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*Original article*

# Development of TaqMan-based real-time PCR assay based on the E1 gene for the quantitative detection of the Getah virus

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## Abstract

To develop a sensitive, specific, and rapid approach for the detection Getah virus (GETV), a set of primers targeting the conserved region of the *E1* gene was created. The TaqMan-based real-time PCR method for GETV detection was developed by optimizing the reaction conditions. The method demonstrated excellent specificity, and amplification did not occur with the causative agents of all prevalent swine viral infections (CSFV, PRRSV, PRV, PEDV, PTV, and JEV), except GETV. Additionally, upon assessing the sensitivity of the method, the minimum detection limit for GETV was found to be 5.94 copies/μL, which is 10 times higher than that of the traditional PCR approach. Further, the intra- and inter-assay variation coefficients were less than 1%, demonstrating good repeatability. Moreover, GETV was found in 10 of the 20 field serum samples using real-time PCR but only in three of the samples using traditional PCR. Consequently, the first GETV TaqMan-based real-time PCR approach based on the *E1* gene was developed for GETV pathogenic diagnoses, and this exhibited high specificity, sensitivity, and repeatability. This assay is practical for the pathogenic diagnosis and epidemiology of GETV.

**Key words:** Getah virus, real-time PCR, TaqMan, detection

## Introdduction

Getah virus (GETV) is a positive-sense, single--stranded RNA virus. According to taxonomy, the *Getah virus* is a member of the *Togaviridae* family and belongs to the genus *Alphavirus* (Chen et al. 2018). GETV has a genome of about 11.7 kb, containing two large open reading frames coding for non-structural polyprotein and structural polyprotein. The non-structural and structural polyproteins are processed to form four nonstructural proteins (nsP1 - 4) and five structural proteins (C, E3, E2, 6K and E1), respectively (Chen et al. 2018).

GETV, an arbovirus, can spread to various mammals via *Culex* and *Aedes* mosquitoes (Fukunaga et al. 2000). As a result, the emergence of mosquitos frequently coincides with GETV epidemic seasons. GETV is currently widely distributed in several nations, including China, South Korea, Japan, Mongolia, Russia, India, and the Philippines (Ksiazek et al. 1981,

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Brown and Timoney 1998, Fukunaga et al. 2000, l'vov et al. 2000, Turell et al. 2003, Kuwata et al. 2018, Lu et al. 2020, Sam et al. 2022).

GETV infects a range of hosts and causes disease. Clinical signs in horses include fever, rashes, and limb swelling in the hind legs (Nemoto et al. 2015). GETV is also pathogenic for pregnant sows and newborn piglets. Infections in fetuses manifest as stillbirths or fetal mummies, and infected piglets experience pyrexia, anorexia, sadness, ataxia, tremor, and sometimes death (Yang et al. 2018). Additionally, GETV infection in young foxes causes rapid fever, anorexia, sadness, neurologic symptoms, and death (Shi et al. 2019). Since 2017, numerous investigations have revealed that GETV is widely prevalent in domestic animals in China (Yang et al. 2018a, Liu et al. 2019, Lu et al. 2019, Shi et al. 2019, Li et al. 2019, Ren et al. 2020, Xing et al. 2020, Shi et al. 2022). Notably, GETV infections in pigs have been steadily increasing in recent years (Yang et al. 2018a, Ren et al. 2020, Xing et al. 2020, Shi et al. 2022). Thus, establishing a rapid, sensitive, and specific approach for GETV detection is crucial for disease monitoring and diagnosis.

Currently, various techniques, including ELISA, virus isolation, and PCR, have been employed to identify viruses (Li et al. 2017, Ge et al. 2021). However, owing to its sensitivity, speed, and accuracy, PCR has been frequently employed for pathogen identification. Therefore, we aimed to develop a TaqMan-based real-time PCR assay based on the *E1* gene for the quantitative detection of GETV. We believe this approach would provide a practical method to test for GETV infections in clinical settings.

## Materials and Methods

### Viral strains and samples

The following viruses were maintained at -80°C in our laboratory: GETV (HuN1 strain, GenBank# MF741771) (Yang et al. 2018a), pseudorabies virus (PRV, XiangA strain, GenBank# KP710981), Japanese encephalitis virus (JEV, HNML1 strain, GenBank# KX774636), and porcine teschovirus (PTV, HuN1 strain, GenBank# MF170905) (Yang et al. 2018b). The porcine reproductive and respiratory syndrome virus (PRRSV) and the classical swine fever virus (CSFV) attenuated live vaccination strains were purchased from Pulike Biological Inc., China. A live, attenuated strain of the porcine epidemic diarrhea virus (PEDV) vaccine was purchased from CAHIC Inc., China. Twenty serum samples collected from healthy sows of a commercial farm in the Hunan province, China, were maintained in our laboratory at -80 °C.

### Viral RNA/DNA extraction and reverse transcription

Viral RNA extraction (Tiandz Inc., Beijing, China) was performed using 0.2 mL of the supernatants of virus strains and serum samples. The RNA extraction process for every clinical sample contained a positive (GETV-HuN1 strain) and a negative (water-only) control in each run. The RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) was used to create the viral cDNA in accordance with the manufacturer's instructions. The reaction mixture had a total volume of 10 µL, including 2 µL of 5X reaction buffer, 0.5 µL of random hexamer primer (100 µmol), 1 µL of 10 mmol dNTP Mix, 0.5 µL of 20 u/µl RiboLock RNase inhibitor, 0.5 µL of 200 u/µl RevertAid M-MuLV reverse transcriptase, 1 µL of RNA extract, and 5.5 µL of RNase free water. Reverse transcription was processed under the following conditions: 5 min at 25°C, 60 min at 42°C, and terminate the reaction by heating at 70°C for 5 min. Use 1 µL of the cDNA to amplify in PCR for the detection of GETV. Viral DNA was extracted using the viral DNA extraction kit (Tiandz Inc., Beijing, China) in accordance with the manufacturer's instructions for DNA viruses.

### Design and synthesis of primers and TaqMan probe

The known genomic sequences of GETV were obtained from GenBank and analyzed using Lasergene software (DNAStar Inc., Madison, WI, USA) to create primers based on the conserved sequences. One set of primers (GETV-rF/GETV-rR) and a probe (GETV-rT), positioned in the conserved region of the *E1* gene, were developed to amplify a 132 base-pair (bp) fragment of GETV DNA after multiple alignments (Table 1). The 5′ end of the GETV TaqMan probe was labeled with FAM, and the 3′ end was labeled with the fluorescence quencher BHQ1. The primers and probes were commercially synthesized (Tsingke Biotechnology Co., Ltd, Beijing, China).

### Standard preparation

An 1871 bp GETV *E1* gene fragment was amplified from the cDNA of the GETV-HuN1 strain using the primers GETV(E1)-F and GETV(E1)-R (Table 1) to create the standard control plasmid. PCR products were separated on 0.8% agarose gel. Specific bands were purified using gel extraction kit (Tiandz Inc., Beijing, China) and cloned into pMD19-T vector (Takara, Otsu, Japan). The recombinant plasmids were then transformed into Trans1-T1 *Escherichia coli*

Table 1. Primers used for Getah virus (GETV) PCR.

| Primers | Oligonucleotide sequence (5'-3')[d] | Genome location[e] | Sequence length |
|---|---|---|---|
| GETV-rF[a] | CAAACCCTGTTTTGGCTGGAGCT | 9831–9853 | 132 bp |
| GETV-rR[b] | GACGGGAGTTCCCAGGCTCAC | 9942–9962 | |
| GETV-rT[c] | CAACGCCGMTCGCYGCCATAATCATA | 9856–9881 | |
| GETV(E1)-F | TGGGATGAGAATCAAACCCTGTT | 9819–9841 | 1871 bp |
| GETV(E1)-R | GTAAAATATTAAAAAAACAAAT | 11668–11689 | |

[a] F: forward primer
[b] R: reverse primer
[c] T: probe
[d] *M*, A or C; *Y*, C or T
[e] Location of primers and probe relative to the complete genomic sequence of GETV-HuN1 (GenBank # MF741771).

bacteria (produced by TransGen, Beijing, China), where they were propagated in accordance with the cloning manual's instructions. Following the manufacturer's instructions, the plasmids were extracted using Fast Mini Plasmid Kit (Tiandz Inc., Beijing, China), quantified using a spectrophotometer (BioDrop, Cambridge, UK), and then sequenced (BioSune, Shanghai, China).

### Optimization of real-time PCR and generation of the standard curve

Real-time PCR tests were conducted using the plasmids as standards. The real-time PCR was optimized using primer and probe volumes of 1.0, 0.5, 0.3, 0.2, and 0.1 µL and annealing temperatures of 50, 55, 60, and 65°C. Each reaction had a total volume of 20 µL, including 10 µL of the AceQ® qPCR Probe Master Mix (Vazyme, Nanjing, China), 1 µL of the standard plasmids, and optimized volumes of 10 µmol/L for each probe and two primers. The remaining 20 µL of the reaction was made up with nuclease-free water. Using the ABI StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), amplification and quantification processes were carried out under the following conditions: 2 min at 37°C, 30 s at 95°C, 40 cycles of 10 s at 95°C, and 30 s at the optimal annealing temperature. The sample was deemed negative if no cycle threshold (CT) was identified after 40 amplification cycles. The initial plasmid standards comprising 10-fold serial dilutions ($5.94\times10^2$ to $5.94\times10^7$ copies/µL) were used to create the standard curve. Each dilution was performed in triplicate.

### Specificity and sensitivity tests

Testing samples positive for other causative agents of prevalent swine viral infections, such as CSFV, PRRSV, PRV, PEDV, PTV, and JEV, has allowed researchers to determine the specificity of primers. Ten-fold serial dilutions ($5.94\times10^{-1}$ to $5.94\times10^8$ copies/µL) of the plasmid standards were evaluated to determine the sensitivity of the real-time PCR. Each diluted plasmid standard served as a template for traditional PCR detection. The traditional PCR reaction mixture had a total volume of 20 µL, including 10 µL of 2X PCR MagicMix 3.0 (Tiandz Inc., Beijing, China), 0.5 µL of two primers (10 µmol), 1 µL of the standard plasmids, and 8 µL of RNase free water. The reaction was performed under the following conditions: an initial denaturation step at 94°C for 5 min; followed by 40 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 10 s; and a final extension at 72°C for 7 min. PCR products were examined using agarose gel electrophoresis, and their sensitivity was assessed by viewing them under a UV light source.

### Reproducibility test

Three 10-fold serial dilutions of the plasmid standards ($5.94\times10^4$ to $5.94\times10^6$ copies/µL) were assessed independently in three separate runs and concurrently in the same run to assess the repeatability of the real-time PCR. Microsoft Excel software (version 2010, USA) was used to compute the average CT, standard deviation (SD), and coefficient of variation (CV) values for both tests.

### Analysis of field samples

To identify 20 serum samples for the presence of GETV, the developed TaqMan-based real-time PCR was used. Real-time PCR results were contrasted with those of traditional PCR. Positive and negative controls were used in both the traditional PCR and real-time PCR techniques. The plasmid standard of GETV and nuclease-free water were used as templates for positive and negative controls, respectively.

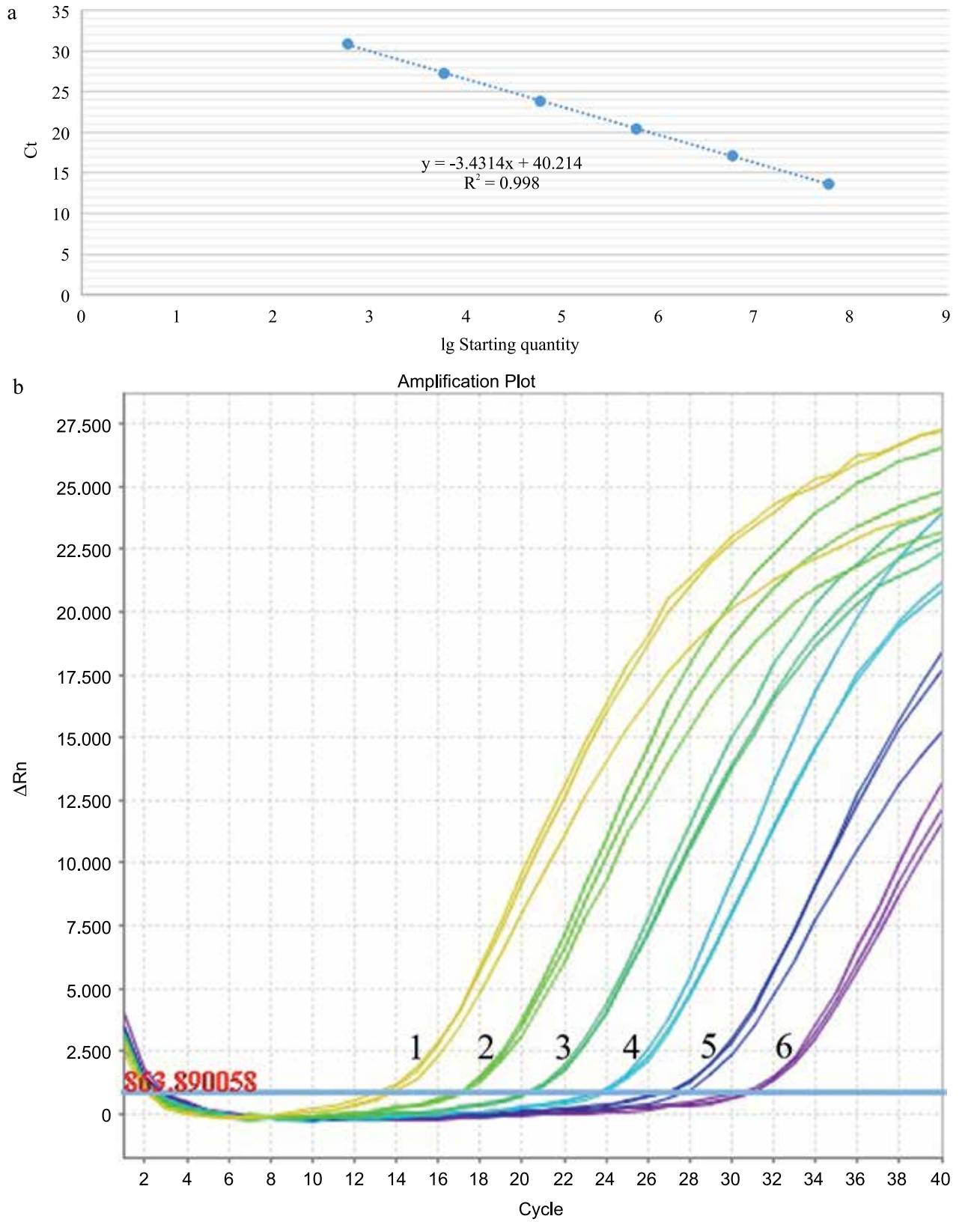


Fig. 1. Standard curve of Getah virus (GETV) real-time PCR assay. (a) Standard curves were generated using the mean cycle threshold (CT) values measured against the diluted plasmid standards ($log_{10}$ copy number). Calculations were made to determine the equation of the regression curve (Y) and correlation coefficient ($R^2$). Equation: $y = -3.4314x + 40.214$; correlation coefficient: $R^2 = 0.9998$. (b) Amplification curve produced using 10-fold successive dilutions of the pMD19-T-GETV plasmids, designated as 1–6, in the range of $5.94\times10^7$ to $5.94\times10^2$ copies/$\mu L$, respectively.

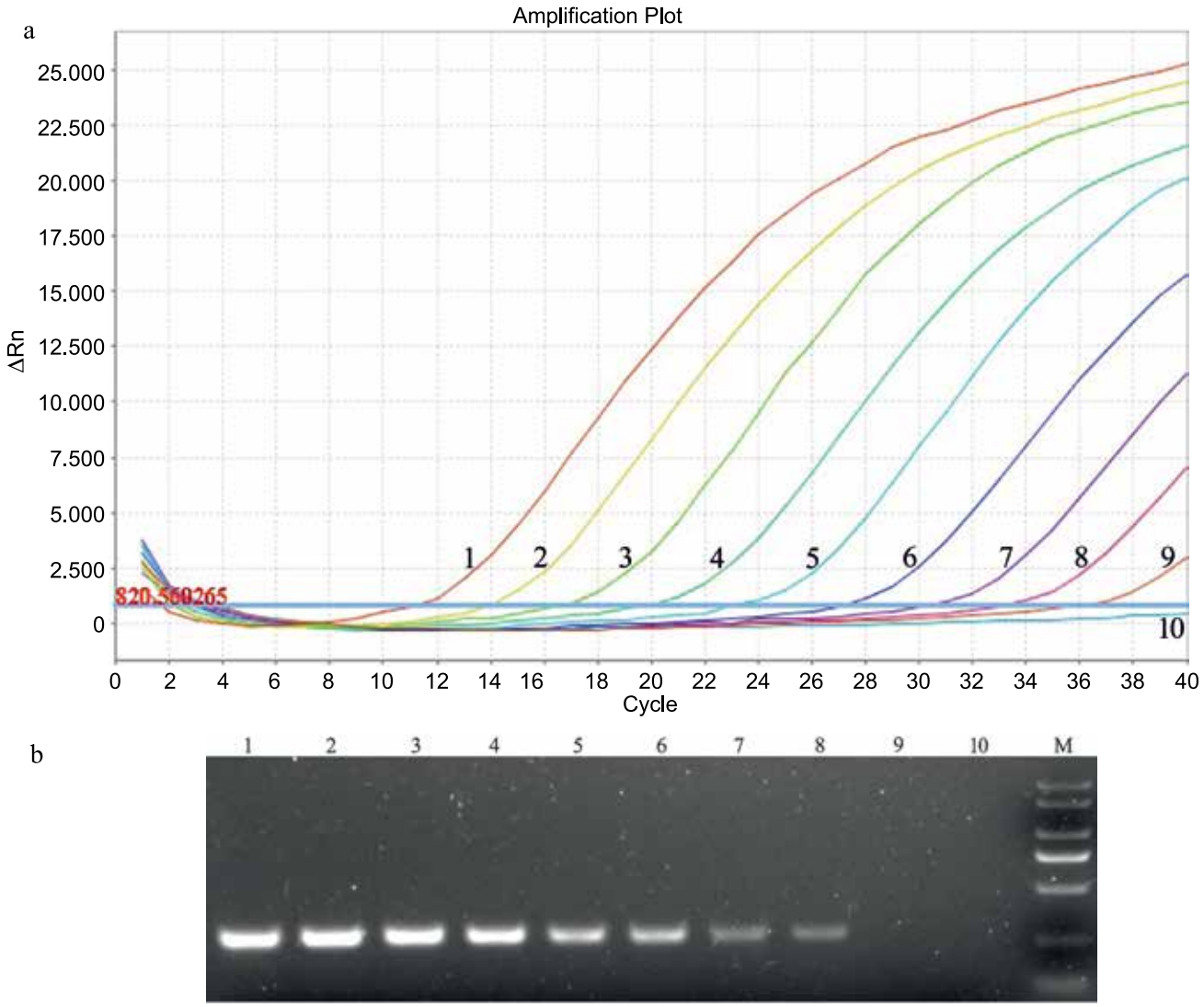


Fig. 2. Sensitivity of the Getah virus (GETV) real-time and traditional PCR. (a) A range of $5.94\times10^{8}$ to $5.94\times10^{-1}$ copies/$\mu L$ of pMD19-T-GETV plasmids, designated as 1–10, respectively, was used. (b) Findings of traditional PCR based on agarose gel electrophoresis. The pMD19-T-GETV plasmids were serially diluted 10-fold in lanes 1–10, ranging from $5.94\times10^{8}$ to $5.94\times10^{-1}$ copies/$\mu L$, respectively; lane M served as a DNA marker measuring 2000 bp.

## Results

### Optimization of real-time PCR

Through determinations of the ideal annealing temperature and primer and probe concentration, the TaqMan-based real-time PCR was optimized, and conditions of annealing at 60°C, 0.5 µL of 10 µM primers, and 0.2 µL of 10 µM probe were determined.

### Standard curve and sensitivity of the real-time PCR

The concentration of GETV plasmid standard in the extracted sample was 297.1 ng/µL, and convert to copy number was $5.94\times10^{10}$ copies/µL. By graphing the plasmid copy number against the observed CT values, standard curves were created using 10-fold serial dilutions of pMD19-T-GETV plasmids. An association was identified based on linear regression with a correlation coefficient ($R^2$) of 0.9998, a slope of −3.4314, and an intercept of 40.214 (Fig. 1). These values indicated that CT values and template concentrations were strongly related.

The real-time PCR method's sensitivity was ascertained by assessing the minimal detection limit of plasmid standards with 10-fold serial dilutions ($5.94\times10^{-1}$ to $5.94\times10^{8}$ copies/$\mu L$). The TaqMan-based PCR had a minimum detection threshold of 5.94 copies/µL (Fig. 2a). Compared with the real-time assay, traditional PCR was 10 times less sensitive, with a threshold of $5.94\times10^{1}$ copies/µL (Fig. 2b).

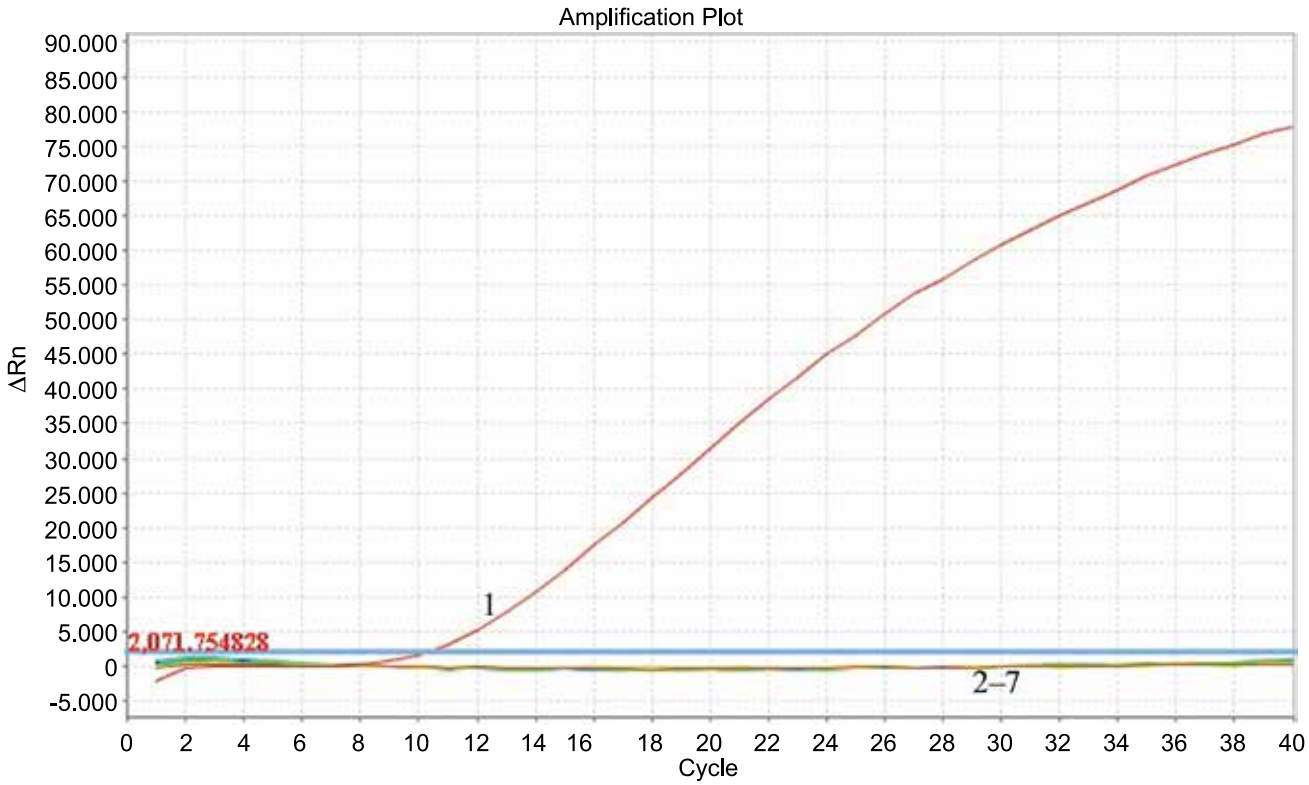


Fig. 3. Specificity of the Getah virus (GETV) real-time PCR. 1: GETV; 2–7: classical swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV), pseudorabies virus (PRV), porcine epidemic diarrhea virus (PEDV), porcine teschovirus (PTV), and Japanese encephalitis virus (JEV).

Table 2. Reproducibility of Getah virus (GETV) real-time PCR.

| Concentration of standard plasmid (copies/μL) | Repeatability of intra-batch assay | | Repeatability of inter-batch assay | |
|---|---|---|---|---|
| | Mean CT±SD | CV(%) | Mean CT±SD | CV(%) |
| $5.94\times10^6$ | 17.05±0.10 | 0.60 | 17.09±0.12 | 0.72 |
| $5.94\times10^5$ | 20.37±0.08 | 0.39 | 20.63±0.18 | 0.86 |
| $5.94\times10^4$ | 23.77±0.15 | 0.65 | 23.89±0.19 | 0.81 |

### Specificity of the real-time PCR

Cross-reactivity against common swine viral infectious agents, such as CSFV, PRRSV, PRV, PEDV, PTV, and JEV, was tested to assess the specificity of the reaction. These viruses were not associated with any distinct positive signals (Fig. 3), demonstrating that the primers and probe were highly specific to GETV.

### Reproducibility of the real-time PCR

The intra- and inter-assay reproducibility of the real-time PCR method, utilizing three plasmid standards with 10-fold serial dilutions ($5.94\times10^4$ to $5.94\times10^6$ copies/μL), was then evaluated. Utilizing these standards in triplicate for each run, the intra-assay CT value was examined, and the inter-assay reproducibility was independently determined over the course of three different runs. Real-time PCR was highly repeatable, as shown by the computed CV values for intra- and inter-assay variations, which ranged from 0.39% to 0.65% and from 0.72 to 0.86%, respectively (Table 2).

### Field sample evaluation using the real-time PCR

Real-time PCR and traditional PCR were used to detect 20 serum samples for the presence of GETV simultaneously. Using GETV real-time PCR, 10 of 20 (50.00%) samples were positive (Table 3). Comparatively, by traditional PCR, only three of 20 (15.00%) samples were GETV-positive (Table 3). These findings demonstrated that the real-time PCR method is suited for field sample detection and is more sensitive than conventional PCR.

Table 3. Detection of Getah virus (GETV) in 20 field samples using real-time PCR and conventional PCR.

| | Conventional PCR | |
|---|---|---|
| | Positive | Negative |
| Real-time PCR | | |
| Positive | 3 | 7 |
| Negative | 0 | 10 |

## Discussion

The first mention of GETV dates back to 1955 in Malaysia. In our country, the virus was initially isolated from *Culex* in Hainan Province in 1964 (Li et al. 1992). After that, GETV has become widely prevalent in mosquitoes and livestock in various Chinese provinces (Zhai et al. 2008, Li et al. 2017, Yang et al. 2018a, Li et al. 2019, Liu et al. 2019, Shi et al. 2019, Ren et al. 2020, Xing et al. 2020). Notably, there has been a sharp increase in reported cases of GETV infections in pigs in recent years. Notably, the signs of GETV disease in pigs are highly similar to those of swine fever, pseudorabies, and porcine reproductive and respiratory syndrome. The differential diagnoses of these diseases by their clinical manifestations are difficult. Additionally, it can be challenging to diagnose GETV disease clinically in pigs owing to co-infection with two or more viruses. Therefore, developing a rapid, accurate, and reliable GETV detection approach is crucial.

After numerous genome sequence alignments of GETV strains from the GenBank database, a set of primers was constructed for the current investigation. It targets the conserved region of the *E1* gene. The reaction conditions were also optimized to create the TaqMan-based real-time PCR approach for GETV detection. This method was found to have the advantage of high specificity and good repeatability. Furthermore, the high sensitivity of the method was demonstrated, and the minimum detection limit for detecting GETV was found to be 5.94 copies/L, which was more than 10 times that of the TaqMan-based real-time PCR method developed by Shi et al. (2018). Field samples were further examined to detect GETV, which was compared with conventional PCR assays to further assess the use of the real-time PCR assay. Positivity was confirmed for all GETV-positive samples identified by conventional PCR using the real-time PCR assay. However, seven GETV-positive real-time PCR samples were not detected using traditional PCR. The fact that most of these samples had copy numbers fewer than 100 suggested that real-time PCR is more sensitive than traditional PCR for identifying field samples with lower copy numbers.

The prevalence of GETV, which has been documented in pigs, horses, foxes, cows, and humans in recent years, has increased in China (Li et al. 1992, Yang et al. 2018a, Liu et al. 2019, Lu et al. 2019, Shi et al. 2019). In the future, GETV could begin to cause a disease that endangers animal and human health. Therefore, it is essential to increase GETV epidemiological surveillance in animal populations using a quick and sensitive method. The GETV real-time PCR assay generated using a TaqMan platform, created in the current work, will be a good option.

In conclusion, we created a real-time PCR approach for GETV detection and quantification with great sensitivity, specificity, and repeatability. The assay performed better than expected when analyzing field samples of porcine serum. The availability of this assay will enable future research on the pathogenic diagnosis and epidemiology of GETV.

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