Simultaneous Detection and Identification of Peste des petits ruminants Virus Types II and IV by MCA-Based Real-Time RT-qPCR Assay within Single Reaction

Jingyu Tang[1], Hanyu Du[1], Nannan Jia[1], Aoxing Tang[1], Chuanfeng Li[1], Chunchun Meng[1], and Guangqing Liu[1]

[1]Shanghai Veterinary Research Institute Chinese Academy of Agricultural Sciences

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Abstract

Peste des petits ruminants (PPR) disease is a cross-species infectious disease that severely affects small ruminants and causes great losses to livestock industries in various countries. Distinguishing vaccine-immunized animals from naturally infected animals is an important prerequisite for the eradication of PPR. [ ](javascript:void(0);)Although peste des petits ruminants virus (PPRV) was currently divided into lineages I-IV and only one vaccine strain N75 belongs to type II, its epidemic strains in China all belong to lineage IV. As to achieve this goal, we developed an SYBR Green I real-time RT-qPCR method for rapid detection and differentiation of PPRV lineage II and IV by analyzing different melting curve analyses. Primers targeting the L gene were highly specific, as evidenced by the negative amplification of closely related viruses, such as Orf virus, goat poxvirus, and Foot-and-mouth disease virus. The sensitivity of the assay was assessed based on plasmid DNA and the detection limit achieved was 10 copies of PPRV-II and PPRV-IV respectively. As the method has high sensitivity, specificity, and reproducibility, which will be significant in effectively controlling the occurrence and transmission of PPRV.

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Jingyu Tang#, Hanyu Du#, Nannan Jia, Aoxing Tang, Chuanfeng Li, Chunchun Meng*, Guangqing Liu*

Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai, 200241, PR China

# These authors contributed equally to this work

* Corresponding authors: Chunchun Meng, *mengcc@shvri.ac.cn* . and Guangqing Liu, *liugq@shvri.ac.cn*

Abstract

Peste des petits ruminants (PPR) disease is a cross-species infectious disease that severely affects small ruminants and causes great losses to livestock industries in various countries. Distinguishing vaccine-immunized animals from naturally infected animals is an important prerequisite for the eradication of PPR. Although peste des petits ruminants virus (PPRV) was currently divided into lineages I-IV and only one vaccine strain N75 belongs to type II, its epidemic strains in China all belong to lineage IV. As to achieve this goal, we developed an SYBR Green I real-time RT-qPCR method for rapid detection and differentiation of PPRV lineage II and IV by analyzing different melting curve analyses. Primers targeting the L gene were highly specific, as evidenced by the negative amplification of closely related viruses, such as Orf virus, goat poxvirus, and Foot-and-mouth disease virus. The sensitivity of the assay was assessed based on plasmid DNA and the detection limit achieved was 10 copies of PPRV-II and PPRV-IV respectively. As the method has high

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KEYWORDS

Peste des Petits Ruminants, real-time qRT-PCR, melting curve analyses, peste des petits ruminants virus types II and IV

Peste des Petits Ruminants (PPR) is a highly contagious, cross-species disease caused by the Peste des Petits Ruminants Virus (PPRV) (Abubakar et al. 2017; Njeumi et al. 2020). It can infect not only small ruminants such as goats and sheep, but also large ruminants such as camels, buffalo, and wild animals (Lembo et al. 2013; Zakian et al. 2016). According to 2018 data from the FAO, there are 2.5 billion small ruminants and over 68% of sheep and goats living in PPR-infected countries (Zhao et al. 2021). The widespread distribution and prevalence of PPR have an important impact on economic trade and biodiversity.

PPRV belongs to the *Morbillivirus* genus, *Paramyxoviridae*family (Gibbs et al. 1979). According to the sequences of the N gene and F gene, PPRV can be classified into four lineages, I, II, III, and IV (Balamurugan et al. 2010). Lineage I and II are predominantly endemic in West Africa (Muniraju et al. 2014b; Tounkara et al. 2018), while lineage III is mainly found in the Arab region, and has also been reported in East Africa (Muniraju et al. 2014a; Rume et al. 2019), Lineage IV is mainly prevalent in the Middle East and Asia, and has also been reported in Africa (Woma et al. 2016; Liu et al. 2018; Tounkara et al. 2018). PPR was first reported in the Tibet region in 2007 (Wang et al. 2009), then re-emerged in Xinjiang Uyghur Autonomous Region in 2013, and then spread to 23 provinces, autonomous regions, and municipalities of China (Bao et al. 2017; Li J. et al. 2017). China has adopted strict compulsory immunization and culling policies, which have effectively controlled the occurrence of the PPR. Currently, the live vaccine named the Nigeria 75/1 is the only vaccine widely used for the prevention of PPR in China, it can provide strong immune protection in goats and sheep, as it can proliferate continuously after injection. In clinical application, as an inability to distinguish vaccine group from PPRV antibody or antigen positive animals is the significant impediment to the eradication of PPR. So, for prevention and eliminating PPR, there is a great need for a simple assay that can detect and distinguish the PPRV vaccinated and infected group at the same time.

Since the Nigeria 75/1 strain PPRV belongs to lineage II, while the PPRV prevalent in China all belong to type IV (Bao et al. 2017), an SYBR Green I real-time RT-qPCR assay was developed by analyzing all the published PPRV sequences of type II and type IV. We use only one pair of primers that can detect all the type II and IV PPRV, and by analyzing different melting curve analyses (MCA), it can differentiate them conveniently and quickly. The parameters were optimized, and the sensitivity, specificity, and reproducibility of the assay were assessed and compared with the traditional methods. The diagnostic application of the assay was carefully evaluated based on the PPRV available sequences.

First, 71 published whole-genome sequences (9 form type II PPRV and 62 form type IV PPRV, including 37 were Chinese strains and 25 were foreign strains) were retrieved from the GenBank database. DNAstar software was used for comparison, and L gene was used to design primers to detect and differentiate type II and IV PPRV, based on the principle that GC content difference in the inner region of two typed PPRV gene sequences can produce different melting curves. The optimized forward primer F was 5'-ACAGGTTCGACAACATTCAAGCCA-3' and reverse primer R was 5'-GCGAAGGTAGGTCAGAGAGCA-3', and an expected 200 bp amplification product was generated. SYBR Green-I RT-qPCR assays were performed using Novozymes Ultra SYBR Mixture (Vazyme), according to the manufacturer's instructions.

Two linear standard curves were constructed using tenfold dilutions of type II and type IV PPRV standard plasmids, using a dilution concentration of 1.0×10^8-1.0×10^2copies/μL. All samples were in triplicate and tested independently. The equations for the type II and IV PPRV standard curves were y = -3.1734x + 31.044 and y = -3.1358x + 32.956, respectively. Standard curves analysis demonstrated that when the copy numbers were in the range of 10^8 and 10^2 copies/μL, the amplification efficiency of type II and IV PPRV

was 106% and 108%, respectively. The correlation coefficient R^2 value for the linear regression equation of type II and IV PPRV was 0.9981 and 0.9984 (Fig. 1A and 1B). In addition, the detection limits for type II and IV PPRV were 10 copies/μL. The melting curves for both type II and IV PPRV were single peaks and melting temperatures (Tm) were 83.39 and 81.92, respectively (Fig. 1C).

To verify the specificity of the assay, type II PPRV, Orf virus (ORFV), goat poxvirus (GTPV), and Foot-and-mouth disease virus (FMDV) genomic cDNA or DNA, and type IV PPRV plasmids were used as templates and amplified using the conditions described above. Only PPRV (type II and IV) was amplified in these samples and no specific peaks appeared in the other samples (Fig. 1D). This indicated that the established real-time RT-qPCR assay was specific for PPRV and does not cross-react with other pathogens.

Standard plasmids (type II and IV of PPRV) were diluted (10^7,10^5,10^3copies/μL) to assess intra- and inter-assay reproducibility. And the coefficients of variation (CV) of CT values were all less than 5%. Specifically, for type II PPRV plasmids, the CV of the inter- and intra- assays was ranged from 0.06% to 0.49% and 0.07% to 6.09%, and for type IV PPRV plasmids, the CVs were ranged from 0.06% to 0.30% and 1.03% to 6.42%, respectively. These results indicate that the real-time RT-qPCR assay had good reproducibility.

To evaluate the assay's potential performance in clinical setting, we calculate the melting temperature in the qRT-PCR target region of all the PPRV lineage II and IV strains(9 lineage II strains, 23 lineage IV strains isolated from foreign countries, and 37 lineage IV strains from Chin) (Table 1). The average, median and standard deviation melting temperature of lineage II (IV) amplified area is 82.34 (80.97), 82.30 (81) and 0.19 (0.14), respectively. Furthermore, there was no significant difference between lineage IV strains isolated from China and foreign countries. The DNA denaturation temperature diversity between the two lineages is about 1.3 which can be easily reflected after MCA, it indicates this assay can efficiently detect and differentiate N75 and PPRV lineage IV isolates when used in the future.

The stability of the DNA duplexes can be affected by the GC content and length of the PCR products. Thus, the fluorescence signal of the SYBR Green I was different and could be used to specifically identify PCR products by MCA. It is very similar to high-resolution melting (HRM) but more convenient(Hou and You 2018).

At present, there are many detection methods for PPRV, which are mainly designed to detect all PPRV strains and can not distinguish between field PPRV strains and vaccine strains (Polci et al. 2015; Mahapatra et al. 2019; Lucas et al. 2020). The real-time RT-PCR method designed by Li et al. (Li L. et al. 2016) could specifically detect lineage IV PPRV, while the other three lineages could not be detected which limits of detection was found to be 10 copies/reaction. Yang established two rapid detection methods for PPRV, real-time and lateral flow strip RT-RPA. The sensitivity of both was as low as 100 copies/reaction at 40 and as low as 150 copies/reaction at 39 , respectively (Yang et al. 2017). All these methods can detect PPRV rapidly and effectively, but cannot distinguish between the PPRV vaccine strain and the prevalent strain in China. In this study, we describe for the first time that only uses one pair of primers to distinguish lineage II and IV of PPRV based on the MCA and without reducing sensitivity. The sensitivity of the method is 10 copies/reaction. Furthermore, it does not have cross-react with other viruses such as ORFV, GTPV, and FMDV.

In conclusion, in this study, we developed an MCA-based SYBR Green I RT-PCR assay to effectively detect and differentiate lineage II and IV of PPRV. The detection method is accurate, rapid, sensitive, reproducible, and easy to perform. Therefore, this detection method can be used for subsequent epidemiological investigations and PPR eradication.

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Conflict of interest The authors report no declarations of interest.

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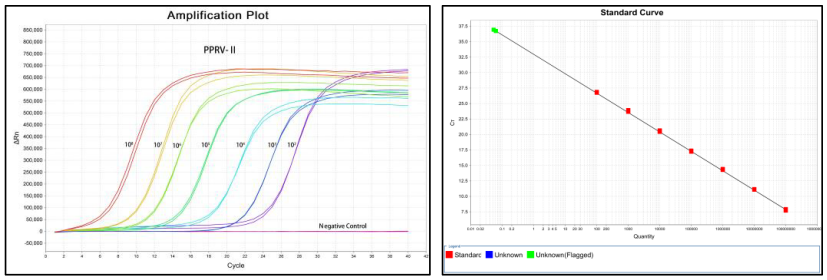
FIGURE LEGENDS

Fig. 1 Simultaneous detection and differentiation of type II and IV PPRV through melting curve analysis by one pair of primer.**A, B** Standard curves of real-time PCR for type II and IV PPRV. The tenfold diluted standard plasmid of type II and IV PPRV tested with the same pair of primer. Regression lines between the Ct values and the input concentrations of type II PPRV (A) and type IV PPRV (B) plasmid DNA using SYBR Green I real-time PCR. **C** Melting curve analysis of type II and IV PPRV. Amplification melting curve and melting temperature values (Tm) after SYBR Green I real-time PCR followed by melting curve analysis

of type II and IV PPRV. **D** Specificity of the real-time PCR assay. The specific fluorescent signals were detected from cDNA of type II PPRV and type IV PPRV plasmids, and the dissociation curves showed that there were two specific product peaks for type II and IV PPRV, respectively, but no specific amplification for negative control (ORFV, GTPV, and FMDV).

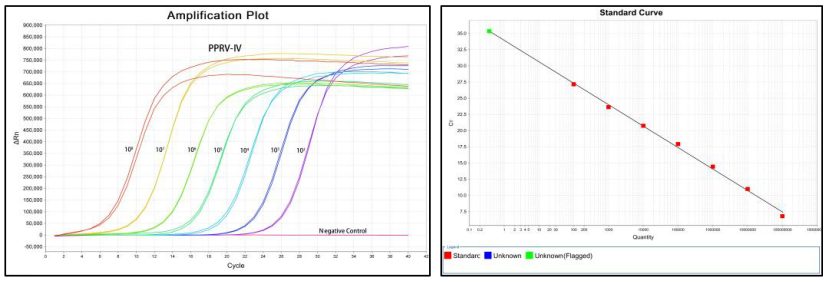
Table 1 Calculation the melting temperature in qRT-PCR amplified region of all the PPRV lineage II and IV strains

1A



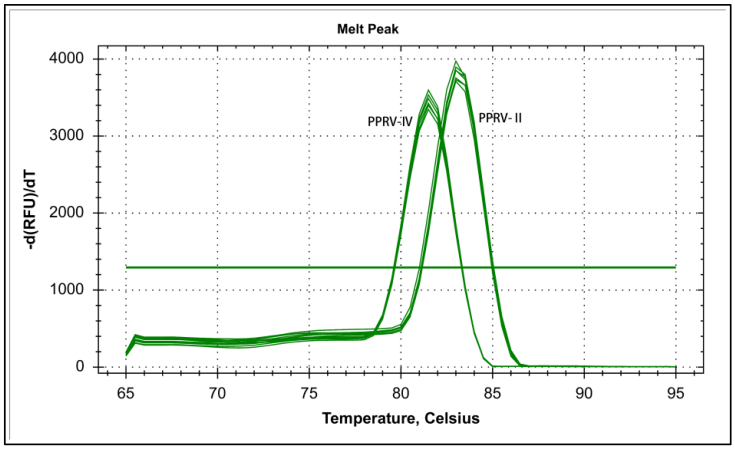
y = -3.1734x + 31.044 R^2 =0.9981 E=106%

1B



y = -3.1358x + 32.956 R^2 =0.9984 E=108%

1C



1D

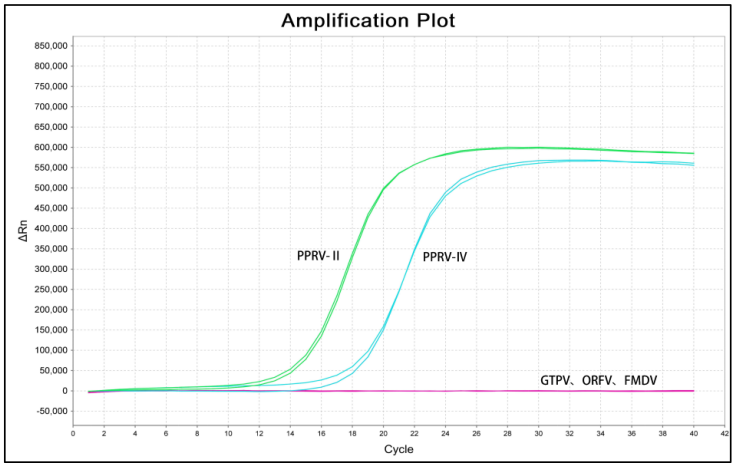


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Table 1.docx available at https://authorea.com/users/740249/articles/713293-simultaneous-detection-and-identification-of-peste-des-petits-ruminants-virus-types-%E2%85%B1-and-%E2%85%B3-by-mca-based-real-time-rt-qpcr-assay-within-single-reaction