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Development and application of a novel TaqMan qPCR assay targeting the *gN* gene for genotyping pseudorabies virus

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Pseudorabies virus (PRV), known as porcine herpesvirus type I, belongs to the α-herpesvirus subfamily of the herpesviridae family. Its virions are oval or round, with a genome of approximately 143,000 bp and a high guanine-cytosine (GC) content of 73%. (Pomeranz et al., 2005). While swine are the natural host, and the virus has caused significant economic loss to the pig industry, PRV can infect other mammals, causing acute infectious diseases characterized by neurological symptoms (He et al., 2019; Cheng et al., 2020). Young pigs are more susceptible to PRV and the infection in piglets is nearly 100% fatal, whereas adult pigs exhibit reproductive disorders such as abortion, stillbirths, and reduced fertility, ultimately decreasing farm productivity (Zuckermann, 2000) . Due to its global impact, PRV is classified as a Class B infectious disease by the World Organization for Animal Health (WOAH).

Widespread use of the Bartha-K61 attenuated vaccine effectively curbed PRV outbreaks for years (Delva et al., 2020; Zheng et al., 2022). However, PRV outbreaks have re-emerged in China even in Bartha-K61-vaccinated herds since 2011 (Tan et al., 2021). The emerging strains, designated PRV genotype II, exhibit significantly higher virulence than the classic genotype I strains (Zhai et al., 2019). Existing vaccines for genotype I offer limited cross-protection against PRV genotype II (An et al., 2013); consequently, the development of a vaccine specifically targeting PRV genotype II represents a milestone for controlling the ongoing epidemic (Jin et al., 2022). An effective control strategy, however, requires an integrated approach that combines effective vaccination with rapid, highly sensitive diagnostic methods capable of differentiating between PRV genotypes.

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Common methods for detecting PRV include virus isolation and cultivation, Polymerase Chain Reaction (PCR), and immunological assays (Morenkov et al., 1997; Wu et al., 2023). Among these, PCR is widely preferred due to its rapidity, high sensitivity, and strong specificity (Tu et al., 2022). It enables high-throughput screening of a large panel of samples and is suitable for live detection, making it ideal for clinical diagnosis (Ding et al., 2024). Of the various PCR-based methods, the TaqMan assay offers superior specificity compared to SYBR Green, establishing it as a reliable technique for clinical applications. Current PRV genotyping predominantly targets envelope glycoprotein genes such as glycoprotein B (*gB*), glycoprotein C (*gC*), glycoprotein D (*gD*), and glycoprotein E (*gE*) (Song et al., 2017). However, whether other envelope glycoprotein genes can serve as effective genotyping markers remains unclear. An ideal genotyping target requires two key features: inter-genotype variability to enable clear differentiation, and strong intra-genotype conservation to ensure reliable detection. In this study, analysis of PRV genomic DNA led to the identification of the glycoprotein N (*gN*) gene and, for the first time, the discovery of conserved, genotype-specific signature

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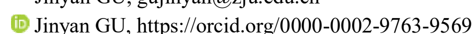
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sequences within it. We aim to utilize these sequences to develop a multiplex TaqMan Probe-based Real-time Quantitative PCR (TaqMan qPCR) assay for rapid and accurate genotyping of PRV.

Based on comparative analysis of complete PRV genotype I and II genome sequences using SnapGene, we identified several conserved regions within the *gN* gene (Figs. 1a and 1b). Specifically, positions 105-123 and 400-418, which are conserved across both genotypes, were selected as the upstream and downstream primers, respectively. The region between positions 195-211, also conserved in both genotypes, was chosen for the universal probe. For genotype-specific detection, positions 321-340 of the *gN* gene, highly conserved and unique to PRV genotype I, were selected as the PRV genotype I probe. Similarly, positions 325-340, which are conserved specifically in PRV genotype II, were selected for designing the PRV genotype II probe. The detailed positions and sequences are shown in Fig. 1a.

The *gN* gene sequences of PRV genotypes I (PRV-BarthaK61) and II (PRV-DX) were amplified with PCR using their respective genomic DNA as templates. The amplified products were then blunt-end ligated into the pUC-19 vector using the pClone007 kit, and then transformed into *E. coli* DH5α competent cells. The positive strains were screened and the plasmids extracted and purified, yielding the recombinant plasmids pUC-PRV-BarthaK61 (pUC-BT) and pUC-PRV-DX (pUC-DX).

Using serial dilutions (10^1 to 10^6 copies/μL) of the standard plasmids pUC-BT and pUC-DX as templates, we generated standard curves for PRV genotypes I and II by plotting the cycle threshold (Ct) values against the logarithms of the plasmid copy numbers (Figs. 1c and 1d). The standard curve of PRV genotype I was $Y=-3.5609X+41.056$, with a coefficient of determination (R^2) of 0.9997. For PRV genotype II, the standard curve was $Y=-3.7331X+43.677$, with $R^2=0.9998$. These results indicated a good linear relationship across the dilution range (10^1 to 10^6 copies/μL). The minimum detection limit of the established method was determined to be 2×10^1 copies/μL.

The analytical sensitivity of the established triplex-probe assay was compared to that of the single-probe format under identical reaction conditions. The TaqMan qPCR assay contained 10.0 μL of 2×AceQ qPCR Probe Master Mix, 2.0 μL of templates, 0.9 μL each of upstream and downstream primer (10 μM), 0.4 μL of each probe (10 μM); and ddH_2O was used to make up to 20.0 μL. The TaqMan-based PCR conditions were as follows: pre-denaturation at 95°C for 30s, and 40 amplification cycles of 95°C for 10s and 60°C for 30s. Both assays achieved a minimum detection limit of 2×10^1 copies/μL, demonstrating that the high sensitivity of the detection system was maintained despite increased probe multiplexing.

The specificity of the triplex qPCR assay was verified by its ability to detect PRV genotypes I and II exclusively in their respective channels. No cross-reactivity was observed with other swine pathogens such as Porcine deltacoronavirus (PDCoV), Classical swine fever virus (CSFV), Porcine reproductive and respiratory syndrome virus (PRRSV), Porcine epidemic diarrhea virus (PEDV), Porcine circovirus type 2 (PCV2) or plasmids containing full-length Porcine circovirus type 3 (PCV3) and Porcine circovirus type 4 (PCV4) genomes (Fig. 1e). These results demonstrated that the established method effectively differentiated PRV genotypes I and II with high specificity.

To evaluate repeatability, we tested tenfold serial dilutions of the standard plasmids (10^1 to 10^6 copies/μL) under the established conditions. Intra-assay variability was assessed by testing each dilution in triplicate within a single run, while inter-assay variability was determined by repeating the experiment on three separate days. As shown in Table S1, the intra-assay coefficients of variation (CVs) ranged from 0.94% to 1.62% for PRV genotype I, and from 1.80% to 2.74% for PRV genotype II. Inter-assay CVs for both genotypes were below 3% (Table S2). Thus, the established triplex qPCR assay exhibited high repeatability and stability for the detection and differentiation of PRV genotypes.

Genomic DNA was extracted from 54 clinical samples and subjected to detection using the established triplex TaqMan qPCR assay. PRV-DX and PRV-BarthaK61 were used as positive controls, and ddH_2O as the negative control. The assay identified 29 positive samples, yielding a positive rate of 53.70%; this was substantially higher than the 25.93% positive rate obtained using the conventional PCR method recommended by the national standard "Diagnostic Method for Pseudorabies" (GB/T18641-2018). Notably, all positive

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samples detected by the multiplex qPCR assay were identified as PRV genotype II. This genotyping result was confirmed by sequencing and phylogenetic analysis, which classified all positive samples as PRV genotype II. These findings demonstrated that, compared to conventional PCR, the established multiplex TaqMan qPCR assay not only exhibited superior sensitivity for detecting PRV-positive clinical samples, but also enabled simultaneous genotype identification, highlighting its practical value for clinical diagnosis and epidemiological surveillance. It should be noted that although virulence may vary among different PRV strains within the same genotype, sequence analysis of the *gN* gene revealed only two distinct sequence types corresponding to genotypes I and II. Nevertheless, further validation using a broader panel of clinically representative PRV strains is necessary to further substantiate the assay's generalizability and reliability for genotyping applications.

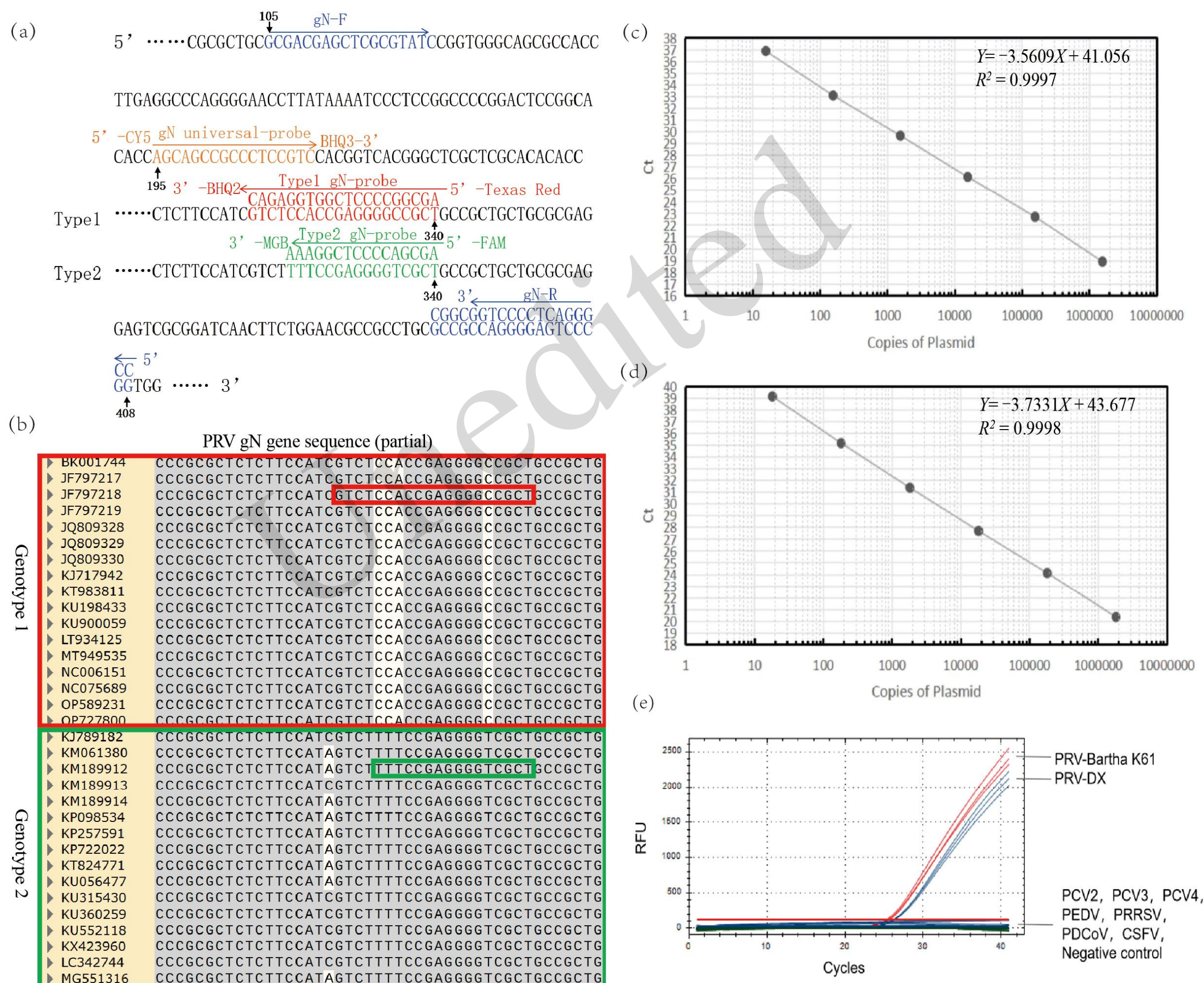

Fig. 1 Diagram of the primers or probes of the *gN* gene for genotyping of PRV; and standard curve or specificity of the TaqMan qPCR assay: (a) Primers and positions of the *gN* gene for genotyping of PRV; (b) Sequence alignment of *gN* genes of PRV genotypes I and II; (c-d) Standard curve of TaqMan qPCR assay of PRV genotypes I and II, respectively; (e) Specificity of the TaqMan qPCR assay. Porcine circovirus type 2 (PCV2), Porcine circovirus type 3 (PCV3), Porcine circovirus type 4 (PCV4), Porcine epidemic diarrhea virus (PEDV), Porcine reproductive and respiratory syndrome virus (PRRSV), Porcine deltacoronavirus (PDCoV), Classical swine fever virus (CSFV).

In this study, we developed a triplex TaqMan qPCR assay for the simultaneous detection and genotyping of PRV, offering high specificity, sensitivity, stability, and user-friendly operation. A key advantage of this method is its capacity to perform PRV identification and genotype discrimination in a single reaction, substantially improving detection efficiency. Notably, this detection assay represents the first application of the *gN* gene for PRV genotyping, which addresses limitations associated with conventional genetic targets. Furthermore, the use of a single pair of specific primers simplifies the reaction system, reduces primer consumption by two thirds, and effectively minimizes the risk of primer mismatches and non-specific amplification. Collectively, these features position the proposed method as a practical and effective tool for PRV genotyping in clinical settings. We expect that it will provide valuable support for PRV epidemiological surveillance and contribute to efforts aimed at eradicating persistently infected swine populations.

Data availability statement

The data used to support the findings of this study are included within the article and its supplementary materials. No additional datasets were generated or analysed during the current study.

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Author contributions

Yulan JIN, Xuan JI, and Weiren DONG performed the experimental research and data analysis, and wrote and edited the manuscript. Yanming HUANG contributed to the software Analysis. Jiyong Zhou and Jinyan Gu contributed to the study conceptualization, supervision, and funding acquisition, and reviewed and edited the manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Yulan JIN, Xuan JI, Weiren DONG, Yanming HUANG, Jiyong ZHOU, and Jinyan GU declared that they have no conflicts of interest.

The collection and use of clinical samples in this study were approved by the Experimental Animal Ethics Committee of Zhejiang University (No. ZJU20240676). All procedures followed were in accordance with the Guidelines for the Use of Experimental Animals of China.

Declaration on the use of generative AI tools

We declare that no generative AI tools were used in the creation of this manuscript. All content, analysis, and writing were performed solely by the authors.

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Supplementary information

Tables S1 and S2

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