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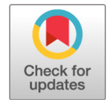

Co-occurrence of Plasmid-Mediated Tigecycline and Carbapenem Resistance in *Acinetobacter* spp. from Waterfowls and Their Neighboring Environment

Chao-Yue Cui,[a,b] Chong Chen,[a,b] Bao-Tao Liu,[d] Qian He,[a,b] Xiao-Ting Wu,[a,b] Ruan-Yang Sun,[a,b] Yan Zhang,[a,b] Ze-Hua Cui,[a,b] Wen-Ying Guo,[a,b] Qiu-lin Jia,[a,b] Cang Li,[a,b] Barry N. Kreiswirth,[e] Xiao-Ping Liao,[a,b,c] Liang Chen,[e] Ya-Hong Liu,[a,b,c] Jian Sun[a,b,c]

[a]National Risk Assessment Laboratory for Antimicrobial Resistance of Animal Original Bacteria, South China Agricultural University, Guangzhou, China

[b]Guangdong Laboratory for Lingnan Modern Agriculture, Guangzhou, Guangdong, China

[c]Guangdong Provincial Key Laboratory of Veterinary Pharmaceutics Development and Safety Evaluation, South China Agricultural University, Guangzhou, China

[d]College of Veterinary Medicine, Qingdao Agricultural University, Qingdao, China

[e]Hackensack-Meridian Health Center for Discovery and Innovation, Nutley, New Jersey, USA

Chao-Yue Cui, Chong Chen, and Bao-Tao Liu contributed equally to this work. Author order was determined on the basis of seniority.

ABSTRACT Tigecycline serves as one of the antibiotics of last resort to treat multidrug-resistant (including carbapenem-resistant) pathogens. However, the recently emerged plasmid-mediated tigecycline resistance mechanism, Tet(X), challenges the clinical efficacy of this class of antibiotics. In this study, we detected 180 *tet*(X)-harboring *Acinetobacter* isolates (8.9%, $n = 180$) from 2,018 samples collected from avian farms and adjacent environments in China. Eighteen *tet*(X)-harboring isolates (10.0%) were found to cocarry the carbapenemase gene bla_{NDM-1}, mostly from waterfowl samples (94.4%, 17/18). Interestingly, among six *Acinetobacter* strains, *tet*(X) and bla_{NDM-1} were found to colocalize on the same plasmids. Moreover, whole-genome sequencing (WGS) revealed a novel orthologue of *tet*(X) in the six isolates coharboring *tet*(X) and bla_{NDM-1}. Inverse PCR suggested that the two *tet*(X) genes form a single transposable unit and may be cotransferred. Sequence comparison between six *tet*(X)- and bla_{NDM-1}-coharboring plasmids showed that they shared a highly homologous plasmid backbone even though they were isolated from different *Acinetobacter* species (three from *Acinetobacter indicus*, two from *Acinetobacter schindleri*, and one from *Acinetobacter lwoffii*) from various sources and from different geological regions, suggesting the horizontal genetic transfer of a common *tet*(X)- and bla_{NDM-1}-coharboring plasmid among *Acinetobacter* species in China. Emergence and spread of such plasmids and strains are of great clinical concern, and measures must be implemented to avoid their dissemination.

KEYWORDS *Acinetobacter* spp., bla_{NDM-1}, coharboring plasmid, *tet*(X), tigecycline resistance, waterfowls

Antibiotic resistance poses a significant threat to public health. Multidrug-resistant (MDR) pathogens cause nearly 700,000 death worldwide each year (1). Carbapenems have been used as last-line antibiotics for the treatment of infections caused by MDR bacteria (2). Unfortunately, carbapenem-resistant Gram-negative bacteria (CRGN), especially carbapenem-resistant *Enterobacteriaceae* (CRE), *Pseudomonas aeruginosa* (CRPA) and *Acinetobacter baumannii* (CRAB), have emerged in recent years, seriously threatening this class of lifesaving drugs (3–5). Effective antibiotics against CRGN remain limited, with polymyxin and tigecycline being the two choices of last resort.

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Address correspondence to Jian Sun, jiansun@scau.edu.cn.

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However, the recent discovery of a plasmid-mediated colistin resistance gene, *mcr-1*, challenges the clinical utility of colistin to treat these infections.

Although colistin and ceftazidime-avibactam have recently been approved for clinical usage in China, tigecycline remains one of the most common antibiotics used to treat complicated CRGN infections in the country. Tigecycline resistance was sporadically reported in clinical isolates, primarily due to chromosome-mediated mechanisms (6, 7). However, two very recent studies described the emergence of a plasmid encoding tigecycline enzymatic inactivation mechanism, Tet(X), in *Acinetobacter* spp. and *Enterobacteriaceae* bacteria, including isolates coharboring *mcr-1*, from animal and human samples in China (8, 9), threatening the clinical efficiency of tigecycline as a last-line antibiotic.

In addition to being common nosocomial pathogens (10, 11), *Acinetobacter* species strains are widely distributed in a variety of environmental sources, including water, soil, foods, arthropods, and livestock. Thus, antimicrobial resistance genes (ARGs), including *tet*(X), should be closely monitored in *Acinetobacter* species isolates from humans, animals (especially livestock), and environment microbiomes. Interestingly, *Acinetobacter* spp. appear to be common bacterial hosts for the mobile tigecycline resistance gene *tet*(X) (8), and a further concern is the convergence of carbapenem and tigecycline resistance in *Acinetobacter* spp., which will simultaneously deactivate the two most important families of antibiotics. Indeed, sporadic cases of *tet*(X)- and bla_{NDM-1}-positive *Acinetobacter* isolates have been reported from livestock samples in Jiangsu province in China (8).

In addition to livestock, poultry and other avian species constitute an important reservoir for the transmission of antimicrobial resistance genes. In this study, we described a high prevalence of *Acinetobacter* isolates coharboring *tet*(X) and bla_{NDM-1} from avian and especially waterfowl samples in different regions in China. Interestingly, genomic sequencing analysis revealed the occurrence of two *tet*(X) genes, *tet*(X) [formerly named *tet*(X3), GenBank accession number MK134375] and a novel *tet*(X) orthologue, in the same *Acinetobacter* isolates cocarrying bla_{NDM-1}. More importantly, we were the first to describe six novel plasmids concomitantly carrying *tet*(X) and bla_{NDM-1} with highly similar backbone structures from various *Acinetobacter* species and different sources. These findings greatly deepened our understanding of the landscape of *tet*(X)-harboring plasmids in *Acinetobacter* spp.

It should be noted that according to the standards of the nomenclature center (http://faculty.washington.edu/marilynr/), all *tet*(X) genes [formerly named *tet*(X1) to *tet*(X5)] at present can only be called *tet*(X). Therefore, the *tet*(X3) (GenBank accession no. MK134375) we detected in this study has been replaced by the name *tet*(X) in the following text.

RESULTS

Molecular epidemiology of *tet*(X) from avian samples and their adjacent environments in China. The mobile tigecycline resistance gene *tet*(X) was detected in 180 (8.9%) *Acinetobacter* isolates from 2,018 samples collected from avian farms and the adjacent environment, including isolates from all seven sampled provinces, namely, Guangdong, Hainan, Guangxi, Fujian, Shandong, Xinjiang, and Liaoning (Fig. 1 and Table 1). The prevalence of *tet*(X)-positive *Acinetobacter* species isolates ranged from 1.6% to 18.3% among the seven provinces, with Xinjiang province having the highest (18.3%) (Table 1). The *tet*(X)-positive *Acinetobacter* species isolates were mainly detected in pigeons (37.5%) and waterfowls (geese, 15.0%; ducks, 8.8%), followed by chickens (8.2%) and environmental samples (sewage, 7.5%; soil, 6.7%) (Table 1).

Antimicrobial susceptibility testing revealed that all 180 *tet*(X)-positive *Acinetobacter* species isolates were resistant to tigecycline and tetracycline and exhibited high MICs to the newly FDA-approved eravacycline (MIC range, 1 to 4 mg/liter) and omadacycline (MIC, 4 to 16 mg/liter). In addition, 98.9% ($n = 178$), 97.8% ($n = 176$), 36.1% ($n = 65$), 22.8% ($n = 41$), and 21.7% ($n = 39$) of isolates also showed resistance to trimethoprim-sulfamethoxazole, ciprofloxacin, ceftazidime, cefotaxime, and gentamicin, respectively.

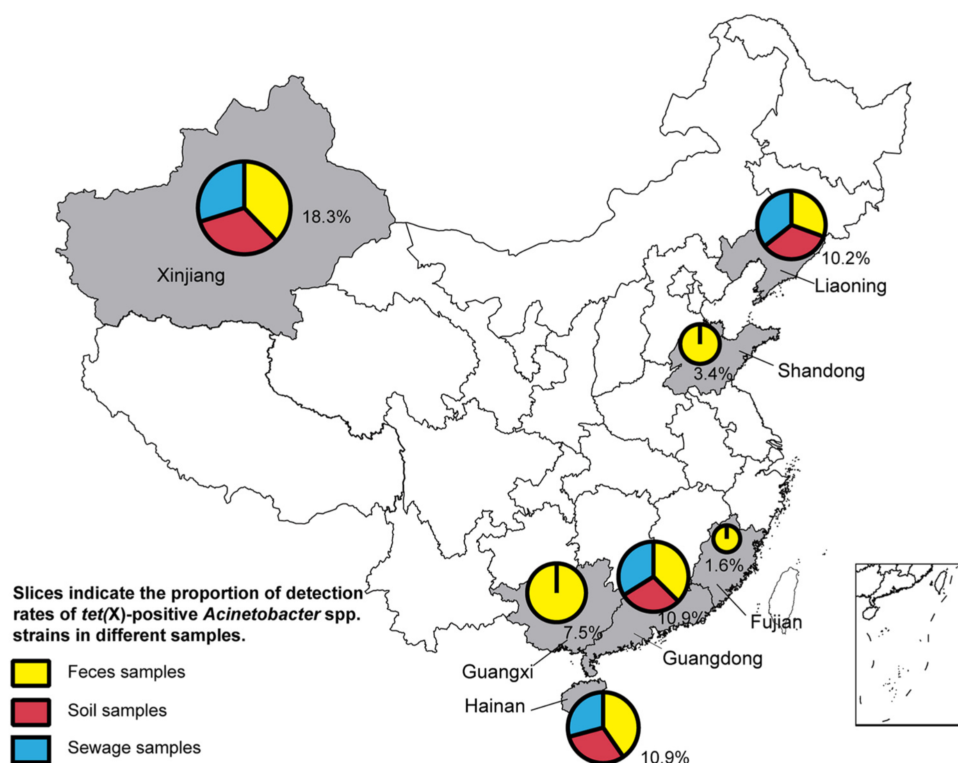

FIG 1 Map of *tet*(X)-positive *Acinetobacter* species strain sampling areas in China. For the seven provinces, the area of each pie graph represents the total detection rate of *tet*(X)-positive *Acinetobacter* species strains in all samples in the respective region. Slices indicate the proportion of detection rates of *tet*(X)-positive *Acinetobacter* species strains in different samples.

Alarmingly, 10.0% ($n = 18$) of *Acinetobacter* species isolates were also resistant to meropenem.

Characteristics of *Acinetobacter* species isolates coharboring *tet*(X) and bla_{NDM-1}. The tigecycline degradation and carbapenem inactivation method (CIM) assays showed that all 18 meropenem-resistant *Acinetobacter* species isolates could hy-

TABLE 1 Prevalence of *tet*(X)-positive *Acinetobacter* species strains in samples from avian farms and adjacent environments in this study

			No. (%) of *tet*(X)-positive strains by sample type (no. positive samples/no. of tested samples)[a]				
Province	**Origin**	**Sampling period**	**Feces**	**Soil**	**Sewage**	**Dust**	**Total overall**
Hainan	Goose farm	2017	8/42 (7.1) (**3**)	1/5 (20.0)	0/5	0/5	9/57 (15.8)
	Chicken farm	2017–2018	16/149 (10.7)	1/16 (6.3)	3/28 (10.7)	0/17	20/210 (9.5)
Guangdong	Pigeon farm	2016	15/40 (37.5)		3/10 (30)	0/4	18/54 (29.6)
	Chicken farm	2017	3/182 (1.6)	1/14 (7.1)	0/6	0/6	4/208 (1.9)
	Duck farm	2017–2018	62/471 (13.2) (**8**)	10/107 (9.3) (**3**)	10/104 (9.6)	0/13	82/695 (11.9)
	Goose farm	2018	5/40 (12.5) (**3**)	0/3	1/13 (7.7)		6/56 (10.7)
Guangxi	Chicken farm	2017	8/60 (13.3)	0/5	0/4		8/69 (11.6)
	Goose farm	2017	3/25 (12.0)				3/25 (12.0)
	Duck farm	2017	2/80 (2.5)				2/80 (2.5)
Xinjiang	Chicken farm	2017–2018	8/38 (20.1)	2/11 (18.2)	1/6 (16.7) (**1**)	0/5	11/60 (18.3)
Shandong	Duck farm	2018	9/193 (4.7)	0/36	0/34		9/263 (3.4)
Fujian	Duck farm	2018	3/120 (2.5)	0/36	0/36		3/192 (1.6)
Liaoning	Chicken farm	2018	2/21 (9.5)	2/19 (10.5)	1/9 (11.1)		5/49 (10.2)
Total			144/1,461 (9.9) (**14**)	17/252 (6.7) (**3**)	19/255 (7.5) (**1**)	0/50	180/2,018 (8.9) (**18**)

[a]Parenthetical numbers in boldface represent the numbers of *tet*(X)- and bla_{NDM-1}-coharboring *Acinetobacter* species strains.

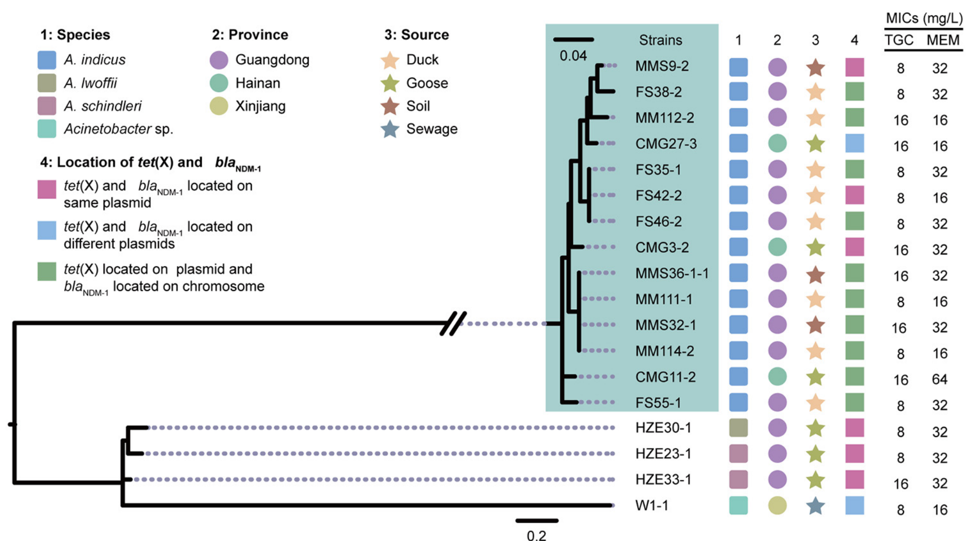

FIG 2 Genetic characteristics of isolates coharboring *tet*(X) and bla_{NDM-1}. (Left) Core genome single nucleotide polymorphism (CG-SNP) phylogenetic tree of 18 *Acinetobacter* spp. coharboring *tet*(X) and bla_{NDM-1}. (Right) The species, isolate source, sampling province, and the locations of *tet*(X) and bla_{NDM-1} in the genomes are indicated according to the color legend. TGC, tigecycline; MEM, meropenem.

drolyze both tigecycline and meropenem, and subsequent PCR and Sanger sequencing indicated that they all carried bla_{NDM-1} (Fig. 2 and 3). The 18 *tet*(X)- and bla_{NDM-1}-coharboring strains showed multidrug resistance phenotypes, including resistance to tigecycline, meropenem, ceftazidime, cefotaxime, ampicillin, ciprofloxacin, trimethoprim-sulfamethoxazole, and florfenicol (Table 2). The isolates were discovered from three provinces (Guangdong, Hainan, and Xinjiang) and were identified as *Acinetobacter indicus* ($n = 14$), *Acinetobacter schindleri* ($n = 2$) and *Acinetobacter lwoffii* ($n = 1$) and one unknown *Acinetobacter* species ($n = 1$) (Fig. 2). They were mainly

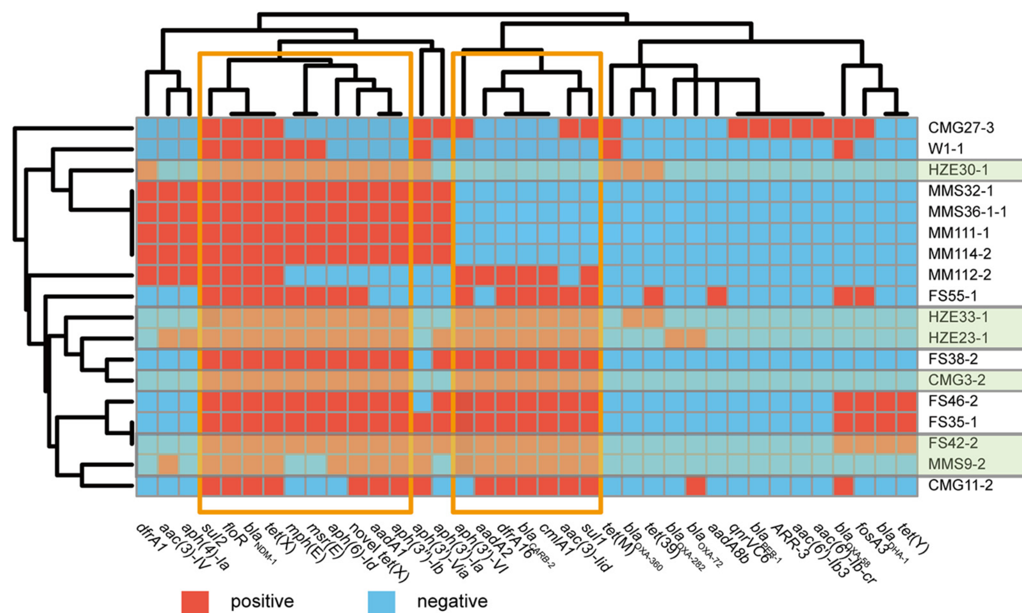

FIG 3 A heat map of the antimicrobial resistance genes for 18 *Acinetobacter* species isolates cocarrying *tet*(X) and bla_{NDM-1}. Genes positive and negative for resistance are indicated. Green shading indicates the strains in which *tet*(X) and bla_{NDM-1} are located on the same plasmids. Areas boxed in orange indicate the two major resistance gene clusters. The heat map was generated using R, version 3.3.2 (R Foundation for Statistical Computing) using the pheatmap package (https://cran.r-project.org/web/packages/pheatmap/).

TABLE 2 MICs of antimicrobials for 18 *Acinetobacter* species isolates coharboring *tet*(X) and bla_{NDM-1}

		MIC (mg/liter)[a]														
Strain	**Province**	**TGC**	**ERA**	**OMA**	**TET**	**MEM**	**CAZ**	**CTX**	**AMP**	**FFC**	**CIP**	**S/T**	**GEN**	**AMK**	**FOS**	**CS**
FS35-1	Guangdong	8	4	8	>256	32	>256	>256	256	64	32	>320	32	32	>256	0.25
FS38-2	Guangdong	8	4	8	>256	32	>256	>256	256	64	32	>320	128	32	256	0.25
FS42-2	Guangdong	8	4	8	256	16	>256	128	256	256	16	>320	1	8	>256	0.25
FS46-2	Guangdong	8	4	16	>256	32	>256	>256	>256	256	64	>320	128	8	>256	0.25
FS55-1	Guangdong	8	4	8	256	32	>256	>256	128	>256	128	320	1	32	>256	0.25
CMG3-2	Hainan	16	4	16	128	32	>256	256	128	>256	256	>320	16	8	256	0.5
CMG11-2	Hainan	16	4	16	128	64	>256	256	256	256	32	>320	128	16	256	0.5
CMG27-3	Hainan	16	4	16	128	16	>256	>256	32	256	128	>320	64	8	256	0.25
HZE23-1	Guangdong	8	4	16	64	32	>256	>256	256	256	64	320	16	32	256	0.25
HZE30-1	Guangdong	8	4	16	>256	32	>256	>256	>256	>256	64	>320	0.25	64	256	0.25
HZE33-1	Guangdong	16	4	16	256	32	>256	>256	>256	256	32	>320	32	32	256	0.5
MM111-1	Guangdong	8	4	16	128	16	>256	256	64	>256	32	160	8	4	128	0.25
MM112-2	Guangdong	16	4	16	256	16	>256	128	64	>256	32	160	64	4	64	0.25
MM114-2	Guangdong	8	4	16	128	16	>256	256	64	>256	32	160	4	4	128	0.25
MMS9-2	Guangdong	8	4	16	128	32	>256	>256	64	>256	16	160	8	4	128	0.25
MMS36-	Guangdong	16	4	16	256	32	>256	256	64	>256	32	160	8	4	128	0.25
MMS32-1	Guangdong	16	4	16	128	64	>256	>256	128	128	16	160	2	16	256	0.25
W1-1	Xinjiang	8	4	16	128	16	>256	>256	128	256	64	160	0.5	16	256	0.25

[a]TGC, tigecycline; ERA, eravacycline; OMA, omadacycline; TET, tetracycline; MEM, meropenem; CAZ, ceftazidime; CTX, cefotaxime; AMP, ampicillin; FFC, florfenicol; CIP, ciprofloxacin; S/T, trimethoprim-sulfamethoxazole; GEN, gentamicin; AMK, amikacin; FOS, fosfomycin; CS, colistin.

isolated from waterfowls (1.4%, 14/971) and their neighboring environmental soil samples (1.6%, 3/187), except that one isolate was from a sewage sample next to a chicken farm (Fig. 2).

Analysis of the localization of *tet*(X) and bla_{NDM-1}. S1 nuclease pulsed-field gel electrophoresis (S1-PFGE) and Southern blot hybridization results of the 18 *tet*(X)- and bla_{NDM-1}-coharboring isolates showed that in 10 isolates the *tet*(X) gene was located on a plasmid and that the bla_{NDM-1} gene was on the chromosome, while in 2 isolates the two genes were located on two different plasmids. Interestingly, we found six isolates, including three *A. indicus*, one *A. lwoffii*, and two *A. schindleri*, in which the *tet*(X) and bla_{NDM-1} genes were located on the same plasmids (~110 kb to ~140 kb) (Fig. 2 and Fig. S1 in the supplemental material).

Transfer of *tet*(X)-carrying plasmids. Conjugation experiments were then conducted in 30 selected *tet*(X)-positive *Acinetobacter* species strains, including the 18 aforementioned *tet*(X)- and bla_{NDM-1}-coharboring isolates. The tigecycline resistance in the 18 *tet*(X)- and bla_{NDM-1}-coharboring isolates failed to transfer to *Acinetobacter baylyi* ADP1 via conjugation. In contrast, the *tet*(X) gene was successfully transferred into *A. baylyi* ADP1 through conjugation in 2 of the remaining 12 *tet*(X)-positive isolates.

Subsequently, we performed natural transformation experiments on 18 *tet*(X)- and bla_{NDM-1}-coharboring isolates in which tigecycline resistance failed to transfer by conjugation. The tigecycline resistance of 2 of 18 strains was successfully transferred to *A. baylyi* ADP1 by natural transformation, including a strain (MMS9-2) in which *tet*(X) and bla_{NDM-1} were located on the same plasmid.

Whole-genome sequencing (WGS) analysis of *Acinetobacter* species isolates coharboring *tet*(X) and bla_{NDM-1}. Core-genome phylogenetic analysis of the 18 *tet*(X)- and bla_{NDM-1}-coharboring isolates showed that the 14 *A. indicus* isolates clustered together, while the other 4 isolates were distinct from *A. indicus* isolates (Fig. 2). Among the *A. indicus* strains, we identified two small clusters of three (FS35-1, FS42-2, and FS46-2) and four (MMS36-1-1, MM111-1, MMS32-1, and MM114-2) strains (Fig. 2) with limited core single nucleotide polymorphism (SNP) differences ($n \leq 10$). Notably, the four strains within one small *A. indicus* cluster were isolated from both waterfowls (MM111-1 and MM114-2) and their adjacent environmental soils (MMS36-1-1 and MMS32-1), and they shared identical resistance genotypes (Fig. 2 and 3). Further analysis of resistance genes showed that the 18 isolates carried some similar resistance genes, such as *sul*, *floR*, bla_{NDM-1}, two *tet*(X) genes, *mph*(E), *msr*(E), *aph*, *aadA*, *aac(3)*,

dfrA, bla_{CARB}, and *cmlA*; most of them were divided into mainly two clusters, indicating that these genes may be cotransferred with *tet*(X) and bla_{NDM-1} (Fig. 3).

Identification of a novel *tet*(X) orthologue. The novel *tet*(X) orthologue encodes a putative monooxygenase enzyme of 378 amino acids, displaying 84.39%, 84.66%, 79.63%, and 87.30% amino acid identities, respectively, to the previously reported tigecycline resistance proteins, all of which are now named Tet(X): GenBank accession no. M37699.1; AJ311171, formerly named Tet(X2); MK134375, formerly named Tet(X3); and CP037909, formerly named Tet(X4). To verify the function of this novel *tet*(X) orthologue in tigecycline resistance, this *tet*(X) gene from pCMG3-2-1 was cloned into plasmid vector pBAD24 as described previously (9). The construct demonstrated a 32-fold increase in the tigecycline MIC (8 mg/liter) in comparison to that of the empty vector control (Table S2), indicating that this newly identified *tet*(X) gene was active and conferred high-level tigecycline resistance. Among the 18 *tet*(X)- and bla_{NDM-1}-coharboring isolates, 15 were found to carry this novel *tet*(X) gene (Fig. 3), including all six strains in which *tet*(X) and bla_{NDM-1} were located on the same plasmids. PCR mapping was performed on the remaining 9 strains cocarrying *tet*(X) and the novel *tet*(X) orthologue using primers hp2-F and tnpF-R (Table S1). It was confirmed that *tet*(X) genes were located downstream of the novel orthologue of *tet*(X) in four strains, as with pCMG3-2-1, while the other five strains could not be amplified by PCR, suggesting that they may have different genetic contexts or two *tet*(X) genes not on the same plasmid.

Characteristics of the genetic contexts of *tet*(X) and bla_{NDM-1} genes. We analyzed the 18 *tet*(X)-carrying contigs showing two types of genetic profiles (type i, $n = 1$; type ii, $n = 17$) (Fig. 4a). Only one isolate, W1-1 belonged to type i, and inverse PCR showed that it formed a 5.1-kb circular intermediate of the structure of *tet*(X)-*parA-hp*-IS*CR2-tnpF*, which was the same as that of *A. baumannii* plasmid 34AB isolated from a pig at slaughter in China (GenBank accession no. MK134375). Type ii contained the sequence ΔIS*CR2*-IS*26*-Δ*tnpF*-*tet*(X)-*parA-hp*-IS*CR2* which was found in isolates from duck feces ($n = 8$), soil of duck farms ($n = 3$), and goose feces ($n = 6$). Notably, IS*CR2* was located downstream of the *tet*(X) gene among all 18 strains, which has been reported to be associated with the mobilization of *tet*(X) in previous studies (8).

From analysis of the 18 contigs carrying bla_{NDM-1}, three genomic backbone profiles were obtained (type I, $n = 5$; type II, $n = 12$; type III, $n = 1$), and they all belong to the Tn*125* or truncated Tn*125* structures. Notably, 3 isolates including *A. indicus* CMG3-2 and *A. lwoffii* HZE30-1 within type I [IS*Aba14-aph(3′)-VI*-IS*Aba125*-bla_{NDM-1}-*ble-trpF-dsbC-tnpR*], 12 isolates within type II (ΔIS*Aba125*-bla_{NDM-1}-*ble-trpF-dsbC-dvt-groS-groL-tnpA-ISAba125*), and 1 isolate within type III (ΔIS*Aba125*-bla_{NDM-1}-*ble-trpF*-Δ*dsbC-hp*-Δ*ISAba14*) were highly similar to the corresponding regions on the plasmid from a human *Acinetobacter* species isolate from China (GenBank accession no. KM210088), on the chromosome of a clinical *A. baumannii* IOMTU 433 isolate from Vietnam (AP014649), and on the chromosome of a human *A. baumannii* ACN21 isolate from India (CP038644) (Fig. 4b), respectively.

Characteristics of *tet*(X)- and bla_{NDM-1}-coharboring plasmids. To obtain the complete chromosome and plasmid structures, the six *tet*(X)- and bla_{NDM-1}-coharboring isolates, including three *A. indicus* (MMS9-2, FS42-2, and CMG3-2), one *A. lwoffii* (HZE30-1), and two *A. schindleri* (HZE23-1 and HZE33-1) with plasmids coharboring *tet*(X) and bla_{NDM-1} were subsequently sequenced by a PacBio RSII system (Nextomics) to obtain the complete chromosome and plasmid structures. The genomes of these 6 isolates varied from 3.00 to 3.16 Mb in chromosome size and carried between three and five plasmids ranging from 10.6 to 140.2 kb (Fig. 5a). In *A. indicus* CMG3-2, *tet*(X) and bla_{NDM-1} genes were found to be on a 120,957-bp untypeable plasmid, pCMG3-2-1, along with multiple resistance genes: *ble*, *sul1*, *sul2*, bla_{CARB-2}, *cmlA6*, *aadA1*, *aadA2*, *aac(3)-IId*, *aph(3′)-VI*, *aph(3′)-Ib*, *aph(6)-Id*, *aph(3′)-Via*, *floR*, *dfrA16*, and the novel *tet*(X) orthologue (Fig. 3 and 5b).

The complete sequences of the six *tet*(X)- and bla_{NDM-1}-coharboring plasmids were further compared. The results showed that the six plasmids contained two similar

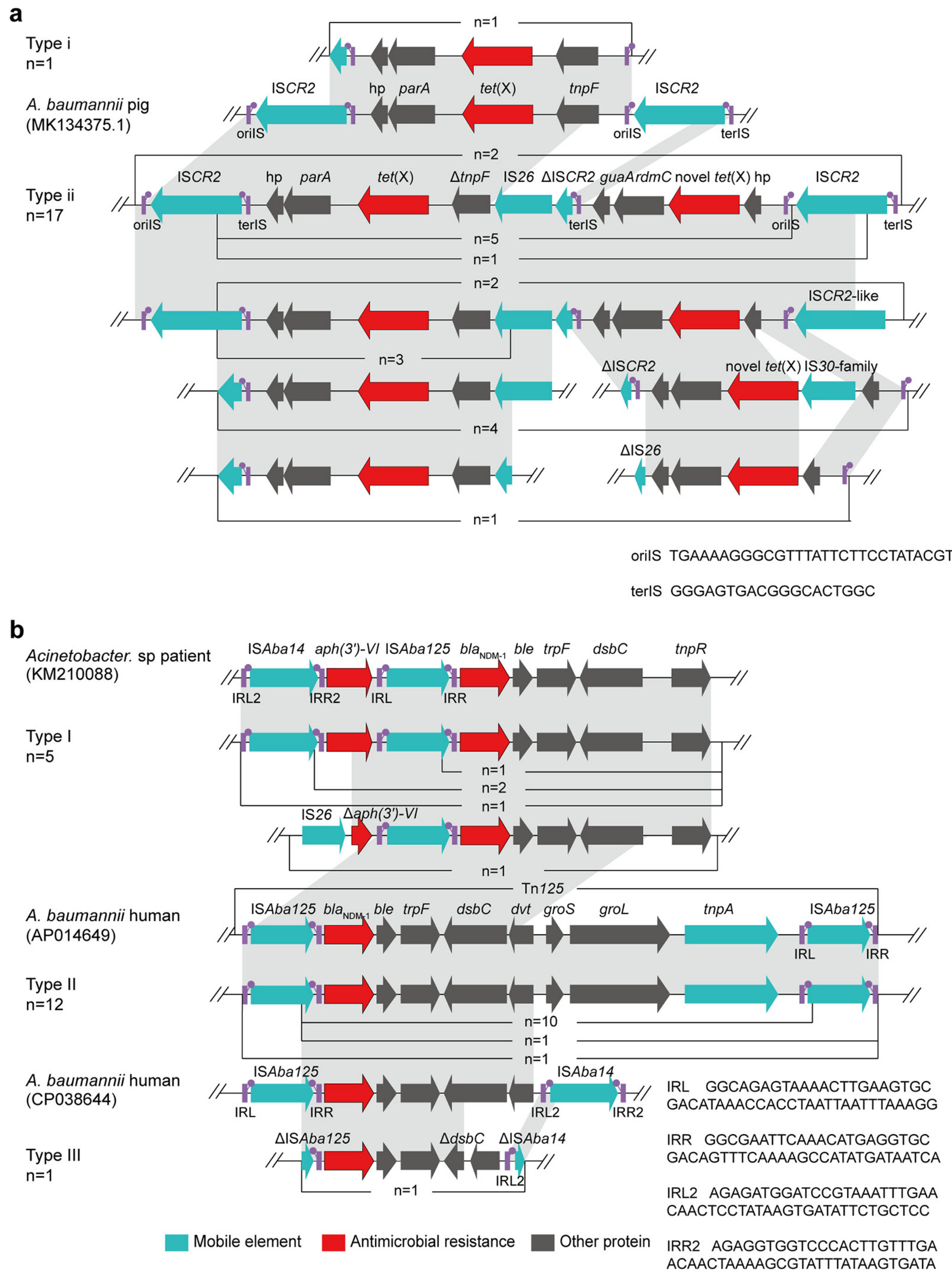

FIG 4 Genetic features of *tet*(X) and bla_{NDM-1}. (a) Linear sequence comparison of genetic context of *tet*(X) in the 18 isolates in this study carrying plasmid 34AB (GenBank accession no. MK134375.1) from *A. baumannii* isolated from a pig in China. (b) Linear sequence comparison of genetic context of bla_{NDM-1} in this study with that of an *Acinetobacter* species isolate from human in China (KM210088) and from chromosome of a clinical *A. baumannii* IOMTU 433 isolate from Vietnam (AP014649). The arrows indicate the positions and directions of transcription for the genes. Regions of >99.0% nucleotide sequence identity are shaded in gray. The delta (Δ) symbol indicates a truncated gene. oriIS, the origin of replication; terIS, the termination sequence; IRL, terminal inverted repeat, left; IRR, terminal inverted repeat, right.

multidrug resistance regions (pHZE30-1-1 contained only one) and plasmid backbone structures, including genes encoding plasmid replication, maintenance, and stability (Fig. 5b). Plasmid pHZE33-1-1 (from an *A. schindleri* isolate from goose), pMMS9-2-1 (*A. indicus* isolate from soil), pHZE23-1-1 (*A. schindleri* isolate from goose), pHZE30-1-1 (*A. lwoffii* isolate from goose), and pFS42-2-1 (*A. indicus* isolate from duck) showed 84% to

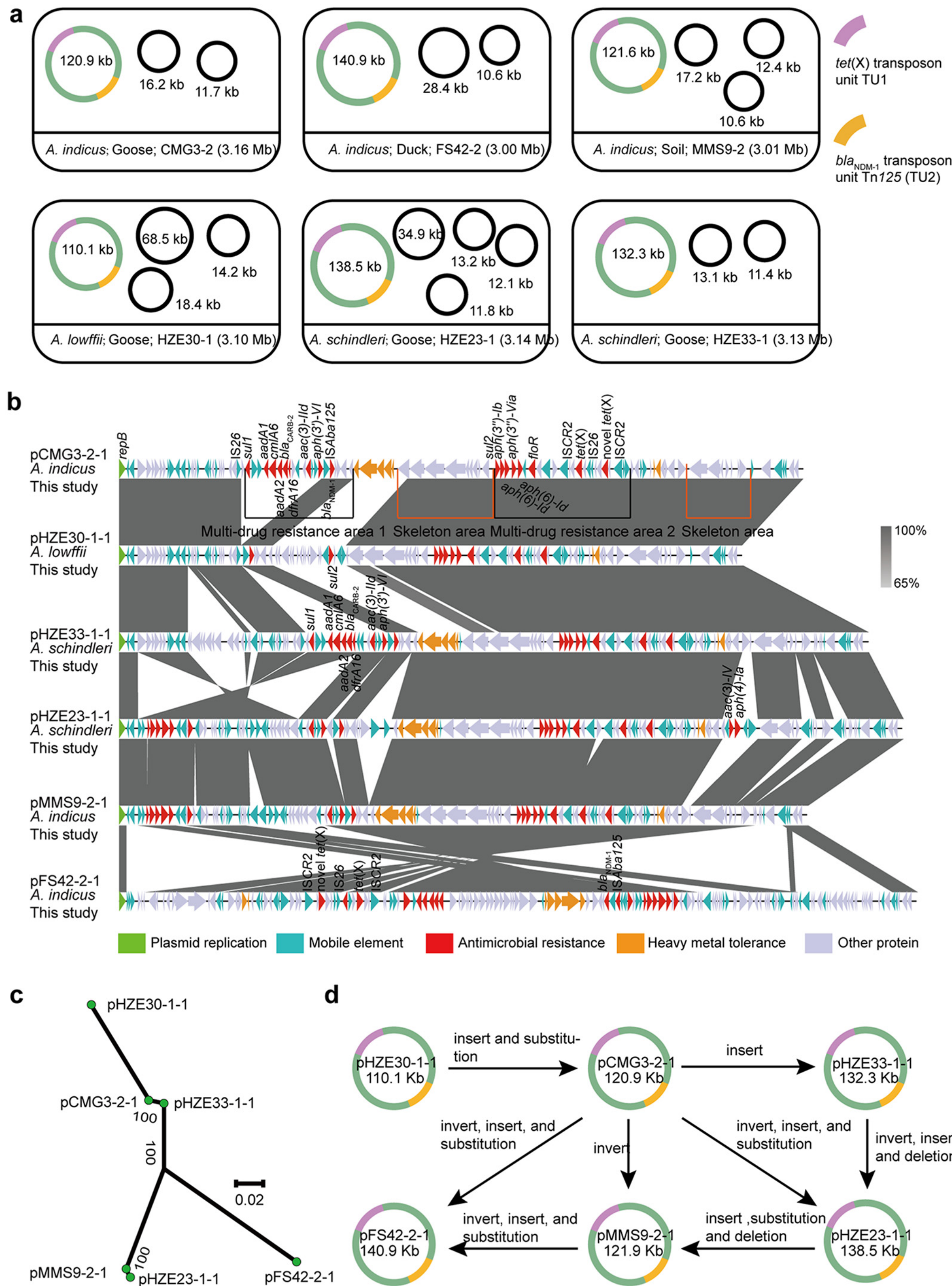

FIG 5 Characterization of the six *tet*(X)- and *bla*$_{NDM-1}$-coharboring plasmids by the PacBio RSII platform. (a) Species, source, and chromosome size of each strain are indicated. The transposition unit TU1 of *tet*(X) and the transposition unit Tn*125* (TU2) of *bla*$_{NDM-1}$ are indicated. (b) Linear sequence comparison of the six *tet*(X)- and *bla*$_{NDM-1}$-coharboring plasmids. The results of sequence alignment were generated with Easyfig, version 2.1. Regions of homology are marked by shading. The delta (Δ) symbol indicates a truncated gene. Genes associated with plasmid replication, antimicrobial resistance, heavy metal resistance, and mobility are marked in green, red, orange, and blue, respectively, while other gene are shown in gray. (c) A radial representation of a phylogenetic tree of six *tet*(X)- and *bla*$_{NDM-1}$-coharboring plasmids and homologs. (d) Hypothesis of the molecular evolution of the six *tet*(X)- and *bla*$_{NDM-1}$-coharboring plasmids.

99% query coverage and 98% to 100% nucleotide identity to the sequence of pCMG3-2-1. Notably, the six plasmids were isolated from different *Acinetobacter* species (*A. lwoffii*, *A. indicus*, and *A. schindleri*) and from various sources (goose, duck, and soil samples) from different regions of China (Guangdong and Hainan). On the basis of

pCMG3-2-1, the other five plasmids may be formed through sequence insertion, substitution, deletion, and inversion (Fig. 5d).

DISCUSSION

The genus *Acinetobacter*, including non-*baumannii Acinetobacter* species, includes opportunistic pathogens and commonly causes nosocomial infections (10). Worrisomely, the *tet*(X) genes have already been reported in *A. baumannii* strains isolated from inpatients. By BLAST analysis, we also detected the *tet*(X)-carrying contigs in *Acinetobacter* species from clinical samples, including *A. pittii* (GenBank accession no. JRQZ01000088.1), *A. nosocomialis* (NNSH01000121.1), and *A. baumannii* (UFMQ01000023.1). All of these indicated the importance of *tet*(X)-positive *Acinetobacter* species strains for human diseases, challenging the clinical effectiveness of the last-resort tigecycline.

In the present study, during our recent antimicrobial surveillance on avian farms and adjacent environments, the *tet*(X) gene was found in 180 (8.9%) *Acinetobacter* species isolates from all seven sampled provinces (Fig. 1), which was significantly higher than the rate (0.1%) in avian samples from a previous report (8). This indicated widespread presence of *tet*(X)-positive *Acinetobacter* spp. in avian farms and their surrounding environments. Surprisingly, 10% ($n = 18$) of these *tet*(X)-bearing strains also carried the carbapenem resistance gene bla_{NDM-1}, mainly from waterfowls and the soils of their neighboring environments (1.6%), suggesting that the environmental soils adjacent to farms may be contaminated by avian feces and could serve as a reservoir for the spread of tigecycline- and carbapenem-resistant *Acinetobacter* spp. Indeed, previous studies showed that the soils from greenhouse and open-field agricultural soil can often contain *tet*(X) genes (12), likely from organic fertilizer with animal feces (13). Additionally, one sample of sewage (0.4%) adjacent to a chicken farm carried one *tet*(X)- and bla_{NDM-1}-coharboring *Acinetobacter* species isolate (Fig. 2); however, none of the chicken samples from the farm were positive for such *Acinetobacter* spp. (Table 1). Therefore, we suspected that this *tet*(X)- and bla_{NDM-1}-coharboring isolate may have been transmitted from somewhere through contaminated water (14). In fact, the high detection rate among *tet*(X)- and bla_{NDM-1}-coharboring isolates in waterfowl samples suggested the transmission of these bacteria through contaminated water systems. Tigecycline is a novel glycylcycline, only approved for the treatment of complex skin and intra-abdominal infections and community-acquired pneumonia, and its use has never been allowed in animal husbandry. However, recent epidemiological studies show that the detection rate of *tet*(X)-positive strains in farmed animals was significantly higher than that in humans (8, 9). Some studies suggest that this is related to the large-scale use of tetracyclines (e.g., tetracycline, oxytetracycline, chlortetracycline, and doxycycline) in the livestock industry (8, 9, 15). The selection pressure from tetracyclines may promote the enrichment and transmission of *tet*(X) genes. Further studies are required to reveal how these clinically significant ARGs [*tet*(X) and bla_{NDM}] were transferred into waterfowl farms.

Notably, in the present study, we found common *tet*(X)- and bla_{NDM-1}-coharboring vectors, pCMG3-2-1-like plasmids, which have been disseminated among three *Acinetobacter* species isolates from diverse sources, including waterfowls, soil, and sewage, in three geographic areas in China. Plasmid sequence phylogenetic analysis showed that the six plasmids have a very close evolutionary relationship with each other (Fig. 5c). Additionally, it is also noted that a pCMG3-2-1-like plasmid carried two copies of *tet*(X) genes, and these two *tet*(X) genes were located in a composite transposon-like unit (TU1), flanked by two intact copies of IS*CR2* in the same orientation, and the gene arrangement of TU1 was IS*CR2*-hp-*tet*(X)-*rmdc*-*guaA*-ΔIS*CR2*-IS*26*-Δ*tnpF*-*tet*(X)-*parA*-hp-IS*CR2*. However, a *tet*(X)-only circular intermediate failed to be amplified by inverse PCR. In contrast, two *tet*(X) genes coharboring a circular intermediate were obtained. Analysis of the bla_{NDM-1}-containing elements among the six plasmids revealed that bla_{NDM-1} genes were harbored by similar Tn*125* elements (1 intact and 5 partial), located approximately

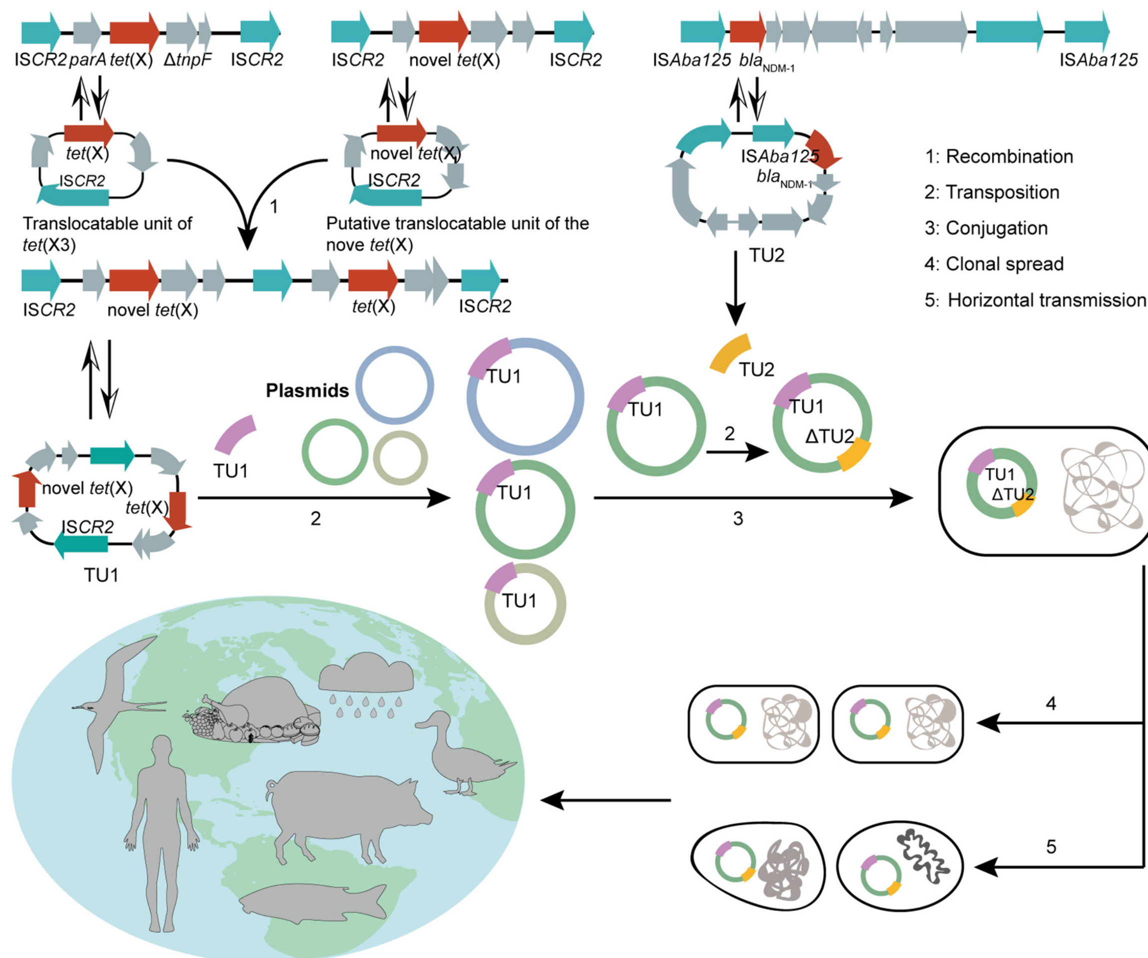

FIG 6 Schematic diagram of the formation, evolution, and spread of the six *tet*(X)- and bla_{NDM-1}-coharboring plasmids.

35 to 45 kb upstream of *tet*(X), further confirming that the Tn*125* transposon is mainly responsible for the horizontal transmission of bla_{NDM-1} in *Acinetobacter* spp. (16). These results prompted us to propose a hypothesis about the formation, evolution, and spread of the six *tet*(X)- and bla_{NDM-1}-coharboring plasmids (Fig. 6). We speculate that the aforementioned TU1 might have originated by the integration of two *tet*(X) translocatable units together (Fig. 6). The bla_{NDM-1}-harboring Tn*125* may be transposed independently into the same pCMG3-2-1-like plasmid background, and under the pressure of antibiotic selection, this type of multidrug-resistant plasmid can be transferred between various pathogens and ecosystems through clonal spread or horizontal transmission of strains.

The discovery of a highly similar pCMG3-2-1-like plasmid in different species, sources, and geographical regions suggested the horizontal gene transfer (HGT) of this plasmid, even though several attempts have failed to demonstrate conjugation of a pCMG3-2-1-like plasmid into the recipient *A. baylyi* ADP1. Accordingly, the plasmid sequence analysis failed to identify any plasmid transfer operons. Other mechanisms, including natural transformation, transduction, and/or outer membrane vesicle (OMV)-mediated transfer, may contribute to the HGT of this plasmid (17–19), which deserves further study. Nevertheless, our study clearly demonstrated that these antibiotic-resistant plasmids or strains are spreading in waterfowls and their neighboring environments in China. A formidable concern is that these resistance genes, plasmids, and strains may eventually spread into human populations via direct contact or consumption of contaminated food products.

It is noteworthy that only the term of *tet*(X) is currently accepted by the nomenclature center, and according to the standard of nomenclature, a series of recently discovered *tet*(X) genes [formerly named *tet*(X3), *tet*(X4), *tet*(X5), and other variants] can only be called *tet*(X). However, the initial *tet*(X) gene is mainly located in obligate anaerobic bacteria (20–23), which could not confer tigecycline resistance due to its requirement for O_2 and NADPH (24). Although this gene was subsequently detected in aerobic bacteria, there is no evidence that it can convey tigecycline resistance at the resistance breakpoint (EUCAST, >2 mg/liter; FDA, ≥8mg/liter). In contrast, one study cloned the initial *tet*(X) into an *Escherichia coli* expression system and found that the MIC of tigecycline is only 0.25 mg/liter (25), which is much lower than that of the recently reported *tet*(X) genes (8 or 16 mg/liter) (8, 9). Similarly, the research by Xu and coworkers (26) showed that different *tet*(X) orthologues have different levels of resistance to tigecycline, so we think that it is very confusing to use only the name *tet*(X) to refer to all *tet*(X) genes. Here, we ask that the nomenclature standard for *tet*(X) be improved as soon as possible to facilitate further research on this important resistance gene.

Conclusion. Our study reported the relative prevalence of tigecycline-resistant *Acinetobacter* species isolates coharboring *tet*(X) genes and bla_{NDM-1} in avian samples, especially in waterfowls and samples from their adjacent environments, in China. In addition, we identified a novel plasmid-borne *tet*(X) gene in *Acinetobacter* spp. which can mediate high levels of resistance to tigecycline. More importantly, we described for the first time a common *tet*(X)- and bla_{NDM-1}-coharboring plasmid in *Acinetobacter* species isolates in avian and adjacent environmental samples from different regions in China, highlighting the mobile nature of these highly resistant genes. Considering the close relationship among animals, environments, and humans, the further spread of these strains and plasmids into human health care settings should be closely monitored. Coordinated efforts should be taken to control the spread of these antibiotic-resistant bacteria in different sectors.

MATERIALS AND METHODS

Sample collection and identification of *tet*(X)-positive strains. From July 2016 to November 2018, a total of 2,018 nonduplicate samples were collected from 34 avian farms in seven provinces of China (Table 1). Briefly, the fecal samples were randomly collected from ducks ($n = 864$), geese ($n = 107$), chickens ($n = 450$), and pigeons ($n = 40$). Samples from the environments surrounding the farms were also collected (Table 1), including soil ($n = 252$), sewage ($n = 255$), and dust ($n = 50$) samples (Table 1). Following selection on CHROMagar *Acinetobacter* plates containing tigecycline (2 mg/liter), the tigecycline-nonsusceptible isolates were screened for *tet*(X) genes using previously reported primers (8). The species of *tet*(X)-positive *Acinetobacter* strains were further determined by 16S rRNA and *rpoB* sequencing analyses (27). This study protocol was reviewed and approved by the South China Agriculture University Animal Ethics Committee.

Antimicrobial susceptibility testing. We determined the MICs of amikacin, gentamicin, tetracycline, meropenem, ceftazidime, cefotaxime, ciprofloxacin, trimethoprim-sulfamethoxazole, and florfenicol for all *tet*(X)-positive strains using the agar dilution method, and the results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Fresh Mueller-Hinton (MH) broth was prepared, and the MICs of tigecycline, eravacycline, omadacycline, and colistin were examined by the broth microdilution method. The breakpoints for tigecycline and colistin were interpreted according to the FDA criteria for *Enterobacteriaceae* bacteria (resistant, ≥8 mg/liter) and the European Committee on Antimicrobial Susceptibility Testing (resistant, >2 mg/liter), respectively. No MIC breakpoints exist of eravacycline and omadacycline for *Acinetobacter* species strains. *E. coli* ATCC 25922 was used as a quality control strain.

Microbiological degradation assays and PCR testing. The tigecycline degradation assays were determined as previously described (9), with little change. First, the MH broth suspension of the test strains was prepared with an optical density of 2.0 at 600 nm. Then, tigecycline was added to the suspension (2.5 mg/liter) and incubated at 37°C for 8 h. Meanwhile, an overnight culture of *Bacillus stearothermophilus* 7953 was spread on an MH agar plate, and three 6-mm-diameter holes were punched. Finally, the culture solution of the previous step was centrifuged and filtered through a 0.22-μm-pore-size filter; 20 μl was dispensed into plate wells and incubated at 60°C for 16 h to observe the results. The *A. baumannii* ATCC 19606 strain was used as a negative control.

Testing using the carbapenem inactivation method (CIM) was performed as previously reported (28) with slight modification. Briefly, a loopful of bacteria (~10 μl) was suspended in 400 μl of sterile water with one 10-μg imipenem disc and incubated for 2 h. The disc was then placed on a Mueller-Hinton agar plate coated with a susceptible *E. coli* indicator strain (ATCC 29522) and reincubated for 18 h. If the bacterial isolate produced carbapenemase, then the antibiotic in the disc

would be inactivated, leading to the appearance of an obviously diminished zone of inhibition. *E. coli* ATCC 25922 was used as a negative-control strain. Moreover, carbapenemase-producing strains were screened for the carbapenemase-encoding genes bla_{NDM}, bla_{IMP}, bla_{VIM}, bla_{SPM}, bla_{AIM}, bla_{DIM}, bla_{GIM}, bla_{SIM}, bla_{KPC}, bla_{BIC}, bla_{OXA-48}, bla_{OXA-23}, bla_{OXA-51}, and bla_{OXA-58} using previously described primers (29, 30). Strains positive for related genes were used as positive controls.

Conjugation experiments and natural transformation experiments. The transferability of tigecycline resistance in selected *tet*(X)-positive *Acinetobacter* isolates was examined by the filter mating method using rifampin-resistant *A. baylyi* ADP1 as the recipient strain. Briefly, the donor and recipient strains were mixed at a ratio of 1:3 and applied to a sterilized 0.22-μm-pore-size filter, followed by culturing at 30°C for 24 h. The transconjugants were selected on selective LB agar containing tigecycline (4 mg/liter) together with rifampin (150 mg/liter). We further confirmed the transconjugants by PCR for *tet*(X) and by M13 PCR (PCR-based fingerprinting for *Acinetobacter* spp.) (31).

For 18 *tet*(X)- and bla_{NDM-1}-coharboring isolates for which tigecycline resistance was not transferred by conjugation, natural transformation experiments were performed as described previously (32). Briefly, the naturally competent strain *A. baylyi* ADP1 was cultured in LB broth at 30°C to 1×10^9 cells ml^{-1}, concentrated to 1×10^{10} cells ml^{-1} in LB broth with 10% glycerol, and stored at −80°C until use. For transformation assays, a fresh bacterial suspension was diluted to 2.5×10^8 cells ml^{-1} in LB broth containing 100 ng ml^{-1} donor DNA. After incubation at 30°C for 2 h, the bacterial suspension was appropriately diluted and plated on LB agar plates containing 4 mg/liter tigecycline. The colonies were selected for PCR screening after 24 h at 30°C.

Localization of *tet*(X) and bla_{NDM-1}. To analyze the chromosomal and plasmid localizations of *tet*(X) and bla_{NDM-1} genes, we performed S1 nuclease-PFGE as previously described (33). Subsequently, Southern blot hybridization was conducted using DNA probes specific for *tet*(X) and bla_{NDM-1} nonradioactively labeled with a DIG (digoxigenin) High Prime DNA labeling and detection kit (Roche Diagnostics, Mannheim, Germany).

Whole-genome sequencing. Total DNA of the 18 *Acinetobacter* species isolates coharboring *tet*(X) and bla_{NDM-1} was extracted using a TIANamp Bacteria DNA kit (Tiangen, China) and then sequenced using an Illumina HiSeq 2500 platform (Bionova Biotech Co.) (Illumina). The sequences were assembled using SPAdes, version 3.12.0 (34), followed by antibiotic resistance gene prediction using ResFinder, version 3.1 (https://cge.cbs.dtu.dk/services/ResFinder/), and transposon and insertion (IS) element mining using ISfinder (https://www-is.biotoul.fr). Functional annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline server and RAST server (35). Core genome SNPs were analyzed using a previously described method (36). In brief, the trimmed, paired-end sequences were mapped to *A. indicus* genome SGAir0564 (GenBank accession number CP024620), followed by the removal of SNPs in prophages and repeated and recombination regions. Concatenated core genome SNP sites were extracted from the recombination-free alignment, and a maximum likelihood phylogenetic tree was generated by IQ-TREE (37). The bla_{NDM-1} and *tet*(X) gene flanking regions were compared by blastn.

To characterize the complete sequences of plasmids cocarrying *tet*(X) and bla_{NDM-1}, isolates with plasmids coharboring the bla_{NDM-1} and *tet*(X) genes were subjected to single-molecule real-time sequencing using a PacBio RSII system (Nextomics), followed by assembly using Unicycler, version 0.4.1 (38). ClustalW was utilized to generate a nucleotide-guided multiple sequence alignment, which was utilized for the subsequent phylogenetic analysis. A maximum likelihood tree with 500 bootstrap replicates was generated by MEGA, version 7 (39). Comparison of these plasmids was performed by Easyfig, version 2.1 (40). To determine the potential transferability of the two *tet*(X)-cocarrying segments, inverse PCR was performed for isolate CMG3-1 using P1 primers (see Table S1 in the supplemental material).

Data availability. Genome assemblies of 18 strains coharboring *tet*(X) and bla_{NDM-1} were deposited in GenBank under BioProject accession number PRJNA558439.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

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We declare that we have no competing interests.

C.-Y.C., C.C., and B.-T.L. contributed equally in this study. Y.-H.L., J.S., L.C., and X.-P.L. designed the study. C.-Y.C., C.C., Q.H., X.-T.W., Y.Z., Z.-H.C., W.-Y.G., Q.-LJ., and C.L. collected the data. C.-Y.C., J.S., L.C., C.C., B.-T.L., and R.-Y.S. analyzed and interpreted the data. Y.-H.L., L.C., J.S., C.C., B.-T.L., X.-P.L., and C.-Y.C. wrote the draft of the manuscript. All authors reviewed, revised, and approved the final report.

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