Global dissemination of *tet*(X3) and *tet*(X6) among livestock-associated *Acinetobacter* is sporadic and mediated by highly diverse plasmidomes

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Abstract

The emergence of plasmid-borne *tet*(X) genes mediated high-level resistance of tigecycline largely threatening its clinical effectiveness. Currently, the dissemination pattern of plasmid-borne *tet*(X) genes remains unclear. In this study, 684 fecal and environmental samples were collected at six livestock farms, and 15 *tet*(X)-positive *Acinetobacter* isolates were recovered, mainly including 9 *tet*(X3)- and 5 *tet*(X6)-positive *A. towneri* strains. A clonal dissemination of *tet*(X3)-positive *A. towneri* was detected in a swine farm, while the *tet*(X6)-positive *A. towneri* strains mainly sporadically disseminated in the same farm. A *tet*(X3)-carrying plasmid (pAT181) was self-transmissible from a tigecycline-susceptible *A. towneri* strain to *A. baumannii* ATCC17978, causing a 128-fold and 64-512-fold increase in the MIC values of tigecycline and the other tetracyclines, respectively. Worrisomely, pAT181 was stably maintained and increased the growth rate of ATCC17978. Further identification of *tet*(X)s in 10,680 *Acinetobacter* genomes retrieved from GenBank revealed that, *tet*(X3) (n=249) followed by *tet*(X5)-like (n=61) and *tet*(X6) (n=53) are the prevalent alleles mainly carried by four species, and most of them are livestock associated. Phylogenetic analysis showed that most of *tet*(X3)- and *tet*(X6)-positive isolates disseminate sporadically. The structures of *tet*(X3) and *tet*(X6) plasmidomes are highly diverse and no epidemic plasmids have emerged yet. However, cross-species and cross-region transmissions of *tet*(X3) might have been mediated by several plasmids in a small proportion of strains. Our study evidence that *tet*(X3) and *tet*(X6)

currently disseminate sporadically in *Acinetobacter*. Continuous surveillance for *tet*(X)s in the context of One Health is necessary to prevent them from transmitting to humans.

Keywords: tigecycline resistance, *tet*(X3), *tet*(X6), *Acinetobacter*, self-transmissible plasmid

Importance

Recently identified plasmid-borne *tet*(X) genes highly challenged the efficiency of tigecycline, a last resort antibiotic for severe infection. Currently, the dissemination pattern of plasmid-borne *tet*(X) genes remains unclear. In this study, we first identified plasmid-borne *tet*(X)-positive *Acinetobacter* spp. strains from fecal and environmental samples collected at six livestock farms. A clonal dissemination of *tet*(X3)-positive *A. towneri* was detected in a swine farm, while the *tet*(X6)-positive *A. towneri* strains mainly disseminated sporadically in the same farm. A *tet*(X3)-carrying plasmid was found self-transmissible resulting in enhanced tigecycline resistance and growth rate. Further exploring a global dataset of *tet*(X)-positive *Acinetobacter* genomes retried from GenBank revealed that most of *tet*(X3) and *tet*(X6)-positive isolates share highly distant relationship, and the structures of *tet*(X3) and *tet*(X6) plasmidomes are highly diverse. Our study evidence that *tet*(X3) and *tet*(X6) disseminate sporadically in *Acinetobacter* and continuous surveillance for *tet*(X)s in the context of One Health is necessary.

Introduction

Tigecycline is used to treat a wide range of clinical infection caused by Gram-positive and Gram-negative bacteria with multidrug resistance (MDR). With the global dissemination of carbapenemases and MCRs in recent years, this broad-spectrum tetracycline-family antibiotic has been raised to be a last line treatment regimen in clinical settings (1-6). However, the recent discoveries of transferable tigecycline inactivation genes [*tet*(X)s] particularly threaten the clinical efficacy of tigecycline (7, 8).

The first flavin-dependent monooxygenase gene *tet*(X) was identified in Tn*4351* and Tn*4400* encoded on the chromosome of *Bacteroides fragilis* in 1990 (9). Subsequently, numerous chromosome-encoded and plasmid-mediated *tet*(X) alleles, *tet*(X1) to *tet*(X14), have been reported in various species originating from animals, humans and environments (10-12). These Tet(X) variants, except Tet(X1), exhibited different levels of activity against almost all tetracyclines, including the fourth generation tetracycline (eravacycline) approved by the Food and Drug Administration (FDA) in 2018 (4, 12, 13). Remarkably, the first findings of plasmid-borne *tet*(X3) and *tet*(X4) identified in livestock-associated *Acinetobacter baumannii* and *Escherichia coli* strains in 2019 (7), respectively, raise the concern of horizontal transfer of tigecycline resistance. Since then, additional *tet*(X) alleles have been reported to be plasmid-borne, including *tet*(X5) and *tet*(X6) and their variants. Epidemiological

studies reveal that these novel *tet*(X) orthologs have mainly circulated in animals in China due to the heavy uses of tetracyclines in husbandry (8). However, plasmids are currently rarely reported to be the transmissible vectors of *tet*(X)s although an increasing number of plasmid-borne *tet*(X)s has been detected. In some pioneer studies, IS*CR2* is highlighted to be the key element facilitating the horizontal transfer of *tet*(X)s through circular intermediates (14-17). Therefore, the role of plasmids in the dissemination of *tet*(X)s remains obscure.

Surveillance studies show that the *tet*(X) alleles have been detected in over 16 bacterial species with *Acinetobacter* spp. to be the predominate one, and *tet*(X4) is the only allele primarily detected in *E. coli* with a low prevalence (7, 11, 17-20). The *tet*(X)-positive *Acinetobacter* spp. isolates are mainly recovered from dairy cows, chickens and pigs in China (16, 21), and plasmid-borne and/or chromosomal-encoded *tet*(X3) and *tet*(X6) are prevalent among *Acinetobacter* spp. strains isolated from both humans and animals (7, 16, 20, 22, 23). A surveillance at avian farms showed that 1.6-18.3% *Acinetobacter* spp. strains were *tet*(X)-positive among seven provinces in China (23). Another surveillance for tigecycline-resistant *Acinetobacter* spp. from 2015 to 2018 in 14 provinces and municipalities in China reported that 2.3-25.3% *tet*(X)-positive isolates from pig farms, migratory birds and human samples were identified in 9 provinces (20). Currently, *tet*(X5) is solitarily detected in an *A. baumannii* strain from humans (22). However, it is unclear how the plasmid-borne *tet*(X)s disseminate among *Acinetobacter* spp., i.e. vertical transfer (clonal dissemination), horizontal transfer, and sporadic dissemination.

In this study, a surveillance of *tet*(X)-positive *Acinetobacter* spp. recovered from livestock and their surrounding environmental sources was performed at six livestock farms locating in Zhejiang province in 2019. The epidemiological and genetic characterizations of *tet*(X)-positive isolates and *tet*(X)-harboring plasmids were dissected. We further comprehensively investigated the population structure and distribution of *tet*(X)-positive *Acinetobacter* strains identified in the public database, as well as the plasmidome of *tet*(X3) and *tet*(X6).

Results

A. towneri was the prevalent species carrying *tet*(X) genes among *Acinetobacter* strains collected in this study

Two hundred and ninety-two strains were recovered from 534 stool samples and 150 environmental samples collected from 2 swine farms, 2 dairy farms and 2 sheep farms, including 215 strains of *Acinetobacter* spp. and 77 strains belonging to other species. PCR screens of *tet*(X)s identified 23 positive isolates (7.88%; 23/292), including 15 *Acinetobacter* spp. isolates (6.88%; 15/218), 3 *Myroides odoratimimus* isolates and 5 *Empedobacter stercoris* isolates (Table 1). The 23 *tet*(X)-positive strains were exclusively isolated from swine farms. Twenty strains were recovered from the fecal samples of swine farm 1, and the 3 *M. odoratimimus* strains were from the soil samples of swine farm 2.

ANI analysis assigned the 15 *tet*(X)-positive *Acinetobacter* spp. isolates to *A. towneri* (n=14) and an unnamed species (n=1) (Table 1), suggesting that *A. towneri*

was the prevalent species carrying *tet*(X)s in *Acinetobacter* spp. population circulating at swine farms. Four different *tet*(X) alleles were detected in the 23 isolates, including *tet*(X2) detected in 5 *E. stercoris* isolates and 3 *M. odoratimimus* isolates, *tet*(X3) in 9 *A. towneri* strains and 1 strain (ZJ199) belonging to the unnamed species, *tet*(X6) in 5 *A. towneri* strains, and *tet*(X14) in 2 *E. stercoris* strains (ES183 has been described previously (10)) (Table 1). One *A. towneri* strain (AT185) carried two copies of *tet*(X6). To our knowledge, this is the first report of two copies of *tet*(X6) identified in single strain. The phylogenetic analysis of 15 *tet*(X)-positive *Acinetobacter* spp. isolates showed that all but one *tet*(X3)-carrying *A. towneri* strains (8 out of 9) clustered together with 3-36 SNPs (Figure 1), suggesting a clonal dissemination of *tet*(X3) occurred in the swine farm. The other *tet*(X3)-carrying *A. towneri* strain AT200 clustered with the *tet*(X6)-carrying strains with 27,664-30,557 SNPs (Figure 1). All but two *tet*(X6)-positive strains showed distant relationship (26,876-31,071 SNPs), indicating that they disseminated sporadically.

Antimicrobial resistance profile of *tet*(X)-carrying isolates

AST results showed that 34.78% (8/23) of *tet*(X)-positive isolates were resistant to tigecycline with MIC values at 1-2 mg/L, and the other 15 isolates showed MIC values at 0.06-0.5 mg/L (Table 2). These tigecycline resistant strains encompass 4 *tet*(X3)-positive *A. towneri* isolates, 1 *tet*(X6)-positive *A. towneri* isolate, 2 *tet*(X2)- and *tet*(X14)-positive *E. stercoris* isolates and 1 *tet*(X2)-positive *M. odoratimimus* isolate. Five tigecycline-resistant strains (3 *A. towneri* isolates and 2 *E. stercoris* isolates)

additionally exhibited resistance to the newly FDA-approved eravacycline with MIC values at 1-4 mg/L. Except that the strain (AT185) carrying 2 copies of *tet*(X6) was susceptible to tetracycline, the other 14 *Acinetobacter* spp. strains were resistant to tetracycline with MIC values ≥ 16 mg/L (Table 2). Strain AT232 showed significantly higher resistance to tetracyclines than the other 13 strains, which might be caused by the presence of a two component system AdeSR involved in the expression of the AdeABC efflux pump (24). In addition, 26.7% (*n* = 4) and 13.3% (*n* = 2) *Acinetobacter* spp. strains showed resistance to ciprofloxacin and doxycycline, respectively (Table 2). All of *tet*(X)-positive *Acinetobacter* spp. isolates were susceptible to colistin and carbapenems. *M. odoratimimus* isolates were resistance to both colistin and carbapenems due to intrinsic resistance (25).

In silico analysis of ARGs among *Acinetobacter* spp. strains showed that the number of ARGs detected in the strain ZJ199 [*sul2* and *tet*(X3)] was much less than that in *A. towneri* strains (Figure 1). All of *A. towneri* strains were MDR, and more ARGs were detected in the *tet*(X6)-carrying clone (mean=8.67; median=9) than in the *tet*(X3)-carrying clone (mean=6; median=6) albeit not significant ($p > 0.05$) (Figure 1). The 8 strains of the *tet*(X3)-carrying clone shared an identical resistome [*aph(3")-Ib*, *aph(3')-Ia*, *aph(6)-Id, cmlB1*, *sul2* and *tet*(X3)], further supporting the clonal dissemination (Figure 1). While the resistome of the *tet*(X6)-carrying strains was highly diverse, including *aacC4*, *ant(3")-Ia* and *aph(4)-Ia* resistant to aminoglycoside; bla_{OXA-58} resistant to beta-lactam; *floR* resistant to phenicol; *dfrA1* resistant to trimethoprim; *erm(B)*, *mph(E)* and *msr(E)* resistant to macrolide; *tet*(X6) and *tet*(Y)

177 resistant to tetracyclines (Figure 1). The resistome of *E. stercoris* and *M.*
178 *odoratimimus* was different from that of *Acinetobacter* spp. (Table S1). *E. stercoris*
179 strains carried *tet*(X2) and *tet*(X14) resistant to tetracyclines; *mef*(C) and *mph*(G)
180 resistance to macrolide; and bla_{EBR-1} resistant to beta-lactam. *M. odoratimimus* strains
181 carried *tet*(X2) and *tet*(36) resistant to tetracyclines; *ereD* resistant to macrolide;
182 bla_{MUS-1} resistant to beta-lactam; and *sul2* resistant to macrolide (Table S1).
183
184 **_tet_(X3) and _tet_(X6) were harbored by various plasmids**
185 To understand the vectors of the two prevalent *tet*(X) alleles, i.e. *tet*(X3) and *tet*(X6),
186 the representative *tet*(X3)- and *tet*(X6)-carrying *Acinetobacter* spp. strains (AT181,
187 AT184, and ZJ199; AT232 and AT235) were chosen additionally for long-read
188 sequencing based on their antimicrobial resistance profiles and genetic environments
189 of *tet*(X)s. The hybrid assembly confirmed that *tet*(X3) and *tet*(X6) were plasmid-borne
190 in the four *A. towneri* strains, and a chromosome-encoded *tet*(X3) was detected in
191 strain ZJ199.
192 The *tet*(X3)-carrying plasmids detected in AT181 (pAT181) and AT184 (pAT184)
193 were identical with a size of 75,969-bp, and were circularized (confirmed by PCR).
194 These two plasmids were untypable with an average GC content of 42.5%. Multiple
195 ARG genes were carried by the two plasmids, including *aph(3')-Ia*, *aph(3")-Ib*,
196 *aph(6)-Id*, *sul2*, and *tet*(X3). Blast analysis of the nucleotide sequence of pAT181 in
197 GenBank showed that the best match was a transferable *tet*(X3)-harboring plasmid
198 p10FS3-1-3 (CP039146) (100% identity; 97% coverage) carried by a novel species of

Acinetobacter (20). Other plasmids sharing a high similarity with pAT181 included a *tet*(X5)-harboring plasmid pAB17H194-1 (99.95% identity; 86% coverage) carried by an *A. pittii* strain and a *tet*(X3)-harboring plasmid p18TQ-X3 (CP045132, 99.99% identity; 80% coverage) carried by an *A. indicus* strain. These data suggest that pAT181-like plasmids have disseminated among various species of *Acinetobacter*.

pAT181 was used as a reference to perform blast comparisons among our *tet*(X3)-carrying strains to evaluate the genetic similarities of the other *tet*(X3)-carrying plasmids. The results revealed a conserved backbone shared by *tet*(X3)-carrying plasmids harbored in the 8 clonal strains with a coverage and nucleotide-acid identity >90% (Figure S1A). The *tet*(X3)-carrying plasmid carried by strain AT200 showed a different plasmid backbone with identity >90% and coverage <50% to pAT181 (Figure S1A). The best match of pAT200 was p10FS3-1-3 with 58.77% coverage and 70% identity, indicating that pAT200 might be a novel plasmid.

The two *tet*(X6)-harboring circularized plasmids pAT232 and pAT235 showed as low as 38% coverage and 99.95% identity between each other, suggesting that they were two different plasmids. pAT232 was 186,508-bp in length with GC content of 41.03%. Blasting in GenBank showed that the best matches of pAT232 were a *tet*(X6)-carrying plasmid pAT205 (CP048015) (76% coverage and 99.99% identity) carried by an *A. towneri* strain AT205 isolated in the same swine farm (26), and a *tet*(X)-negative plasmid p19110F47-2 (CP046044) (70% coverage; 99.99% identity) carried by an *A. towneri* strain isolated from the pig. pAT235 was 124,466-bp in length with GC content of 41.16%. The best matches of pAT235 were pAT205 (49%

coverage; 100% identity) and a *tet*(X3)-harboring plasmid pGX7 (CP071772) (44% coverage and 99.95% identity) detected in an *A. towneri* strain isolated from the pig in China. These data suggest that pAT232 and pAT235 might originate from *A. towneri* associated with pigs.

When pAT232 was used as a reference to identify the plasmids of *tet*(X6) in the other *tet*(X6)-positive strains collected here, AT208 showed the highest similarity with pAT232 (77.84% coverage; 99.16% identity) (Figure S1B). When pAT235 was used as a reference, AT185 shared 100% coverage and 94.51% identity (Figure S1C), suggesting that a pAT235-like *tet*(X6)-encoding plasmid was harbored in AT185. Of note, AT185 was genetically distant from AT235 with 30,097 SNPs (Figure 1), suggesting that the horizontal transfer of pAT235-like plasmid might have occurred between the two strains. A pAT205-like *tet*(X6)-harboring plasmid was detected in AT208 when pAT205 was used as a reference (100% coverage; 96.48% identity) (Figure S1D). These results reveal that horizontal transfers of *tet*(X6)-carrying plasmids might have occurred in few strains.

Genetic environment of *tet*(X3) and *tet*(X6)

The genetic environment of plasmid-borne *tet*(X3) [ΔIS*CR2*-*xerD*-*tet*(X3)-*res*-IS*CR2*] detected in the 8 of 9 *A. towneri* strains was identical, which was highly similar with that of the prototype detected in *A. baumannii* strain 34AB (7) (Figure 2A). To fully understand the distribution of this genetic environment among *tet*(X3)-carrying *Acinetobacter* strains, we blasted it against 249 *tet*(X3)-carrying *Acinetobacter*

243 genomes retrieved from GenBank (see below), and results showed that 21.3%
244 (53/249) genomes carry the fragment ΔIS*CR2*-*xerD*-*tet*(X3)-*res*-IS*CR2* locating on a
245 single contig with >90% identity and >90% coverage. The proportion increased to
246 86.35% (215/249) when matches on different contigs were counted together, implying
247 that this might be the major structure encoding *tet*(X3) in *Acinetobacter* spp.. A
248 different genetic environment of *tet*(X3) [IS*4*-IS*4*-*tet*(X3)-*res*-ΔIS*CR2*] was detected on
249 the chromosome of strain ZJ199, in which IS*CR2* and *xerD* located at the upstream of
250 *tet*(X3) were replaced by two copies of IS*4* (Figure 2A). Inspection of the wider context
251 of *tet*(X3) in strain ZJ199 showed that two copies of IS*4* adjacent to *sul2* and *glmM*
252 located at the downstream of ΔIS*CR2* as found in an *A. indicus* strain AI2 (16) (Figure
253 2A). This results in a putative IS*4* bracketed transposon, which might be responsible
254 for the mobilization of *tet*(X3) and *sul2.*

255 The genetic environment of *tet*(X6) was much more diverse than that of *tet*(X3)
256 detected in our collection (Figure 2B). A 7,270-bp composite structure
257 [ΔIS*CR2*-IS*30*-*tet*(X6)-*abh*-*guaA*-IS*CR2*] was detected in pAT232, which is similar
258 with the prototype [ΔIS*CR2*-*tet*(X6)-*abh*-*guaA*-IS*CR2*] identified in pAT205 and a
259 *Proteus* genomospecies 6 strain (26, 27), except for the insertion of an IS*30* (Figure
260 2B). The *tet*(X6) located within a 6,885-bp region [IS*CR2*-*fabF*-*tet*(X6)-*abh*-*glmM*-*sul2*]
261 in pAT235 (Figure 2B), which shares 100% coverage and 99.58% identity with that
262 detected on the chromosome of an *A. indicus* strain Q186-3_T and 100% coverage
263 and 98.70% identity with pABF9692 carried by an *A. baumannii* strain (CP048828). In
264 strain AT185, the genetic context of one copy of *tet*(X6) was identical to that detected

in pAT235, and a truncated structure was found for the other copy (Figure 2B). The IS*CR2-fabF-tet*(X6)-*abh* fragment was also found on the chromosome of *A. indicus* strain LYS68A (CP070997) and *A. baumannii* strain 31FS3-2 (CP0445177), indicating that this structure might mediate the mobilization of *tet*(X6) on the plasmid and chromosome of *Acinetobacter* spp..

A *tet*(X3)-carrying plasmid is self-transmissible from *A. towneri* to *A. baumannii* and increased the resistance to tetracyclines and growth rate

Conjugation assay was performed to test the transferability of *tet*(X)-encoding plasmids. We only obtained tigecycline-resistant transconjugants of *A. towneri* strain AT181 with frequencies at 1.85×10^{-6} per recipient cell. Multiple attempts of plasmid transfers failed when *E. coli* strain EC600 was used as a recipient. Compared with that of the recipient strain ATCC17978, the MIC value of tigecycline and the other tetracyclines against the transconjugant ATCC17978-pAT181 increased by 128-fold and 64~512-fold, respectively (Table S2). To understand the transmission pattern of *tet*(X3) (i.e., by plasmid or by a circular form), WGS were performed for ATCC17978-pAT181 and ATCC17978 to detect the transferrable structure of *tet*(X3). A unique plasmid pAT181 was detected in the transconjugant ATCC17978-pAT181, demonstrating that the transmission of tigecycline resistance was mediated by pAT181 (Figure S2). This is different from another self-transmissible *tet*(X3)-harboring plasmid p10FS3-1-3 that the transfer of p10FS3-1-3 into *A. baylyi* ADP1 did not bring significant additive effect on the resistance to tetracyclines (20). To our best

knowledge, this is the first report showing that the horizontal transfer of *tet*(X3)-carrying plasmid conferring tetracyclines resistance to the recipient.

tet(X3) was stable in the recipient strain ATCC17978 without antibiotic stress after 10-day passage, with 100% retention rate, indicating that pAT181 is able to be stably maintained in ATCC17978. The growth rate of the transconjugant ATCC17978-pAT181 increased compared with that of ATCC17978, and the doubling time shortened from 4.59 h to 2.91 h (Figure 3). These results suggest that pAT181 could facilitate the dissemination of *tet*(X3) among *Acinetobacter* spp..

tet(X3) and _tet_(X6) are the prevalent alleles of _tet_(X) family and mainly sporadically disseminate in four species of _Acinetobacter_ spp.

As shown in this and other studies (7, 16, 17, 20, 23), *Acinetobacter* spp. is the major host of *tet*(X)s. To fully understand the distribution of *tet*(X)s among *Acinetobacter* spp., the nucleotide-acid sequences of 15 known *tet*(X) alleles and their variants were blasted against 10,680 *Acinetobacter* genomes retrieved from GenBank. *tet*(X3) was found in 249 strains; *tet*(X4) in 9 strains; *tet*(X5), *tet*(X5.2) and *tet*(X5.3) in 61 strains; *tet*(X6) in 53 strains; *tet*(X13), an one-residue variant of *tet*(X6), was found in 4 strains. These data reveal that *tet*(X3), *tet*(X5.2) and *tet*(X6) are the prevalent *tet*(X) genes among *Acinetobacter* spp..

Species identification showed three predominant *Acinetobacter* species carrying *tet*(X3), i.e. *A. indicus* (27.71%; 69/249), *Acinetobacter* sp002018365 (26.51%; 66/249) (an unnamed species with *Acinetobacter* sp. ANC 4845 as the reference) and

A. towneri (13.65%; 34/249). Except for *A. variabilis* (11.32%; 6/53), *A. indicus* (22.64%; 12/53), *Acinetobacter* sp002018365 (20.75%; 11/53) and *A. towneri* (11.32%; 6/53) are also the predominant species carrying *tet*(X6). The species distribution of *tet*(X5.2) was similar with *tet*(X6), and the major species include *A. indicus* (22.64%; 12/53), *Acinetobacter* sp002018365 (20.75%; 11/53), and *A. towneri* (11.32%; 6/53), *A. variabilis* (11.32%; 6/53) and *A. lwoffii* (11.32%; 6/53). These results indicate that *A. indicus* and *Acinetobacter* sp002018365 are the most prevalent species carrying *tet*(X) genes.

To further evaluate the dissemination pattern of *tet*(X3) and *tet*(X6) among *Acinetobacter* population, we performed phylo-genomic analysis for *tet*(X3)/*tet*(X6)-positive strains of four major hosts as representatives, i.e. *A. indicus*, *Acinetobacter* sp002018365, *A. towneri* and *A. variabilis* (Figure 4; Figure S3). Most strains of each species shared a distant relationship, and no epidemic clones were detected. Two inter-regional transmission events were detected for 4 (no SNPs) and 5 (0-1 SNP) strains of *A. indicus,* and one cross-host event (pig and environment) was detected for 4 (1-44 SNPs) strains of *Acinetobacter* sp002018365 (Figure 4). The data suggest that *tet*(X3) and *tet*(X6) mainly sporadically disseminate among *Acinetobacter* population.

The structures of *tet*(X3)/*tet*(X6) plasmidome are highly diverse and no epidemic plasmids have emerged among *Acinetobacter* population yet

To explore the role of plasmids in the disseminations of *tet*(X3) and *tet*(X6) in

Acinetobacter spp., we here intended to dissect the genetic relatedness of *tet*(X3) and *tet*(X6)-harboring plasmids. Four circularized *tet*(X3)/*tet*(X6)-harboring plasmids obtained in this study and all finished *tet*(X3)/*tet*(X6)-harboring plasmids deposited in GenBank [n = 30; 18 for *tet*(X3), 6 for *tet*(X6), and 6 for *tet*(X3) and *tet*(X6)] were analyzed at first. All but two of these publicly available plasmids were collected between 2009 and 2020 in China, and 25 were identified in *Acinitobacter* spp. (Figure 5). Pairwise comparisons using nucleotide-acid sequences revealed that most of the 26 *tet*(X3)-harboring plasmids (including the 6 *tet*(X3)-*tet*(X6)-harboring plasmids) share a coverage lower than 65%, indicating a highly diverse structure for the plasmidome of *tet*(X3) (Figure 5A). Four of the 6 *tet*(X3)-*tet*(X6)-positive plasmids share a high similarity (>89.83% coverage; >85% identity), suggesting that they were derived from an ancestor. The 4 plasmids were hosted in *A. schindleri* and *A. indicus* isolated from goose and soil collected in different provinces of China (Figure 5A), indicating that cross-species, cross-sector (poultry and environment) and/or cross-region transmission has occurred for these plasmids. A similar transmission event was observed for another three *tet*(X3)-encoding plasmids (pAT181, pAT184 and p10FS3-1-3) carried by *A. towneri* and a novel species of *Acinetobacter* as aforementioned (Figure 5A).

The pairwise sequence comparison of 14 *tet*(X6)-harboring plasmids (including the 6 *tet*(X3)-*tet*(X6)-harboring plasmids) showed that, the 5 *tet*(X6)-harboring plasmids carried by *Acinetobacter* and an unknown species share a low similarity, except for pAT232 and pAT205 as aforementioned (Figure 5B). They are different from the 3

353 *tet*(X6)-harboring plasmids (pAZ25, pZN3 and pZN2) carried by *Proteus* species, and
354 the 6 *tet*(X3)-*tet*(X6)-harboring plasmids (Figure 5B). This suggests that the
355 *tet*(X3)-*tet*(X6)-harboring plasmids might be resulted from the capture of *tet*(X6) by
356 *tet*(X3)-harboring plasmids.

357 To further understand the distribution of *tet*(X3)-harboring plasmids among
358 *Acinetobacter* spp., we selected 17 plasmids out of 26 *tet*(X3)-harboring plasmids as
359 reference according to their similarities (< 80% coverage and identity). The 17
360 plasmids were blasted against the 243 *tet*(X3)-positive genomes (6 genomes with
361 chromosome-encoding *tet*(X3) were excluded), and no epidemic plasmids were found
362 (Figure 6A). To evaluate structural conservation of plasmids amongst *tet*(X3)-positive
363 isolates, we mapped the 243 genomic sequences against the 17 representative
364 plasmid sequences (Fig. 6B). This revealed that plasmid structures were highly
365 diverse amongst isolates (mean plasmid coverage range 12.09-55.05%). Using a
366 cutoff range of >80% coverage and >90% identity, we found that a pGX5-like plasmid
367 was hosted in 36 strains belonging to different species (20 *A. towneri* strains, 10 *A.*
368 *variabilis* strains, 4 *Acinetobacter* sp002018365 strains and 2 *A. indicus* strains), and
369 a p34AB-like, a p94-2-*tet*X3-like, a pXM9F202-2-*tet*X-90k-like and a p10FS3-1-3-like
370 plasmid were found in 17, 9, 8, and 7 strains belonging to different species,
371 respectively (Figure 6A). These data suggest that the current dissemination of *tet*(X3)
372 in *Acinetobacter* is mainly mediated by various plasmids, and cross-species
373 transmissions mediated by few of them might have occurred in a small proportion of
374 strains.

375

376 **The promoter of *tet*(X3) and *tet*(X6) is interchangeable**

377 Our previous study showed that *tet*(X3) confers higher tigecycline resistance than
378 *tet*(X6) (26). In order to understand whether this difference is resulted from the
379 different promoters of the two genes, constructions of promoter-exchanged
380 overexpression plasmids were performed. Transformants carrying the expression
381 cassette promoter$_{tet(X6)}$-ORF$_{tet(X3)}$ [*tet*(X3) ORF followed *tet*(X6) promoter] exhibited the
382 same level of resistance with that of the original cassette promoter$_{tet(X3)}$-ORF$_{tet(X3)}$
383 (Table 3). Likewise, the reconstruction of expression cassette promoter$_{tet(X3)}$-ORF$_{tet(X6)}$
384 did not alter the activity of *tet*(X6) either (Table 3). These results suggest that the
385 tigecycline resistance activity of *tet*(X3) and *tet*(X6) could be determined by the
386 sequence of ORFs and the promoter of *tet*(X3) and *tet*(X6) is interchangeable.

387

388 **Discussion**

389 A total of 15 *tet*(X) alleles have been reported since 1990 (9), and they have spread
390 to cover 5 of the 7 continents (8). Recent surveillance for *tet*(X)s reveals the wide
391 range of ecosystems, including soil, sewage, animals, hospitals, livestock farms and
392 human gut (14-16, 28). The *tet*(X)-positive isolates are especially prevalent in
393 livestock and poultry, like pigs, cows, chicken, and less in shrimp, migratory birds and
394 waterfowls (7, 16, 18, 23, 28-31). In this study, we comprehensively characterized
395 *tet*(X)-positive strains collected from different livestock farms (swine farms, dairy
396 farms and sheep farms), and we found that *tet*(X)-positive strains were exclusively

397 isolated from swine farms (Table 1). A similar finding has been reported recently that
398 *tet*(X3)-positive *Acinetobacter* spp. isolates were exclusively detected in the intensive
399 pig farms in China (20). These results suggest that the dissemination risk of *tet*(X)
400 genes to human from pigs could be much higher than from other livestock.

401 Current surveillances show that *Acinetobacter* spp. is the major reservoir of *tet*(X)
402 genes (17, 20, 23). In this study, *A. towneri* was found to be the major host of *tet*(X3)
403 and *tet*(X6). A recent surveillance of *tet*(X)-positive *Acinetobacter* isolates from human,
404 animal, and their surrounding environments conducted between 2015 and 2018
405 shows that *A. towneri* and *A. indicus* following a novel species of *Acinetobacter* were
406 the major hosts of *tet*(X3), *tet*(X4) and *tet*(X5) (20). This indicates that the diversity of
407 *tet*(X) hosts may be source- and/or geographic-dependent. To fully understand the
408 distribution of *tet*(X)s in *Acinetobacter* population, we searched 15 *tet*(X) alleles and
409 their variants in all *Acinetobacter* genomes available in GenBank, and results
410 revealed that *tet*(X3) and *tet*(X6) are the predominant alleles mostly associated with
411 livestock, and *A. towneri* is the third prevalent species carrying *tet*(X3) and *tet*(X6)
412 following *A. indicus* and *Acinetobacter* sp002018365. Further analysis showed that
413 the population structure of the four major species is highly diverse (Figure 4 and S3),
414 suggesting that *tet*(X3) and *tet*(X6) are mainly sporadic dissemination. However, few
415 inter-regional transmission events were detected here, highlighting the needs for
416 controlling the dissemination of *tet*(X3) and *tet*(X6) positive *Acinetobacter* spp.,
417 especially from livestock to humans.

418 First identification of plasmid-borne *tet*(X3) and *tet*(X4) causing the horizontal

419 transfer of tigecycline resistance has highly aroused the public attention. Since then,
420 numerous *tet*(X) alleles have been continuously identified either on chromosomes or
421 on plasmids in various bacterial species. However, whether plasmids are the major
422 vectors of plasmid-borne *tet*(X)s remains unclear. Pioneer studies have shown the
423 importance of IS*CR2*-mediated *tet*(X) transposition structure (7, 17). The rolling-circle
424 transposition has been experimentally confirmed by using the cassette
425 "Δ*tpnF-tet*(X3)-hp-hp-IS*CR2*" clone, and inverse PCR assays identified
426 "IS*CR2-xerD-tet*(X3)-*res*-ORF1" and "IS*CR2*-ORF2-*abh-tet*(X4)" minicircles in
427 different studies (7, 20). In our study, IS*CR2* was found upstream or downstream of
428 *tet*(X3) and *tet*(X6) genes. Albeit we did not test the transferability of the
429 IS*CR2*-mediated *tet*(X) transposition structure, the genetic context of *tet*(X3) carried
430 by 249 genomes of *Acinetobacter* species were comprehensively compared. The
431 proportion of the structure IS*CR2-xerD-tet*(X3)-*res*-IS*CR2* might be up to 86.35%
432 (215/249), implying the critical role of IS*CR2* in the dissemination of *tet*(X3).

433 Of note, we found that a *tet*(X3)-encoding plasmid pAT181 was self-transmissible
434 from *A. towneri* to *A. baumannii*, and conferred tetracyclines resistance to the
435 recipient. Currently, very few studies have identified self-transmissible plasmids
436 carrying *tet*(X)s. Chen *et al*. reported the conjugability of a *tet*(X3)- and
437 *tet*(X5.3)-harboring plasmid pYH12207-2 from *A. piscicola* to *A. baylyi* ADP1, and the
438 conjugability of a *tet*(X3)-harboring plasmid p10FS3-1-3 from an *Acinetobacter* novel
439 species to *A. baylyi* ADP1. However, these two plasmids did not enhance the
440 resistance to tetracyclines in the recipient strain (20). This is different from our findings

that the transfer of pAT181 to the recipient resulted in a 64-512-fold increase of tetracyclines resistance (Table S2). Remarkably, the donor strain of pAT181 is a tigecycline-susceptible *A. towneri* strain, and the recipient strain is *A. baumannii*, suggesting that the expression of *tet*(X3) could be species-dependent. More than half of *tet*(X3)/*tet*(X6)-positive *A. towneri* strains were tigecycline-susceptible in this study, indicating the silent transmission of *tet*(X3)/*tet*(X6) in *A. towneri.* Concerningly, pAT181 with a relatively high transfer frequency (10^{-6}) did not impose fitness cost but increased the growth rate of the recipient. It is suggested that successful disseminations of resistance plasmids largely depends on the fitness cost imposed on hosts (32). No fitness cost imposed on hosts by obtaining pAT181-like plasmids would greatly facilitate their spread, thus may contribute to the propagation of *tet*(X3) gene in the future. Additionally, although no epidemic plasmids of *tet*(X3) have been detected currently, several plasmids were found circulating in a small proportion of strains. It is possible that these plasmids could become epidemic after transmitting to other hosts in the future.

Conclusions

Our study evidence that the predominate *tet*(X) alleles, *tet*(X3) and *tet*(X6), disseminate sporadically in *Acinetobacter* population. Currently, the dissemination of *tet*(X3) and *tet*(X6) is mainly limited among livestock-associated sites. Continuous surveillance for *tet*(X)s in the context of One Health is necessary to prevent them from transmitting to humans.

Materials and Methods

Screenings of *tet*(X)-positive *Acinetobacter* spp. strains

Five hundred and thirty-four non-repetitive fecal samples were collected from 6 livestock farms locating in Zhejiang province in 2019, including 2 swine farms, 2 dairy farms and 2 sheep farms. Environmental samples were collected from soil (n=72) and water (n=78) surrounding the farms in parallel. These samples were initially enriched in LB medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl) for 6 hours and spread on CHROMagar™ *Acinetobacter* medium plates (CHROMagar, Paris, France) to recover *Acinetobacter* spp. strains. PCR screens of *tet*(X) alleles were performed as previously described (26).

Antimicrobial susceptibility testing (AST)

The minimum inhibitory concentration (MICs) for all *tet*(X) positive strains were determined using microbroth dilution method according to the guideline of Clinical and Laboratory Standards Institute (CLSI) (29th edition) (33). The tested drugs included tigecycline, tetracycline, eravacycline, minocycline, doxycycline, demeclocycline, chlortetracycline, oxytetracycline, colistin, cefoperazone-sulbactam, trimethoprim-sulfamethoxazole, gentamicin, amikacin, levofloxacin, ciprofloxacin, meropenem, cefepime, ceftriaxone, and ceftazidime. The breakpoint for tetracycline was interpreted as ≥ 16 mg/L for *Acinetobacter* spp., *Enterobacteriacea* and non-*Enterobacteriaceae* according to CLSI (33). The breakpoint for tigecycline and

485 eravacycline was interpreted as > 0.5 mg/L for *Enterobacteriaceae* according to
486 EUCAST V10 (34). *E. coli* ATCC25922 was used as the quality control strain.
487
488 **Whole genome sequencing (WGS) and bioinformatic analysis**
489 Genomic DNAs of *tet*(X)-positive isolates were extracted by using Puregene
490 Yeast/Bact Kit B (Qiagen, Gaithersburg, MD, Germany) according to the instruction of
491 the manufacture, and were sequenced by using Hiseq 4000 system (Illumina, San
492 Diego, United States). The average nucleotide identity (ANI) was calculated by using
493 FastANI (35). Sequence similarity of *tet*(X)-harboring plasmids was analyzed by using
494 BRIG v0.95 (36). Representative strains with various genetic context of *tet*(X) genes
495 were selected out to be further sequenced by using PromethION platform (Nanopore,
496 Oxford, UK). Hybrid assembly of short reads and long reads sequencing data was
497 performed by using Unicycler version 0.4.8 (37).
498 Phylogenetic analysis was performed by using Parsnp v1.2 (38), and the number
499 of SNPs (single nucleotide polymorphisms) among the core genomes were
500 determined by MEGA X (39). Functional annotation was performed using RAST
501 server (40). Antibiotic resistance genes (ARGs) were identified by using ResFinder
502 4.0 (41) and CARD (https://card.mcmaster.ca/) with the threshold of nucleotide-acid
503 identity >90% and coverage >90%. Synteny analysis was performed by using Easyfig
504 (42).
505
506 **Compilation of genomic data set and plasmidome analysis**

All assembled genomes of *Acinetobacter* spp. (n= 10,680) deposited in GenBank (as of 31th May 2021) were downloaded to search *tet*(X) alleles. The fifteen *tet*(X) alleles were queried in these genomes by blasting against their nucleotide-acid sequences using a cutoff as 99% identity and 100% coverage.

Conservation of reference plasmid genes was calculated as previously described (43). Briefly, RedDog pipeline (https://github.com/katholt/RedDog) was used to simulate 100-bp reads from *tet*(X3)-carrying genomes. To calculate the coverage of each representative plasmid in each genome, those 100-bp reads were mapped against representative *tet*(X3)-harboring plasmids by using Bowtie2 v2.2.9 (44). The proportion of *tet*(X3)-carrying genomes containing annotated genes of each reference plasmid was calculated according to the gene presence/absence table reported by Red-Dog (at least five reads covering ≥ 95% of the length of the gene was defined as presence) and plotted as circular heatmaps using ggplot2 in R (geom_tile for heatmap grid and coord_polar for circularise).

Pairwise sequence comparison of circularized plasmids was performed as previously described (45). Briefly, the length of nucleotide-acid sequence that could be aligned between pairs of plasmids and the number of SNPs among the aligned regions were determined by NUCmer v3.1 (46) from the MUMmer package. The percentage of aligned bases between pairs of complete plasmids was showed in heatmap generated by the "gplots" package (v3.1.1) in R v4.0.5 (https://www.r-project.org/).

Conjugation assay

The transmissibility of *tet*(X3) and *tet*(X6) was evaluated by conjugation assay. Briefly, *tet*(X)-carrying *Acinetobacter* strain as a donor strain was mixed with rifampicin-resistant *A. baumannii* ATCC17978 or rifampicin-resistant *E. coli* EC600 as a recipient strain at the ratio of 1:1 by conjugational mating at 37^{o}C without shaking for overnight. The transconjugants were selected on LB agar plates containing rifampicin (600 mg/L) and tigecycline (2 mg/L). The species of all putative transconjugants were verified by using MALDI-TOF mass spectrometry (Hexin, Guangzhou, China). PCR verifications of *tet*(X) genes were performed for the putative transconjugants of which the species was confirmed as *A. baumannii* or *E. coli*. Transfer frequency was calculated as the number of transconjugants obtained per donor. Growth of donor strain and transconjugants were measured by determining the optical density at 600 nm (OD_{600}) every 30 min.

Plasmid stability testing

Plasmid stability was estimated according to a previous study with minor modifications (47). Transconjugants were cultured in antibiotic-free LB broth at 37°C for 24 h. The 24h-growth cultures were diluted with the ratio of 1:100 in fresh LB medium. These freshly inoculated cultures constituted time point zero, and cultures were grew at 37°C in a shaking bath (200 rpm) and went on serial passages for 10 days (approximately 200 generations). Cultures were diluted and plated onto antibiotic-free LB plates every 24 h. The colonies growing on antibiotic-free LB agar

plates were randomly selected (~50 colony per day) for *tet*(X)-specific PCRs to determine the proportion of *tet*(X)-positive bacteria in each population. Plasmids were considered stable when the retention rates were still over 80% at the end of the experiment. The plasmid stability was evaluated in triplicate.

Functional cloning

Predicted promoters of *tet*(X3) and *tet*(X6) according to softberry (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb) were fused with open reading frames (ORFs) of *tet*(X3) and *tet*(X6) to construct promoter exchanged clones, respectively. Briefly, the promoter region of *tet*(X3) was amplified using primers PstI-*tet*(X3)-F-P (5'-cgctgcagTACCACCAAGGGAATGGAAC-3') and X3P+X6O-R (5'-GTTCGCTGGTTTTAATGTCAATCAAAAATGGCACATAACAAG-3'), and the ORF of *tet*(X6) was amplified using primers X3P+X6O-F (5'-CTTGTTATGTGCCATTTTTGATTGACATTAAAACCAGCGAAC-3') and XbaI-*tet*(X6)-R (5'-cgtctagaTTTCTCTTTCATTTCCTCGCC-3'). The derived amplicons were fused by the fusion PCR using primers PstI-*tet*(X3)-F-P and XbaI-*tet*(X6)-R, resulting in an X3P-X6O fragment. The digested X3P-X6O fragment was cloned into pUC19 to construct pUC19-X3P-X6O. Likewise, primers PstI-*tet*(X6)-F-P (5'-cgctgcagATGGTTGCAGACCTTGACGA-3') and X6P+X3O-R (5'-CGTATCTATTCGCATTGTCATCTAATGTCTGTCAATTTAATC-3') were used to amplify the promoter region of *tet*(X6). Primers X6P+X3O-F

(5'-GATTAAATTGACAGACATTAGATGACAATGCGAATAGATACG-3') and XbaI-*tet*(X3)-R (5'-cgtctagaGCAAAACTGCTTGTTAGTAGC-3') were used to amplify the ORF of *tet*(X3).The fused amplicon X6P-X3O was ligated into pUC19 to construct pUC19-X6P-X3O. The recombinant plasmid was transformed into *E. coli* DH5*α* competent cells by heat shock. Transformants were selected on LB agar plates containing 100 mg/L ampicillin. *tet*(X3) and *tet*(X6) with parental promoters were individually cloned into pUC19 as positive controls.

Statistical analysis

Statistical analysis was performed using unpaired *t*-test analysis, and statistical significance is taken as $p < 0.05$.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Data availability

The genome sequences of *tet*(X) positive strains have been submitted to GenBank, and the accession numbers are listed in Table 1.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KZ, Y-YC, Y-HX, and R-CC designed the study. Y-YC, YC, and F-MH collected the data. Y-YC and YL analyzed and interpreted the data. Y-YC and KZ wrote and revised the manuscript. All authors reviewed, revised, and approved the final report

Acknowledgements

This work was supported by the National Key Research and Development Program of China (grant number 2017YFC1200200); the National Natural Science Foundation of China (grant numbers 81902029); Shenzhen Basic Research Key projects (grant numbers JCYJ20200109144220704) and Shenzhen Basic Research projects (grant numbers JCYJ20190807144409307; JCYJ20190807150401657).

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Figure legends:

Figure 1. Phylogenetic analysis of *tet*(X)-positive *Acinetobacter* isolates collected in this study. The core-genome SNPs of *tet*(X)-encoding strains were used to generate the phylogenetic tree. The tree is rooted at strain ZJ199. The ARGs of each strain are exhibited by heatmap, and the existence of ARGs is in red. MIC values of each strain against tetracyclines are listed. AT205 has been reported previously [24].

Figure 2. Comparison of genomic context of *tet*(X3) (A) and *tet*(X6) (B) identified in *Acinetobacter* spp. isolates. Genes are indicated by colour-coded arrows dependent on the functional annotations and direction of transcription. ARGs are in red; mobile genetic element genes are in green; other function genes are in blue; hypothetical genes are in orange. The **g**enomic context of *tet*(X3) identified in *A. baumannii* 34AB (MK134375) and *A. indicus* AI2 (GCA012366935) are as the reference for the comparison of *tet*(X3); the **g**enomic context of *tet*(X6) identified in *P.* genomospecies 6 T60 (CP043925) and *A. indicus* CMG3-2 (CP044446) are as the reference for the comparison of *tet*(X6).

Figure 3. The growth curve of the recipient strain *A. baumannii* ATCC17978 and the transcongjutant ATCC17978-pAT181. The optical density at 600 nm was record every 30 min at 37°C. The assay was in triplicate.

Figure 4. Phylogenetic analysis of *tet*(X3)/*tet*(X6)-encoding *A. indicus* (A) and *Acinetobacter* sp002018365 (B) genomes retrieved from GenBank. The core-genome SNPs of *tet*(X)-encoding strains were used to generate the phylogenetic tree. The tree is mid-point rooted. The *tet*(X) genes (group), isolate source (host), sampling location (location) and years (date) of strains are shown at the right side of the tree in different colors. Two inter-regional transmission events for 4 and 5 strains of *A. indicus*, and one cross-host event for 4 strains of *Acinetobacter* sp002018365 are highlighted by shading. The scale bar represents the number of SNPs.

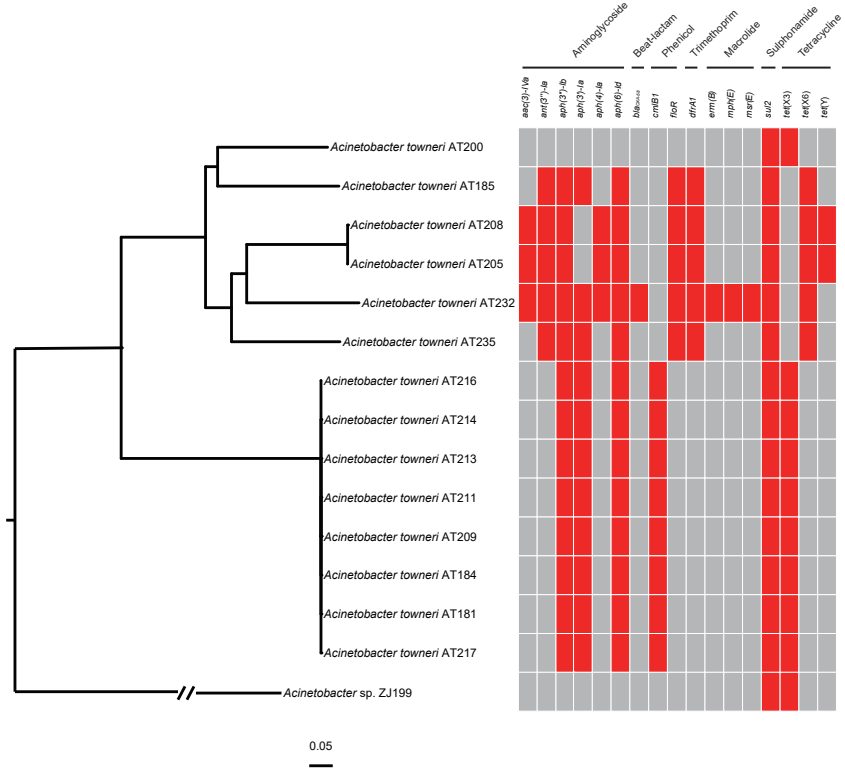
Figure 5. Pairwise sequence comparisons between circularized *tet*(X3)/ *tet*(X6)-carrying plasmids. The heat map shows the percentage of aligned bases between pairs of *tet*(X3)-carrying plasmids **(A)** and *tet*(X6)-carrying plasmids **(B)**. The row and column orders are the same. The information of host species, sampling source, sampling location and isolation years are indicated by colored graphic subsequent the phylogenetic tree. The 6 plasmids co-harbored *tet*(X3) and *tet*(X6) genes are boxed.

Figure 6. Analysis of *tet*(X3) Plasmidome. (A) Blasting results of the 17 representative *tet*(X3)-carrying plasmids against 243 *tet*(X3)-positive genomes. The heat map shows the percentage of aligned bases between pairs of *tet*(X3)-positive plasmids and genomes. (B) Conservation of reference plasmid genes amongst 243 genome sequences of *tet*(X3)-carrying *Acinetobacter* spp.. The frequency of each gene in the reference plasmid is shown in circularized heatmaps. Genes order in the corresponding reference plasmid are around the cell. The mean coverage of the reference plasmids sequence is indicated in percentages after the plasmid name.

Supplementary figure 1. Comparative analysis of *tet*(X)-encoding plasmids in this study. (A) The inner ring represents the circularized *tet*(X3)-encoding plasmid pAT181 as reference; **(B)** The inner ring represents the circularized *tet*(X6)-encoding plasmid pAT232 as reference; **(C)** The inner ring represents the circularized *tet*(X6)-encoding plasmid pAT235 as reference; **(D)** The inner ring represents the circularized *tet*(X6)-encoding plasmid pAT205 as reference.

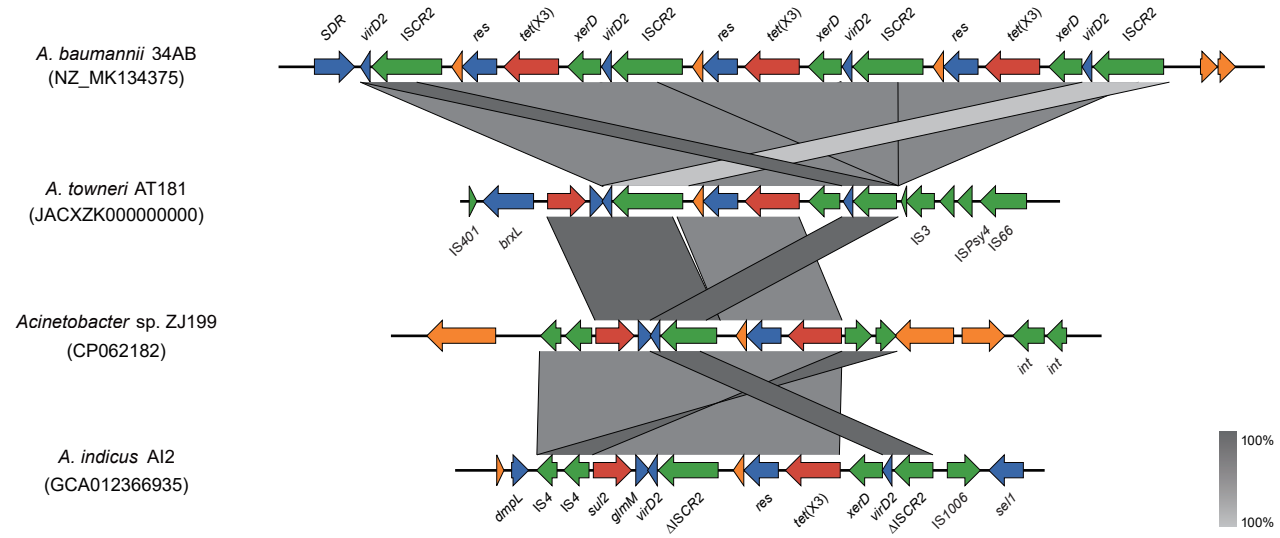
Supplementary figure 2. Verification of horizontal transfer of *tet*(X3)-encoding plasmid pAT181 in the transconjugant ATCC17978-pAT181. The inner ring represents pAT181 as reference. The outer ring represents the mapping result of exogenous DNA in the tansconjugant AB181 compared with the recipient strain ATCC17978.

Supplementary figure 3. Phylogenetic analysis of *tet*(X3)/*tet*(X6)-encoding *A. towneri* (A) and *A. variabilis* (B) genomes retrieved from GenBank. The core-genome SNPs of *tet*(X)-encoding strains were used to generate the phylogenetic tree. The tree is mid-point rooted. The *tet*(X) genes (group), isolate source (host), sampling location (location) and years (date) of strains are exhibited at the right side of phylogenetic tree in different colors.

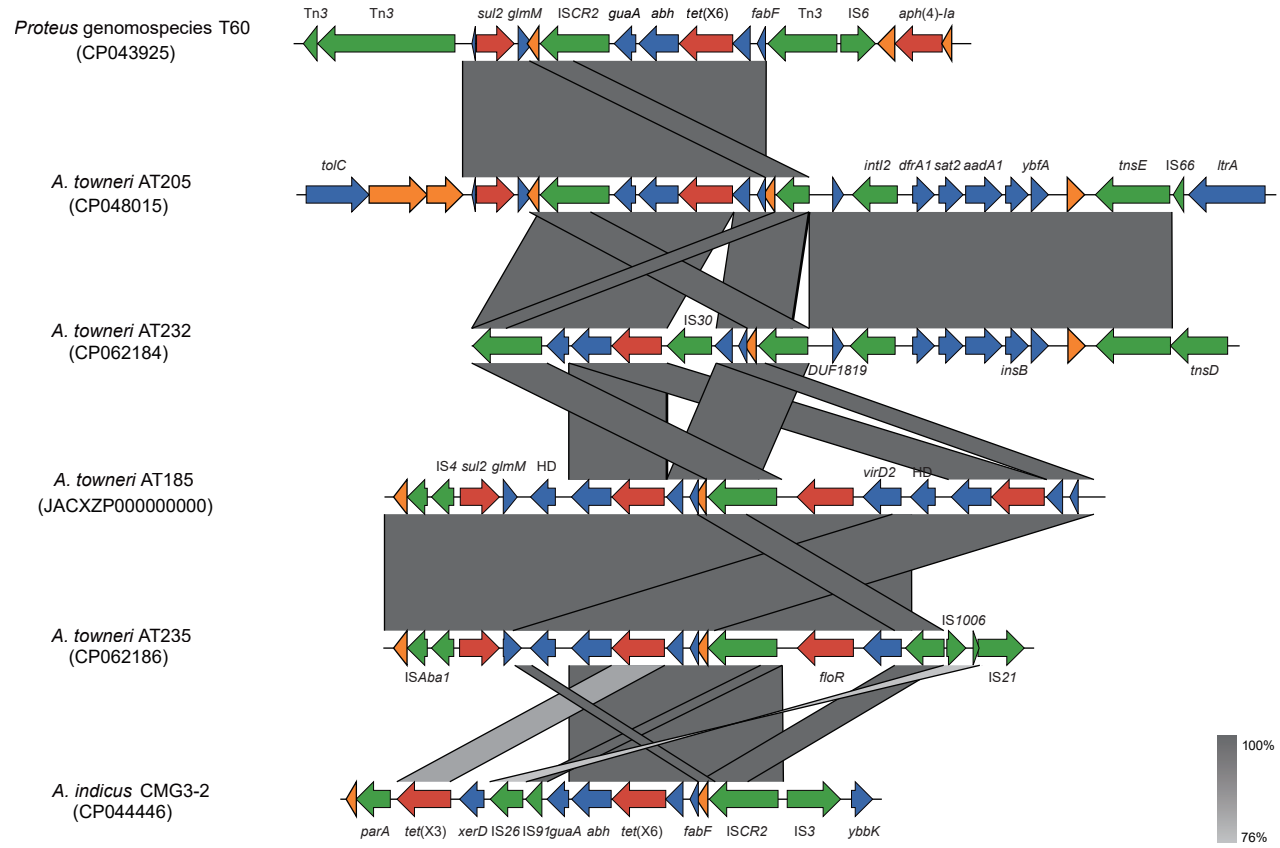


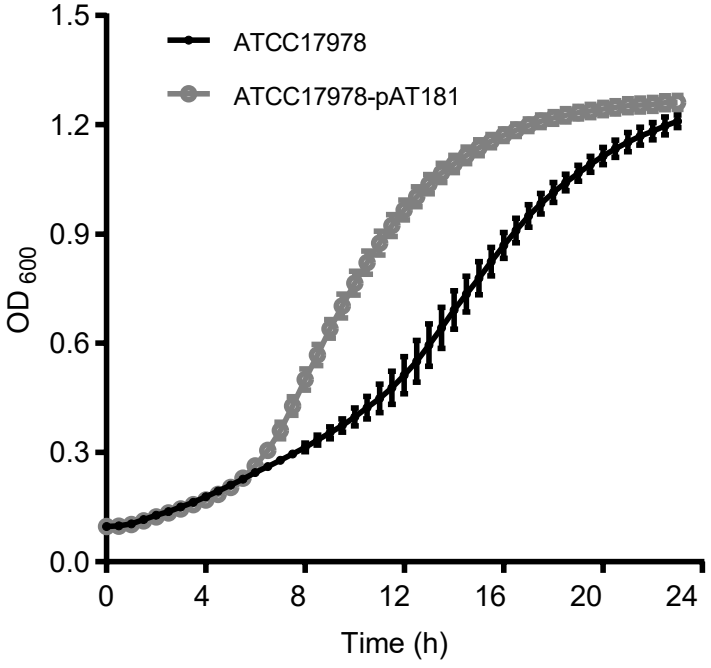
	MIC(mg/L)		
	Tigecycline	Eravacycline	Tetracycline
Acinetobacter towneri AT200	0.25	0.25	32
Acinetobacter towneri AT185	0.12	0.25	8
Acinetobacter towneri AT208	0.12	0.25	>32
Acinetobacter towneri AT205	0.12	0.25	128
Acinetobacter towneri AT232	2	4	64
Acinetobacter towneri AT235	0.06	0.125	16
Acinetobacter towneri AT216	1	0.5	32
Acinetobacter towneri AT214	1	0.5	32
Acinetobacter towneri AT213	0.5	0.25	32
Acinetobacter towneri AT211	1	1	32
Acinetobacter towneri AT209	1	0.5	32
Acinetobacter towneri AT184	0.5	1	32
Acinetobacter towneri AT181	0.5	1	32
Acinetobacter towneri AT217	0.5	0.5	32
Acinetobacter sp. ZJ199	0.25	0.5	32

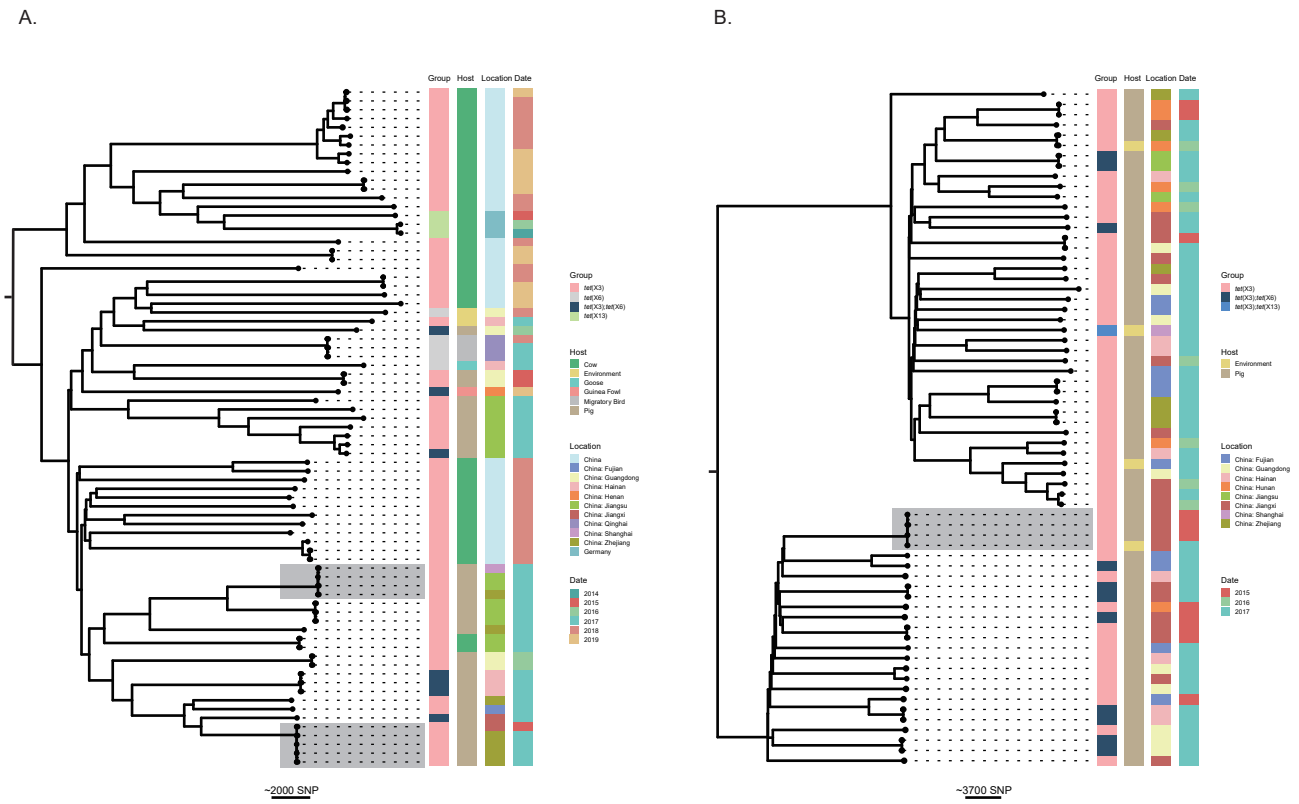
A.



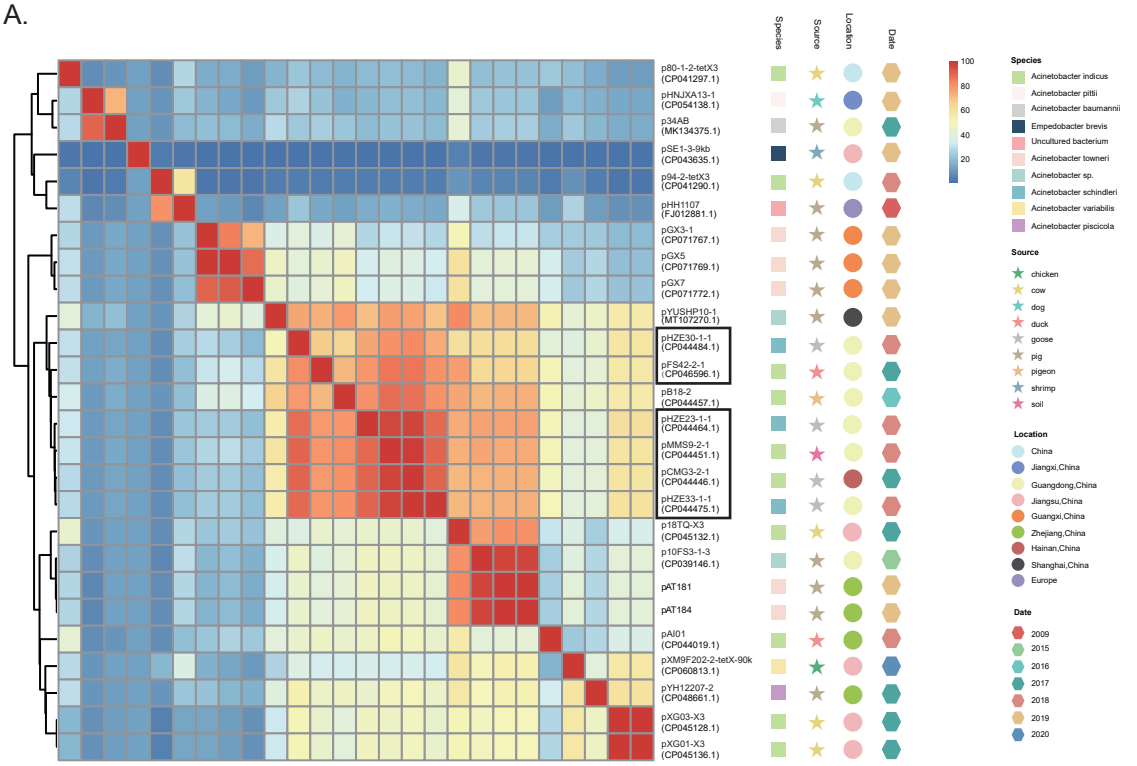
B.



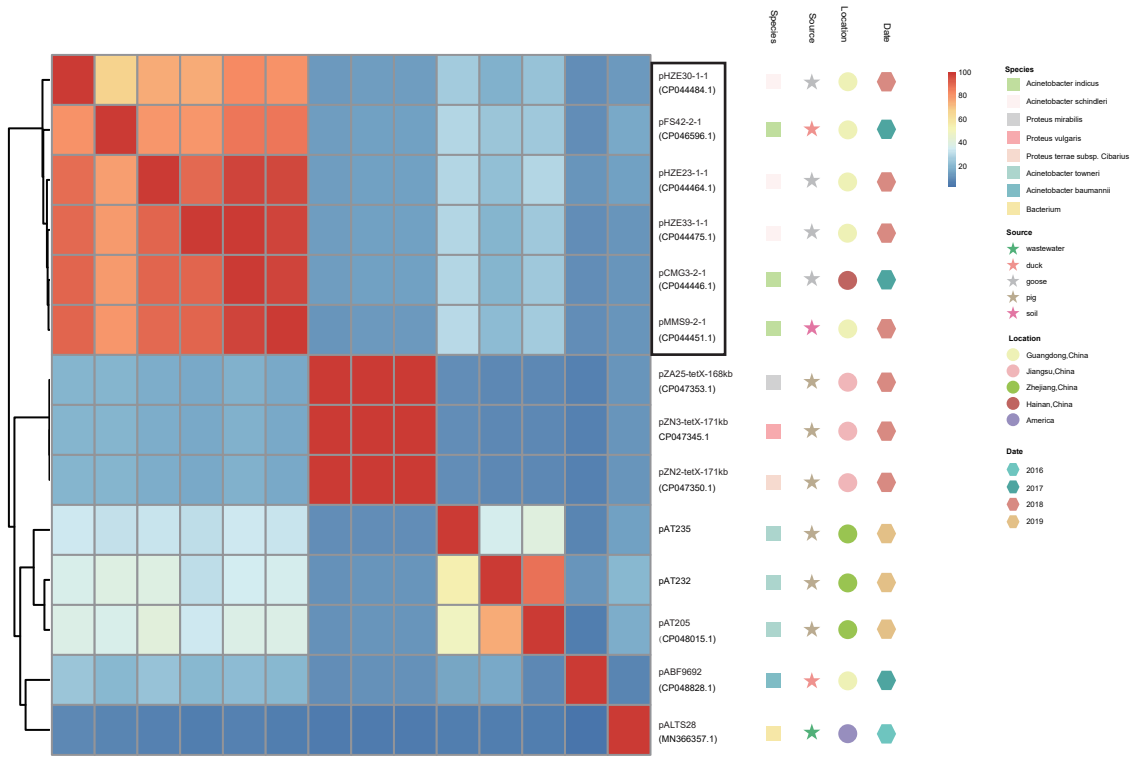

ATCC17978
ATCC17978-pAT181
OD 600
1.5
1.2
0.9
0.6
0.3
0.0
0
4
8
12
16
20
24
Time (h)


A.
B.
Group
Host
Location
Date
tet(X3)
tet(X6)
tet(X3);tet(X6)
tet(X13)
Cow
Environment
Goose
Guinea Fowl
Migratory Bird
Pig
China
China: Fujian
China: Guangdong
China: Hainan
China: Henan
China: Jiangsu
China: Jiangxi
China: Qinghai
China: Shanghai
China: Zhejiang
Germany
2014
2015
2016
2017
2018
2019
~2000 SNP
tet(X3);tet(X13)
Environment
Pig
China: Fujian
China: Guangdong
China: Hainan
China: Hunan
China: Jiangsu
China: Jiangxi
China: Shanghai
China: Zhejiang
2015
2016
2017
~3700 SNP

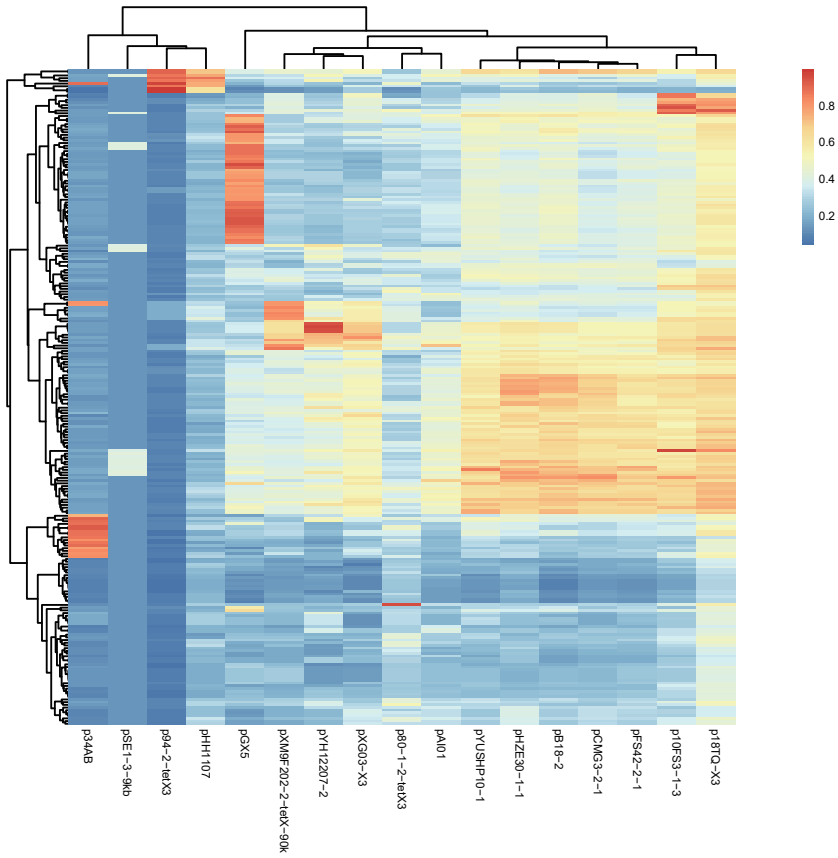
A.



B.



A.


0.8
0.6
0.4
0.2
p34AB
pSE1-3-9kb
p94-2-tetX3
pHH1107
pGX5
pXM9F202-2-tetX-90k
pYH12207-2
pXG03-X3
p80-1-2-tetX3
pAI01
pYUSHP10-1
pHZE30-1-1
pB18-2
pCMG3-2-1
pFS42-2-1
p10FS3-1-3
p18TQ-X3

B.


p18TQ-X3
55.05%
p10FS3-1-3
46.49%
pHZE30-1-1
44.40%
pB18-2
43.53%
pYUSHP10-1
43.00%
pFS42-2-1
41.80%
pCMG3-2-1
41.59%
pGX5
40.64%
pXG03-X3
36.35%
pAI01
34.79%
pYH12207-2
34.09%
pXM9F202-2-tetX-90k
33.47%
p80-1-2-tetX3
33.42%
pHH1107
25.86%
p34AB
19.72%
pSE1-3-9kb
15.17%
p94-2-tetX3
12.09%
value
1.00
0.75
0.50
0.25
0.00

Table 1. *tet*(X)-positive strains isolated in this study.

Strains	Species	*tet*(X) GENE	*tet*(X) Gene Location	Source	Sequencing Platform	Genome accession
ZJ202	*Empedobacter stercoris*	*tet*(X2)	chromosome	fecal, swine farm 1	Illumina	JABFOQ000000000
ZJ180	*Empedobacter stercoris*	*tet*(X2)	chromosome	fecal, swine farm 1	Illumina	JACXZB000000000
ZJ215	*Empedobacter stercoris*	*tet*(X2)	chromosome	fecal, swine farm 1	Illumina	JACXZC000000000
ZJ286	*Myroides odoratimimus*	*tet*(X2)	NA	soil, swine farm 2	Illumina	JACXZD000000000
ZJ291	*Myroides odoratimimus*	*tet*(X2)	NA	soil, swine farm 2	Illumina	JACXZE000000000
ZJ295	*Myroides odoratimimus*	*tet*(X2)	NA	soil, swine farm 2	Illumina	JACXZF000000000
AT184	*Acinetobacter towneri*	*tet*(X3)	plasmid	fecal, swine farm 1	nanopore	JACXZG000000000
ZJ199	*Acinetobacter sp.*	*tet*(X3)	chromosome	fecal, swine farm 1	nanopore	CP062182
AT200	*Acinetobacter towneri*	*tet*(X3)	plasmid	fecal, swine farm 1	Illumina	JACXZH000000000
AT216	*Acinetobacter towneri*	*tet*(X3)	plasmid	fecal, swine farm 1	Illumina	JACXZI000000000

AT217	*Acinetobacter towneri*	*tet*(X3)	plasmid	fecal, swine farm 1	Illumina	JACXZJ000000000
AT181	*Acinetobacter towneri*	*tet*(X3)	plasmid	fecal, swine farm 1	nanopore	JACXZK000000000
AT209	*Acinetobacter towneri*	*tet*(X3)	plasmid	fecal, swine farm 1	Illumina	JACXZL000000000
AT211	*Acinetobacter towneri*	*tet*(X3)	plasmid	fecal, swine farm 1	Illumina	JACXZM000000000
AT213	*Acinetobacter towneri*	*tet*(X3)	plasmid	fecal, swine farm 1	Illumina	JACXZN000000000
AT214	*Acinetobacter towneri*	*tet*(X3)	plasmid	fecal, swine farm 1	Illumina	JACXZO000000000
AT185	*Acinetobacter towneri*	*tet*(X6), *tet*(X6)	plasmid	fecal, swine farm 1	Illumina	JACXZP000000000
AT208	*Acinetobacter towneri*	*tet*(X6)	plasmid	fecal, swine farm 1	Illumina	JACXZQ000000000
AT232	*Acinetobacter towneri*	*tet*(X6)	plasmid	fecal, swine farm 1	nanopore	CP062183-CP062184
AT235	*Acinetobacter towneri*	*tet*(X6)	plasmid	fecal, swine farm 1	nanopore	CP062185-CP062186
AT205	*Acinetobacter towneri*	*tet*(X6)	plasmid	fecal, swine farm 1	nanopore	CP048014-

						CP048018
ZJ183	*Empedobacter stercoris*	*tet*(X14), *tet*(X2), *tet*(X2)	chromosome	fecal, swine farm 1	nanopore	CP053698-CP053701
ZJ182	*Empedobacter stercoris*	*tet*(X14)-tet(*X2*)	chromosome	fecal, swine farm 1	Illumina	JACXZR000000000

Table 2. MIC values of antibiotics tested in this study

Strains	MIC (mg/L)																		
	CAZ	CRO	FEP	IPM	MEM	CIP	LVX	AMK	GEN	SXT	CSL	COL	TGC	OTC	CTC	DMC	DOX	MIN	ERV	TET
ZJ202	4	2	0.125	0.25	0.125	1	0.5	16	8	0.25	2	16	0.5	32	4	4	1	0.5	0.5	16
ZJ180	2	2	0.125	0.5	0.25	1	0.5	16	4	0.06	4	32	0.5	16	4	2	0.5	0.25	1	8
ZJ215	2	2	0.25	0.5	0.125	0.125	0.5	2	4	>8	0.25	16	0.5	32	4	4	1	0.5	1	16
ZJ286	64	>64	8	>32	2	>32	8	>128	>128	1	>128	>32	0.5	>128	>128	>128	>128	128	1	>128
ZJ291	64	>64	8	>32	2	>32	16	>128	>128	>8	>128	>32	2	>128	>128	>128	64	32	1	>128
ZJ295	64	>64	8	>32	2	>32	8	>128	>128	0.5	>128	>32	0.5	>128	>128	>128	>128	16	0.5	>128
AT184	2	4	0.5	0.125	0.03	1	1	1	1	>8	1	0.5	0.5	128	16	16	1	0.5	1	32
ZJ199	0.25	0.25	0.06	0.03	0.03	4	2	0.06	0.125	>8	0.06	1	0.25	128	16	8	2	0.25	0.5	32
AT200	2	4	0.25	0.125	0.03	0.03	0.06	0.25	0.125	>8	0.5	2	0.25	64	8	4	0.5	0.5	0.25	32
AT216	2	4	0.5	0.125	0.06	2	0.5	1	0.25	>8	0.25	1	1	64	16	8	0.5	0.25	0.5	32

AT217	2	4	0.5	0.125	0.06	2	0.5	1	0.25	8	0.25	1	0.5	128	16	16	0.5	0.25	0.5	32
AT181	2	4	0.25	0.125	0.06	1	0.5	1	0.5	>8	1	1	0.5	128	16	16	1	0.5	1	32
AT209	2	4	0.25	0.125	0.03	0.03	0.5	1	0.5	>8	1	0.5	1	128	16	8	0.5	0.5	0.5	32
AT211	2	4	0.25	0.125	0.03	0.03	0.5	1	0.5	>8	1	1	1	128	16	8	1	0.25	1	32
AT213	2	4	0.25	0.125	0.03	0.03	0.5	2	0.5	>8	1	1	0.5	128	16	8	0.5	0.5	0.25	32
AT214	2	4	0.25	0.125	0.03	0.03	0.5	2	0.5	>8	1	1	1	64	8	8	0.25	0.5	0.5	32
AT185	2	4	0.5	0.25	0.03	1	0.5	0.5	0.25	>8	1	2	0.12	32	8	4	0.25	0.25	0.25	8
AT208	2	4	0.25	0.25	0.03	0.03	1	1	8	>8	1	2	0.12	>128	128	128	16	2	0.25	>32
AT232	2	4	0.5	0.25	0.06	4	1	0.5	4	8	0.5	2	2	128	64	32	4	2	4	64
AT235	2	4	0.5	0.125	0.03	4	1	0.5	0.125	8	0.25	2	0.06	32	4	2	0.25	0.25	0.125	16
AT205	4	8	0.5	0.5	0.06	4	1	1	8	>8	1	2	0.12	128	128	128	32	0.5	0.25	128
ZJ183	2	4	0.5	0.25	0.125	1	1	32	16	0.06	4	32	1	128	8	8	4	0.125	1	16
ZJ182	1	1	0.06	0.125	0.125	2	1	16	8	0.06	2	32	1	64	8	8	2	1	2	16

Abbreviation: CAZ, Ceftazidime; CRO, Ceftriaxone; FEP, Cefepime; IPM, Imipenem; MEM, Meropenem; CIP, Ciprofloxacin; LVX, Levofloxacin; AMK, Amikacin; GEN, Gentamycin; SXT, Sulfamethoxazole-Trimethoprim; CSL, Cefoperazone-Sulbactam; COL, Colistin; TGC, Tigecycline; OTC, Oxytetracycline; CTC, Chlortetracycline; DMC, Demeclocycline; DOX, Doxycycline; MIN, Minocycline; ERV, Eravacycline; TET, Tetracycline.

Table 3. MIC values of tetracyclines tested in this study

Strains	MIC (mg/L)							
	TGC	TET	OTC	CTC	DMC	DOX	MIN	ERV
DH5α-*tet*(X3)promoter-*tet*(X6)ORF	8	>128	>128	>128	>128	>128	8	8
DH5α-*tet*(X6)promoter-tet(X6)ORF	8	>128	>128	>128	>128	>128	16	8
DH5α-*tet*(X6)promoter-tet(X3)ORF	32	>128	>128	>128	>128	>128	64	32
DH5α-*tet*(X3)promoter-tet(X3)ORF	16	>128	>128	>128	>128	>128	32	32

Abbreviation: TGC, Tigecycline; TET, Tetracycline; OTC, Oxytetracycline; CTC, Chlortetracycline; DMC, Demeclocycline; DOX, Doxycycline; MIN, Minocycline; ERV, Eravacycline.