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Genome Diversity and Molecular Detection of PRRS Field Strains and Vaccine Strains, and PCV3 and PCV2 Strains

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Summary

Molecular diagnosis of porcine reproductive and respiratory syndrome virus (PRRS) and porcine circo virus (PCV) are challenging due to high genetic diversity in the viral genomes. Differentiating PRRS vaccine strains is even more challenging and is currently done by DNA sequencing, which is expensive and time-consuming. A multiplexed system (Luminex) allowing multiple detection targets in the same reaction is available. However, this system is not fully developed for common swine pathogens. Therefore, an assay was built to detect the majority of field PRRS strains by using different pairs of primers, and at the same time, to provide differentiation of the four PRRS vaccine strains used in the US by using vaccine-specific primers. Two sets of detection primer pairs were used that detect 85.4% and 91.2% of the 694 full genomes of the current PRRS collection in the GenBank. The combination of the 2 primer pairs will detect 98.1% of the genomes in the GenBank. Testing a limited number of field strains and the four vaccine strains (PrimePac, Ingelvac MLV, Ingelvac ATP, and Fostera) available in the US indicated that the assay detected all strains and identified each of the four vaccine strains correctly.

Recently, clinical signs of porcine dermatitis and nephropathy syndrome, reproductive failure and multisystemic inflammation have been associated with PCV3. A real-time PCR assay is developed based on 67 PCV3 full-genome sequences with 100% detection rate. Also, 1,907 available PCV2 genomes were analyzed. Based on this analysis, 2 primer pairs were designed to detect an estimated 94.8% and 90.5% field strains, respectively, with a combined detection rate of 99%. The PCV3 and PCV2 assays were then combined into one reaction with an internal control to monitor the DNA extraction efficiencies. The combined multiplex assay detected all PCV3 and 99% of PCV2 strains with no cross-detection observed.

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Introduction

Real-time polymerase chain reaction (PCR) is the most-used platform for molecular diagnostics for animal and zoonotic pathogens. Simplicity of operation, fast turnaround time, and exponential amplification features have made PCR-based diagnostic applications widely applied. Most real-time PCR systems though, are limited to 5 channels of detections, and practically only 3 to 4 channels are used in multiplexed assays. In addition, viral genomes such as PRRS and PCV2 are constantly changing. It has been challenging to keep the PCR assays current with the increasingly divergent viral genomes. The fluorescent bead-based Luminex assays allow the use of 100 or more primer pairs in one reaction, providing the opportunity to build highly multiplexed assays for multiple pathogen detection with a single assay.

The most practical way to design a PCR assay to detect most field strains is to perform a thorough analysis on all genomic information that is available at the time of design. Then update the assay as needed based on viral genome changes over time. In this study, a multiplex Luminex assay was developed to detect and differentiate field PRRS strains and the 4 PRRS vaccine strains used in the US (Prime Pac, Ingelvac MLV, Ingelvac ATP, and Fostera). In addition, primers were included to detect PCV2 and PCV3 strains.

Procedures

There were 694 PRRS full genome sequences were available from the NCBI GenBank (https://www.ncbi.nlm.nih.gov/) at the time of design. The 694 sequences were downloaded and aligned in CLC Genomic Workbench (https://www.qiagenbioinformatics.com/products/clc-genomics-workbench). The aligned sequences were used to identify conserved regions for the detection primer design and used to identify regions specific to each vaccine strain for vaccine differentiation designs. Two pairs of detection primers were identified that could detect 85.4% and 91.2% of the 694 full genomes that when combined would detect 98.1%. Also, a pair of primers for each vaccine strain were identified. Magnetic beads with capture sequences that matched our selected primers were purchased from Luminex (https://www.luminexcorp.com/). Each capture sequence on the beads is synthesized for each primer pair. Also, the reverse primer for each designed primer pair is synthesized with a biotin attached to generate a signal for detection. The primers were then pooled and used for amplification. The beads were washed to remove unused primers, and amplified PCR products were purified and hybridized to the magnetic beads. Hybridized beads were run on a BioRad Bio-Plex 200 system to generate detection results.

The 35 PCV3 full-genome sequences that were available in the GenBank and the 32 PCV3 full genomes sequenced at the K-State lab[1] were aligned together with selected PCV2 genomes in CLC Genomic Workbench. The PCV3-unique regions that are conserved within PCV3, but have low homology to PCV2 were used for primer and probe designs. Currently 1,907 full genomes of PCV2 sequences are available in the GenBank. Due to the high diversity of the genomes, an assay designed from ORF1 that can detect 94.8% of the strains, and another assay designed in ORF3 that can cover 90.5% of the strains, were used. When used together, the two designs can detect 99% of the 1,907 sequences. A swine housekeeping gene that is always present in the pig genomes, SB2M,

was also used as an internal control to monitor DNA extraction efficiencies. The PCV2, PCV3, and SB2M assays were then multiplexed into a single reaction and tested with viral isolates and field samples.

Results and Discussion

The two pairs of detection primers collectively detected all four PRRS vaccine strains, and the four PRRS positive diagnostic samples. They did not detect either of the four PRRS negative diagnostic samples or the negative control (Figure 1).

The PCV3/PCV2/SB2M triplex real-time PCR was analyzed under multiplexed conditions. The PCR amplification efficiency for PCV3 is 95.5%, and for PCV2 is 91.6%, which are within the general guidelines of 90-110%. The correlation coefficient for both PCV3 and PCV2 were greater than 0.99, which also meet the general criteria. Testing on 717 diagnostic samples indicated that the PCV3 was detected in 156 (21.8%) of the samples. Sequencing of 32 full genomes indicated that the PCV3 strains in the US have undergone changes in the genomes, yet at a slow pace. The current PCV3 diversity level is 2.8% with a minimal homology of 97.2%. Figure 2 shows the phylogenetic relationship among these strains. From 125 diagnostics samples, 18 were positive to PCV2 reflecting a 14.4% prevalence.

Based on these results the PRRS and PCV assays can be combined. An assay can detect many more targets than PCR-based technology in one assay. One limitation though, is that the assay is not able to quantify the viruses, and only a presence or absence of pathogen is reported. Also, as these pathogens evolve, or new vaccines are available, more primer pairs can be added to update the system. Additionally, with adequate genetic information, detection of other pathogens such as swine influenza virus can be added to the system.

Type	Detection1	Detection2	MLV	ATP	Prime Pac
NTC	371	594	719	359.25	808.5
MLV	7685.5	13041	15112.5	350	652
Fostera	7240.5	15609	594.5	612.5	835
ATP	2022.5	13542	506	13151	461
Prime Pac	225	7622	290.5	205.5	5953
Positive1	10257.5	4296.5	765	682	1966.5
Positive2	376.5	8149	8811	327	3232.5
Positive3	5773	11306	289	637	524
Positive4	7117	12501	538	886.5	1004
Negative1	454	1213.5	745	425	1859
Negative2	372.5	862	778	413.5	877.5
Negative3	570	1127	1101	635	1256
Negative4	399	1031.5	795	438	988.5

Figure 1. Luminex assay result for detection of PRRS field and vaccine strains.
* NTC: No template control.

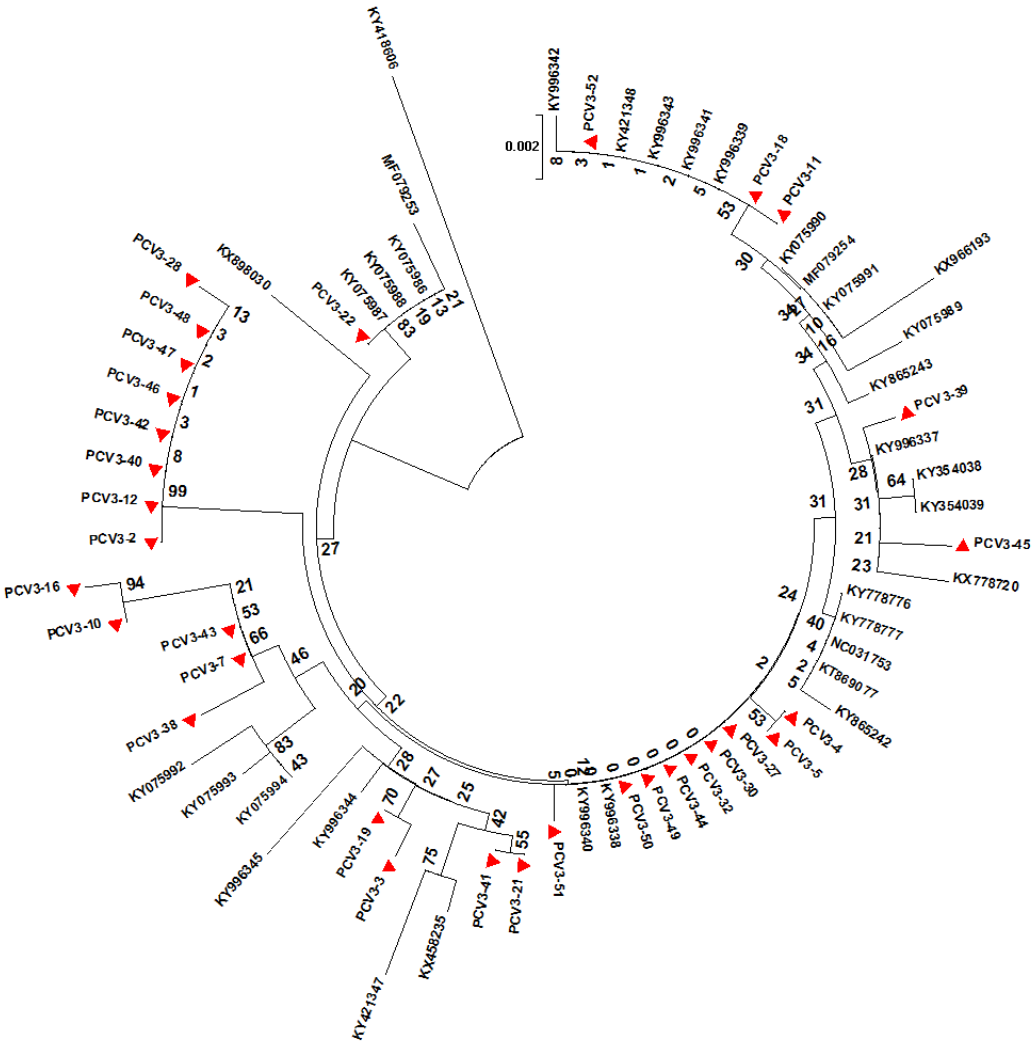

Figure 2. Phylogenetic tree generated with 67 (35 were from the NCBI GenBank; 32 labeled with red triangle were sequenced by our lab) PCV3 full genomes indicating limited genetic diversity in current strains.