 2021). Species identification was performed via 16s rRNA gene sequencing (Wang, Peng et al., 2021). The polymerase chain reaction products were confirmed using Sanger sequencing and Basic Local Alignment Search Tool (BLAST) analysis. MexCD-OprJ genes were detected using a universal primer as previously described (Hirabayashi et al., 2021).

2.2 Antimicrobial susceptibility testing

TmexCD3-toprJ3-positive isolates were subjected to antimicrobial susceptibility testing for nine antimicrobial agents (ciprofloxacin, ampicillin, meropenem, cefotaxime, gentamicin, trimethoprim/sulfamethoxazole, ceftazidime, tetracycline and tigecycline) using the agar dilution method and interpreted according to the American Clinical and Laboratory Standards (CLSI 2020). Escherichia coli strain ATCC 25922 served as the quality control strain (Wang, Peng et al., 2021).

2.3 Genomes sequencing and bioinformatic analysis

The isolates containing tmexCD3-toprJ3 were subjected to whole-genome sequencing. DNA was extracted from the isolate containing tmexCD3-toprJ3 using the TIANamp Bacteria DNA kit DP302 (Tiangen Biotech). The whole-genome sequence of strains was determined using Illumina HiSeq 2500 (Annoroad Genomics Co.) and the MinION platform (Oxford Nanopore Technologies). Unicycler software was used for the hybrid assembly of short and long reads (Wick et al., 2017).

2.4 Bioinformatics analysis

Antibiotic resistance genes were identified by the Center for Genomic Epidemiology (CGE) (https://cge.cbs.dtu.dk/services/ResFinder/). Moreover, the presence of the efflux pump gene cluster, tmexCD3-toprJ3, was confirmed by the BLAST at the National Center of Biotechnology Information Center genome (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and virulence genes were identified using the Virulence factors of Bacterial Pathogens database (http://www.mgc.ac.cn/VFs/main.htm). The complete genome sequence was annotated using PATRIC3.6.9 (https://patricbrc.org/). Finally, the genomic environment of tmexCD3-toprJ3 was determined using Easyfig 2.2.3 (http://mjsull.github.io/Easyfig/).

3 RESULTS

3.1 Bacterial isolation and antimicrobial susceptibility profiles

Two tmexCD3-toprJ3-positive isolates were identified as P. juntendi and P. terrae, using the 16s RNA gene, collected from the vagina of a sow in the farrowing house and pig faecal samples in the finishing house, respectively. To the best of our knowledge, this is the first report of the presence of tmexCD3-toprJ3 in P. juntendi and P. terrae. Both strains showed high minimum inhibitory concentration (MIC) for...
3.2 | Genetic context of *tmexCD3-toprJ3* gene

The complete genome size of *P. juntendi* K37-3 and *P. terrae* 20Q172tw is 5,582,866 and 4,200,367 bp, with 62.3% and 38.5% Guanine and Cytosine content, respectively. The fragments of *tmexCD3-toprJ3* located in the chromosome of *P. juntendi* K37-3 and *P. terrae* 20Q172tw were 100% or 99.98% identical to the *tmexCD3-toprJ3* gene cluster in *Pseudomonas* sp. (Genbank accession no. CP045554) or *Aeromonas caviae* (Genbank accession no. CP039832.1) chromosome. In comparison, they were 97.8% identical to the first reported *tmexCD1-toprJ1* in the *K. pneumonia* plasmid AMH7V81 (Genbank accession no. MK347425) and only 78.1% similar to *mexCD1-oprJ1* in the chromosome of *P. aeruginosa* strain PAO1 (Dean et al., 2003; Genbank accession no. AE00409). Compared to *tmexCD2-toprJ2* (Genbank accession no. MN175502) in *R. ornithinolytica*, they showed 96.8% identity (Figure 1). Furthermore, they were 100% identical to the *tmexCD3-toprJ3* in *P. mirabilis*. Although our reported *tmexCD3-toprJ3* gene cluster existed on chromosomes, these two strains were identical to the *tmexCD3-toprJ3* gene cluster reported on plasmids rather than PAO1.

Using the ISfinder tool (https://www-is.biotoul.fr/), two genes encoding site-specific integrases, which can capture and co-transfer large DNA fragments (Chen et al., 2018), were located upstream of the *tmexCD3-toprJ3* gene (Figure 1). Meanwhile, two IS belonging to the *Tn* family (*TnAas2, TnAas3*) and one insertion sequence belonging to the *IS1182* family (*ISPa7*) were identified in *P. juntendi*, while two IS, *ISCfr1* and *ISVsa3*, were found in *P. terrae* originating from the *IS1182* and *IS91* families, respectively (Figure 1).

3.3 | Antimicrobial resistance genes and virulence factor genes

Through analysis at the CGE, sulphonamide resistance genes (*sul1* and *sul2*), streptomycin resistance genes (*aadA2*), tetracycline resistance genes [tet(H)] and florencil resistance genes (floR) were identified in *P. terrae* 20Q172tw. Meanwhile, tetracycline resistance genes [tet(A) and tet(R)], sulphonamide resistance genes (*sul1*) and streptomycin resistance genes (aadA13) were identified in *P. juntendi* K37-3. The tet(A) gene segments of *P. juntendi* K37-3 isolate showed 100% identity to the tet(A) gene from *P. aeruginosa* RP4 (NG_048148.1), and tet(A) was the critical influencing factor for increasing the resistance ability of RND efflux pumps according to a previous study (Xu et al., 2021). Additionally, a major facilitator superfamily efflux pump gene, floR, conferring
TABLE 1 Antimicrobial resistance genes and virulence genes in tmexCD-toprJ3-positive isolates

| Strain     | Species      | Source          | GC content | Antimicrobial resistance genes | Virulence genes |
|------------|--------------|-----------------|------------|-------------------------------|-----------------|
| K37-3      | Pseudomonas  | Pig vagina      | 62.31%     | tet(A), aph(6')-Ib, aph(3')-Ib, aac(6')-Ila, sul1, aadA13, sulI | pilA, pilC, pilD, pilE, pilF, pilM, pilN, pilP, pilQ, pilR, pilT, flgG, flgI, flaA, flil, flIP |
| 20Q172tw   | Proteus terrae | Pig feces       | 38.54%     | tet(H), sul1, sul2, adaA2, dfrA12, aph(3')-Ib, aph(6')-Ib, aph(3')-Vla, aph(3')-Ia, aph (4)-Ia, insU(F), aac(3)-IV, floR | pilR, pilT, flgG, flgI, flaA, flil, flIP |

In conclusion, Proteus terrae, resistance to chloramphenicol and florfenicol resistance (Hua et al., 2014), was identified in both P. juntendi K37-3 and P. terrae 20Q172tw.

Genes encoding the pilus (pilA, pilC, pilD, pilE, pilF, pilM, pilN, pilP, pilQ, pilT) were found in P. juntendi, and pilR and pilT genes were detected in P. terrae 20Q172tw. Adherence factors (flgG, flgI, flaA, flil, flIP) were observed in P. juntendi K37-3 and P. terrae 20Q172tw-2 (Table 1). Fim operon genes encoding type IV fimbrilae were detected in both isolates. These fimbrilae, common in P. aeruginosa and essential in establishing respiratory tract infection, may act as a tether in initial interactions with epithelial membranes (Feldman et al., 1998). Furthermore, type IV pili are an efficient and versatile device for bacterial surface motility and are multifunctional complexes that can act as bacterial virulence factors because pilus-based motility can be used to spread pathogens over a tissue’s surface or build multicellular structures such as biofilms and fruiting bodies (Nudleman & Kaiser, 2004).

4 | DISCUSSION

In this study, we initially aimed to investigate the epidemiology of efflux pump resistance genes at different stages of pig growth and under different growth environments; however, we found only two positive strains. One of the strains, P. juntendi K37-3, was isolated from a freshly collected pig faecal sample, and the other strain, P. terrae 20Q172tw strain, was isolated from the vagina of a sow. It is worth noting that despite our strict aseptic sampling, positive strains from pig faecal/vaginal samples are also likely to originate from environmental flora, which poses a more serious challenge for the monitoring and control of drug resistance in pig farms. Furthermore, both tmexCD-toprJ3-positive isolates exhibited high resistance to tigecycline. To the best of our knowledge, tigecycline is not a commonly used drug for clinical treatment in pig farms in China. In addition, 20 breeders and six technicians on the farm did not have any records of antibiotic use within half a year. Therefore, we speculated that this may be due to the widespread use of tetracycline for disease prevention and treatment due to its broad antimicrobial spectrum and low price, which leads to cross-resistance. In recent reports, several RND efflux pumps, including MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM have been suggested as important mechanisms underlying drug resistance in P. aeruginosa (Yaghoubi et al., 2021), and we report for the first time the presence of tmexCD-toprJ3 in P. juntendi and P. terrae. IS are abundant mobile genetic elements on bacterial genomes, responsible for the mobilisation of many antimicrobial resistance genes (Razavi et al., 2020). The IS close to tmexCD-toprJ3 provides the potential to transfer the resistance gene cluster (Chen et al., 2018). In addition, we speculate that P. juntendi and P. terrae may be intermediate carriers of MexCD-oprJ transmission to other P. aeruginosa or P. chromosomes; however, more experiments and data are required to support this speculation. Furthermore, the presence of multiple virulence factors in the resistant isolates studied may indicate an increased potential for these strains to colonise the human host and cause infection (Ratajczak et al., 2021). This can significantly enhance the difficulty of clinical drug selection and treatment with intense antibiotic resistance and high pathogenicity (Zwama & Nishino, 2021). Genetic data alone are insufficient to determine the virulence of these isolates, particularly as the contribution of each virulence factor is difficult to determine; therefore, these data should be interpreted carefully.

The irrational use of antibiotics can lead to a large amount of drug resistance in the pig intestine. The residual antibiotics and drug-resistant bacteria can be discharged into the environment via faeces, placing vast antibiotic pressure on the environment owing to the spread of the resistance gene (Karkman et al., 2019). The emergence of tmexCD-toprJ3 in pig farms will further escalate the increasingly severe problem of drug resistance.

5 | CONCLUSION

In conclusion, tmexCD-toprJ3 efflux pump cluster conferred MDR, including resistance to tigecycline, demonstrating pathogenicity and transmission. Our research expands the existing knowledge of tmexCD-toprJ3 resistance genes in P. juntendi and P. terrae strains. Further studies should focus on the characterisation and metastability of tmexCD-toprJ3 gene and investigate practical approaches to eliminate resistance.

AUTHOR CONTRIBUTIONS

Conceptualisation, data curation, formal analysis, software, writing—original draft, writing—review and editing: Jie Yang.

Data curation, formal analysis: Ziyue Zeng. Data curation, investigation: Jufang Hu. Data curation: Zhihong Liu.

Software: Jinrong Gu. Investigation, supervision: Xiaojun Chen. Conceptualisation, funding acquisition, project administration, resources, writing—original draft, writing—review and editing: Zhiliang Sun. Conceptualisation, data curation, formal analysis, investigation, methodology, software, writing—original draft, writing—review and editing: Jiyun Li.
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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All genome assemblies of the Pseudomonas juno"ntendi K37-3 and Proteus terrea 20Q172tw strains were deposited in GenBank under the BioSample accession number SAMN20206206 and SAMN20206207.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal’s author guidelines page, have been adhered to. No ethical approval was required, as this is a review article with no original research data.

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