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Harnessing efficient multiplex PCR methods to detect the expanding Tet(X) family of tigecycline resistance genes

Kai Ji[a,b#], Yongchang Xu[b,c#], Jian Sun[d], Man Huang[b], Xu Jia[c], Chengjian Jiang[a,e], and Youjun Feng[a,b,c,d]

[a]State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, Guangxi Microorganism and Enzyme Research Center of Engineering Technology, College of Life Science and Technology, Guangxi University, Guangxi, China; [b]Department of Pathogen Biology & Microbiology and Department of General Intensive Care Unit of the Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, China; [c]Non-coding RNA and Drug Discovery Key Laboratory of Sichuan Province, Chengdu Medical College, Chengdu, Sichuan, China; [d]National Risk Assessment Laboratory for Antimicrobial Resistance of Animal Original Bacteria, South China Agricultural University, Guangzhou, China; [e]Guangxi Key Laboratory of Mangrove Conservation and Utilization, Guangxi Mangrove Research Center, Guangxi Academy of Sciences, Guangxi, China

ABSTRACT

A growing number of *tet(X)*-type tigecycline resistance determinants [*tet(X1)* to *tet(X5)*] constitutes an expanding family of tetracycline-inactivating enzymes, posing a potential risk to global public health. Here, we report the development of an efficient multiplex PCR method to detect the family of *tet(X)* variants. This method is successfully applied in the screen and validation of *tet(X)* genes in the field and clinic bacterial samples. In addition, we found that the formerly proposed *tet(X1)* is a premature truncated version by the inappropriate annotation, and fixed this error. Overall, it might be the first genetic tool for the detection of different Tet(X) members.

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Introduction

Antimicrobial resistance is one of the biggest challenges to one health covering environmental, animal and human sectors [1]. Tigecycline, the third generation of tetracycline [2], is regarded as a final line of defense (somewhat next to polymyxin) against multidrug-resistant pathogens [3]. Because that tigecycline primarily targets protein synthesis, the sporadic cases of tigecycline resistance are generally associated with ribosome protection as well as active efflux pumps [3,4]. In fact, the discovery of Tet(X)-type enzyme represents a distinct mechanism of tigecycline resistance in that they can destruct/inactivate all the known derivatives of tetracycline [5–7]. Among them, the paradigm version refers to Tet(X) [1167 bp, 388aa] that is an NADP-dependent oxidoreductase carried by a transposon of the obligate anaerobe *Bacteroides fragilis* [8,9]. Subsequently, Whittle et al. [10] reported two additional *tetX* variants [designated as *tet(X1)* and *tet(X2)*] are neighbored in an operon encoded by the conjugative transposon Tn4351 of *Bacteriodes*. Of note, the multiple sequence alignment analyses suggested that the formerly annotated *tet(X1)* [1080 bp] is a truncated version lacking its 60 bp at 5′-end (**Fig. S1**) [10], therefore we fixed this error here by extending it from 1080 bp to 1140 bp (a polypeptide of 379aa, **Fig. S2**). Unlike that *tet(X1)* (1140 bp) has only 68.2% similarity to *tet(X)* at the level of nucleotide acids, *tet(X2)* (1167 bp) is almost identical to *tet(X)* [99.8%, with only two substitutions A280G and G1077C in **Fig. S3**, and E94K and I359M in **Fig. S4**]. As for Tet(X3) [378aa], it was proposed by Nakamura *et al.* to be a polypeptide product of *tet(X3)* [1137 bp] that is only detected in the clinical isolate of *Pseudomonas aeruginosa* (Acc. no.: AB097942).

Very recently, two different research groups in China reported the emergence of plasmid-borne *tet(X4)* [and *tet(X5)*] conferring the appreciably modest level of phenotypic tigecycline resistance [5,6]. Whole-genome sequencing indicated that i) *tet(X4)* is carried by two distinct plasmids in *Escherichia coli*, namely the small IncQ1 plasmid pLHM10-1-p6 [12,783 bp] [6] and the mega IncHI2-type plasmid pEC47 [170,312 bp] [5]; ii) *tet(X5)* [renamed from its formerly duplicated designation of *tet(X3)*] is harbored by the un-typable megaplasmid p34AB (277,384 bp) in *Acinetobacter baumannii* strain 34AB [5]. In relative to *tet(X)* [1167 bp], *tetX4* [1158 bp] and *tet(X5)* [1167 bp] separately displays 94.3% and 87.2% similarity. Despite the limited distribution with a low percentage of detection ratio right now [11], the transferability of plasmid-mediated Tet(X4) [and Tet(X5)] tigecycline resistance still

CONTACT Youjun Feng fengyj@zju.edu.cn; Chengjian Jiang jiangcj0520@vip.163.com
[#]These two authors contribute equally to this work.
Supplemental data for this article can be accessed here.

constitutes a risky challenge to clinical anti-infection therapies. Also, we are concerned with the appearance of new *tet(X)* variants and its expanded dissemination in the near future. Retrospectively, a similar scenario is seen with the MCR family of polymyxin resistance [12–14]. Not only have the new *mcr* variants already been extended from *mcr-1* [1,14,15] to *mcr-9* [16,17], but also some of them, like *mcr-1* [1,12] and *mcr-3* [1,18], have disseminated globally. Thus, it is in great demand to gain insights into the molecular epidemiology of *tet(X)*-mediated tigecycline resistance.

Here, we report a set of multiplex PCR approaches designed to detect the ongoing Tet(X) family. This method has been successfully applied in the screen of field/clinic samples, validating its efficacy/efficiency. Overall, our work (to some extent) pioneers the development of genetic toolbox used in detection of the expanding family of *tet(X)* determinants.

Results and discussion

Design and specificity of *tet(X)* primers

Except that *tet(X1)* gives the modest level of similarity (68.2%) to *tet(X)*, all the other four variants [*tet(X2)* to *tet(X5)*] exhibit relatively high level of identity (85.1% to 99.8%) when compared with *tet(X)*. Though that we recently reported a pair of unique primers specific for the detection of *tet(X4)* [and *tet(X5)*] [11], the high level of similarity amongst different *tet(X)* variants does raise a challenge to design specific primers for each *tet(X)* variant. In particular, the fact that *tet(X2)* is almost identical to that of *tet(X)* with the only two nucleotides' substitution (A280G and G1077C, in **Fig. S3**) might render the routine PCR method ineffective to distinguish them. Following rounds of practical trials, we developed an array of combinations of *tet(X)* primers (Figure 1(a)) and optimized the system of PCR reactions (Figure 1(b)). As for the five *tet(X)* [*X1* to *X5*] target genes, the length of resultant DNA fragments ranges from 204 bp to 685 bp (Table 1 and Figure 1(a)). The trials of optimization revealed that the annealing temperature of 59°C is suitable for the efficient amplification of all the *tet(X)* variants in single PCR tests, in which bacterial cultures with the *tet(X)*-bearing plasmid act as templates (Figure 1(b)). After separation with an electrophoresis of 2% agarose gel, the various *tet(X)*-specific PCR fragments with expected sizes are clearly visualized (Figure 1(b)). This verified the specificity of all the *tet(X)* primers we designed (Table 1 and Figure 1).

Development of multiplex PCR

The key issue to develop an efficient multiplex PCR is the eliminated possibility that the mixture of multiple sets of primer pairs cross-react. Prior to multiplex PCR, we utilized single PCR to rule out the potential cross-reaction within the pool of *tet(X)* primers (Figure 2). In these PCR assays, all the five different bacterial cultures, one of which only harbors a single *tet(X)* variant on the engineered plasmid (Table 2), are tested for each pair of *tet(X)* primers (Figure 2). As predicted, the target fragment (486 bp) of the only *tet(X1)* gene is amplified in the PCR screen containing the pair of primers *tet(X1)*-F/*tet(X1)*-R (Table 1 and Figure 2(a)). In general agreement with that of *tet(X1)*-F/*tet(X1)*-R (Figure 2(a)),

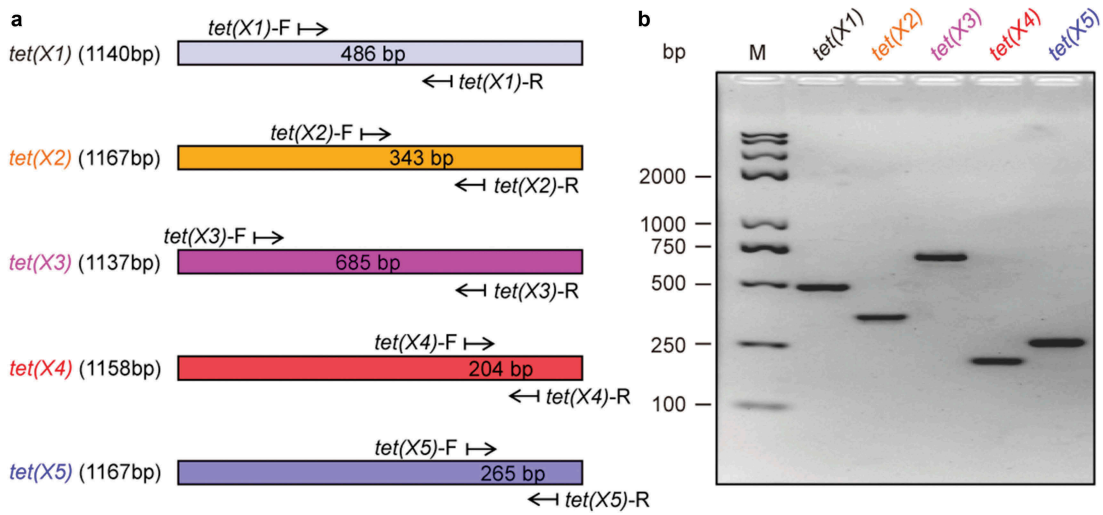

Figure 1. Design and validation of primers specific for five different variants of *tet(X)* [X1 to X5] (a). Cartoon scheme for physical locations of five pairs of *tet(X)* primers. The rectangle represents a given *tet(X)* gene, and an arrow denotes the primer (b). Use of routine PCR to verify the feasibility of *tet(X)* primers. The PCR products were separated by an electrophoresis with 2.0% agarose gel. Designations: bp, base pair; M, DNA marker (Trans 2K Plus II); the symbol of "–", negative control.

Table 1. Primers used in this study.

Primers	Primer sequences	Length (bp)
tet(X1)-F	5′-CGA AAA ATG TTG CTT GGC AGC TT-3′	486
tet(X1)-R	5′-AGT TGT TGA ACG AAT TAA CTC C-3′	
tet(X2)-F	5′-CGG GAT GTC CAA GGT AAG AAA A-3′	343
tet(X2)-R	5′-TGA CAA CGT CGT ATG AAT CAA-3′	
tet(X3)-F	5′-GAC ACT TGA TCT GCA CAG GGA TT-3′	685
tet(X3)-R	5′-CCC TAC AAA AGA TGA TGT CAA AC-3′	
tet(X4)-F	5′-CTG ATT CGT GTG ACA TCA TCT TTT G-3′	204
tet(X4)-R	5′-GTT AAA TTT CCC ATT GGT CAG ATT A-3′	
tet(X5)-F	5′-GGT ATC AAC ATT TCA ATG CTT G-3′	265
tet(X5)-R	5′-CGA TTC GTC CTG CGT ATC TTT TG-3′	

a unique band is exclusively present in our single PCR detections with *tet(X2)*-F/*tet(X2)*-R [343 bp] for *tet(X2)* in Figure 2(b), and *tet(X3)*-F/*tet(X3)*-R [685 bp] for *tet(X3)* in Figure 2(c). In addition, similar scenarios were also seen in the trials with *tet(X4)*-F/*tet(X4)*-R [204 bp] for *tet(X4)* in Figure 2(d), and *tet(X5)*-F/*tet(X5)*-R [265 bp] for *tet(X5)* in Figure 2(e). Evidently, these data supported that our *tet(X)* primers are qualified to be utilized in the subsequent multiplex PCR assays, ruling out the possibility of cross-reaction.

We then attempted to develop a series of multiplex PCR systems. In total, it includes four types of different orientations that separately target double genes, triple genes, quadruple genes, and quintuple genes (Figure 3). In principle, 10 different sets of primer combinations are set up in the multiplex PCR-based detection of both double genes [*tet(X1/2)* to *tet(X4/5)*] in Figure 3(a), and triple genes [*tet(X1/2/3)* to *tet(X3/4/5)*] in Figure 3(b). By contrast, five kinds of

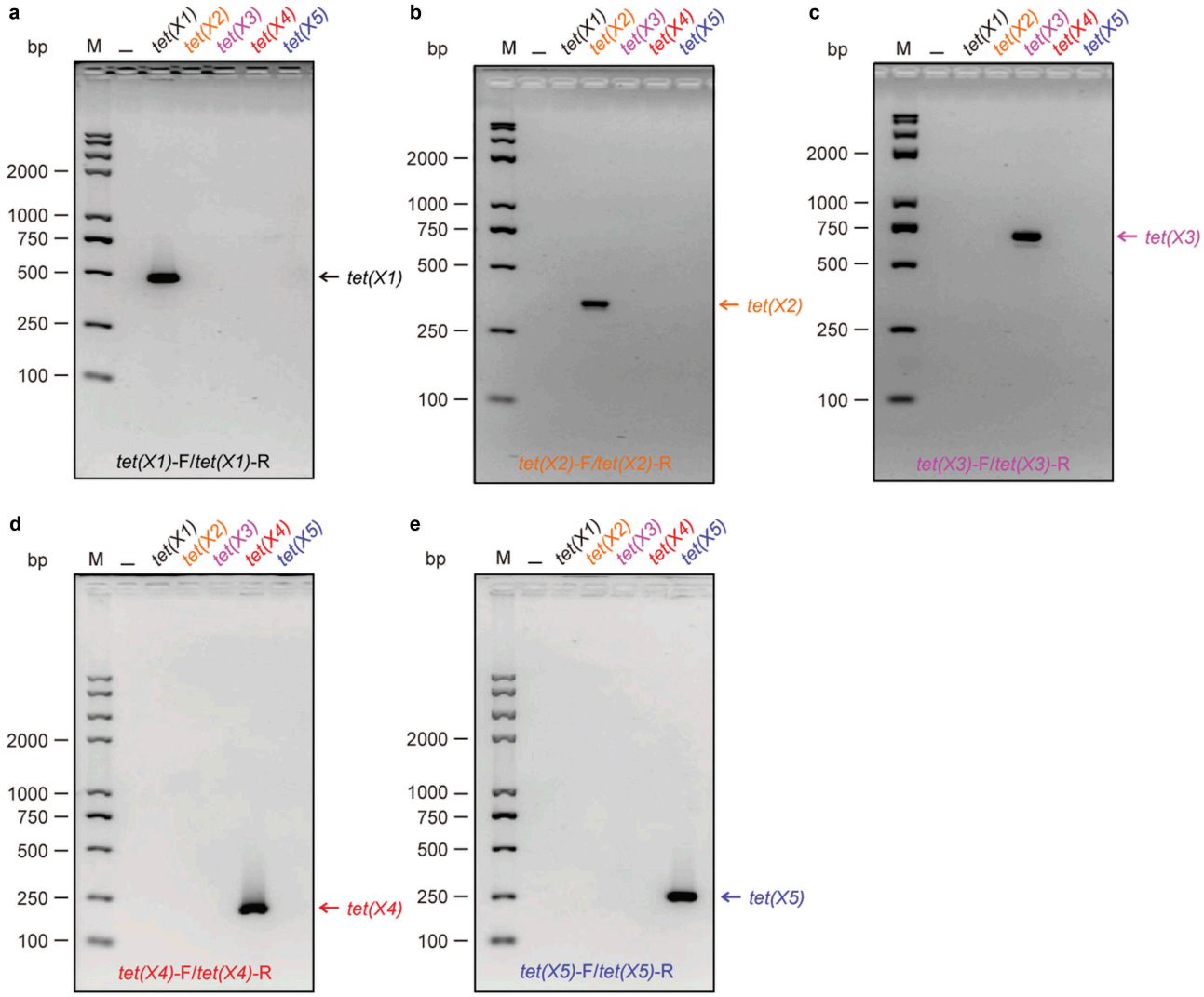

Figure 2. Evaluation of specificity of the five sets of *tet(X)* [X1 to X5] primers (a). PCR assay suggests that the pair of primer [*tet(X1)*-F plus *tet(X1)*-R] is exclusively specific for *tet(X1)*-containing sample (b). The specificity of the primer [*tet(X2)*-F plus *tet(X2)*-R] is validated with PCR assays supplemented with the template of a single colony carrying different *tet(X)* gene (c). Among five kinds of different samples, only *tet(X3)*-bearing one is positive in PCR detection with the specific primers of *tet(X3)*-F plus *tet(X3)*-R (d). The *tet(X4)*-harboring sample is exclusively recognized by a single PCR with a pair of primers [*tet(X4)*-F plus *tet(X4)*-R] (e). The specificity of the primers [*tet(X5)*-F plus *tet(X5)*-R] is unique to the *tet(X5)*-positive sample PCR products were separated with electrophoresis of 2% agarose gel. Designations: bp, base pair; M, DNA marker (Trans 2K Plus II); the symbol of "–", negative control.

Table 2. Field and clinical isolates examined in this study.

Isolates	Genotype	Species	Resources
FYJ4014	*tet(X1)*	*E. coli* JM109	Lab stock, [6]
FYJ4015	*tet(X2)*	*E. coli* JM109	Lab stock, [6]
FYJ4016	*tet(X3)*	*E. coli* JM109	Lab stock, [6]
FYJ4017	*tet(X4)*	*E. coli* JM109	Lab stock, [6]
FYJ4018	*tet(X5)*	*E. coli* JM109	Lab stock, [6]
SSS2-2	*tet(X4)*	*E. coli*	Soils, Guangdong
SSS3-1	*tet(X4)*	*E. coli*	Soils, Guangdong
SS13-1-1	*tet(X4)*	*E. coli*	Duck feces, Guangdong
WF90-1	*tet(X4)*	*E. coli*	Duck feces, Guangdong
LHM10-1	*tet(X4)*	*E. coli*	Swine, Guangdong
G3X16-2	*tet(X4)*	*E. coli*	Swine, Guangdong
YSP8-1	*tet(X4)*	*E. coli*	Swine, Guangdong
FS35-1	*tet(X3/5)*	*Acinetobacter*	Duck feces, Guangdong
CMG11-2	*tet(X3/5)*	*Acinetobacter*	Goose feces, Guangdong
Q477-2	*tet(X5)*	*Acinetobacter*	Feces of migratory birds
Q478-2	*tet(X5)*	*Acinetobacter*	Feces of migratory birds
Q479-2	*tet(X5)*	*Acinetobacter*	Feces of migratory birds
Q480-2	*tet(X5)*	*Acinetobacter*	Feces of migratory birds
CQW11-1	*tet(X5)*	*Acinetobacter*	Water, Guangdong
CQW13-2	*tet(X5)*	*Acinetobacter*	Water, Guangdong
CQS29-1	*tet(X5)*	*Acinetobacter*	Soils, Guangdong
CQS35-1	*tet(X5)*	*Acinetobacter*	Soils, Guangdong
KP1	/	*Klebsiella pneumoniae*	Patient, The Second Affiliated Hospital, Zhejiang University School of Medicine (TSAH/ZJSU)
KP2	/	*K. pneumoniae*	Patient, TSAH/ZJSU
KP3	/	*K. pneumoniae*	Patient, TSAH/ZJSU
KP4	/	*K. pneumoniae*	Patient, TSAH/ZJSU
KP5	/	*K. pneumoniae*	Patient, TSAH/ZJSU
KP6	/	*K. pneumoniae*	Patient, TSAH/ZJSU
KP7	/	*K. pneumoniae*	Patient, TSAH/ZJSU
KP8	/	*K. pneumoniae*	Patient, TSAH/ZJSU

primer combinations [*tet(X1/2/3/4)* to *tet(X2/3/4/5)*] are designed in the multiplex PCR for quadruple genes (Figure 3(c)). The only one set of combined primers [*tet(X1/2/3/4/5)*] is available as for an assay for quintuple genes (Figure 3(c)). Of particular being noteworthy, the primers are added in the system of multiplex PCR (50 μl in total) as follows: 10μM for *tet(X1)*-F/R, 60μM for *tet(X2)*-F/R, 10μM for *tet(X3)*-F/R, 60μM for *tet(X4)*-F/R, and 10μM for *tet(X5)*-F/R (Table 1), respectively. As predicted, the sensitivity of multiplex PCR is determined to be qualified at appreciable level (**Figs S5A-B**). Consistent with that of single PCR, an annealing temperature (59°C) works well in the aforementioned sets of multiplex PCR experiments with the template of a single colony and/or bacterial cultures (Figure 3). In general, the mixture of resultant PCR fragments is well separated by the electrophoresis with 2% agarose gel, ranging from 2 bands to 5 bands (Figure 3(a–c)). Here, all the PCR products were also demonstrated with direct DNA sequencing. Therefore, we believe that we have success in the development of the multiplex PCR methods suitable for the efficient detection of a family of *tet(X)* genes.

Application of multiplex PCR

To further address the feasibility in routine screen, we screened field/clinical samples with this approach of multiplex PCR (Figure 4). Briefly, bacterial samples examined here consist of 17 field isolates of environmental/animal origins and 8 clinical isolates (Table 2). Among the 17 field isolates, the multiplex PCR results suggested that i) seven *E. coli* isolates are positive for *tet(X4)*; ii) eight *Acinetobacter* isolates contain *tet(X5)*; and iii) two isolates of *Acinetobacter* surprisingly coharbor *tet(X3)* and *tet(X5)* (Table 2 and Figure 4(a,b)). The coexistence of *tet(X3)* and *tet(X5)* that initially discovered in multiplex PCR (Figure 4(a)) was further consolidated with a single *tet(X3)* [or *tet(X5)*]-specific PCR (Figure 5). By contrast, none of the clinical isolates are positive in the *tet(X)*-specific multiplex PCR assays (Figure 4(b)). It seems likely that *tet(X4)* is preferentially occupied in *E. coli*, and *tet(X5)* is carried in tendency by *Acinetobacter* (Table 2). Indeed, this is generally consistent with recent observations by He et al. [5]. Since we assigned the different genotype of *tet(X3)* [*tet(X4)* and *tet(X5)*] to the field isolates (Table 2 and Figure 4), we furthered the genotype-to-phenotype relationship in the context of tigecycline resistance. The level of tigecycline resistance in different field isolates varied from 32μg/ml to 64μg/ml (**Fig. S6**). Together with the fact that expression of *tet(X4)* only renders the *E. coli* JM109 resistant to 16μg/ml of tigecycline [11], significantly lower than that of *E. coli* MG1655 (32 μg/ml, **Fig. S6**), we favored to believe the possibility that Tet(X)-mediated tigecycline resistance can be strains (and/or species)-dependent, which is somewhat similar to the performance of *eptA* and *arnT* in the phenotypic colistin resistance [12]. Not surprisingly, our approach of multiplex PCR was successfully applied in the confirmation of the cooccurrence of *tet(X3)* and *tet(X5)* in *Acinetobacter*. To some extent, it highlights its efficacy in routine surveillance. Despite that we verified the sequence identity of *tet(X3)* [*tet(X5)*] in the field isolates, subsequent whole-genome sequencing is needed to finely map the distinct location of *tet(X3)* [*tet(X5)*] in plasmids or chromosomes in the near future.

Conclusions

The data shown here represents a timely follow-up study in response to the newly-identification of *tet(X4)* [*tet(X5)*] [5,6,19]. It provides an efficient and powerful approach to detect the prevalence of *tet(X)* family of variants. Similarly, we developed an efficient multiplex PCR method to detect the family of *mcr*-like genes [20]. As of reporting our method, *tet(X4)* is only detected in non-human *E. coli* isolates [5,6,19], whereas *tet(X5)* is exclusively present in *Acinetobacter baumannii* [5]. The current situation of *tet(X4)* [*tet(X5)*] epidemiology in China is generally consistent with our observation with the multiplex PCR method we developed (Table 2 and Figure 5). The unusual scenario in our case is the cooccurrence of *tet(X3)* and *tet(X5)* in

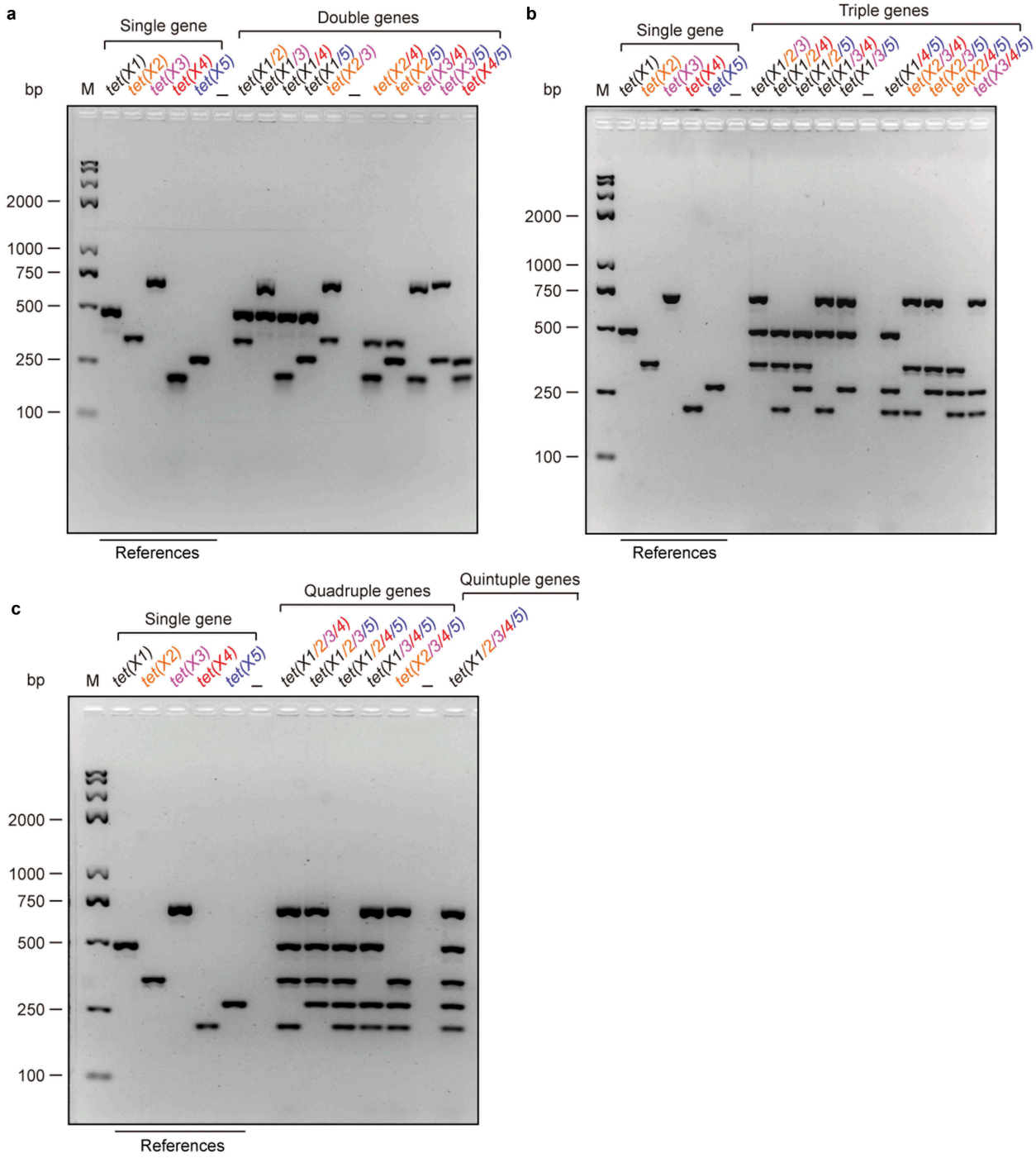

Figure 3. Establishment of different multiplex PCR assays for the *tet(X)* family genes (a). Development of multiplex PCR approaches to detect different combinations of double *tet(X)* genes in a single colony (b). Harnessing multiplex PCR approaches for detection of three *tet(X)* genes in a single colony (c). Developing efficient multiplex PCR assays to detect quadruple and even quintuple variants of *tet(X)* from a single colony. The specific PCR products with known sizes in a single PCR act as references. Namely, it denotes 486 bp for *tet(X1)*, 343 bp for *tet(X2)*, 685 bp for *tet(X3)*, 204 bp for *tet(X4)*, and 265 bp for *tet(X5)* (Table 1), respectively. Two percent of agarose gel was applied to separate the mixture of PCR products. Designations: bp, base pair; M, DNA marker (Trans 2K Plus II); the symbol "–", negative control.

Acinetobacter (Table 2 and Figure 5), which indicates the complexity and dynamics of tigecycline resistance conferred by Tet(X5) [or Tet(X3)]. Because of the unavailability of *tet(X4)* [or *tet(X5)*] distribution beyond China, harnessing this multiplex PCR method to detect *tet(X)* genes facilitates updating global

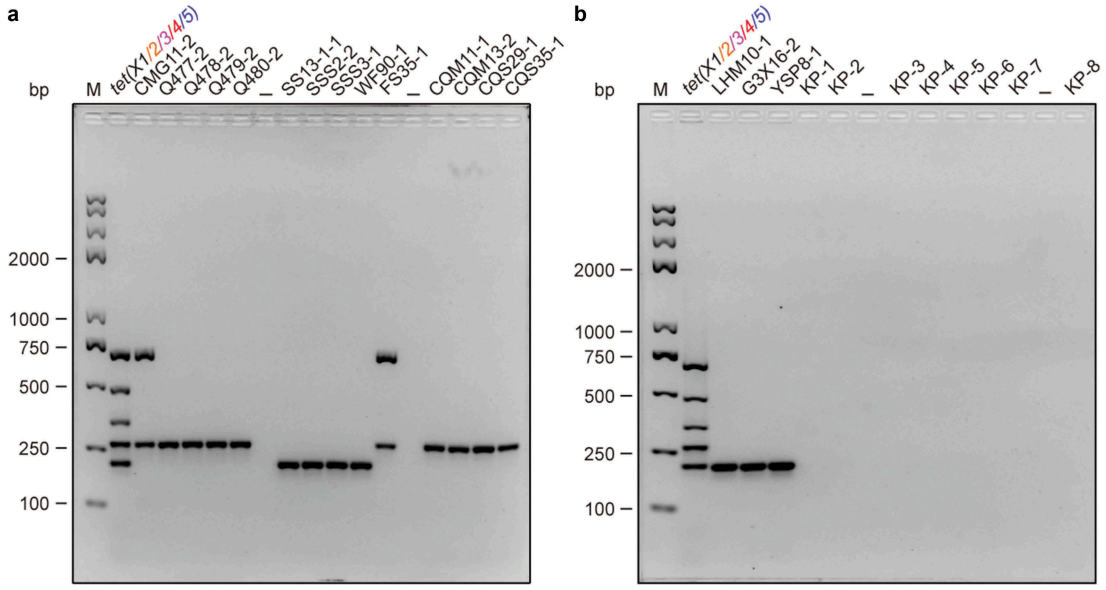

Figure 4. The use of multiplex PCR to screen *tet(X)* variants in field/clinical isolates. The stains examined here include field isolates and clinical isolates. All the field isolates were provided by the collaborator Dr. Jian Sun from South China Agriculture University, Guangzhou, China (Table 2). Among them, 17 are detected to be positive for *tet(X)* variants, including 8 *tet(X5)*-positive *Acinetobacter* isolates (Q477-2, Q478-2, Q479-2, Q480-2, CQW11-1, CQW13-2, CQS29-1 & CQS35-1), and 7 *tetX4*-bearing *E. coli* isolates (LHM10-1, G3X16-2, YSP8-1, SS13-1-1, SSS2-2, SSS3-1 & WF90-1), and 2 *Acinetobacter* isolates coharboring *tet(X3)* and *tet(X5)* (Table 2). The remaining eight strains (KP-1 to KP-8) refer to clinical isolates of *Klebsiella pneumoniae* with tigecycline resistance, collected from The Second Affiliated Hospital, Zhejiang University School of Medicine. Electrophoresis of 2% agarose gel was applied to separate the mixture of PCR products. Designations: bp, base pair; M, DNA marker (Trans 2K Plus II); the symbol "–", negative control; the mixture of *tet(X)* [X1 to X5] PCR products, positive control.

epidemiology of Tet(X) tigecycline resistance in the context of one health (environmental, animal and clinical sectors).

Both colistin and tigecycline are one of the few clinical options used to combat lethal infections with carbapenem-resistant pathogens [12,21,22]. The resistance to colistin arises primarily from the remodeling of lipid A anchored on the bacterial surface [1], in which EptA/MCR is one of the central players having the activity of lipid A phosphoethanolamine transferase [12]. In contrast, the majority of newly-emerging tigecycline resistance is due to the action of Tet(X)-type tigecycline destructases [5,6,23]. As a member of FAD-dependent, NADP-requiring oxidoreductase [23,24], Tet(X) represents a new resistance mechanism [5,6], threatening the renewed interest of tigecycline [and even the two newly-approved alternatives by FDA (eravacycline [25] and omadacycline [26])] in clinical settings. Moreover, we defined that the FAD-binding cavity and tigecycline-loading channel both are essential for Tet(X4) action and its phenotypic resistance [11]. Because that diversified variants of the MCR family have almost spread to the whole world [27], we are seriously concerned with its potential large-scale dissemination of plasmid-mediated Tet(X) family tigecycline resistance determinants, in spite of its current limited distribution of *tet(X4)* [11]. Worrisomely, the fact that the members of MCR family have been extended from MCR-1 [15] to MCR-9 [16,17] prompted us to believe that new *tet(X)* variants [e.g. *tet(X6)*] other than *tet(X4)* [or *tet(X5)*] might be discovered soon. Since that MCR can co-exist with New Delhi Metallo-β-lactamase [NDM-1 [28] or NDM-5 [29,30]] forming a potential superbug insusceptible to both colistin and carbapenem, it is in rational to predict that *tet(X)* coexists with *mcr*-like variants and/or bla_{NDM}. However, it requires further experimental demonstration, though that antibiotic exposure can be driving force for the ongoing superbugs with multiple-drug resistance [30].

Materials and methods

Bioinformatic analyses and primer design of tet(X) genes

Five tigecycline resistance genes separately included *tet(X1)* [Acc. no.: AJ311171], *tet(X2)* [Acc. no.: AJ311171], *tet(X3)* [Acc. no.: AB097942], *tet(X4)* [Acc. no.: MK134376], and *tet(X5)* [Acc. no.: MK134375]. Multiple alignment of nucleotide sequences was carried out with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/), which provides hints to design primers

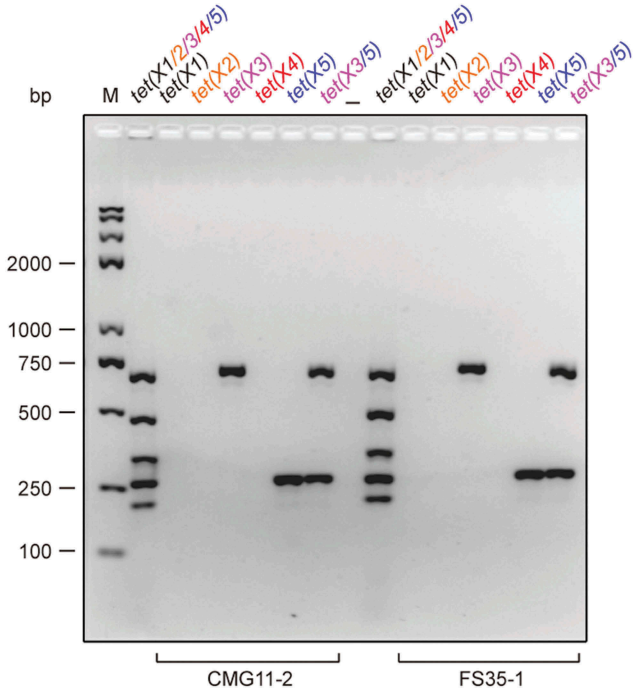

Figure 5. Use of single PCR to re-verify the co-carriage of *tet(X3)* and *tet(X5)* in two isolates of CMG11-2 and FS35-1 PCR results of single gene verified that the two strains (CMG11-2 and FS35-1) are consistently positive in *tet(X3)* [and *tet(X5)*]-specific molecular detection, whereas negative in *tet(X1)* [or *tet(X2)/tet(X4)*]-specific PCR assays. The expected size of PCR products is 685 bp for *tet(X3)*, and 265 bp for *tet(X5)* (Table 1). PCR products were separated with electrophoresis of 2% agarose gel. Designations: bp, base pair; M, DNA marker (Trans 2K Plus II); the symbol "–", negative control; the mixture of *tet(X)* [X1 to X5] PCR products, positive control.

with primer premier 5.0. The resultant primers (Table 1) were verified to amplify various target *tet(X)* fragments in distinct sizes.

Establishment of single and multiplex PCR systems

The mixture of a single PCR reaction (50 μl in total) contained 2× Rapid Taq Master Mix (P222-AA) (Vazyme, China) (25 μl), 10 mM of each primer (1 μl), the template of colony solution (1 μl), and nuclease-free ultra-pure water (22 μl). As we described with *mcr*-like genes with little change [20], different combinations of various primer pairs [*tet(X1)*-F/R to *tet(X5)*-F/R] were utilized in the series of multiplex PCR. Of note, multiple sets of primers were adjusted to different ratio [*tet(X1)*-F/R, 10μM; *tet(X2)*-F/R, 60μM; *tet(X3)*-F/R, 30μM; *tet(X4)*-F/R 60μM; *tet(X5)*-F/R,10μM] in all the multiplex PCR trials (50 μl in total). The reaction condition of both single PCR and multiplex PCR comprises i) 3 min of predenaturation at 95℃ followed by 30 cycles of amplification (15 s of denaturation at 95℃, 15 s of annealing of *tet(X)* primers with target genes at 59℃, and 20 s of target fragment elongation at 72℃), and a final elongation at 72℃ for 5 min. The PCR products were separated by the electrophoresis with 2.0% agarose gel (110 voltages, 35 min). The total time cost in these PCR reactions and electrophoresis is estimated to be around 2 h.

Determination of phenotypic tigecycline resistance

To test the phenotypic tigecycline resistance, all the field isolates that were determined to carry *tet(X)* genes were evaluated with the method of solid LBA supplemented with varied level of tigecycline (0, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, 64.0, 128.0 and 256.0μg/ml). Log-phase cultures were subjected to series of dilution (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}), and then spotted on the aforementioned LBA plates (5 μl each). Following the overnight incubation at 37℃, bacterial viability was visualized.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Author contributions

YF and CJ designed research; KJ, YX, SS, and YF performed research; YF, CJ, MH, KJ, and YX analyzed data; XJ, and JS contributed reagents and analytic tools; YF, and KJ wrote this draft.

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