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RESEARCH ARTICLE

Source Tracking and Global Distribution of the Tigecycline Non-Susceptible *tet*(X)

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ABSTRACT The emergence of *tet*(X) genes has compromised the clinical use of the last-line antibiotic tigecycline. We identified 322 (1.21%) *tet*(X) positive samples from 12,829 human microbiome samples distributed in four continents (Asia, Europe, North America, and South America) using retrospective data from worldwide. These *tet*(X) genes were dominated by *tet*(X2)-like orthologs but we also identified 12 samples carrying novel *tet*(X) genes, designed *tet*(X45), *tet*(X46), and *tet*(X47), were resistant to tigecycline. The metagenomic analysis indicated these *tet*(X) genes distributed in anaerobes dominated by *Bacteroidaceae* (78.89%) of human-gut origin. Two mobile elements IS*Bf11* and IS*4351* were most likely to promote the transmission of these *tet*(X2)-like orthologs between *Bacteroidaceae* and *Riemerella anatipestifer*. *tet*(X2)-like orthologs was also developed during transmission by mutation to high-level tigecycline resistant genes *tet*(X45), *tet*(X46), and *tet*(X47). Further tracing these *tet*(X) in single bacterial isolate from public repository indicated *tet*(X) genes were present as early as 1960s in *R. anatipestifer* that was the primary *tet*(X) carrier at early stage (before 2000). The *tet*(X2) and non-*tet*(X2) orthologs were primarily distributed in humans and food animals respectively, and non-*tet*(X2) were dominated by *tet*(X3) and *tet*(X4). Genomic comparison indicated these *tet*(X) genes were likely to be generated during *tet*(X) transmission between *Flavobacteriaceae* and *E. coli*/*Acinetobacter* spp., and IS*CR2* played a key role in the transmission. These results suggest *R. anatipestifer* was the potential ancestral source of *tet*(X). In addition, *Bacteroidaceae* of human-gut origin was an important hidden reservoir and mutational incubator for the mobile *tet*(X) genes that enabled spread to facultative anaerobes and aerobes.

IMPORTANCE The emergence of the tigecycline resistance gene *tet*(X) has posed a severe threat to public health. However, reports of its origin and distribution in human remain rare. Here, we explore the origin and distribution of *tet*(X) from large-scale metagenomic data of human-gut origin and public repository. This study revealed the emergency of *tet*(X) gene in 1960s, which has refreshed a previous standpoint that the earliest presence of *tet*(X) was in 1980s. The metagenomic analysis from data mining covered the unculturable bacteria, which has overcome the traditional bacteria isolating and purificating technologies, and the analysis indicated that the *Bacteroidaceae* of human-gut origin was an important hidden reservoir for *tet*(X) that enabled spread to facultative anaerobes and aerobes. The continuous monitoring of mobile tigecycline resistance determinants from both culturable and unculturable microorganisms is imperative for understanding and tackling the dissemination of *tet*(X) genes in both the health care and agricultural sectors.

KEYWORDS tigecycline resistance, *tet*(X), source tracking, human microbiome, *Riemerella anatipestifer*, *Bacteroidaceae*

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The first generation of tetracycline antibiotics consisted of tetracycline, chlortetracycline, and oxytetracycline and were put into clinical practice in 1952 (1), while the second generation derivatives doxycycline and minocycline were put into use in 1976 (2). These antibiotics have been incorporated into animal feed to improve growth and feed efficiency (3). However, bacterial resistance to the tetracyclines was observed from the very beginning of their usage. To date, more than 65 specific resistant determinants and nine MDR efflux pump genes of the root nodulation-division superfamily have been confirmed including AdeABC, AcrAB-TolC and MexAB-OprM (2). These determinants confer resistance to first and second generation tetracyclines and are widely distributed among 130 Gram-negative and Gram-positive bacteria (2).

A third-generation tetracycline (tigecycline) was approved in the United States in 2005 and its use in the European Union and China was authorized in 2006 and 2010, respectively (4, 5). Tigecycline has a robust treatment range and includes bacteria resistant to first- and second-generation tetracyclines (6) and is a last resort antibiotic used to treat severe infections caused by carbapenem- and colistin-resistant pathogens (5). Thus, this antibiotic was classified as a critically important antimicrobial by the World Health Organization and its usage is restricted (7). However as early as 1984, the transferable gene *tet*(X) displaying tigecycline insusceptibility was discovered on an R plasmid from a *B. fragilis* isolate of human origin. This was the earliest occurrence of an antibiotic resistance gene (ARG) that directly inactivated tetracyclines (8). The *tet*(X) gene was only functional under aerobic growth conditions because it is a flavin dependent monooxygenase that requires FAD, NADPH, Mg^{2+}, and O_2 to inactive almost all of the tetracycline class (9). In 2001, the existence of *tet*(X1) and *tet*(X2) were confirmed on a transposon from *Bacteroides thetaiotaomicron* of human origin and shared 61.7 and 99.5% amino acid identity with *tet*(X), respectively. To date, *tet*(X)/*tet*(X2) genes have already spread to 16 countries/regions covering five continents (Asia, Europe, North America, South America and Africa) (2). A small comfort was that these ARGs displayed low level tigecycline resistance (MIC $\leq$ 2 μg.ml^{-1}) (2).

The emergence of plasmid-mediated high-level tigecycline resistance encoded by the *tet*(X3) and *tet*(X4) genes in 2019 posed a severe threat to public health (7, 10). In addition, 10 more *tet*(X) orthologs have been identified and include *tet*(X5)–*tet*(X14) (11–14). These orthologs were primarily found in food animals especially swine, including *tet*(X3), *tet*(X4), *tet*(X6), and *tet*(X14), first detected in *Acinetobacter baumanii*, *Escherichia coli*, *Myroides phaeus*, and *Empedobacter stercoris*, respectively. The *tet*(3.2) and *tet*(X5) gene were identified from an *Empedobacter brevis* isolate of shrimp origin and an *Acinetobacter baumannii* isolate of human origin, respectively. All the *tet*(X7)–*tet*(X13) orthologs were identified directly from gut-derived metagenomic libraries, but their host bacteria were unknown. Epidemiological studies (2, 15, 16) indicated the dissemination of these *tet*(X) orthologs were dominated by *tet*(X3) and *tet*(X4) that were primarily detected from *Acinetobacter* spp. and *E. coli*, respectively. Furthermore, *tet*(X3)/*tet*(X4) samples from humans, animals, and meat for consumption revealed a prevalence of 0.3–66.7% and the highest level of 66.7% was detected from pig cecum samples from abattoirs (7). Compared with the *tet*(X3)/*tet*(X4) in animal isolates (6.9%, 73/1,060) (7), lower prevalence from human (0.32%, 4/1,250) were observed in a retrospective screening of *tet*(X)-carrying clinical isolates (5).

The *tet*(X) genes have been primarily identified using traditional cultural methods and this imposed limitations on their identification including the loss of uncultured bacteria, low-throughput and long processing times. Although the *tet*(X7)–*tet*(X13) orthologs were found directly from gut-associated samples using metagenomic sequencing, the bacterial hosts, relative abundance and propagative characteristics were absent (14). Nonetheless, public repositories are a promising high-throughput resource for exploring antibiotic resistomes. For instance, a retrospective epidemiological study based on the available public bacterial gene data sets revealed that the food chain was a potential dissemination pathway for *mcr-1* (17). Additionally, a metagenomic screening study based on public metagenome data sets revealed a high detection rate of *tet*(X3) (25.4%) in poultry samples (18). However,

there are few studies that utilize data mining for *tet*(X) in public databases (18, 19). In addition, public data repositories including GenBank are a valuable resource for the exploration of novel bacterial species. For instance, a recent study utilized 9,428 metagenomes to reconstruct 154,723 microbial genome bins that generated 4,390 species-level genome bins including 77% of which were not present in public repositories (20). Identification of *tet*(X) genes from these species-level genome bins and tracing their distribution in assembly isolates from public repository may provide a new perspective for source tracking of the global spread of *tet*(X).

In the current study, we utilized these data mining techniques and discovered that *tet*(X) had emerged as early as 1960 and the *Riemerella anatipestifer* was its potential ancestral source. In addition, *Bacteroidaceae* of human gut origin were a hidden reservoir and mutational incubator for mobile *tet*(X) genes that enabled spread to facultative anaerobes and aerobes.

RESULTS

Identification of *tet*(X) orthologs. A total of 202,265 metagenome-assembled genomes (MAGs) that were reconstructed from 12,829 metagenomic samples of human-microbiome origin in previous studies (20–22). We downloaded these MAGs and found a total of 322 (1.21%) encoded a 388 aa protein with 96.13–100% similarity with *tet*(X) orthologs reported in a previous study (11) (Text S1 and Table S2 in the supplemental material). All the assembled contigs in these MAGs have been constructed using a single-sample assembly strategy and passed strict quality control tailored at maximizing the quality. Of these, 96.27% (310/322) harbored 15 types of *tet*(X)-like orthologs that shared 98.71–100% identity with *tet*(X2) (Acc. No. AJ311171) (Fig. S1 and S2, Table S3). The remaining 3.73% (12/322) shared $<$ 98.20% identical with the known *tet*(X). Since there was not a criterion for assignment of *tet*(X) orthologs in previous studies (11, 14), we temporarily designated these *tet*(X2)-like orthologs as *tet*(X2.2) to *tet*(X2.16). These orthologs represented a large proportion of samples (n = 12,829) from 19 countries, and they likely represent a relatively comprehensive assessment of *tet*(X2)-like orthologs. We therefore tentatively used the lowest cutoff of 98.20% between *tet*(X2)-like orthologs [*tet*(X2.15) *vs* *tet*(X2.16)] for assignment of novel *tet*(X) orthologs. In addition, when *tet*(X)s were annotated as one group of *tet*(X), such as *tet*(X2) (Fig. S1), they should be grouped into one clade using the neighbor joining typing method (23). According to data, 44 *tet*(X)s have been assigned (24). Therefore, we found three new *tet*(X) orthologs and their subtypes shared less than 98.20% amino acid identity with their closest neighbors in the phylogeny designed *tet*(X45), *tet*(X45.2), *tet*(X45.3), *tet*(X46), *tet*(X46.2), and *tet*(X47) (Fig. S1, Table S3). Most of these *tet*(X) orthologs found from metagenomic analysis were not present in the NCBI database with the exceptions of *tet*(X2) (Acc. No. AJ311171), *tet*(X2.4) (Acc. No. JQ990987) and *tet*(X46.2) (Acc. No. KU547718.1) (Table S3).

Resistance phenotypes of *tet*(X) orthologs. All the *tet*(X45), *tet*(X46), and *tet*(X47) groups found from metagenomic analysis of human-gut origin (Fig. 1c) in the *E. coli* JM109 were resistant to tigecycline (MICs 8 to 16 ml/liter) and exhibited high MIC to the fourth-generation antibiotics omadacycline (MICs 16 to 32 ml/liter) and eravacycline (MICs 2 to 4 ml/liter) (Table S4 in the supplemental material). In addition, all the non-*tet*(X2) exhibited resistance to tetracycline (MICs 128 to 256 ml/liter), doxycycline (MICs 32 to 64 ml/liter), and minocycline (MICs 16 to 32 ml/liter) (Table S4). All the *tet*(X2) like orthologs were susceptible to tigecycline (MICs 0.25 to 1 ml/liter) but resistant to tetracycline (MICs 8 to 256 ml/liter). Among the 23 *tet*(X) variants, 22 of them were resistant to doxycycline (MICs 8 to 64 ml/liter) and minocycline (MICs 2 to 32 ml/liter), excluding *tet*(X2.10) that intermediate to doxycycline (MIC 1 ml/liter) and minocycline (MIC 0.5 ml/liter) (Table S4). All of these *tet*(X2) like orthologs exhibited MIC of omadacycline from 0.5 to 16 ml/liter, and eravacycline from 0.0125 to 4 ml/liter (Table S4). Three *tet*(X2) like orthologs *tet*(X2), *tet*(X2.4) and *tet*(X2.15) showed relatively higher MICs to omadacycline (8 to 16 ml/liter) and eravacycline (1 to 4 ml/liter).

Global distribution and taxonomic assignment of *tet*(X) carrying MAGs. The 322 *tet*(X)s carrying MAGs were detected in 19 countries including Europe (n = 12), Asia (n = 4) and America (n = 3) but were absent in Oceanica and Africa (Fig. 1a, Table S5 in the supplemental material). The prevalence of *tet*(X) in European countries (0–9.40%) was more

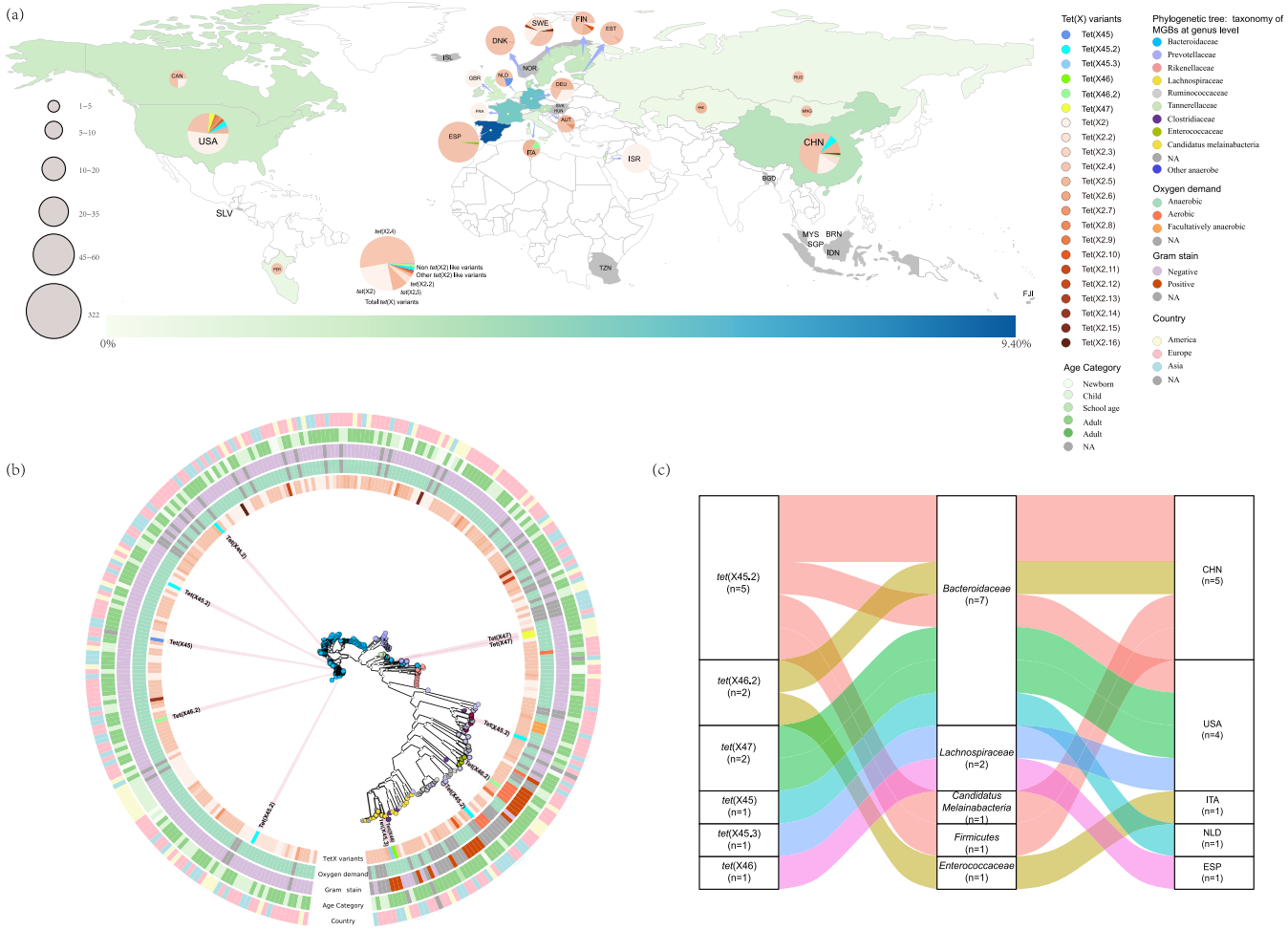

FIG 1 Global distribution of *tet*(X)s from human microbiome. (a) World map showed the positive rates of *tet*(X) gene in 19 countries and the colored countries represented the positive rates of *tet*(X) according to the hot map (>0–9.40%) at the bottom. The gray countries indicated they were negative for *tet*(X) gene. The size of the pie charts represented the numbers of *tet*(X)-positive MAGs and the colors in the pie charts indicated the composition of *tet*(X) variants. (b) PhyloPhlAn analysis of the *tet*(X) carrying MAGs. The taxonomic assignments of the *tet*(X)-carrying MAGs were depicted with colored circles in the phylogenetic tree. The *tet*(X) variants carried by the MAGs, as well as oxygen demand, Gram stain, age category and the countries of the *tet*(X)-carrying MAGs were showed in the five colored rings surrounding the phylogenetic tree. (c) Distribution of the 12 MAGs carried non-*tet*(X2) genes with tigecycline inactivate function.

complex than Asian (0–3.17%) and American countries (0–2.44%) although the positive rates for *tet*(X) in these three continents were not significantly ($P > 0.05$) different (Fig. S3). The prevalence of *tet*(X) was highest in Spain (9.40%), followed by Germany (5.14%), France (3.82%), Denmark (3.34%) and China (3.17%) and the remaining countries were < 3% (Fig. 1a and Table S5).

The 322 *tet*(X)-carrying MAGs carried a range of 82–108,870 contigs (Table S8) and based on these contigs in each MAG the bacterial taxonomy assignment indicated that all the MAGs could be assigned to five phyla and was dominated by *Bacteroidetes* (78.89%, 254/322) followed by *Firmicutes* (16.78%, 47/322), *Proteobacteria* (1.24%, 4/322), *Candidatus Melainabacteria* (1.24%, 4/322), and *Fusobacteria* (0.62%, 2/322) (Fig. 1b). Of which, 96.68% (301/322) were classified to the family level and most of them were also belonging to *Bacteroidaceae* (70.10%, 211/301) (Fig. 1b and Table S6). Furthermore, 72.30% (154/211) of these *tet*(X) carrying *Bacteroidaceae* MAGs could be further divided into 14 *Bacteroides* species that were dominated by *Bacteroides vulgatus* (37.67%, 58/154), *Bacteroides uniformis* (20.78%, 32/154), *Bacteroides dorei* (14.94%, 23/154), *Bacteroides ovatus* (5.84%, 9/154), *Bacteroides fragilis* (3.90%, 6/154) and *Bacteroides caccae* (3.25%, 5/154) (Table S7).

The *tet*(X2)-like positive MAGs were distributed across these 19 *tet*(X) positive countries. *tet*(X2.4) (55.02%, 170/309), *tet*(X2) (26.86%, 83/309), *tet*(X2.5) (9.32%, 30/309), and *tet*(X2.2)

(4.53%, 14/309) totaled 96.11% (297/309) and prevailed over other *tet*(X2)-like orthologs (≤0.6%, 2/309) (Fig. S4). Only 81.08% (240/296) of these four predominant *tet*(X) carrying MAGs could be assigned at genus level and most of them were also identified in *Bacteroides* (71.67%,172/240), followed by *Prevotella* (6.67%, 16/240) and *Alistipes* (5%, 12/240) (Table S8). In addition, the non-*tet*(X2) orthologs included 75% (9/12) that were distributed in China and United States. Of which, *tet*(X45.2) was the most prevalent ortholog (Fig. 1c). The remaining three non-*tet*(X2) carrying MAGs were distributed in Europe and the single *tet*(X46.2) ortholog was carried by an *Enterococcaceae* from Italy. Almost all *tet*(X)-carrying MAGs were collected from human stools excluding only one *tet*(X2.4)-carrying MAG identified as an *Enterococcus* spp. from an oral cavity sample (Table S8).

The 322 *tet*(X) carrying MAGs included 196 from the 4,390 MAGs and their average abundance in human microbiomes has been calculated in previous study (Table S8) (20). The average abundance of these 196 *tet*(X)-positive MAGs was 5.97 ± 3.89 and significantly higher than that of the total 4,390 MAGs (1.76 ± 3.74) (20).

Culturable isolates from public repository insight into the distribution and evolutionary timescale of *tet*(X)s. To further trace the distribution of these *tet*(X)s in culturable isolates, we examined 774,435 whole genome sequences of bacterial isolates present in GenBank and only 0.12% (896/774,435) carried an ORF with > 70% amino acid identity with the known *tet*(X) genes including the novel ones found in the current study (Table S9 in the supplemental material). The 70% sequence identity cutoff was chosen to ensure that no other gene family would incorrectly annotate as new *tet*(X) members (24). The PhyloPhlAn analysis indicated that the facultative anaerobe clade was phylogenetically distinct between anaerobes and aerobes (Fig. 2a). These *tet*(X) genes were found in 17 bacterial families that were dominated by aerobes including *Moraxellaceae* (279/896, 31.17%), *Enterobacteriaceae* (208/896, 23.24%), and *Weeksellaceae* (16.20%, 145/896). The *Bacteroidaceae* (20.67%, 185/896) were also an important anaerobic carrier for *tet*(X2) like orthologs (Fig. 2a and Table S9). Three *tet*(X) orthologs were most prevalent and included *tet*(X2)-like (35.71%, 320/896), *tet*(X3) (27.34%, 245/896) and *tet*(X4) (21.99%, 199/896) (Table S9). Interestingly, different bacterial families from variant hosts were preference for carrying specific *tet*(X) ortholog (Fig. 2a). Almost all the *Bacteroidaceae* (98.91%, 182/184) and most *Weeksellaceae* (63.45%, 92/145) isolates carried *tet*(X2)-like orthologs and were primarily from human samples (38.64%, 114/295). In addition, all *tet*(X3) were detected from *Acinetobacter* spp. and almost all *tet*(X4) (88.89%, 176/197) were carried by *E. coli* and these were primarily from food animals (66.59%, 295/443) including pigs, chickens, ducks, cattle, and geese.

The *tet*(X2)-like orthologs were detected prior to 8.41 ± 6.17 years ago and earlier than *tet*(X3) (4.00 ± 1.12) and *tet*(X4) (2.38 ± 1.34). To be noted, the collection dates of three *R. anatipestifer* isolates from U.K. duck samples were prior to 1980. One isolate (BioSample: SAMN09912225) was collected in 1966 and was positive for *tet*(X12) and a *tet*(X2)-like gene that only differed in a single amino acid from *tet*(X2) was designed *tet*(X2.17). The two other isolates were collected in 1976. One (BioSample: SAMN09912224) carried a *tet*(X2.17) gene and another (BioSample: SAMN09912221) carried a *tet*(X) gene shared 99.45% similarity with *tet*(X47), designed *tet*(X47.2).

Date for lineage divergence of the earliest occurred *tet*(X) orthologs were produced by Bayesian phylogenetic inference (Fig. 2b). The analysis indicated a mean rate of 0.29 SNP per year for these *tet*(X) during 1966–2018. The most recent common ancestor (MRCA) of all *tet*(X) from this study was approximately from 1944 to1964, and the tracer analysis indicated that the presence of *tet*(X) most likely occurred in 1956 A.D. (95% highest posterior distributor). Two main lineages that originated from *tet*(X2.17) and *tet*(X12), respectively, were observed in this phylogeny and both *tet*(X) orthologs were collected from the *Riemerella anatipestifer* of duck origin from the United Kingdom in 1966 A.D.

Annotation and comparison of the *tet*(X) genomic environment. A total of 1,218 *tet*(X)-carrying contigs ranging from 1,190 to 931,600 bp were retrieved from the metagenome and bacterial-isolate data. These contigs were grouped into 455 clusters that carried a range of 1–48 contigs (Table S10 in the supplemental material). In each cluster, longer contig shared more than 97% coverage and more than 97% similarity with shorter contig.

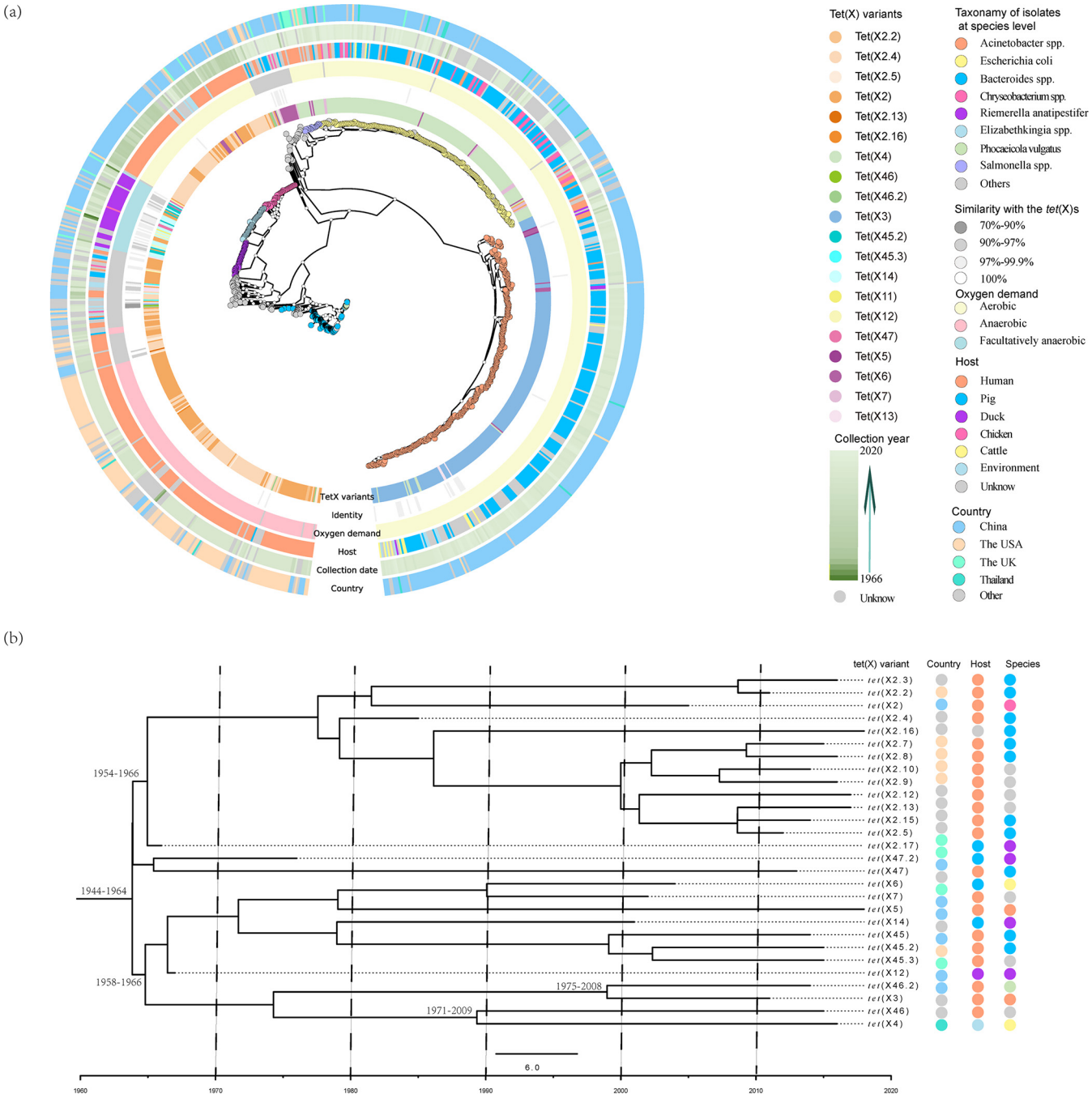

FIG 2 Culturable isolates insight into *tet*(X) distribution patterns. (a) PhyloPhlAn analysis of the *tet*(X)-carrying isolates from the public repository. The species of the *tet*(X)-carrying isolates were depicted with colored circles in the phylogenetic tree. The information of the *tet*(X) carrying isolates including *tet*(X) variants, oxygen demand, host, collection date and country were showed in the six colored rings surrounding the phylogenetic tree. (b) Dates of lineage divergence of the earliest *tet*(X) orthologs as determined using Bayesian phylogenetic inference. The *tet*(X) variants, countries, host and species of these isolates were shown at the right region.

The high coverage and similarity of these contigs indicated that these *tet*(X) could spread among each cluster (Table S10). This indicated *tet*(X) orthologs could spread among a great diversity of hosts including human, animal and environment (Fig. 3). Both humans and pigs were the primary *tet*(X) hosts. *tet*(X2)-like *tet*(X3), *tet*(X4), *tet*(X7), and *tet*(X47) have been found in humans as well as *tet*(X2)-like, *tet*(X3), *tet*(X4), and *tet*(X6) in pigs. However, only *tet*(X2)-like and *tet*(X3) orthologs could transfer between these two hosts (Fig. 3a). Interestingly, *tet*(X2)-like orthologs could hitch a great diversity of vehicles to spread between humans and pigs and these included *Bacteroides* spp., *R. anatipestifer* and *Chryseobacterium* spp. The remaining

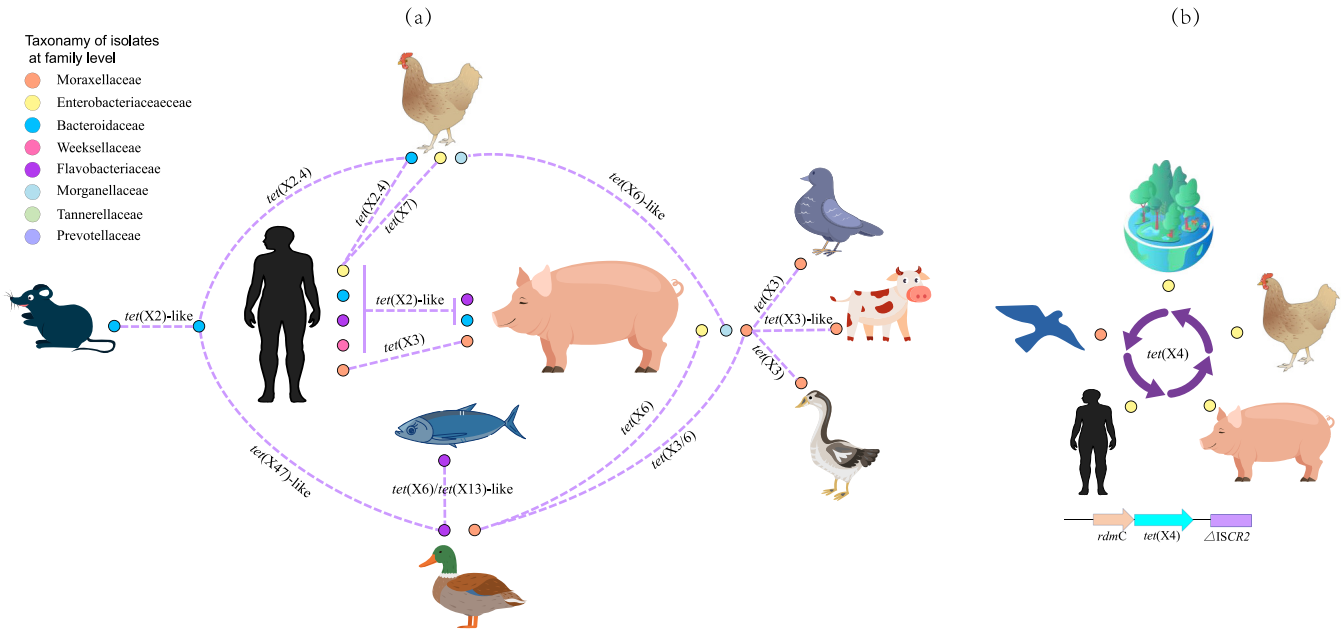

FIG 3 Possible transmission routes of the *tet*(X) genes. The colored circles surrounding the hosts represented the tet(X) carrying bacterial families. The dotted lines represented the possible transmission routes of the tet(X) genes between different hosts. (a) The transmission routes of the *tet*(X) gene between different hosts. (b) The transmission routes of the *tet*(X4) gene and their flanking genomic environment (*rdmC-tet*(X4)-△*ISCR2*) between different hosts.

tet(X) genes were spread only *via* special species between different hosts. For instance, the *tet*(X3) gene could only be transited by *Acinetobacter* spp. and spread between pigs and other hosts including pigeons, cattle, geese, ducks and humans (Fig. 3a). In addition, the *tet*(X4) in the genomic array *rdmC-tet*(X4)-△I*SCR2* could spread among wild birds, humans, pigs, chickens and the environment (Fig. 3b).

Genomic annotation and comparisons indicated that other ARGs frequently flanked the *tet*(X) gene and these included *erm*F, *aad*K, *tet*(Q), and $bla_{OXA-347}$ that conferred resistance to erythrocin, streptomycin, tetracycline, and ampicillin, respectively (Fig. 4a and b). Of these, the *erm*(F) gene was the most frequent to flank the *tet*(X) gene ($n = 152$), and their genomic environment were clustered into two types according to their relative position: *erm*F located upstream (15.79%, 24/152) and downstream (80.92%, 123/152) of *tet*(X) gene (Fig. 4a and b). The upstream *erm*F always formed a conserved structure *tnpF-erm*F-*tet*(X1)-*tet*(X2)/*tet*(X2.2)-*aad*K ($n = 24$) (Fig. 4a). This structure was also present in a conjugative transposon CTnDOT of *Bacteroides* origin (25), but the *aad*K (930 bp) was replaced by another aminoglycoside ARG *aad*S (903 bp) in CTnDOT and these two ARGs shared a 96.77% identity at the nucleotide level. Interestingly, the *tet*(X) orthologs and their genomic contexts were more diverse when *erm*F was located immediately downstream of *tet*(X). These *tet*(X) genes included 12 orthologs that were dominated by *tet*(X2.4) and *tet*(X2.5) (Fig. 4b), Text S1 in the supplemental material). In addition, these *tet*(X) genes were able to spread among anaerobes, aerobes and facultative anaerobes (Supplemental Text S1).

DISCUSSION

Source tracking of the *tet*(X) orthologs. One of the most significant findings in current study was that *tet*(X) emerged as early as 1960s in *R. anatipestifer* of duck origin, which was earlier than that had been previously reported in 1980s (8). A previous epidemiological study indicated the MRCA of these *tet*(X)s orthologs likely occurred 9900 years ago (7887 BC) (15). However, we also estimated the MRCA of these *tet*(X) genes but speculated it occurred only 65 years ago (1956 A.D.) that was 4 years following the introduction of tetracycline in clinics. This was consistent with the streptomycin, erythrocin and florfenicol, in which their resistance also emerged over the ensuing years following their introduction in clinic (4). In this study, we screened for the presence of *tet*(X) orthologs from public repositories on a large scale (774,435 isolates) and the earliest emergence of 28 *tet*(X) variants was

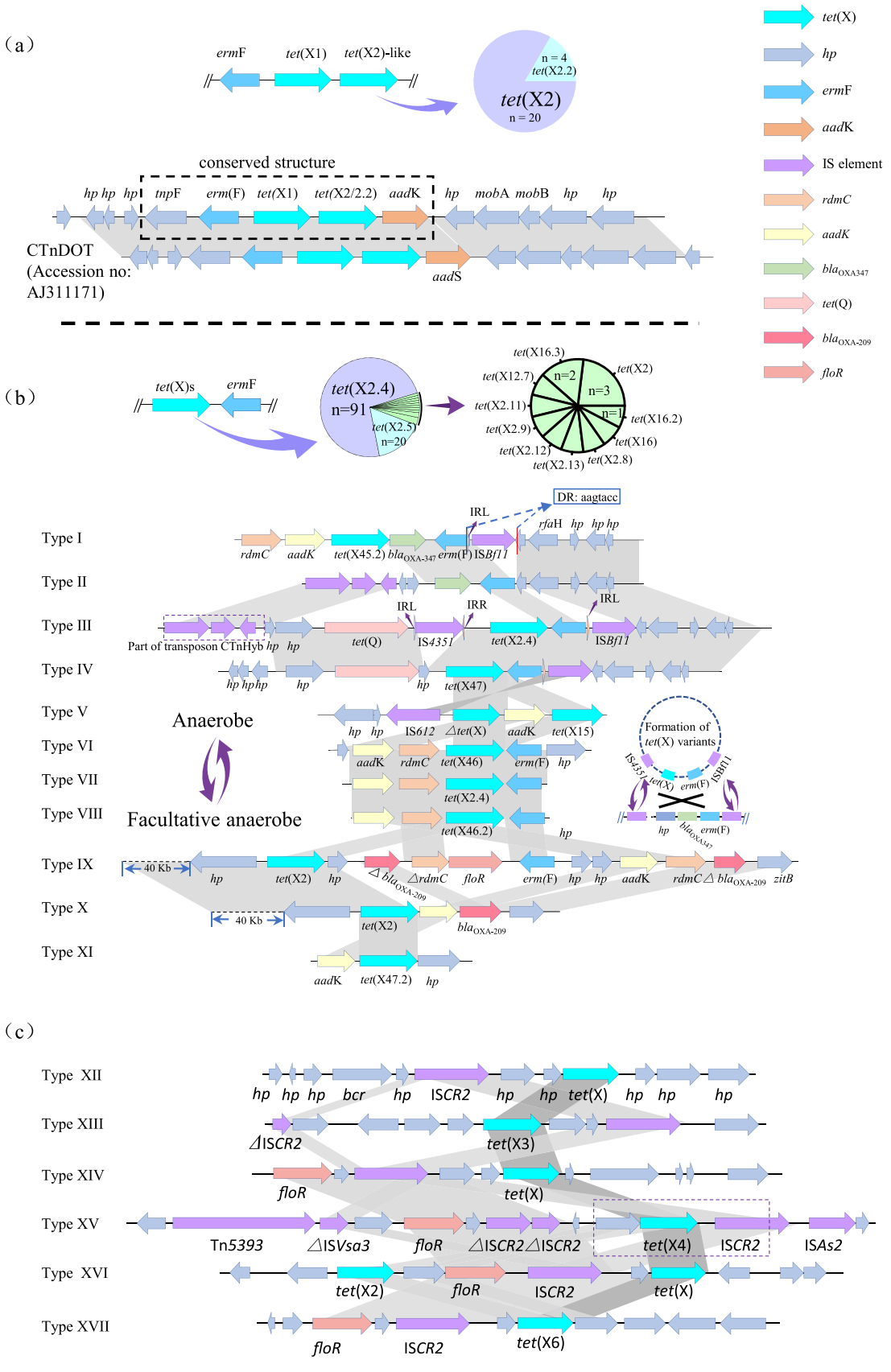

FIG 4 Comparison of *tet*(X) genomic environments. (a) The genomic comparison of *erm*(F) gene located upstream of *tet*(X2)-like genes. The proportions of the *tet*(X2) and *tet*(X2.2) located downstream *tet*(X1) were showed in the pie chart. (b) The

(Continued on next page)

selected to perform MRCA estimations (Fig. 2b). The time of the earliest occurrences of *tet*(X) variants was an important factor for the chronogram phylogeny construction using the Bayesian evolutionary analysis, which should be more reasonable than previous study (15). Meanwhile, this was likely to cause the discrepancy for the estimation of the *tet*(X) emergency between current and previous studies (15). However, this analysis was limiting in covering more *tet*(X) variants that spanned a longer time, and the coverages of some *tet*(X) variants carrying contigs were unknown (Table S3), which may affect the identification accuracy. Thus, these results could only provide an alternative model for the emergence of *tet*(X).

The *Flavobacteriaceae* have been recognized as a potential ancestral source of the tigecycline resistance gene *tet*(X) (5). We found that both the two earliest (1966) occurring *tet*(X) orthologs [*tet*(X2.17) and *tet*(X12)] harbored by *R. anatipestifer* that is also a bacterial species member belonging to *Flavobacteriaceae* family. Meanwhile, before 2000 only a total of seven isolates were confirmed as *tet*(X) gene carrier and five of them were also identified as *R. anatipestifer* (Table S9 in supplemental material). Among the 896 *tet*(X)-carrying isolates from public repository, 71 *tet*(X)-carrying were *Flavobacteriaceae* including *R. anatipestifer* that accounted for a large number (64.79%, 46/71) versus all other *Flavobacteriaceae* members (Fig. 2a). Additionally, *R. anatipestifer* harbored a great diversity of *tet*(X) variants and only 23.94% (17/71) carried the known *tet*(X)s including *tet*(X12), *tet*(X14), *tet*(X2.17) and *tet*(X47.2) (Fig. 2a). The remaining carried other *tet*(X) orthologs that shared 94.9–99.7% similarity with their most closely related *tet*(X) ortholog (Table S9). A recent study indicated the poultry pathogen *R. anatipestifer* appears to be a reservoir for *tet*(X) tigecycline resistance (26). These indicated that *R. anatipestifer* was most likely the ancestral source of the tigecycline resistance gene *tet*(X).

A great diversity of *tet*(X) and their flaking genomic contexts was observed when the *erm*F gene was present downstream of *tet*(X) (Fig. 4b and Text S2 in the supplemental material). A comparison of the *tet*(X) genomic contexts from MAGs and culturable isolates yielded 11 genomic backbones that were associated with the formation of non-*tet*(X2) orthologs found in the current study (Fig. 4b). The non-*tet*(X2) orthologs were likely to be generated from *tet*(X2)-like orthologs during their transmission between anaerobes or between anaerobes and facultative anaerobes (Fig. 4b and Text S2). IS*Bf11* and IS*4351* played important roles in their transmission between anaerobes that was dominated by *Bacteroides* spp. where a mobile cyclic structure was speculated based on genomic Types I - IV (Fig. 4b and Text S2), and the *tet*(X45), *tet*(X46), and *tet*(X47) groups were likely to be generated during the transmission of these mobile structures. In addition, genomic comparisons indicated that these *tet*(X) genes were also able to spread between *Flavobacteriaceae* and *E. coli* as well as between *Flavobacteriaceae* and *Acinetobacter* sp. (Fig. 4c and Text S2). We have demonstrated that *R. anatipestifer*, a *Flavobacteriaceae* family member, was a potential ancestral source of *tet*(X) and the new *tet*(X) orthologs were likely to be produced during their transmission. The high similarity of the nucleotide sequences flanking *tet*(X3) and *tet*(X4) (Fig. 4c and Text S2) suggested that these two genes were also derived from *Flavobacteriaceae* and IS*CR2* played a key role in this process (Fig. 4c and Text S2).

Global distribution of *tet*(X) orthologs. The human microbiome plays an important role in public health. Here, we first determine the *tet*(X) prevalence in the human microbiome using a large-scale survey of 12,829 samples (20–22). A total of 16 *tet*(X2)-like and two new non-*tet*(X2) orthologs have been identified directly in the human stool samples. Since there was not standard for assignment of the newfound *tet*(X) orthologs, certain conflict for *tet*(X) numbering have been published in previous papers (13, 27), and it was necessary to

FIG 4 Legend (Continued)
genomic comparison of *tet*(X) genes located downstream *erm*(F). The proportions of the *tet*(X) variants located downstream of *erm*(F) were showed in the pie chart. The possible mechanisms of non-*tet*(X2) formations were showed in the two circles plotted with dotted line. (c) Genomic comparison of the regions flanking *tet*(X3) and *tet*(X4) among *Flavobacteriaceae*, *Acinetobacter* and *E. coli*. Arrows indicate the directions of transcription of the genes, and different genes are shown in different colors. Regions of ≥ 99.0% nucleotide sequence identity are shaded light gray. Regions of 77%–91% nucleotide sequence identity are shaded dark gray. The Δ symbol indicates a truncated gene. IS, insertion sequence. See Table S11 in the supplemental material for genomic Type I–XVII definitions.

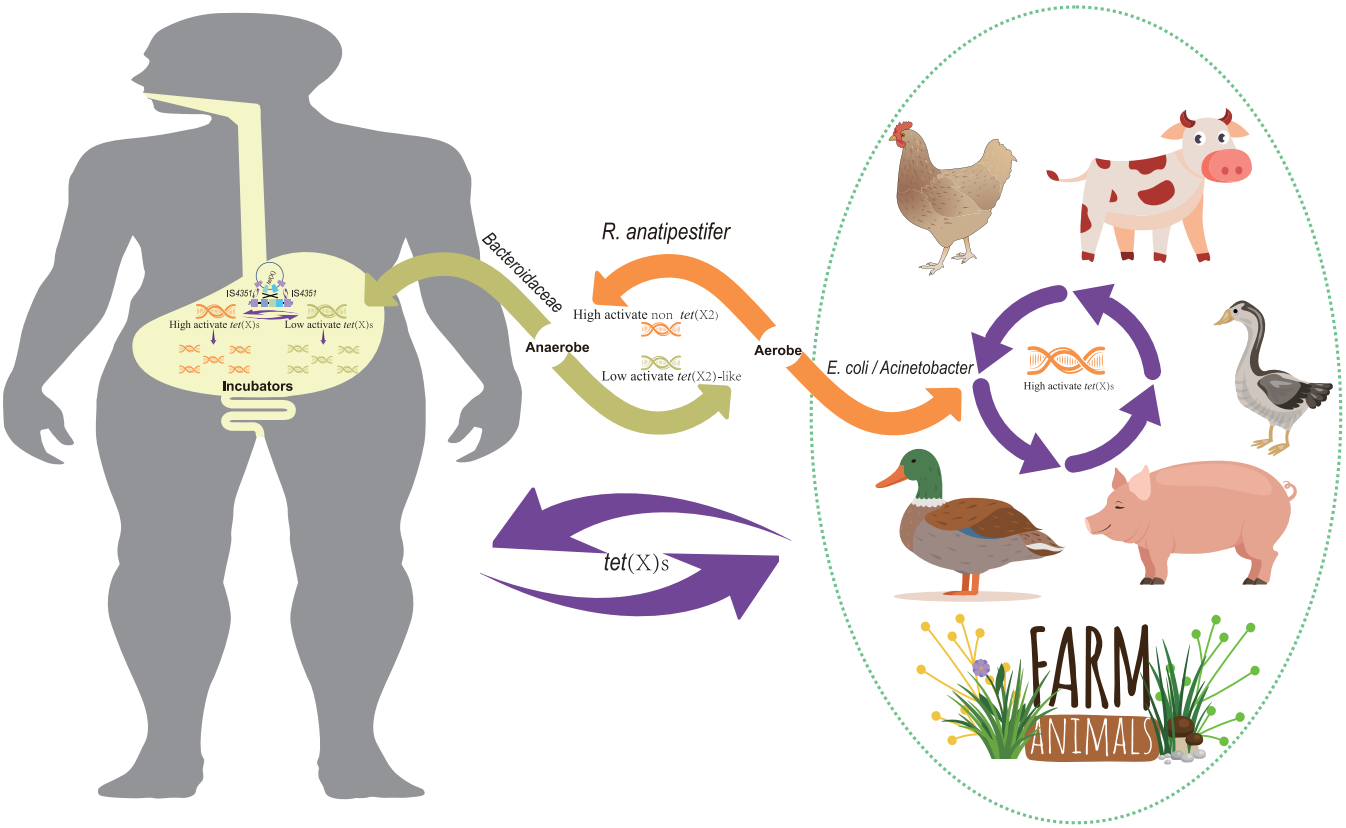

FIG 5 Potential origin and main transmission routes of the *tet*(X) genes.

distinguish the different *tet*(X) orthologs in current study. Thus, we temporarily set up a criterion for the assignment of *tet*(X) orthologs. This maybe not comprehensive as the assignment for mobile colistin resistance (*mcr*) genes (28) which have established a platform in NCBI (pd-help@ncbi.nlm.nih.gov) to confirm and allocate the allele numbers for new *mcr* gene. The assignment of new *mcr* alleles would not be confused in this platform and an allele numbers assignment platform for *tet*(X)s should be established urgently.

We found a prevalence for *tet*(X) at 1.21% (322/26,548) that was higher than for *E. coli* and *K. pneumoniae* from hospital isolates (0.32%, 4/1520) (5) indicating that traditional culture methods have underestimated the prevalence of *tet*(X). Our results were similar to an epidemiological study that detected the bla_{NDM} and *mcr-1* genes directly from samples that was higher than for the *E. coli* isolates (29).

The *tet*(X2)-like genes carrier in human microbiomes were dominated by the *Bacteroidaceae* in contrast to previous epidemiological studies where *tet*(X3) and *tet*(X4) were primarily carried by *A. baumannii* and *E. coli*, respectively (2, 15). The *Bacteroides* are predominant anaerobes estimated to account for 25–30% of human gut microflora (30) while the *Enterobacteriaceae* normally constitutes only 0.1–1% (31). We also found that the average abundance of *tet*(X)-carrying MAGs (5.97 $\pm$ 3.89) annotated as *Bacteroidaceae* prevailed over species-level genome bins (1.76 $\pm$ 3.74) in the human microbiome. This was likely the reason for the absence of *tet*(X3) and *tet*(X4) in our human microbiome analyses. Although *tet*(X) genes are inactive in anaerobes, the high abundance of *tet*(X2)-carrying MAGs and a variety of non-*tet*(X2)-like orthologs found in the current study indicated that the *Bacteroidaceae* were an important reservoir and mutational incubator for the mobile *tet*(X) orthologs in the human microbiome (Fig. 5). Furthermore, the *Bacteroidaceae* could generate new non-*tet*(X2) orthologs with tigecycline inactivation functions, and the comparison of the *tet*(X) genomic environment suggested that these non-*tet*(X2) enabled transfer to facultative anaerobes and aerobes (Fig. 5).

We have demonstrated *R. anatipestifer* as potential ancestral source of *tet*(X) genes, and the earliest emergence of high-level tigecycline resistance genes *tet*(X3) and *tet*(X4)

were likely to be 1975 and 1971, respectively, both of which were earlier than the clinical introduction of tigecycline in 2005. This was further evidence that the use of even older antibiotic tetracycline may contribute to the resistance to newer antibiotics (4). The *tet*(X) distributions from culturable isolates indicated that *tet*(X2)-like and non-*tet*(X2) orthologs were prevalent in anaerobes and aerobes respectively (Fig. 5). Since *tet*(X) is active only in an aerobic environment, the non-*tet*(X2)-like orthologs with tigecycline inactivate function tended to be captured by aerobes under tetracycline selective pressure (Fig. 5). This was likely to be the reason that non *tet*(X2) orthologs primarily distributed in aerobes but the high prevalence of *tet*(X2)-like orthologs in *Bacteroidaceae* from human microbiome need to be further explored.

Taxonomic assignments for *tet*(X)-carrying MAGs were estimated using bioinformatic methods that may not be as precise as cultural methods, but such an approach has proved feasible (20, 32). Another limitation of this approach was challenging to combine the chromosome with their respective plasmid sequences (33). Therefore, these *tet*(X)-carrying contexts from MAGs in the current study were likely to be chromosome-borne. This differed with *tet*(X3)/*tet*(X4) that are present in a variety of plasmids and IS*CR2* was an essential element for their mobilization (2, 15). Transmission between *Bacteroidaceae* of these *tet*(X)s orthologs was primarily mediated by the CTnDOT-like conjugative transposon and *erm*F-related IS elements including IS*Bf11* and IS*4351*. CTnDOT has been reported to harbor *erm*F, *tet*(X1) and *tet*(X2) in *Bacteroides* (25) and conjugative transposons can also insert into co-resident plasmids in addition to the chromosome (34). Therefore, conjugative transposons have been found in numerous genera including *Enterococcus*, *Streptococcus*, *Lactococcus*, *Butyrivibrio*, *Clostridium*, *Salmonella*, *Pseudomonas*, *Mezorhizobium*, and *Vibrio* (25). The erythromycin resistance *erm*F gene is frequently reported in *R. anatipestifer* and *Bacteroides* spp. isolates (35, 36). The IS *Bf11* and IS*4351* flanking *tet*(X) in the Type III genomic contexts have also been previously identified (37) and reveals that this mobile structure has spread to China, the USA, France, Denmark, Sweden, and Belgium. In addition, the IS*CR2* element belonging to the IS*91* family has been described in the first report of *tet*(X3) and *tet*(X4), both downstream and upstream of *tet*(X3). This inserted sequence (IS) element could form a mobile amplicon and this was demonstrated using inverse PCR experiments (7) and in an *Acinetobacter towneri* isolate flanking region of *tet*(X6) (38). We also identified this IS element located upstream of *tet*(X6) and indicated that this IS element can play an important role in transmission of non-*tet*(X2) orthologs with tigecycline inactivation functions in the *Flavobacteriaceae*, *E. coli* and *Acinetobacter*.

In conclusion, we concluded an analysis on integrated human gut meta genome and global bacterial isolates to trace the origin and distribution of *tet*(X) gene. The *tet*(X) gene emerged as early as 1960 and the *R. anatipestifer* was an ancestral source of *tet*(X). The *tet*(X3)-carrying *Acinetobacter* spp. and *tet*(X4)-carrying *E. coli* were prevalent in food animals and these two *tet*(X)s were likely formed during the transmission of *tet*(X)s between *Flavobacteriaceae* and *E. coli*/*Acinetobacter*, and IS*CR2* played a key role in the transmission. The *tet*(X2)-like orthologs enriched in the anaerobes that was dominated by *Bacteroidaceae* of human-gut origin and could transfer between these anaerobes. The mobile elements CTnDOT, IS*Bf11*, and IS*4351* played important roles in the transmission. The low-level tigecycline resistance *tet*(X2)-like gene could mutate to high-level tigecycline resistant determinants that could spread to facultative anaerobes and aerobes. *Bacteroidaceae* of human-gut origin was an important reservoir and mutational incubator for *tet*(X) that could spread to facultative anaerobes and aerobes.

MATERIALS AND METHODS

Collection of microbial genomic sequences from human microbiome in retrospective data. A total of 26,728 metagenomic samples of human-microbiome origin deposited in public repositories were *de novo*-assembled and binned into metagenome-assembled genomes (MAGs) for the exploration of new bacterial species in previous studies (20–22). We removed the duplicative samples from these metagenomic samples according to their accession number and found a total of 12,829 non-duplicate metagenomic samples from 31 countries. These samples were reconstructed into 202,265 MAGs and online released in previous studies (20–22) (Table S1 and S2). We screened for the presence of all known *tet*(X) orthologs from these 202,265 MAGs using BLAST using an 80% identity and 70% hit length cutoff. The prevalence of *tet*(X) in 31 countries were plotted using R version 3.5.3. Phylogenetic analysis for amino acid sequences of all *tet*(X) gene

products was constructed using neighbor joining with the default parameters in Mega X Version 10.0.5 (23) and alignments were constructed using ESPript 3 (39).

Functional identification of *tet*(X)s. Tigecycline resistance for these gene products was assessed by synthesis of full-length nucleotide sequences of all detected *tet*(X) genes. EcoRI and a SalI sites were then added 5′ and 3′ respectively (Tsingke Biological Technology, Beijing, China). The synthesized *tet*(X) genes were cloned into plasmid vector pBAD24 and transformed into competent *E. coli* JM109 as described in our previous study (40). The transconjugants *E. coli* JM109+pBAD24-*tet*(X4) and *E. coli* JM109+pBAD24, were used as positive and negative controls, respectively, as previously described (40). The MIC for tetracycline, doxycycline, minocycline, tigecycline, eravacycline and omadacycline were determined by the broth microdilution method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. Tetracycline, doxycycline, and minocycline breakpoints were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (http://www.eucast.org/clinical_breakpoints). The United States FDA criteria was employed to interpret tigecycline breakpoints for *E. coli* and MIC $\geq$4 mg L^{-1} was considered non-susceptible while eravacycline and omadacycline were uninterpreted with no breakpoint. *E. coli* ATCC 25922 was used as the quality control strain.

Taxonomic assignment and phylogenetic analysis of *tet*(X)s-carrying MAGs. We obtained 322 *tet*(X)-carrying MAGs from three previous studies (20–22). This group included taxonomic assignments for 196 that had been previously annotated (20), while the remaining 126 were annotated using metaWRAP-Annotate-bins module using the MetaWRAP pipeline and default parameters (41). Briefly, the assembly contigs from each *tet*(X)-carrying MAG was taxonomically profiled using Kraken2 (42) and then this entire metagenomic bin could conservatively and accurately estimate the taxonomic profiles (41).

The phylogenetic structure for the *tet*(X)-carrying MAGs were performed using an automatic PhyloPhlAn (3.0) pipeline (20, 43), through which the phylogeny in Fig. 1b was built using 400 universal PhyloPhlAn markers with parameter: "–diversity high –accurate –min_num_markers 80." This pipeline integrates diamond (version 0.9.32), mafft (version 7.464) (44), trimal (version 1.4.rev15) (45) and RAxML (version 8.2.12) (46), and the parameters of these software were set as described previously (20). The phylogenetic tress in Fig. 1b were plotted using GraPhlan (version 1.1.3) (47).

Phylogenetic analyses of the *tet*(X) carrying isolates and evolutionary timescale for the *tet*(X)s from isolates. To further trace the spread of all *tet*(X)s in culturable bacteria isolates, a total of 774,435 bacteria assembled whole genome sequences were downloaded from the NCBI database as of 7 November 2020. *tet*(X)-like open reading frames (ORFs) were determined using BLATX against all the *tet*(X)s variants mentioned in current study with a minimum similarity of 70 and 100% coverage. The collection date, origin, countries, and the bacterial host of the *tet*(X)-positive isolates were retrieved according to their Biosample Number. The phylogenetic structure for the *tet*(X)-carrying isolates were also performed using PhyloPhlAn (3.0) pipeline mentioned above.

To determine the evolutionary history of *tet*(X)s, the earliest emergency of *tet*(X) variants (with collection date) with a 388 amino acid (aa) length were applied to generate a chronogram using Bayesian evolutionary analysis version 1.10 (48). For all model combinations, three independent chains of 100 million generations each were run to ensure convergence with sampling every 1,000 iterations. Tracer v1.7.1 was used to assess convergence using all parameter effective sampling sizes of $>$ 200 (49). LogCombiner v2.6.1 was used to combine tree files and a maximum clade credibility tree was created using TreeAnnotator v2.6.0 (49). Tree annotation was visualized using iTOL (50) and FigTree version 1.4.2.

Annotation and comparison of the genomic region flanking the *tet*(X) gene. The *tet*(X)-carrying contig were extracted from the MAGs of metagenomic analysis and isolates from public repository. CD-HIT was employed to group *tet*(X)-carrying full length contigs using a cutoff with a minimum similarity of 97% over 97% of the query coverage (51). These *tet*(X)-carrying contigs were annotated using Prokka (52) and in conjunction with standalone BLAST analyses against the ResFinder (53) and ISfinder (54) databases to cross-validate ARGs and mobile genetic elements, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.6 MB.

SUPPLEMENTAL FILE 2, PDF file, 2.1 MB.

SUPPLEMENTAL FILE 3, XLSX file, 1.8 MB.

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