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

Orlando Perez[1], Mathieu Pinette[1], Jianfa Bai[2], Lalitha Peddireddi[3], John Schiltz [4], Karthik Shanmuganatham[4], Rachel Tell[4], Alfonso Clavijo[5], and Aruna Ambagala[1]

[1]Canadian Food Inspection Agency
[2]Kansas State University
[3]Kansas State Veterinary Diagnostic Laboratory, KansasState University
[4]USDA National Veterinary Services Laboratories
[5]USDA

October 26, 2020

Abstract

Pseudorabies virus (PRV) causes Aujeszky's disease or pseudorabies (PR), which is characterized by 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, 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.

Orlando Perez[1], Mathieu Pinette[1], Jianfa Bai[4], Lalitha Peddireddi[4], John Schiltz[5], Karthik, Shanmuganatham[5], Rachel M. Tell[5], Alfonso Clavijo[1,+], Aruna Ambagala[1, #]

[1]National Centre for Foreign Animal Disease, Canadian Food Inspection Agency, Winnipeg, MB, Canada.[2]Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai, China.[3]College of Veterinary Medicine, Northwest A & F University, Yangling, China. [4]College of Veterinary Medicine, Kansas State University, Kansas, KS, USA.[5]National Veterinary Services Laboratories, VS, APHIS, USDA, Ames, IA, USA.

[#] Corresponding author

+ Current address: USDA, Agricultural Research Service, Manhattan, KS, USA

Abstract

Pseudorabies virus (PRV) causes Aujeszky's disease or pseudorabies (PR), which is characterized by 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, 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, Aujeszky's, Variant, Real-time quantitative PCR, 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 the 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 the 1990s, more than 80% of pigs in China have been vaccinated with Bartha-K61 vaccine; and PR has been 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, or classical strains, are assigned to Genotype I (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 quantitative PCR (qPCR) assays allow detection of PCR amplification during early phases of amplification, are quantifiable, and can be automated. A number of qPCR 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 Adiagène, 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 and 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 (Genotype I) PRV strains, and they may not provide complete protection against highly virulent Chinese strains (Genotype II) (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 qPCR 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 Zoetis Inc. in Lincoln, NE, USA. PRV genomic DNA was extracted from tissue culture amplified virus, PRV positive clinical samples, or from vaccines reconstituted in PBS. Cross reactivity (analytical specificity or exclusivity) of the PCR with other swine viruses was tested with a genomic panel comprised of swine viruses extracted from cell culture amplified African and classical swine fever viruses and foot and mouth disease virus (FMDV); clinical samples positive for porcine respiratory and reproductive syndrome virus (PRRSV), porcine epidemic diarrhea virus (PEDV), and porcine circovirus 2

(PCV2); and herpesviruses originating in bovine, elk , caprine, *Rangifer* , and an unknown cervid . PRV pUC57 plasmid (545 ng/μL) was purchased from Genscript.

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 six partial classical strains, as well as 13 full-length and three 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 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, were designed (Table 1A). 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 employed (Ma et al., 2008). To facilitate the triplex assay development, determine specificity, and to generate positive controls for the assay, synthetic gene fragments (gBlocks) of the target regions of PRV classical and variant genomes and the β-actin gene were synthesized and procured from Integrated DNA Technologies, Inc. (Coralville, Iowa) (Table 1B).

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 three weeks of age and eight weaner pigs at seven 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 seven days of acclimatization, pigs were randomly assigned to three 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^{o}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^{TM} 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 qPCR assay development and optimization:

Three separate qPCR assays (classical PRV RT-PCR, variant PRV qPCR and β-Actin qPCR) 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^{o}C for 20 seconds, and 45 cycles of 95^{o}C for 3 seconds and 60^{o}C for 30 seconds. All primers and probe working stocks were maintained at 10 µM concentration. The primers and probe for the gB qPCR 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 net 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 on PK-15 cells grown in 96-well tissue culture plates. Briefly, PK-15 cells were seeded at 500,000 cells per well in 96 well tissue culture plates using ∝-MEM supplemented with 2 mM L-glutamine, 5mg/mL gentamicin and 2% γ-irradiated Fetal Bovine Serum (FBS). Ten percent (W/V) homogenates were prepared from each tissue and 25 µl of each suspension was added to each well of the PK-15 cells. A positive control plate was inoculated with a PRV variant strain (JS-2012). The plates were incubated for 48 hours in a 5% CO_2 humid air incubator at 37°C and were checked daily for cytopathic effect (CPE) and possible contamination using a light microscope. Two days post infection, the cells were fixed using 200 µl of fixation fluid (Acetone, PBS, and Bovine serum albumin fraction V) per well, and stained using porcine-origin PRV antiserum. 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 the triplex assay along with the gB assay were transferred to Kansas State University Veterinary Diagnostic Laboratory (KSU-VDL) for inter-laboratory comparison using a negative cohort study

comprised of 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, for 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 of which had been challenged with the PRV HeN1 (NVSL, unpublished data).

Results:

Development and optimization of a single-tube, triplex real-time qPCR 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; 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 was 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. 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, including ASF, CSF, and FMD viruses (Table 2). 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.

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 dilutions of PRV plasmid with net copy number of 7.34×10^{11} for both classical and variant strains of PRV (Figure 2). 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 [?] CV [?] 3.64 and the intra-assay CV ranging from 1.74 [?] CV [?] 2.02 for both strains. The diluted samples of both PRV strains were 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 qPCR

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 (Luoe 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-week 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 seizers). The remaining two piglets developed mild neurological signs by 14 dpi and were euthanized on 16 dpi. The JS-2012 inoculated 7-week 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-week 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-week old piglets as early as 4 dpi and starting at 6 dpi in 7-week old pigs (Table 3). Low levels of PRV nucleic acid were detected earlier in some animals by both assays (on 4 dpi in some 7-week old pigs and on 2 dpi in some 3-week old piglets). Of the 102 samples tested, 62 samples were positive, 36 negative, and 4 were suspect (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 suspect. While the gB PCR as published did not define a suspect range, for this project, we designated Ct greater than 35.99 as suspect (Table 4).

Virus isolation was performed on a subset of samples representing different Ct values and sample types (Table 5). PRV-like cytopathic effect (rounding up of cells and occasional 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 VDL, 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 which 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 qPCR 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 qPCR 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 can 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 detect and differentiate when tested against a number of classical PRV strains and two different variant PRV 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, since the assay will not detect PRV gE-deletion mutant marker vaccines, if used.

Acknowledgment: This project was supported by the Swine Health Information Centre Grant #16-250 and the CFIA. Authors would like to thank the animal care staff at the NCFAD-Winnipeg and the USDA National Centers for Animal Health, Ames, Iowa, for helping with the animal experiments. Authors would also like to thank Zoetis Inc. for providing the PR-VAC PLUS (PRV Bucharest strain) vaccine. Additionally, the authors thank Dr. Guang-Zhi Tong at the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai, China for providing the PRV-JS2012 strain, and Dr. En-Min Zhou at the College of Veterinary Medicine, Northwest A & F University, Yangling, China, Dr. Sabrina Swenson at the National Veterinary Services Laboratories, VS, APHIS, USDA, Ames, IA, and Melissa Goolia at the National Centre for Foreign Animal Disease, Canadian Food Inspection Agency, Winnipeg, MB, Canada for their support during this project and Drs. John Copps and Shawn Babiuk for reviewing the manuscript.

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.

References:

An, T. Q., J. M. Peng, Z. J. Tian, H. Y. Zhao, N. Li, Y. M. Liu, J. Z. Chen, C. L. Leng, Y. Sun, D. Chang and G. Z. Tong, 2013: Pseudorabies virus variant in Bartha-K61-vaccinated pigs, China, 2012. Emerg Infect Dis, 19, 1749-1755.

Gaskamp, J. A., K. L. Gee, T. A. Campbell, N. J. Silvy and S. L. Webb, 2016: Pseudorabies Virus and Brucella abortus from an Expanding Wild Pig (Sus scrofa) Population in Southern Oklahoma, USA. J Wildl Dis, 52, 383-386.

Gu, Z., C. Hou, H. Sun, W. Yang, J. Dong, J. Bai and P. Jiang, 2015: Emergence of highly virulent pseudorabies virus in southern China. Can J Vet Res, 79, 221-228.

Hahn, E. C., B. Fadl-Alla and C. A. Lichtensteiger, 2010: Variation of Aujeszky's disease viruses in wild swine in USA. Vet Microbiol, 143, 45-51.

Hu, D., L. Lv, Z. Zhang, Y. Xiao and S. Liu, 2015a: Seroprevalence and associated risk factors of pseudorabies in Shandong province of China. J Vet Sci.

Hu, D., Z. Zhang, L. Lv, Y. Xiao, Y. Qu, H. Ma, Y. Niu, G. Wang and S. Liu, 2015b: Outbreak of variant pseudorabies virus in Bartha-K61-vaccinated piglets in central Shandong Province, China. J Vet Diagn Invest, 27, 600-605.

Lee, J. Y. and M. R. Wilson, 1979: A review of pseudorabies (Aujeszky's disease) in pigs. Can Vet J, 20, 65-69.

Luo, Y., N. Li, X. Cong, C. H. Wang, M. Du, L. Li, B. Zhao, J. Yuan, D. D. Liu, S. Li, Y. Li, Y. Sun and H. J. Qiu, 2014: Pathogenicity and genomic characterization of a pseudorabies virus variant isolated from Bartha-K61-vaccinated swine population in China. Vet Microbiol, 174, 107-115.

Ma, W., K. M. Lager, J. A. Richt, W. C. Stoffregen, F. Zhou and K. J. Yoon, 2008: Development of real-time polymerase chain reaction assays for rapid detection and differentiation of wild-type pseudorabies and gene-deleted vaccine viruses. J Vet Diagn Invest, 20, 440-447.

Meng, X. Y., Y. Luo, Y. Liu, L. Shao, Y. Sun, Y. Li, S. Li, S. Ji and H. J. Qiu, 2016: A triplex real-time PCR for differential detection of classical, variant and Bartha-K61 vaccine strains of pseudorabies virus. Arch Virol, 161, 2425-2430.

Mettenleiter, T. C., 2000: Aujeszky's disease (pseudorabies) virus: the virus and molecular pathogenesis–state of the art, June 1999. Vet Res, 31, 99-115.

Moniwa M, Clavijo A, Li M, Collignon B, Kitching PR. Performance of a foot-and-mouth disease virus reverse transcription-polymerase chain reaction with amplification controls between three real-time instruments. J Vet Diagn Invest. 2007 Jan; 19(1):9-20.

Perez, L. J., C. L. Perera, M. T. Frias, J. I. Nunez, L. Ganges and H. D. de Arce, 2012: A multiple SYBR Green I-based real-time PCR system for the simultaneous detection of porcine circovirus type 2, porcine parvovirus, pseudorabies virus and Torque teno sus virus 1 and 2 in pigs. J Virol Methods, 179, 233-241.

Pol, F., C. Deblanc, A. Oger, M. Le Dimna, G. Simon and M. F. Le Potier, 2013: Validation of a commercial real-time PCR kit for specific and sensitive detection of Pseudorabies. J Virol Methods, 187, 421-423.

Pomeranz, L. E., A. E. Reynolds and C. J. Hengartner, 2005: Molecular biology of pseudorabies virus: impact on neurovirology and veterinary medicine. Microbiol Mol Biol Rev, 69, 462-500.

Rao, P., H. Wu, Y. Jiang, T. Opriessnig, X. Zheng, Y. Mo and Z. Yang, 2014: Development of an EvaGreen-based multiplex real-time PCR assay with melting curve analysis for simultaneous detection and differentiation of six viral pathogens of porcine reproductive and respiratory disorder. J Virol Methods, 208, 56-62.

Tong, W., F. Liu, H. Zheng, C. Liang, Y. J. Zhou, Y. F. Jiang, T. L. Shan, F. Gao, G. X. Li and G. Z. Tong, 2015: Emergence of a Pseudorabies virus variant with increased virulence to piglets. Vet Microbiol, 181, 236-240.

Tong W, Li G, Liang C, Liu F, Tian Q, Cao Y, Li L, Zheng X, Zheng H, Tong G. A live, attenuated pseudorabies virus strain JS-2012 deleted for gE/gI protects against both classical and emerging strains. Antiviral Res. 2016 Jun;130:110-7.

United States Department of Agriculture, A. a. P. H. I. S., 2008: Pseudorabies (Aujesky's Disease) and its Eradication: A review of the U.S. Experience., Technical Bulletin No. 1923.

van Rijn, P. A., G. J. Wellenberg, R. Hakze-van der Honing, L. Jacobs, P. L. Moonen and H. Feitsma, 2004: Detection of economically important viruses in boar semen by quantitative RealTime PCR technology. J Virol Methods, 120, 151-160.

Wang CH, Yuan J, Qin HY, Luo Y, Cong X, Li Y, Chen J, Li S, Sun Y, Qiu HJ. A novel gE-deleted pseudorabies virus (PRV) provides rapid and complete protection from lethal challenge with the PRV variant emerging in Bartha-K61-vaccinated swine population in China. Vaccine. 2014 Jun 5;32(27):3379-85.

Wang J, Song Z, Ge A, Guo R, Qiao Y, Xu M, Wang Z, Liu Y, Zheng Y, Fan H, Hou J. Safety and immunogenicity of an attenuated Chinese pseudorabies variant by dual deletion of TK & gE genes. BMC Vet Res. 2018 Sep 21;14(1):287.

Wernike, K., M. Beer, C. M. Freuling, B. Klupp, T. C. Mettenleiter, T. Muller and B. Hoffmann, 2014: Molecular double-check strategy for the identification and characterization of Suid herpesvirus 1. J Virol Methods, 209, 110-115.

Wu, H., P. Rao, Y. Jiang, T. Opriessnig and Z. Yang, 2014: A sensitive multiplex real-time PCR panel for rapid diagnosis of viruses associated with porcine respiratory and reproductive disorders. Mol Cell Probes, 28, 264-270.

Xiang, S., Z. Zhou, X. Hu, Y. Li, C. Zhang, J. Wang, X. Li, F. Tan and K. Tian, 2016: Complete Genome Sequence of a Variant Pseudorabies Virus Strain Isolated in Central China. Genome Announc, 4.

Yang, Q. Y., Z. Sun, F. F. Tan, L. H. Guo, Y. Z. Wang, J. Wang, Z. Y. Wang, L. L. Wang, X. D. Li, Y. Xiao and K. G. Tian, 2016: Pathogenicity of a currently circulating Chinese variant pseudorabies virus in pigs. World J Virol, 5, 23-30.

Yoon, H. A., S. K. Eo, A. G. Aleyas, S. O. Park, J. H. Lee, J. S. Chae, J. G. Cho and H. J. Song, 2005: Molecular survey of latent pseudorabies virus infection in nervous tissues of slaughtered pigs by nested and real-time PCR. J Microbiol, 43, 430-436.

Yu, X., Z. Zhou, D. Hu, Q. Zhang, T. Han, X. Li, X. Gu, L. Yuan, S. Zhang, B. Wang, P. Qu, J. Liu, X. Zhai and K. Tian, 2014: Pathogenic pseudorabies virus, China, 2012. Emerg Infect Dis, 20, 102-104.

Zanella, E. L., L. C. Miller, K. M. Lager and T. T. Bigelow, 2012: Evaluation of a real-time polymerase chain reaction assay for Pseudorabies virus surveillance purposes. J Vet Diagn Invest, 24, 739-745.

Zhang, M., Z. Xie, L. Xie, X. Deng, Z. Xie, S. Luo, J. Liu, Y. Pang and M. I. Khan, 2015: Simultaneous detection of eight swine reproductive and respiratory pathogens using a novel GeXP analyser-based multiplex PCR assay. J Virol Methods, 224, 9-15.

Zhao, L., B. Cui, H. Chen, Z. Wei, L. Zheng, X. Lu, Y. Jia and X. Zhao, 2008: [Diagonsis establishment of fluorescen quantitative PCR assay for pseudorabies wild-type virus and vaccine virus]. Sheng Wu Gong Cheng Xue Bao, 24, 1149-1154.

Hosted file

`Figure 1.pdf` available at https://authorea.com/users/370320/articles/488999-a-single-tube-triplex-real-time-quantitative-pcr-assay-for-differential-detection-of-highly-virulent-chinese-strains-of-pseudorabies-virus

Hosted file

`Figure 2.pdf` available at https://authorea.com/users/370320/articles/488999-a-single-tube-triplex-real-time-quantitative-pcr-assay-for-differential-detection-of-highly-virulent-chinese-strains-of-pseudorabies-virus

Hosted file

`Tables 1.pdf` available at https://authorea.com/users/370320/articles/488999-a-single-tube-triplex-real-time-quantitative-pcr-assay-for-differential-detection-of-highly-virulent-chinese-strains-of-pseudorabies-virus

Hosted file

Table 2.pdf available at https://authorea.com/users/370320/articles/488999-a-single-tube-triplex-real-time-quantitative-pcr-assay-for-differential-detection-of-highly-virulent-chinese-strains-of-pseudorabies-virus

Hosted file

Table 3.pdf available at https://authorea.com/users/370320/articles/488999-a-single-tube-triplex-real-time-quantitative-pcr-assay-for-differential-detection-of-highly-virulent-chinese-strains-of-pseudorabies-virus

Hosted file

Table 4.pdf available at https://authorea.com/users/370320/articles/488999-a-single-tube-triplex-real-time-quantitative-pcr-assay-for-differential-detection-of-highly-virulent-chinese-strains-of-pseudorabies-virus

Hosted file

Table 5.pdf available at https://authorea.com/users/370320/articles/488999-a-single-tube-triplex-real-time-quantitative-pcr-assay-for-differential-detection-of-highly-virulent-chinese-strains-of-pseudorabies-virus