

A single-tube triplex real-time PCR assay for differential detection of highly virulent Chinese strains of pseudorabies virus

Running title: Triplex real-time PCR for detection of Chinese strains of pseudorabies virus

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Summary (words 292. Limit 300)

Pseudorabies virus (PRV) causes Aujeszky's disease or pseudorabies (PR), fatal encephalitis in newborn piglets, respiratory infection in growing and fattening pigs, and reproductive failures in pregnant sows. It establishes a lifelong latent infection in the peripheral nervous system followed by subsequent intermittent shedding of infectious virus. Since 2011, highly virulent PRV strains that are genetically different from the classic PRV strains surfaced in pig herds in China. Availability of a highly sensitive and specific polymerase chain reaction (PCR)-based diagnostic assay for rapid differential detection of PRV variants is critical to prevent huge economic losses to the U.S. and Canadian pork industries if these strains enter North America and cause an outbreak. Here we describe the development and evaluation of a single-tube triplex real-time-PCR assay for differential detection of variant strains of PRV. The assay targets the intergenic region between the US2 and US6 genes in the PRV genome and is highly sensitive and specific and it did not detect other non-target viruses including related herpesviruses. The clinical specificity and sensitivity of the assay was evaluated using whole blood, serum, tissue and swab samples collected from known negative and experimentally inoculated pigs with either classical (Bristol) or variant (JS-2012 and HeN1) PRV strains. The targeted genomic region of this assay is also deleted in commonly used PRV gE-deleted marker vaccines, and therefore, the triplex assay did not detect viral DNA extracted from two commercial vaccine strains Bartha K-61 and Bucharest. This single-tube triplex assay can be used for routine diagnostics and epidemiological studies for detection and differentiation of classical strains from variant strains of PRV, and as a differentiation of infected and vaccinated animals (DIVA) assay when PRV gE- deletion mutant marker vaccines are used.

Keywords:

Pseudorabies Virus, Variant, highly pathogenic, Triplex PCR, detection, differentiation, DIVA

Introduction:

Pseudorabies virus (PRV), or suid herpesvirus-1, is a double stranded DNA virus of the genus *Varicellovirus*, subfamily *Alphaherpesvirinae*, and family *Herpesviridae* (Lee and Wilson, 1979; Mettenleiter, 2000; Pomeranz et al., 2005). It causes pseudorabies (PR) or Aujeszky's disease in livestock and wild animals including ruminants, carnivores and rodents. However, pigs are the primary host and reservoir for this virus. PRV causes fatal encephalitis in newborn piglets, respiratory signs in growing and fattening pigs, and reproductive failures in pregnant sows. Like other herpesviruses, PRV establishes a lifelong latent infection in the peripheral nervous system, which increases the difficulty in eradicating this virus (Gu et al., 2015).

Pseudorabies has spread throughout the world, but Canada, Greenland, and Australia are considered free of this disease. PRV emerged as a significant pathogen in the USA in 1960s as a result of emergence of virulent strains or the increase in confinement swine housing. In 2004, PRV was eradicated from the U.S. commercial swine herds, but the virus remains in some localized feral swine populations (United States Department of Agriculture, 2008). The presence of PRV in U.S. feral swine poses an on-going threat to the North American swine industry (Hahn et al., 2010; Gaskamp et al., 2016). Pseudorabies is a reportable disease in the U.S. and Canada.

China is considered the largest pork producer in the world. The earliest documented PRV outbreak in China was in 1947. Since 1990s, more than 80% of pigs in China have been vaccinated with Bartha-K61 vaccine and PR was well controlled. In late 2011, a newly emerged PRV variant with clinical manifestations of high fever, depression, anorexia, cough, shivering, diarrhea, and systemic neurological symptoms with high mortality surfaced in Bartha-K61 vaccinated pig herds in Northern China (An et al., 2013; Hu et al., 2015a, Hu et al., 2015b; Tong et al., 2015; Yang et al., 2016; Yu et al., 2014). Since then, this virus has spread across China

causing severe economic losses. These new PRV variants when inoculated into naïve pigs show earlier onset of clinical signs, severe pathological lesions in the brains (subarachnoid hemorrhages, cerebral edema), lungs (pulmonary congestion and edema) and higher mortality compared to other PRV strains. Partial and whole genome analyses of these viruses show many amino acid mutations and insertions in functionally important structural (gE, gC, UL36 etc.) and non-structural (US1) virial proteins (Lee and Wilson, 1979; Luo et al., 2014; Xiang et al., 2016; Yoon et al., 2005). Based on phylogeny, these newly identified highly virulent PRV variants from China have been assigned to Genotype II whereas PRV strains from other countries are assigned to the Genotype I or classical strains (Gu et al., 2015).

The gold standard of PR laboratory diagnosis is virus isolation. However, it is labor intensive and time consuming. Alternatively, polymerase chain reaction (PCR) assays (conventional and real-time) can rapidly detect PRV DNA in nasal and oropharyngeal swabs and organ samples from infected animals. The PCR-based assays are highly sensitive, and they can even detect viral DNA in latently infected animals from which virus can be difficult to isolate.

Conventional PCR assays require electrophoresis of the PCR products at the end of the amplification, and, therefore, are time consuming, labor-intensive, and cannot be automated. In contrast, real-time PCR (RT-PCR) assays allow detection of PCR amplification during early phases of amplification and are quantifiable and can be automated. A number of RT-PCR assays for detection of classical PRV strains (pan-PRV assays) have been developed. The majority of these are TaqMan probe-based assays (Ma et al., 2008; van Rijn et al., 2004; Wernike et al., 2014; Wu et al., 2014; Zhang et al., 2015; Zhao et al., 2008), while a few were intercalating dye-based (SYBR® Green/EvaGreen®) assays (Perez et al., 2012; Rao et al., 2014). The probe-based assays are more specific and therefore preferred over dye-based assays. A TaqMan probe-assay has been validated by the OIE for use in surveillance and routine diagnosis by local veterinary laboratories. This assay (ADIAVET®) PRV REALTIME from Adiaçène, Saint-Brieuc, France) targets the PRV gD glycoprotein-encoding gene and is commercially available as a duplex assay containing an

exogenous positive control (Pol et al., 2013). The United States Department of Agriculture's (USDA) Agricultural Research Service has also developed a set of real-time PCR assays, which can be multiplexed, that can differentiate wildtype classical PRV strains from commercial gE-deleted modified live virus marker vaccines (Zanella et al., 2012). None of these assays, however, can differentiate pigs infected with highly pathogenic PRV strains (Genotype II) from those infected with classical (Genotype I) viruses.

Highly pathogenic variant PRV strains, if they enter North America, could cause huge economic losses to the U.S. and Canadian pork industries. Availability of a rapid, highly-sensitive, PCR-based diagnostic assay for simultaneous detection and differentiation of PRV variants could be critical to prevent such losses. The currently available vaccines in North America for PRV are based on classical PRV strains, and they may not provide complete protection against highly virulent Chinese strains (Yu et al., 2014, An et al., 2013, Wang et al., 2014, Wang et al., 2018) As a result it is critical to rapidly detect and differentiate the highly pathogenic Chinese strains if they spread to North America.

Here we describe development and evaluation of a novel single-tube triplex real-time RT-PCR assay with a built-in internal control for rapid and simultaneous detection and differentiation of highly pathogenic Genotype II PRV strains from Genotype I strains.

Materials & Methods:

Viruses:

Highly pathogenic PRV JS-2012 variant strain (Tong et al., 2015) was obtained from Shanghai Veterinary Research Institute, Shanghai, China. The classical PRV strain Bristol (Classical strain), Shope, Becker, and Bartha strains were available at the National Centre for Foreign Animal Diseases (NCFAD)-Winnipeg. All these viruses were propagated and titrated in PK-15 cells. The PRV modified live vaccine AUSKIPRA - GN (PRV Bartha-K61 strain) was purchased from Laboratorios HIPRA S.A in Spain and PR-VAC PLUS (PRV Bucharest strain) was kindly provided

by the Zoetis Inc. in Lincoln, NE, USA. PRV genomic DNA was extracted from culture supernatant from tissue culture amplified virus, clinical samples, or from vaccines reconstituted in PBS. For specificity testing, nucleic acid extracted from cell culture amplified African and classical swine fever viruses, foot and mouth disease virus, and a number of herpesviruses (bovine, elk, caprine, Ruminant, and cervid), and nucleic acid extracted from clinical samples containing porcine respiratory and reproductive syndrome virus, porcine epidemic diarrhea virus and porcine circovirus 2 were used. PRV pUC57 plasmid was purchased from Genscript with concentration of 545 ng/μL and total length of 688 base pairs.

Primers and Probes:

Primers and probes to detect and differentiate classical and variant PRV strains were designed using Geneious v10.1.1 (Biomatters Inc. Newark, NJ, USA) bioinformatics software sequence analysis tool. To identify the target regions for the primers and probes an alignment of 34 PRV sequences was compiled, consisting of 12 full-length and 6 partial classical strains, as well as 13 full-length and 3 partial strains of variant PRV. The sequence data for these viruses were downloaded from the NIAID Virus Pathogen Resource (ViPR - <http://www.viprbrc.org>) and GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>). The six partial sequences were included as they were used in the design of the PRV Triplex assay recently reported by Meng et al., 2016. Once the sequences were aligned, genomic regions showing multiple mutations or insertions between PRV classical and variant strains were identified. Of those targeted regions, the untranslated region between the US9 gene and the US2 gene was selected (from 127,349bp to 127,485bp nucleotides in JS-2012 PRV strain) as the preferred region for primer/probe design. Within this region, a 21-nucleotide insertion was identified in all variant strain sequences available, and it was used as the target for the PRV US9/US2 UTR variant probe. Downstream of the insertion, we identified a region that shows 13 nucleotide polymorphisms between classical and variant PRV strains. The PRV US9/US2 UTR classical probe was designed to capture 10 out of the

13 polymorphisms observed in this region. It was also observed that the targeted region is deleted in Bartha-K61 and Bucharest strains that are commonly used in commercial PRV glycoprotein E (gE)-deleted marker vaccines (Figure 1).

Two primers, PRV US9/US2 UTR RRT Forward (5'-ACACAGCAGCCTTCCT-3') and PRV US9/US2 UTR RRT Reverse (5'-GCGTGACCACGGTGA-3') were designed to amplify a 137bp long PCR product from variant PRV strains and a 116bp PCR product from Classical strains. In order to differentiate classical and variant PRV strains, two strain specific probes PRV US9/US2 UTR classical and PRV US9/US2 UTR variant Probes were designed (Table 1). As the internal control, primers and a probe specific for β -Actin (β -Actin 1036R 831FP, β -Actin 1036RP and β -Actin-probe-880) were used (Moniwa et al., 2007). For detection of all PRV strains, primers and probes from a widely used, highly sensitive and specific PRV gB real-time PCR assay were used (Ma et al., 2008). To facilitate triplex assay development, determining specificity, and to generate positive controls for the assay, synthetic gene fragments (gBlocks) resembling the target regions of PRV classical and variant virus genomes and the β -actin gene were synthesized and procured from Integrated DNA Technologies, Inc. (Coralville, Iowa) (Table 1).

Animal Experiments:

To generate clinical samples for validation of the triplex assay, a total of 19 Landrace x large white crossbred pigs (11 piglets at 3 weeks of age and 8 weaner pigs at 7 weeks of age) were purchased from a commercial swine farm in Southern Manitoba and transported to the National Centre for Foreign Animal Disease (NCFAD) animal facility. After 7 days of acclimatization, pigs were randomly assigned to 3 groups: Group 1 (eight 7-weeks old pigs for PRV-JS-2012, Pig# 5-12), Group 2 (eight 3 weeks-old piglets for PRV-JS-2012, pig # 13-20) and Group 3 (three 3 weeks old piglets for PRV-Bristol, Pig # 24-26). Each group was housed in an individual pen. Each pig in Group 1 and 2 received 2 ml of PRV Strain JS-2012 (10^5 TCID₅₀/ml, 1 ml in each nostril) and each pig in Group 3 received 2 ml of PRV Bristol strain (10^5 TCID₅₀/ml, 1 ml in each nostril). After the challenge, all animals were monitored twice daily for clinical signs (fever, neurological, respiratory

etc.), and oral fluid, nasal and oropharyngeal swabs were collected every other day starting at 2 days post infection (dpi) until 14 dpi. Pigs that reached humane endpoint were euthanized and tissue samples (tonsils, spleen, lung, brain, trigeminal ganglia, kidney, and lymph nodes) were collected. The remaining animals were euthanized at 14 days post infection and similar samples as above were collected. All the samples were stored at -80°C until tested. The animal experiment was approved by the Animal Care Committee at the Canadian Science Centre for Human and Animal Health. The guidelines from the Canadian Council for Animal Care were observed during all procedures with animals.

Nucleic Acid extraction:

Total nucleic acid was extracted from 55 µl of each sample (cell culture supernatant, nasal and oral swabs, serum, whole blood or 10% tissue suspensions) using 5X MagMAX™ Pathogen RNA/DNA Kit on a MagMAX™ Express-96 Deep well magnetic particle processor (Applied Biosystems™) following manufacturer's protocol. The nucleic acid was eluted in 50 µl of elution buffer. The MagMAX-96 magnetic particle processor was used to extract samples per appropriate script for the sample volume tested.

PRV triplex RT-PCR assay development and optimization:

Three separate RT-PCR assays (classical PRV RT-PCR, Variant PRV RT-PCR and β-Actin RT-PCR) were first developed and later combined into a triplex assay. The triplex assay was optimized on the Applied Biosystems 7500 Real-Time PCR System using detection channels for FAM (absorbance at 495nm, emission at 520nm), HEX (absorbance at 535nm, emission at 556nm), and Q670 (absorbance at 647-670 nm). The optimized PCR reaction (20 µl) contains 5.5 µL sterile PCR grade water, 5 µL 4X TaqMan FAST Virus 1-Step Master Mix, 1 µL of PRV forward and 1 µL PRV reverse primers (0.5 µM final), 0.5 µL PRV variant probe (0.25 µM final), 0.5 µL PRV classical probe (0.25 µM final), 2 µL of β-Actin forward and 2 µL of β-Actin reverse primers (1 µM final), 0.5 µL of β-Actin probe (0.2 µM final) and 2 µL extracted nucleic acid from the sample. The optimized triplex assay conditions are: 50°C for 5 minutes, 95°C for 20 seconds, and 45 cycles

of 95°C for 3 seconds and 60°C for 30 seconds. All primers and probe working stocks were maintained at 10 µM concentration. The primers and probe for the gB RT-PCR assay was adapted from Ma et al, (2008) and the forward and reverse primers were used at 0.4 µl per reaction (0.4 µM final) with the probe at 0.4 µl per reaction (0.2 µM final) with the stock solutions being 20 µM for primers and 10 µM for the probe. The same PCR cycling conditions were used for the Triplex and the gB assays.

Analytical specificity, sensitivity and variability testing:

Analytical specificity of the assay was determined using DNA extracted from cell culture amplified PRV strains Bartha, Becker, Shope, Bristol C61, and JS-2012, common swine viral pathogens encountered in modern swine herds, and bovine herpesvirus-1 (BHV-1), a related herpesvirus. For evaluating the intra-assay variability, 20 replicates of a single sample were tested in a single run. The inter-assay variability was calculated by running the same assay three times over 3 days. The analytical sensitivity of the triplex assay was determined using known concentration of PRV plasmid with neat copy number of 7.34×10^{11} . The limit of detection is interpreted as the lowest copy number that can be detected by the assay.

Virus Isolation

Virus isolation was performed by inoculation of PK-15 cells (25 µL tissue suspension per 500,000 cells well) in 96-well tissue culture plates. The growth medium contained α -MEM liquid medium supplemented with 2 mM glutamax, 5mg/mL gentamicin and 2% γ -irradiated Fetal Bovine Serum (FBS). Briefly 10% (W/V) homogenates were prepared from each tissue, and 25 µl of each suspension was added to the PK-15 cells grown in 96-well plates. A positive control plate was inoculated with a PRV variant strain (JS-2012) isolate of known titer. The plates were incubated for 48 hours in a 5% CO₂ humid air incubator at 37°C and were checked daily for cytopathic effect (CPE) and possible contamination using a light microscope. After 2-days of incubation, the cells were fixed using 200 µl of fixation fluid (Acetone, PBS and Bovine serum albumin fraction V) per

well, and stained using a PRV hyperimmune porcine serum. Briefly, the plates were rehydrated for 30 minutes (using 150 µl of PBS at room temperature), incubated with PRV antibody positive polyclonal serum (diluted 1:100 in PBS-0.1%Tween 20) for 30 minutes at 37°C, followed by a 30-minute incubation with rabbit Anti-Swine IgG HRP conjugate at 37°C and finally for 15 minutes at 37°C with t3-Amino-9-ethylcarbazole (AEC) substrate solution. After the substrate incubation, the plates were washed, and positive staining recorded under a light microscope. PRV JS-2012 and Bristol strains with known titers were used as positive controls.

Inter-laboratory comparison and additional characterization:

The optimized PRV triplex assay along with the gB assay were transferred to Kansas State University Veterinary Diagnostic Laboratory (KSU-VDL) for inter-laboratory comparison and a negative cohort study using negative clinical samples collected from U.S. swine herds. A few known-negative clinical samples were spiked with synthetic PRV specific DNA fragments (gBlocks) and tested on the triplex assay to mimic positive clinical situations.

For the negative cohort study, KSU-VDL used 400 known PRV-negative clinical samples collected from healthy pigs in the U.S. As positive controls, 22 clinical samples (serum, oral fluids, and fecal swabs) were spiked with PRV classical or PRV variant gBlocks. The assay was also evaluated at the U.S. Department of Agriculture's National Veterinary Services Laboratories (NVSL) in Ames, Iowa, on its ability to detect a second PRV variant strain, HeN1 (An et al., 2013) using archived vaccine challenge study samples (58 nasal swabs and 58 oral swabs) which were collected from seven pigs vaccinated with a USDA licensed, commercially available PRV DIVA vaccine and five unvaccinated pigs, all challenged with the PRV HeN1 (NVSL, unpublished data).

Results:

Development and optimization of a single-tube, triplex real-time PCR assay.

A triplex assay that can differentiate highly virulent PRV variant strains from classical strains was successfully designed targeting the US2- US9 intergenic region. This region is deleted in Bartha-

K61 and Bucharest PRV strains which are commonly used in commercial PRV marker vaccines, and therefore the assay can be used to differentiate animals vaccinated with these vaccines from those infected with wild-type PRV strains (DIVA assay). For the assay development, genomic DNA extracted from PK-15 cells infected with PRV classical strain Bristol, PRV Variant strain JS-2012, and PRV gE- deleted DIVA vaccine strain Bartha-K61 were used. Prior to use for assay characterization, the sequence identity of these three virus strains was confirmed by PCR amplification of the region flanking the gE deleted region of the Bartha-K61 strain using forward primer 5'-GTACCGGCGTCGATGATGAT-3'" and reverse primer 5'-GCCCAGGATCCACAGGTG-3', followed by Sanger sequencing.

The triplex assay conditions were optimized using the synthetic gene fragments (gBlocks) resembling the target regions of PRV classical and variant virus genomes and the β -actin gene (data not shown) according to the cycling conditions recommended for the TaqMan FAST Virus 1-Step Master Mix kit. The optimized triplex assay was able to accurately detect and differentiate the PRV classical strains Bristol, Shope and Becker and the variant strain JS-2012. The triplex assay did not detect any of the closely related herpesviruses or other high consequence swine viral pathogens tested. As expected, the triplex assay was unable to detect the gE-deleted PRV Bartha-K61 Vaccine strain and the PRV Bucharest vaccine strain. The gB assay, in contrast, detected all tested PRV strains, confirming the presence of PRV viral DNA in these samples (Table 3).

Once the triplex assay was optimized, the standard curves of the assay were generated and PCR efficiency was determined by plotting Ct value against log copy number from ten-fold serial dilution of PRV plasmid with neat copy number of 7.34×10^{11} for both classical and variant strains of PRV. The assay values with Ct values >40 were not plotted on the graph. The regression line equation for PRV Variant (FAM channel) was $y = -3.3563x + 39.416$ with a correlation coefficient (R^2) of 0.9941. The regression line equation for PRV Classical (HEX channel) was $y = -3.5375x + 41.126$ with a correlation coefficient (R^2) of 0.9907. The PCR efficiencies were determined to be 98.59% and 91.73% for Variant and Classical strain targets respectively.

To determine the limit of detection of the assay, PRV plasmid was diluted ten-fold to extinction. The triplex assay was able to detect as low as 0.86 copies for PRV Variant (FAM) and 1.86 copies for PRV Classical (HEX) targets.

The repeatability of the triplex assay was evaluated by intra-assay and inter-assay variability testing. The coefficient of variation (CV) within each replicate was determined as a percentage of the ratio of standard deviation and mean of Ct values from FAM (specific for variant strain) and HEX (specific for classical strain) channels. Inter-assay variability testing was done with the triplex run three times over three days with one replicate for each dilution of PRV Bristol and JS-2012 viruses. Intra-assay variability was calculated with 20 replicates in a single run using the 10^{-2} dilution of PRV JS-2012 and Bristol viruses. All variability testing was performed on the Applied Biosystems 7500 FAST real-time PCR machine. The triplex assay demonstrated good repeatability for the diluted samples of PRV JS-2012 and PRV Bristol with the inter-assay ranging from $0.52 \leq CV \leq 3.64$ and the intra-assay CV ranging from $1.74 \leq CV \leq 2.02$ for both strains. The diluted samples of both PRV strains were also run on three different real-time PCR instruments including the Light Cycler 480, Applied Biosystems 7500, and Bio-Rad CFX 96. The results were consistent across the real-time PCR platforms tested (data not shown).

Diagnostic sensitivity and specificity of the PRV triplex real-time PCR

To evaluate the diagnostic sensitivity and specificity of the PRV triplex assay, 114 nasal swab samples were collected from pigs experimentally inoculated with PRV JS-2012 or PRV Bristol at the NCFAD-Winnipeg laboratory animal facility. Severe disease was expected in pigs inoculated with the highly virulent PRV strain JS-2012 (Luo et al., 2014); therefore 16 pigs representing two different age groups, 3-weeks and 7-weeks, were used for PRV JS-2012 inoculations. Out of the eight JS-2012 inoculated 3-weeks old piglets, six developed fever, depression and respiratory signs (sneezing and nasal discharge) by 3 dpi. Two of the inoculated 3-weeks old piglets were euthanized on 5 dpi; four were euthanized on 7 dpi due to severe neurological signs (tremors and seizures). The remaining two piglets developed mild neurological signs by 14 dpi and were

ethanized on 16 dpi. The JS-2012 inoculated 7-weeks old pigs survived the infection until the end of the experiment and were euthanized on 14 dpi. The majority of those pigs (6 of 8) developed fever by 4 dpi, as well as mild respiratory (sneezing, nasal discharge) and neurological signs (lameness, prostration) but by 11 dpi they all had recovered. The three 3-weeks old piglets inoculated with PRV Bristol developed clinical signs (fever, lack of appetite) by 3 dpi and they were euthanized on 7 dpi as they had developed severe neurological signs.

Both the PRV gB assay and the triplex assay detected PRV nucleic acid in nasal swabs from 3-weeks old piglets as early as 4 dpi and starting at 6 dpi in 7 weeks old pigs (Table 4). Low levels of PRV nucleic acid were detected earlier in some animals by both assays (on 4 dpi in some 7 weeks old pigs and on 2 dpi in some 3-weeks old piglets). Of the 102 samples tested, 62 samples were positive, 36 negative, and 4 were suspicious (Ct >35.99 were determined as suspect) on the triplex assay for PRV nucleic acids. The PRV gB assay identified 57 samples as a positive, 35 samples as negative, and 10 samples as suspicious (Ct >35.99 Ct were determined as suspect)(Table 5).

Virus isolation was performed on a subset of samples representing different Ct values and sample types. PRV-like cytopathic effect (rounding up of cells, and, occasionally, formation of syncytia) and positive staining of cells was observed for the positive control wells and no staining was observed in negative wells as expected. The Ct values of the triplex assay and the gB assay were comparable for all the tissue samples from PRV JS-2012 and PRV Bristol infected animals. No cross-reactivity was observed on the triplex assay. Virus isolation was successful from all samples with Ct values less than 30 on both the triplex and gB assays.

Inter-laboratory comparison and additional characterization:

Inter-laboratory comparison of the triplex assay was performed at the KSU Veterinary Diagnostic Laboratory and the NVSL in Ames, Iowa. At the KSU, 440 negative samples including oral fluids, serum, feces, lung, tissue and nasal swabs collected from the U.S. commercial swine herd were tested on the triplex real-time PCR and the gB real-time PCR (data not shown). All clinical samples were negative for both classical and variant PRV nucleic acid, as expected. Fluorescent signal for Q670 (endogenous internal control, β -actin) was detected on all samples, with Ct values ranging

from 18.73 to 35.85, confirming efficient DNA extraction and absence of PCR inhibitors in the samples (data not shown).

As positive controls, 11 negative serum samples and one negative oral fluid sample were spiked with the PRV classical gBlock, nucleic acid was extracted and tested on the triplex assay. All of these spiked samples were detected in the HEX and Q670 channels, and no detection was reported in the FAM channel of the triplex assay. Similarly, seven negative serum samples, four negative fecal swab samples and one negative oral fluid samples were spiked with the PRV variant gBlock, nucleic acid was extracted and tested on the triplex assay. All of these spiked samples were detected in the FAM and Q670 channels, and no detection was reported in the HEX channel of the triplex assay.

The triplex assay was further evaluated at the NVSL using archived oral and nasal swabs collected from a vaccine-challenge experiment conducted in 2015 (NVSL, unpublished data). In the experiment, seven weaned piglets were vaccinated with a USDA licensed, commercial gE/gI deleted PRV marker vaccine and five piglets were used as non-vaccinated controls. All pigs were challenged with the highly virulent PRV variant strain HeN1. A total of 116 nasal and oral swabs from this experiment were tested on the triplex assay (24 samples each from -14, 0, 2 and 5-days post challenge and 20 samples each from 8-days post challenge). The assay detected variant PRV starting 2 days post challenge in all the samples. With the exception of two samples that were near the limit of detection on the triplex assay, the results were in agreement with the NVSL-run gB real-time PCR (Ma et al. 2008; Zanella et al. 2012). The triplex assay did not detect any classical PRV genomes in any of these samples tested (data not shown). As positive controls for the triplex assay, known negative nasal and oral swab samples were spiked with gBlocks representing PRV variant, PRV classical and beta-actin.

Discussion:

Pseudorabies (PRV) outbreaks have resulted in devastating economic losses to affected countries and the introduction of highly pathogenic PRV variants from China into North America could cause severe economic losses to the swine industry. The vaccines currently available in North America for PRV are based on classical PRV strains and may not provide complete protection against the

highly virulent variant strains. Therefore, it is important for the veterinary diagnostic laboratories in North America to have highly sensitive and specific diagnostic assays that can rapidly detect and differentiate animals infected with highly pathogenic PRV variant strains.

In September 2016, a group of researchers from China reported development of a single-tube triplex TaqMan probe-based RT-PCR assay that can differentiate classical, variant, and Bartha-K61 vaccine strains (Meng et al., 2016). The assay used three target specific probes: one for Bartha-K61 vaccine strain, the second for PRV classical strains, and the third for variant PRV strains. The latter two probes were designed based on a single nucleotide difference in the gE/gI regions of these viruses. The assay was highly specific and evaluated using 234 field clinical samples (all from Bartha-K61 vaccinated animals). This assay however did not have a built-in internal control. PCR based assays are sensitive to inhibitors present in extracted nucleic acids. Therefore, having an internal control in PCR-based assays is critical to confirm the absence of PCR inhibitors in each sample. An internal control also ensures adequate efficiency of nucleic acid extraction, which can vary from sample-to-sample and the method of extraction used.

In this study we have developed and characterized a new single-tube triplex real-time PCR assay with a built-in internal control for detection and differentiation of nucleic acid from highly virulent PRV variant strains. The assay can be automated and be completed within a few hours once the samples are received in the laboratory. It has a built-in internal control to determine assay performance. The assay was able to detect and differentiate when tested against a number of classical PRV strains and two different PRV variant strains from China. The assay did not detect PRV genomic DNA from commercially available, modified live gE-deleted DIVA vaccine strains, related herpesviruses, and other non-PRV porcine viruses tested. The assay was able to detect 0.86-1.86 copies of PRV plasmid of known DNA length and concentration and was highly reproducible and effective on different real-time PCR machines with comparable sensitivity on different real-time PCR platforms.

To obtain clinical samples for assessment of diagnostic performance at the NCFAD, we collected nasal swabs every other day from pigs inoculated with PRV JS-2012 or PRV Bristol. The assay was highly specific and sensitive, detecting PRV nucleic acid by 4 dpi in 3-weeks old piglets and by

6 dpi in 7-weeks old pigs. This is consistent with higher PRV susceptibility of 3-weeks old pigs compared to 7-weeks old pigs. The assay was further characterized using over 440 PRV negative clinical samples collected from the U.S. and Canadian national herds as well as positive clinical samples collected from pigs experimentally infected with PRV classical (Bristol) and variant strains (JS-2012 and HeN1).

When compared to virus isolation, the gold standard for PRV diagnostics, the real-time triplex assay was equally sensitive in most of the samples, excepting two of the three trigeminal ganglia samples which tested positive by triplex real time PCR assay but were negative by virus isolation. This could be due to rapid establishment of latent stage by these viruses in trigeminal ganglia in these two animals. However, this could also indicate that the triplex real-time PCR assay is more sensitive than the virus isolation method for PRV detection.

The triplex real-time PCR assay developed in this project could be used as a rapid diagnostic tool for foreign animal disease detection in North America; for routine surveillance and in epidemiological studies in countries, like China, where both classical and variant strains are endemic; and the assay will not detect PRV gE-deletion mutant marker vaccines, if used.

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Conflict of Interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Data Availability statement

All data related to this manuscript will be made available upon request from the corresponding author.

Ethics Statement (required during submission process to Transboundary)

The authors confirm that the ethical policies of the journal have been adhered to. The animals used for experimental trials was approved by the Animal Care Committee at the Canadian Science Centre for Human and Animal Health. The guidelines from the Canadian Council for Animal Care were observed during all procedures with animals.

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Table 1. Primer and probe sequences used in the single-tube triplex real-time PCR assay (A) and gBlocks (B) used in the study

(A)

Name	Sequence (5'-3')	Florescent Marker	Quencher
PRV US9/US2-FP	ACACAGCAGCCTTCCT	-	
PRV US9/US2-RP	ACACAGCAGCCTTCCT	-	
PRV US9/US2-CP	TGTTCCCTCTCGTCGGTCTTCTCCCAC	HEX	BHQ-1
PRV US9/US2-VP	TTCCGCCCGTCTCTCGCC	FAM	BHQ-1
β-ACTIN 831-FP	BTCCTTCCTGGGCATGGA	-	
β-ACTIN 1036-RP	GRGGSGCGATGATCTTGAT	-	
β-ACTIN-P-880	TCCATCATGGAAGTGYGACGTSGACATCCG	Q670	BHQ-2

(B)

Name	Sequence (5`-3`)
PRV classical	ACAATAAATCGCACACAGCAGCCTTCCTCGTCTCCGCGTCTCTGCTGTTCCCTCTCGTCGGTCTTCTCCCCTCCGCGTTCGCGAATGCTTCATTGCGAACTGCTTCGCAGACCGCGCTCGCGCCATGGGGGTGACGGCCATCACCGTGGTCACGCTGATGGAC
PRV variant	ACAATAAATGGGCCCGGCGTGTCCGGGCGCACACAGCAGCCTTCCTCTCCTCCGCGTCTCTGTTCCGCGCGTCTCTCGCGGACTCTTCTTCTCCACCGCTCCACCGTCGCAGTTGTGCGGAGCGGTTTCGCACCATGGTGTTCATTGCGAACTGCTTCGCAGACGGGTGACGGCCATCACCGTGGTCACGCTGATGGACGGGGCCGGGCGCAT
β-actin	CTTCCAGCCCTCCTTCCTGGGCATGGAGTCTGCGGCATCCACGAGACCACCTTCAACTC GATCATGAAGTGCAGCGTGGACATCAGGAAGGACCTCTACGCCAACACGGTGCTGTCTG TGCTTCATTGCGAACTGCTTCGCAGACGCGGGACCACCATGTACCCCGGCATCGCCGAC AGGATGCAGAAGGAGATCACGGCCCTGGCGCCAGCACCATGAAGATCAAGATCATCGC CCCTCCCGAGCGCAAGTACT

Table 2. Specificity of the triplex assay using nucleic acid extracted from cell culture amplified PRV JS-2012, Bristol, Bartha K-61, Bucharest, Shope and Becker strains and differential viruses. ND: Not detected

Virus	PRV triplex Assay			PRV gB Assay
	FAM	HEX	Q670	FAM
JS-2012	22.74	0	22.77	22.49
Bristol	0	26.63	26.71	22.65
Bartha K-61	0	0	27.79	22.07
Bucharest	0	0	22.97	20.67
Shope	0	22.01	24.18	22.34
Becker	0	20.97	25.09	21.66
BHV-1	0	0	34.06	ND
ElkHV	0	0	27.95	ND
CaprHV	0	0	35.42	ND
RanHV	0	0	32.17	ND
CervHV	0	0	30.79	ND
ASF	0	0	25.00	ND
CSF	0	0	20.83	ND
PEDV	0	0	21.90	ND
PRRSV	0	0	31.04	ND
PCV-2	0	0	27.88	ND
FMD	0	0	27.80	ND
VSV (Indiana)	0	0	28.32	ND
VSV (New Jersey)	0	0	27.00	ND
SVD	0	0	27.97	ND
NTC	0	0	0	0

Table 4. Clinical sensitivity and specificity of the PRV triplex real-time PCR assay using nasal swabs collected from pigs inoculated with PRV JS-2012 or PRV Bristol. Pigs 24, 25 and 25 inoculated with PRV Bristol developed severe neurological signs on 6dpi and were euthanized. Two piglets inoculated with JS-2012 (#15 and #20) were euthanized on 5 dpi, and #13, 17, 18 and 19) were euthanized on 7 dpi. NA = Not available; S = Suspected, (+) = Positive; (-) = Negative

Group (Age)	PRV JS2012	PRV triplex assay/gB assay (DPI)							
		0	2	4	6	8	10	12	14
1 (7 weeks)	Pig 5	-/-	-/-	-/-	+/+	S/S	+/+	+/S	+/S
	Pig 6	-/-	-/-	S/S	+/+	+/+	+/+	+/+	+/+
	Pig 7	-/-	-/-	-/S	+/+	+/+	+/+	+/+	+/+
	Pig 8	-/-	-/-	-/-	+/S	+/+	+/+	+/+	+/+
	Pig 9	-/-	-/-	-/-	+/+	+/+	+/+	+/+	+/+
	Pig 10	-/-	-/-	S/S	+/+	+/+	+/+	+/+	+/S
	Pig 11	-/-	-/-	-/-	+/+	+/+	+/+	+/+	+/+
	Pig 12	-/-	-/-	+/S	+/+	+/+	+/+	+/+	+/+
2 (3 weeks)	Pig 13	-/-	-/-	+/+	+/+	NA	NA	NA	NA
	Pig 14	-/-	S/S	+/+	+/+	+/+	+/+	+/+	+/+
	Pig 15	-/-	-/-	+/+	NA	NA	NA	NA	NA
	Pig 16	-/-	-/-	+/+	+/+	+/+	+/+	+/+	+/+
	Pig 17	-/-	-/-	+/+	+/+	NA	NA	NA	NA
	Pig 18	-/-	-/-	+/+	+/+	NA	NA	NA	NA
	Pig 19	-/-	-/-	+/+	+/+	NA	NA	NA	NA
	Pig 20	-/-	-/-	+/+	NA	NA	NA	NA	NA

Group	PRV Bristol	PRV triplex assay/gB assay (DPI)			
		0	2	4	6
3 (3 weeks)	Pig 24	-/-	-/S	S/+	+/+
	Pig 25	-/-	-/-	+/+	+/+
	Pig 26	-/-	-/S	+/+	+/+

Table 5. Summary of the PRV triplex and PRV gB real-time PCR assay results of nasal swab samples collected from the PRV JS-2012 and PRV Bristol-infected pigs.

PRV Strain	PRV triplex assay			PRV gB assay		
	Positive	Suspicious	Negative	Positive	Suspicious	Negative
Bristol	5	1	6	6	2	4
JS-2012	62	4	36	57	10	35

Table 6. Comparison between single-tube triplex assay, PRV gB assay and virus isolation results using a selected number of clinical samples collected from PRV JS-2012 and PRV Bristol infected pigs.

PRV strain	Pig #	Sample type	PRV triplex assay			PRV gB assay	Virus isolation
			FAM	HEX	Q670	FAM	
JS-2012	17	Brain	25.79	0	23.15	25.9	+
		Tonsil	28.82	0	24.63	29.17	+
		Trigeminal	31.9	0	25.51	31.78	-
	18	Brain	26.99	0	22.3	26.8	+
		Tonsil	22.72	0	25.61	23.4	+
		Trigeminal	28.81	0	28.96	28.72	+
	19	Brain	24.29	0	19.65	23.98	+
		Tonsil	24.35	0	27.27	24.51	+
		Trigeminal	27.32	0	26.99	27.17	+
Bristol	24	Brain	0	23.18	20.77	23.83	+
		Tonsil	0	28.31	26.99	29.48	+
		Trigeminal	0	27.46	28	28.16	+
	25	Brain	0	24.92	20.16	26	+
		Tonsil	0	23.76	25.48	24.86	+
		Trigeminal	0	27.11	26.21	28.01	+
	26	Brain	0	28.94	25.39	29.99	+
		Tonsil	0	20.78	26.87	21.85	+
		Trigeminal	0	30.77	28.14	31.4	-

Figure 2. Triplex real-time PCR assay Ct value against log copy number plot of PRV Variant (FAM) and PRV Classical (HEX) plasmid of known length and concentration. The standard curve shows the correlation between \log_{10} copy number and Ct values obtained from the diluted PRV plasmid.

