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Development of a Multiplex Real-Time PCR Assay for Rapid Detection of Tigecycline Resistance Gene *tet*(X) Variants from Bacterial, Fecal, and Environmental Samples

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ABSTRACT We developed a multiplex real-time SYBR green-based PCR assay for rapid detection of *tet*(X) and its variants, including *tet*(X1) and *tet*(X2) and high-level tigecycline resistance genes *tet*(X3), *tet*(X4), and *tet*(X5). We showed that the real-time PCR assay developed had high linearity ($R^2 \geq 0.996$), sensitivity (low detection limit), and specificity (only the target gene could be amplified significantly) and further evaluated it using bacterial, fecal, and environmental samples.

KEYWORDS *tet*(X), SYBR green, tigecycline resistance, real-time PCR

Tigecycline is a parenteral glycylcycline antibiotic with broad-spectrum antimicrobial activity against both Gram-negative and Gram-positive pathogens (1, 2). It is now regarded as a last-resort treatment option for serious bacterial infections caused by pathogens with resistance to one or more classes of antibiotics (3, 4), especially colistin and carbapenems (5, 6). *tet*(X) and its variants, *tet*(X1) and *tet*(X2), encode flavin-dependent monooxygenases that inactivate all tetracyclines, including tigecycline (4, 7–9). *tet*(X) did not receive more attention with no evidence of plasmid-borne *tet*(X) variants in clinical pathogens until the emergence of plasmid-mediated high-level tigecycline resistance genes *tet*(X3), *tet*(X4), and *tet*(X5) in numerous members of the *Enterobacteriaceae* family and *Acinetobacter* genus from animals, meat, and humans (10, 11). Moreover, *tet*(X) variants also exist in a wide variety of environments like livestock manure, farmland soil, air aerosols, river water, ponds, river sediments, sewage plant inlet and outlet water, activated sludge, and residual sludge (12). Therefore, there is an urgent need to develop a fast and specific method for the simultaneous detection of *tet*(X) and its variants in different types of samples. Here, we developed a multiplex SYBR green-based real-time PCR assay for rapid, sensitive, and specific detection of six *tet*(X) variant genes from different samples, including *tet*(X) (GenBank accession no. GU014535.1), *tet*(X1) GenBank accession no. AJ311171), *tet*(X2) (GenBank accession no. AJ311171), *tet*(X3) (GenBank accession no. MK134375), *tet*(X4) (GenBank accession no. MK134376), and *tet*(X5) (GenBank accession no. CP040912.1).

Due to the high degree of similarity between *tet*(X) and *tet*(X2) with only three nucleotide substitutions, we designed universal primers to amply both genes simultaneously. Primer premier 5.0 (PRIMIER Biosoft International, Palo Alto, CA, USA) was used to design specific primers for *tet*(X1), *tet*(X3), *tet*(X4), and *tet*(X5), as well as a universal *tet*(X)/*tet*(X2) primer set (Table 1). To guarantee the specificity, the designed primers were checked using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Preliminary specificity verification of the designed primers was conducted by conventional simplex PCR analysis and agarose gel electrophoresis. Further specificity verification was then performed using the QuantStudio 7 Flex real-time PCR system (Q7;

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TABLE 1 Primers for real-time PCR detection of *tet*(X), *tet*(X1), *tet*(X2), *tet*(X3), *tet*(X4), and *tet*(X5) genes

Primer[a]	Sequence (5′→3′)	Product size (bp)	T_m (°C)[b]
tet(X1)-F	CAGCGTTTCCGAGTTCTTGA	141	80.6
tet(X1)-R	GGACGATTACTCTTCCAAGGCT		
tet(X/X2)-F	TGCGGCTAATGGCATCTCAC	227	81.6
tet(X/X2)-R	GCTGCTACACATGACAACGTCGT		
tet(X3)-F	GTGGATGCTTTGCTATTGTCTGA	125	79.5
tet(X3)-R	TCTGTTGATTCGTCCTGCGTAT		
tet(X4)-F	TCGCTACAAAGAACTGATTCGTG	93	81.3
tet(X4)-R	GGTCGCTTACTTCTCCAAGACTTAC		
tet(X5)-F	TGCCGTTGACCTACACAAAGG	161	80.9
tet(X5)-R	TGTCAAAACGATTTTCGGGTC		

[a]Forward and reverse primers are indicated by F and R, respectively, at the end of the primer designation.
[b]T_m, annealing temperature.

Applied Biosystems, Foster City, CA, USA) with the following conditions: 50°C for 2 min, 95°C for 3 min, and 40 cycles, with 1 cycle consisting of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, following by a melt curve stage. Real-time PCR assays were conducted in 20-μl reaction volumes containing 10 μl of 2× PowerUp SYBR green master mix (Thermo Fisher Scientific, Waltham, MA, USA), 0.8 μl of each primer (10 μmol/liter), 6.4 μl of nuclease-free water, and 2 μl of DNA template. Three technical replicates were conducted for each sample. Six *Escherichia coli* DH5α strains containing the *tet*(X), *tet*(X1), *tet*(X2), *tet*(X3), *tet*(X4), or *tet*(X5) gene were used as positive controls, while *E. coli* DH5α was used as the negative control to confirm the specificity of the assay. Because *tet*(X)- or *tet*(X1)-harboring isolates were unavailable, the complete nucleotide sequences of the *tet*(X) and *tet*(X1) genes were synthesized (by Biomed Biotechnology) based on the corresponding sequences from the GenBank database, then cloned into pET-28a(+), and further transformed into *E. coli* DH5α. The results of conventional PCR (see Fig. S1 in the supplemental material) and SYBR green-based real-time PCR (Table 2) assays were 100% consistent and showed that all primer sets were highly specific for their target gene(s). Amplification was not observed either in the DH5α negative control or in isolates that harbored the target gene for any of the primer sets. Then all *tet*(X) genes amplified using the designed primers were cloned into pMD19-T (TaKaRa Bio, Kusatsu, Japan) and then transformed into *E. coli* DH5α. The amplification efficiency, linearity, and detection level of the SYBR green-based real-time PCR detection assay were investigated by generating standard curves using 10-fold serial dilutions of the recombinant plasmids, including pMD19-T-*tet*(X/X2), pMD19-T-*tet*(X1), pMD19-T-*tet*(X3), pMD19-T-*tet*(X4), and pMD19-T-*tet*(X5) as the amplification templates. Real-time PCR conditions were as described above. We established the following criteria for each set of primers used in the assay: (i) R^2 value of $\geq$0.980, which indicated linearity of the standard curve; (ii) amplification efficiency within the range 90% to 110%; (iii) a single peak obtained in the melting curve assay. Finally, the detection range (number of gene copies) of the assay for *tet*(X/X2), *tet*(X1), *tet*(X3), *tet*(X4), and *tet*(X5), were 1.31×10^1 to 1.31×10^9, 1.09×10^1 to 1.09×10^9, 1.07×10^1 to 1.07×10^9, 8.42×10^0 to 8.42×10^8, and 5.28×10^1 to 5.28×10^8, respectively. The cycle threshold (C_T) ranges were 8.193 to 31.891, 8.958 to 34.889, 8.783 to 35.255, 10.209 to 35.397, and 9.682 to 32.491, respectively. All R^2 values were $\geq$0.996, and all amplification efficiencies were within the range of 90% to 110% (Fig. 1). Standard curves, amplification curves, and melting curves are shown in Fig. 1.

Genomic DNA extracted from bacterial, fecal, and environmental samples was then used to further validate the specificity and detection level of the assay. HiPure bacterial DNA kit (Magen, Guangdong, China) was used to extract the bacterial genome. DNeasy PowerSoil (Qiagen, Hilden, Germany) and DNeasy PowerWater (Qiagen, Hilden, Germany) kits were used to extract metagenomic DNA from the solid and water samples, respectively. Twenty-one isolates, including 2 *tet*(X2) isolates, 4 *tet*(X3) isolates, 14 *tet*(X4) isolates, and 1 *tet*(X5) isolate, were correctly identified using the real-time PCR

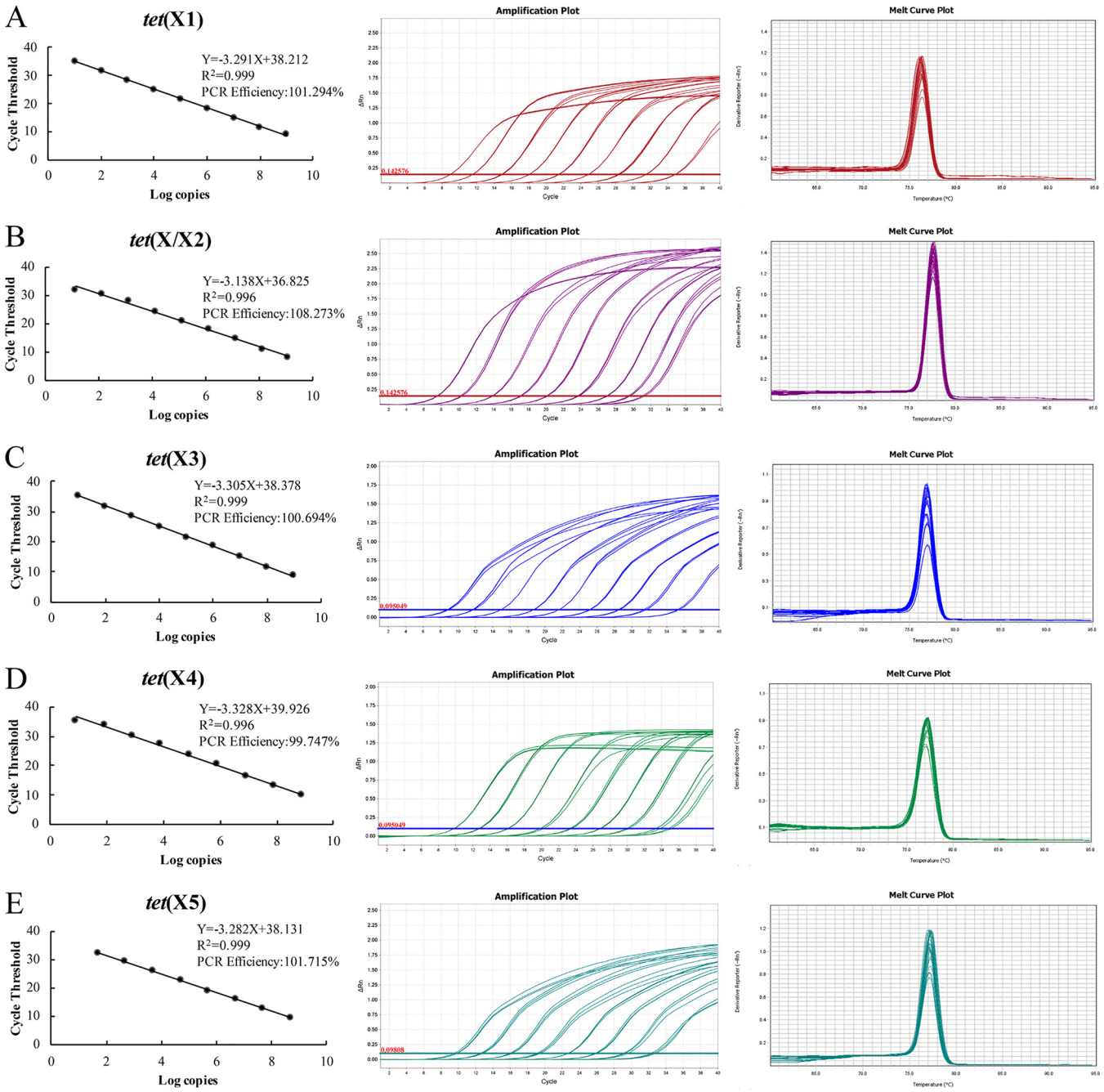

FIG 1 Real-time PCR standard curves, amplification curves, and melting curves. (A to E) Real-time PCR standard curves, amplification curves, and melting curves for *tet*(X1), *tet*(X/X2), *tet*(X3), *tet*(X4), and *tet*(X5), respectively.

assay (Table 1). For these assays, the C_T ranges for *tet*(X2), *tet*(X3), *tet*(X4), and *tet*(X5) were 20.698 to 22.975, 13.858 to 24.423, 11.862 to 21.558, and 13.238, respectively (Table 1). Amplification of nontarget genes was not found for all test strains, suggesting a high specificity for the developed detection method. Moreover, a total of 15 metagenomes that were extracted from feces, compost, soil, sewage, and biogas slurry samples collected from a pig farm in Henan Province, China, were then screened to further evaluate the method. The relative abundance of *tet*(X) and its variants in each of the metagenomes (extracted from 0.25 g of solid sample or 50 ml of water sample) was normalized against the copies of the 16S rRNA gene (gene copies per 10^6 copies of the 16S rRNA gene) (13, 14). The real-time PCR assay revealed normalized gene copy

TABLE 2 Specific verification results of SYBR green real-time PCR detection assay

Isolate or sample type	Genotype	Species	Origin	Gene location[a]	Real-time PCR result[b] for:					
					tet(X/X2)	*tet*(X1)	*tet*(X3)	*tet*(X4)	*tet*(X5)	C_T ± SD
Isolates										
DH5α		*E. coli*			−	−	−	−	−	
DH5α-*tet*(X)	*tet*(X)	*E. coli*	Synthetic	Pl	+	−	−	−	−	22.641 ± 0.121
DH5α-*tet*(X1)	*tet*(X1)	*E. coli*	Synthetic	Pl	−	+	−	−	−	12.817 ± 0.233
DH5α-*tet*(X2)	*tet*(X2)	*E. coli*	Pig	Pl	+	−	−	−	−	17.083 ± 0.017
DH5α-*tet*(X3)	*tet*(X3)	*E. coli*	Pig	Pl	−	−	+	−	−	15.461 ± 0.148
DH5α-*tet*(X4)	*tet*(X4)	*E. coli*	Pig	Pl	−	−	−	+	−	15.689 ± 0.086
DH5α-*tet*(X5)	*tet*(X5)	*E. coli*	Human	Pl	−	−	−	−	+	13.482 ± 0.041
111	*tet*(X2)	*Myroides odoratimimus*	Pig	Chr	+	−	−	−	−	22.975 ± 0.216
141	*tet*(X2)	*Myroides odoratimimus*	Pig	Chr	+	−	−	−	−	20.698 ± 0.312
44-1-3	*tet*(X3)	*Acinetobacter baumannii*	Human	Pl	−	−	+	−	−	13.858 ± 0.151
118	*tet*(X3)	*Acinetobacter lwoffii*	Pig	Pl	−	−	+	−	−	24.423 ± 0.189
159	*tet*(X3)	*E. coli*	Pig	Pl	−	−	+	−	−	21.558 ± 0.315
176	*tet*(X3)	*E. coli*	Pig	Pl	−	−	+	−	−	24.230 ± 0.056
28-1	*tet*(X4)	*E. coli*	Human	Pl	−	−	−	+	−	14.025 ± 0.074
114-1	*tet*(X4)	*E. coli*	Pig	Pl	−	−	−	+	−	15.152 ± 0.142
120	*tet*(X4)	*Sphingobacterium*	Pig	Pl	−	−	−	+	−	17.389 ± 0.031
132-2	*tet*(X4)	*E. coli*	Pig	Pl	−	−	−	+	−	11.862 ± 0.067
133-2	*tet*(X4)	*E. coli*	Pig	Pl	−	−	−	+	−	14.302 ± 0.030
137-1	*tet*(X4)	*E. coli*	Pig	Pl	−	−	−	+	−	13.439 ± 0.037
138	*tet*(X4)	*E. coli*	Pig	Pl	−	−	−	+	−	13.332 ± 0.102
147	*tet*(X4)	*Proteus vulgaris*	Pig	Pl	−	−	−	+	−	14.712 ± 0.251
156	*tet*(X4)	*E. coli*	Pig	Pl	−	−	−	+	−	14.147 ± 0.048
161-2	*tet*(X4)	*E. coli*	Pig	Pl	−	−	−	+	−	17.270 ± 0.067
182	*tet*(X4)	*E. coli*	Pig	Pl	−	−	−	+	−	12.299 ± 0.268
186	*tet*(X4)	*E. coli*	Pig	Pl	−	−	−	+	−	20.220 ± 0.042
188	*tet*(X4)	*E. coli*	Pig	Pl	−	−	−	+	−	13.608 ± 0.029
190-B	*tet*(x4)	*Proteus mirabilis*	Pig	Pl	−	−	−	+	−	12.288 ± 0.075
5AB	*tet*(X5)	*Acinetobacter baumannii*	Human	Pl	−	−	−	−	+	13.238 ± 0.069
Sample types										
Feces			Pig		$1.361 \times 10^3 - 1.731 \times 10^3$	$3.120 \times 10^2 - 1.220 \times 10^3$	$5.112 \times 10^0 - 1.565 \times 10^3$	$1.243 \times 10^1 - 1.629 \times 10^3$	$7.086 \times 10^0 - 5.003 \times 10^2$	
Compost			Pig		$4.122 \times 10^1 - 7.136 \times 10^2$	$8.136 \times 10^3 - 1.932 \times 10^4$	$2.398 \times 10^1 - 2.716 \times 10^3$	$1.818 \times 10^1 - 1.668 \times 10^3$	$8.114 \times 10^0 - 1.150 \times 10^3$	
Savage			Pig		$5.022 \times 10^2 - 3.177 \times 10^3$	$6.900 \times 10^2 - 6.541 \times 10^3$	$1.234 \times 10^1 - 1.996 \times 10^3$	$1.630 \times 10^0 - 1.268 \times 10^2$	$6.215 \times 10^0 - 2.740 \times 10^2$	
Biogas slurry			Pig		$3.554 \times 10^2 - 2.679 \times 10^3$	$3.102 \times 10^1 - 7.317 \times 10^2$	$3.744 \times 10^0 - 9.042 \times 10^2$	$3.091 \times 10^1 - 4.946 \times 10^1$	$6.153 \times 10^0 - 1.632 \times 10^1$	
Soil			Pig		$2.507 \times 10^0 - 2.139 \times 10^2$	$4.415 \times 10^3 - 6.014 \times 10^4$	$2.628 \times 10^0 - 1.163 \times 10^1$	$3.058 \times 10^1 - 1.339 \times 10^3$	$3.630 \times 10^2 - 7.759 \times 10^3$	

[a]Pl, plasmid; Chr, chromosome.

[b]For isolates, the PCR results for *tet*(X) genes for isolates (−, negative; +, positive) and cycle threshold are shown for the isolates. For samples, the range of relative abundance of the *tet*(X) gene normalized by 10^6 copies of 16S rRNA for metagenome is shown.

numbers of 2.507×10^0 to 3.177×10^3, 3.102×10^1 to 6.014×10^4, 2.628×10^1 to 2.716×10^4, 1.630×10^0 to 1.688×10^3, and 6.153×10^0 to 7.759×10^3 for *tet*(X1), *tet*(X/X2), *tet*(X3), *tet*(X4), and *tet*(X5), respectively (Table 2 and Fig. S2).

Since the first report of plasmid-mediated tigecycline resistance genes *tet*(X3) and *tet*(X4) a few months ago, there have already been a number of follow-up reports (10, 15–18). Recently, a novel plasmid-mediated tigecycline resistance gene, *tet*(X5) variant, was reported in *Acinetobacter baumannii* in a clinic (11). It is necessary to establish a rapid detection method of *tet*(X) variants. The method developed in this study is highly effective for the screening of six *tet*(X) variants not only in cultured bacteria but also directly from feces, compost, soil, sewage, and biogas slurry. In particular, the plasmid-mediated tigecycline resistance gene *tet*(X3) can be detected at levels as low as one copy per 10^4 copies, with an upper limit of 10^3 copies per 10^4 copies. Meanwhile, *tet*(X4) and *tet*(X5) can be detected at an abundance as low as one copy per 10^6 copies, with an upper limit of 10^3 copies per 10^6 copies. However, the current method is limited by the fact that we could not confirm the specificity of the assay for *tet*(X)- or *tet*(X1)-carrying cultured bacteria, as we were unable to find strains carrying these genes. Also, all six *tet*(X) genes could not be detected in a single reaction.

In conclusion, we developed a multiplex SYBR green-based real-time PCR assay for the rapid screening and quantification of various *tet*(X) variants, including *tet*(X), *tet*(X1), *tet*(X2), *tet*(X3), *tet*(X4), and *tet*(X5). It is highly sensitive and specific for the detection of *tet*(X) and its variants from samples, including cultured bacteria, feces, compost, sewage, biogas slurry, and soil.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 2.8 MB.

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