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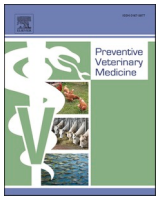

Pseudorabies (Aujeszky's disease) virus DNA detection in swine nasal swab and oral fluid specimens using a gB-based real-time quantitative PCR

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ABSTRACT

In this study, the detection of PRV DNA in nasal swab ($n = 440$) and oral fluid ($n = 1,545$) samples collected over time from experimentally PRV vaccinated and/or PRV inoculated pigs ($n = 40$) was comparatively evaluated by real-time PCR. Serum samples ($n = 440$) were tested by PRV gB/gE blocking ELISAs (Pseudorabies Virus gB Antibody Test Kit and Pseudorabies Virus gpI Antibody Test Kit, IDEXX Laboratories, Inc., Westbrook, ME) to monitor PRV status over time. Following exposure to a gE-deleted modified live vaccine (Ingelvac® Aujeszky MLV, Boehringer Ingelheim, Ridgefield, CT) and/or a wild-type virus (3 CR Ossabaw), PRV gB DNA was detected in oral fluid specimens in a pattern similar to that of nasal swabs. For quantitative analyses, PRV PCR quantification cycle (Cq) results were re-expressed as "efficiency standardized Cqs (ECqs)" as a function of PCR efficiency using plate-specific positive amplification controls. ROC analyses of the PRV gB PCR ECqs results showed a similar performance of the PRV gB PCR for nasal swab and oral fluid specimens (area under the ROC curve = 85 % vs 83 %) and, based on an ECq cutoff of 0.01 a diagnostic specificity of 100 % and diagnostic sensitivities for oral fluid and nasal swab specimens of 53 % (95 % CI: 43 %, 62 %) and 70 % (95 % CI: 55 %, 83 %), respectively. Thus, the results described herein demonstrated the detection of PRV gB DNA in swine oral fluid and supported the use of this specimen in PRV diagnosis and surveillance.

1. Introduction

Suid alphaherpesvirus 1, a DNA virus in Family *Herpesviridae*, is also referred to as pseudorabies virus (PRV) because of rabies-like clinical signs produced by the infection; and as Aujeszky's disease virus because of Aladàr Aujeszky's work demonstrating the transmissibility of the virus to rabbits via contaminated tissues (Kohler and Kohler, 2003; Mettenleiter, 2020). Beginning in the 1960s, PRV was increasingly associated with clinical disease in commercial swine populations in Europe, the Americas, and Southeast Asia, e.g., in the United States, annual losses to swine producers were estimated at $21 to $25 million (USD) (Miller et al., 1996; Neumann et al., 2005). Regional/national PRV control and elimination was achieved by exploiting the molecular biology of the virus using a PRV DIVA (differentiation of infected from vaccinated animals) strategy (van Oirschot et al., 1986; Quint et al., 1987). In brief, among 11 PRV viral envelope glycoproteins, glycoprotein gB is highly conserved, consistently expressed, and required for PRV replication (Mettenleiter, 2000). Therefore, the detection of the gB gene or anti-gB antibody is diagnostic for PRV regardless of the isolate or strain involved (Katz and Pedersen, 1992; Ma et al., 2008). In contrast, the recognition of non-essential envelope glycoproteins and the discovery of naturally-occurring gE-deleted strains, e.g., Bartha and Bucharest, led to the development of higly effective gene-deleted vaccines and companion DIVA serologic and molecular assays (Lomniczi et al., 1984; Ma et al., 2008; Freuling et al., 2017).

Through the 1980s, PRV detection relied on the isolation of virus from infected animals (Gutekunst and Pirtle, 1979; Wittmann et al., 1980; Mettenleiter, 2020). Typically, PRV can be isolated 2–15 days post

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inoculation (dpi) from nasal swabs collected from pigs infected with wild-type PRV isolates (Maes et al., 1983; Wang et al., 2015). In pigs vaccinated with gene-deleted MLVs, shorter periods of detection and lower positivity rates occur after exposure to wild-type virus (Vilnis et al., 1998). As first described by Belák et al. (1989), virus isolation was superseded by the development of PRV PCRs. This development improved both the analytical sensitivity and the efficiency of PRV detection, providing for the detection of PRV gB DNA in specimens from both acutely diseased pigs and from latently infected animals (Belák and Linné, 1988). Later, PCR methods targeting PRV gE DNA made it possible to differentiate gE-deleted MLV vaccinated from wild-type PRV infected animals (Katz and Pedersen, 1992; Scherba et al., 1992; Hasebe et al., 1993; Ma et al., 2013). Subsequently, a commercial PCR assay targeting the PRV gD glycoprotein gene (ADIAVET® PRV REALTIME kit, Bio-X Diagnostics, Rochefort, Belgium) was released and its use has been reported in several studies (Leschnik et al., 2012; Pol et al., 2013; Deblanc et al., 2019). Recently, PRV PCRs have moved toward multiplex formats that simultaneously differentiate among PRV vaccine viruses, typical wild-type viruses, and highly pathogenic strains (Meng et al., 2016), or screen for other swine pathogens, e.g., porcine circoviruses (PCVs), porcine parvoviruses, porcine reproductive and respiratory syndrome virus (PRRSV) (Yue et al., 2009; Tian et al., 2020). The systematic application of highly effective DIVA vaccines and ELISAs proved highly effective in the control and/or elimination of PRV.

Although PRV has been widely eliminated from domestic swine, it remains relevant to swine health because it continues to circulate in feral swine populations. Thus, PRV infection in commercial swine populations after contact with feral pigs has been reported in Minnesota (2002), Wisconsin (2007), and France (Gauntlett et al., 2019). In addition, the emergence of highly pathogenic PRV variants in China raises concerns for global swine health (An et al., 2013; Wu et al., 2013; Luo et al., 2014; Yu et al., 2014; Bo et al., 2020). Under this scenario, PRV surveillance remains important, e.g., ~197,000 individual swine were tested through the US national PRV surveillance program in 2017 (United States Department of Agriculture National Animal Surveillance System (USDA-NAHSS, 2018) and improvements in PRV surveillance remain a legitimate area of investigation.

Contemporary surveillance methods are moving away from individual animal specimens and toward the use of aggregate samples, e.g., processing fluid and oral fluid samples, as a mean of improving surveillance efficiency and lowering costs (Bjustrom-Kraft et al., 2018; Lopez et al., 2018). In particular, oral fluids have been widely adapted to diagnostic technologies and routinely used by producers for the surveillance of PRRSV, influenza A virus, PCV, and many others (Henao--Diaz et al., 2020). Oral fluid specimens from > 23,000 cases were tested for PRRSV RNA by the veterinary diagnostic laboratories at Iowa State University, the University of Minnesota, South Dakota State University, and the Kansas State University in 2018; a 96 % increase relative to 2009 (Trevisan et al., 2019). In 2019, the veterinary diagnostic laboratory at Iowa State University alone performed ~ 240,000 tests on swine oral fluid specimens submitted by clientele (R Main, personal communication). In the present study, the detection of PRV gB DNA in oral fluid and nasal swab specimens was explored in the context of PRV antibody responses in serum using samples from animals of known PRV infection status and the assay's performance (diagnostic sensitivity and specificity) estimated.

2. Material and methods

2.1. Experimental design

In this study, the detection of the PRV gB gene in swine nasal swab and oral fluid specimens using a TaqMan® probe-based PRV gB quantitative PCR (qPCR) was evaluated over time post-inoculation in PRV-inoculated, PRV modified live virus (MLV) vaccinated, and vaccinated/challenged pigs. PRV PCR quantification cycle (Cq) results were standardized by PCR efficiency and plate positive amplification controls and re-expressed as "efficiency standardized Cqs (ECqs)", as derived from a mathematical model described by Pfaffl (2001). Qualitative (positive or negative) and relative quantitative (efficiency standardized Cq; ECq) results were analyzed by specimen to characterize and compare the kinetics of PRV shedding. Finally, ECqs were used to estimate the gB PCR diagnostic sensitivity and specificity using receiver operating characteristic (ROC) curve analysis. Commercial PRV blocking ELISAs (gB and gE) were used to monitor animal PRV infection status over time.

2.2. Pseudorabies virus propagation and titration

PRV 3CR Ossabaw was propagated on swine testicular (ST) cells in 75 cm^2 flasks (Pirtle et al., 1989; Zanella et al., 2012). When cytopathic effect (CPE) was observed in 80–90 % of the infected cell monolayer, flasks were subjected to two freeze-cycles (−80 °C), the harvested contents clarified by centrifugation at1,000 × *g* for 10 min, and the supernatant containing the virus was harvested and stored at −80 °C. Prior to and after animal inoculation, virus titration was performed on monolayers of ST cells cultured in 96-well plates by inoculating wells with 100 μL of serially 10-fold diluted inoculum ($10^{-1} - 10^{-9}$) (Zanella et al., 2012). Inoculated monolayers were incubated at 37 °C with 5 % CO_2 for 72 h and then examined for CPE. Virus titers and 95 % confidence intervals (95 % CI) were calculated using the Spearman-Kärber method (Spearman, 1908; Kärber, 1931).

2.3. Animal treatments and sample collection

Individually penned 12- to 16-week-old pigs were assigned to 4 treatment groups (Table 1): 1. Negative control (NC) 2. Wild-type PRV inoculation (PRV) 3. PRV modified live virus (MLV) vaccination (Ingelvac® Aujeszky MLV, Boehringer Ingelheim, Ridgefield, CT), and 4. MLV vaccination and PRV challenge at 3 weeks post vaccination (MLV-PRV). Pigs in the PRV ($n = 10$), MLV ($n = 10$), and MLV-PRV ($n = 10$) treatment groups were housed in a biosafety level 3 (BSL3-Ag) large animal facility at the USDA National Animal Disease Center (USDA-NADC, Ames, IA). Pigs in the NC group ($n = 10$) were held in a biosafety level 2 (BSL-2) large animal facility at Iowa State University (Ames, IA) (Table 1) (Cheng et al., 2020). Inoculation or challenge was done by intranasally exposing pigs to 4 mL (2 mL per naris) of an inoculum containing $1 \times 10^{2.9}$ $TCID_{50}$/mL (95 % CI: $1 \times 10^{2.5}$, $1 \times 10^{3.3}$) PRV 3CR Ossabaw (Zanella et al., 2012). Vaccine was administered as per the manufacturer's instructions. All animals were humanely euthanized at the end of the observation period by captive bolt or pentobarbital injection followed by exsanguination, as specified in the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (American Veterinary Medical Association (AVMA, 2020). The study protocols were approved by the Institutional Animal Care and Use Committee of Agricultural Research Service, United States Department of Agriculture (USDA-ARS) (protocol number: ARS-2017-689) and Iowa State University (protocol number: 10-17-8622-S).

Blood was collected from the external jugular vein following the timeline listed in Table 1 using 20-gauge needles (Exelint International Co., Saint Petersburg, FL), reusable hubs (Becton, Dickinson and Company, Franklin Lakes, NJ), and 12.5 mL serum separator tubes (Covidien, Minneapolis, MN). After a 15 min centrifugation (1,000 × *g*), serum samples were aliquoted and stored at −20 °C. Nasal swab samples were collected (Table 1) by swabbing each naris with a cotton-tipped swab (Puritan Medical Products Co. LLC., Guilford, ME) which was then placed into 1 mL phosphate-buffered saline (PBS, pH 7.2, Life Technologies Co., Grand Island, NY). Oral fluid samples were collected from individual pigs (Table 1) by allowing pigs to chew on cotton rope (30–45 min) and then harvesting the oral fluid (White et al., 2014; Henao-Diaz et al., 2018).

Table 1
Experimental design: description of treatments and sample collection by study day.

Treatment groups	Pigs (n)	Treatments		Sampling schedule		Total samples	
		PRV vaccine *	PRV inoculum†	Serum, nasal swab	Oral fluid (daily)	Serum, nasal swabs	Oral fluid
NC (negative control)	10	NA	NA	0, 4, 7, 11, 14, 17, 21, 24, 28, 35, 42, 49	0 - 49	120 each	500
PRV inoculation	10	NA	Day 28	23, 27, 30, 34, 37, 41, 48, 55, 62	23 - 62	90 each	283
MLV vaccination	10	Day 7	NA	2, 7, 11, 13, 16, 20, 23, 27	2 - 27	80 each	213
MLV-PRV	10	Day 7	Day 28	2, 7, 11, 13, 16, 20, 23, 27, 30, 34, 37, 41, 48, 55, 62	2 - 62	150 each	549

NA = Not applicable; S = number of samples collected.
* Intramuscular inoculation of PRV modified live virus (MLV) vaccine (Ingelvac® Aujeszky MLV, Boehringer Ingelheim, Ridgefield, CT).
† Intranasal inoculation of PRV 3CR Ossabaw ($1 \times 10^{3.5}$ $TCID_{50}$ per pig).

2.4. PRV gB and gE antibody ELISAs

Serum samples were separately tested for PRV gB and gE antibody using commercial blocking ELISAs (Pseudorabies Virus gB Antibody Test Kit and Pseudorabies Virus gpI Antibody Test Kit, IDEXX Laboratories, Inc., Westbrook, ME). Testing and quality control was performed as directed by the manufacturer. ELISA Results were expressed as sample-to-negative (S/N) ratios (Eq. 1) with S/N ratios ≤ 0.6 considered positive.

$$S/Nratio = \frac{(sampleOD)}{(negativecontrolmeanOD)} \quad (1)$$

2.5. DNA extraction protocols

Nucleic acid extraction from nasal swab and oral fluid samples was done with the MagMax™ Pathogen RNA/DNA Kit (Life Technologies, Carlsbad, CA) on the KingFisher™ Flex Purification System (Thermo Fisher Scientific Inc., Waltham, MA) using specimen-specific high-volume modified lysis procedures provided by the manufacturer (Molecular, Bioinformatics, and Health Assurance Testing Services Section (MVHS, 2018).

For nasal swab samples, the modified lysis solution consisted of 250 μL lysis concentrate, 250 μL 100 % isopropanol (Fisher Scientific, Fair Lawn, NJ), 2 μL carrier RNA, and 0.5 μL Xeno™internal control (XIPC) RNA template at 100,000 copies/μL (Schroeder et al., 2013). Thereafter, 500 μL of the prepared lysis solution was mixed with 200 μL of vortexed nasal swab-PBS sample and 20 μL of magnetic bead mix, and then loaded into the purification system. Purification procedures were performed using Kingfisher program AM1836_DW_HV_v3 (Thermo Fisher Scientific, Inc.) using high volumes in both wash I (300 μL) and wash II (450 μL). Thereafter, the purified nucleic acids were eluted into 90 μL elution buffer.

For oral fluid samples, the lysis solution was prepared by mixing 450 μL of lysis concentrate, 2 μL carrier RNA, and 0.5 μL XIPC RNA template (100,000 copies/μL). For cell lysis, oral fluid samples (300 μL) were added to 450 μL of prepared lysis solution, after which 600 μL of this mixture was combined with 350 μL of isopropanol and 20 μL of magnetic bead mix. Thereafter, DNA purification was performed as described for nasal swab samples.

2.6. gB real-time polymerase chain reactions

To detect the presence of PRV DNA, nasal swab and oral fluid samples were tested using a qPCR (primers and probes listed in Table 2). The gB qPCR was based on an assay described by Ma et al. (2008). In brief, each of the gB PCR reactions contained 5 μL of TaqMan® Fast Virus 1-Step Master Mix (Thermo Fisher Scientific, Inc. Waltham, MA), 7.45 μL of nuclease-free water (Invitrogen™, Carlsbad, CA), 5 μL of DNA extract, 0.8 μL of PRV gB forward primer, 0.8 μL of PRV gB reverse primer, 0.4 μL of PRV gB probe, 0.2 μL of XIPC forward primer, 0.2 μL of XIPC reverse primer, and 0.15 μL of XIPC probe. All primers and probes were used at working concentration of 10 μM. PRV Shope strain DNA extract was included in each PCR run as a positive amplification control (PAC). Nuclease-free water before and after purification procedure were used as negative amplification control and reference amplification control.

Table 2
Primers and probes used in the gB screening real-time PCR.

Primers and probes		Sequences
gB real-time RT-PCR	PRV-gB-F	5′-ACAAGTTCAAGGCCCACATCTAC-3′
	PRV-gB-R	5′-GTCYGTGAAGCGGTTCGTGAT-3′
	PRV-gB-probe	FAM-5′-ACGTCATCGTCACGACC-3′-BHQ1
	XIPC-F	5′-TTCGGCGTGTTATGCTAACTTC-3′
	XIPC-R	5′-GGGCTCCCGCTTGACAATA-3′
	XIPC-probe	Cy5−5'-CTCCGCAGA-TAO-AATCCAGGGTCATCG-3'-IAbRQSp*

* Iowa Black® Dark Reverse Quencher.

PCR reagents and sample extracts were loaded into MicroAmp™ Fast Optical 96-well reaction plates (Applied Biosystems™, Waltham, MA) and sealed with MicroAmp™ optical adhesive films (Applied Biosystems™, Waltham, MA). The gB PCRs were performed using the ABI 7500 Real-Time PCR System (Thermo Fisher Scientific, Inc. Waltham, MA) with the fast mode under the following conditions: 50 °C for 5 min, 95 °C for 20 s, and 45 cycles of 95 °C for 3 s and 60 °C for 30 s. 7500 Fast System Sequence Detection Software (v1.5.1, Applied Biosystems™, Waltham, MA) was used to analyze the PCR results. The Cq values were generated with a threshold of 0.1 for the target gene and 10 % of the maximum amplitude of the sigmoidal amplification curves for XIPC controls. Test results were interpreted as follows: 1) samples with Cqs < 40 for both gB and XIPC were considered positive; 2) samples with gB Cq ≥ 40 or undetermined and XIPC Cq < 40 were considered negative; and 3) samples with XIPC Cq ≥ 40 or undetermined were retested.

2.7. Statistical analysis

Statistical analyses were performed in *R* 4.0.2 (R core team, 2020) and the CRAN (Comprehensive R Archive Network) packages specified below. Initially, raw Cq results were standardized by calculating the fold change in test samples relative to the plate-specific PACs, as shown in Eq. 2 (Livak and Schmittgen, 2001; Pfaffl, 2001; Liu and Saint, 2002; Schmittgen and Livak, 2008):

$$\text{Efficiency standardized Cq} = \text{ECq} = \left(E_{target}\right)^{-\Delta Cq_{target\ (sample\ -\ PAC)}} \quad (2)$$

In Eq. 2, E_{target} represents the PCR amplification efficiency and $\Delta Ct_{target\ (sample\ -\ control)}$ is the difference in Cq values between a test sample and the plate PACs. For each run, the PCR amplification efficiencies of PRV gB genes were estimated from the amplification plots as a function of baseline-corrected fluorescence and number of cycles. Baseline fluorescence due to inefficient quenching of fluorescence-labelled probes was measured prior to amplification-specific signals.

Baseline estimation and correction were done by determining the linear ground and early exponential phases of the PCR reactions using LinRegPCR 2020.0 software (Ramakers et al., 2003; Ruijter et al., 2009, 2013; Ruijter et al., 2014). Efficiency was estimated by fitting baseline-corrected fluorescence data into four-, five-, six-, or seven-parameter log-logistic models and selecting the one with the lowest Akaike's Information Criterion (AIC) using the CRAN package *qpcR* (Ritz and Spiess, 2008). Data generated prior to 10 PCR cycles were excluded from efficiency estimations to avoid background interference. By definition, the AIC selected the simplest model best able to fit the data (goodness of fit) based on the model-maximized log likelihood and the dimension of the model parameter space. For robustness, the efficiency of all reactions on a plate was based on the average of the efficiencies estimated for the PAC and all samples on the plate (Cikos et al., 2007), as shown in Eq. 3:

$$E_{target} = \frac{F_n}{F_{n-1}} \quad (3)$$

where E_{target} is the PCR amplification efficiency, n the number of cycles at the second derivative maximum of the sigmoidal fit of the PCR amplification curve, and F_n and F_{n-1} the fluorescence at n and $n-1$ cycles. Perfect PCR amplification (100 %) implies that the fluorescence of PCR products doubles each cycle, i.e., $E_{target} = 2$.

Qualitative analyses were performed on binary (positive, negative) results of PRV gB qPCR and gB/gE blocking ELISAs. The independency of oral fluid and nasal swab specimen PCR positivity was evaluated using Fisher's exact tests by study day and animal, that is, whether a pig tested positive/negative to PRV gB qPCR in oral fluid was more likely to obtain the same binary result in nasal swabs on given study days. Likewise, the same approach was applied to evaluate the association between binary results determined by serum gB and gE blocking ELISAs. The effect of vaccination on gB DNA detection was further evaluated by comparing post-inoculation qPCR positivity rates (oral fluid and nasal swabs) between unvaccinated (PRV group) and vaccinated animals (MLV-PRV group) using generalized linear mixed effects models that included the number of positive/negative animals (response), pig treatment (independent variable), and study day (random effect).

For quantitative analyses, because pigs were housed individually, animals were assumed to be independent and ECqs clustered by animal over time. The assumption of data normality was violated (Shapiro-Wilk test, $p < 0.001$) for both the raw and transformed (square root, cube root, and log) ECqs. Therefore, the nonparametric clustered Wilcoxon signed rank test was performed on 322 oral fluid and nasal swab samples matched by animal and study day using CRAN package (*clusrank*) and the "DS method" (Datta and Satten, 2008; Jiang et al., 2017). The null hypothesis was that the distribution of the difference between PRV ECqs in oral fluid and nasal swabs were symmetrical at mean zero.

ROC curve analyses were conducted to evaluate the diagnostic performance of the gB qPCR for nasal swab and oral fluid samples and derive diagnostic sensitivity and specificity estimates for specific ECq cutoffs using CRAN package, *pROC* (Robin et al., 2011). ROC analyses require designation of the true status (positive or negative) of the sample. Consistent with prior reports (Mettenleiter et al., 2012; Zanella et al., 2012; Wang et al., 2015; Panyasing et al., 2018a), PRV negative samples were defined as nasal swab and oral fluid samples collected in the PRV group prior to inoculation (nasal swab: $n = 20$; oral fluid: $n = 41$) and all samples in the NC group (nasal swab: $n = 80$; oral fluid: $n = 80$). PRV positive samples were derived from the PRV treatment group and were defined as nasal swabs ($n = 40$) collected 2–14 days after inoculation (study day 30–42) and oral fluid (n = 118) collected 5–17 days after inoculation (study day 33–45). The non-parametric DeLong method was used to estimate the 95 % CIs for the area under the curve (AUC) and compare nasal swab and oral fluid AUCs (DeLong et al., 1988). Estimation of diagnostic sensitivity and specificity confidence intervals was done using a nonparametric stratified bootstrapping method with 10,000 iterations (Carpenter and Bithell, 2000; Robin et al., 2011). For iteration *i*, a sample (*Xi*) was created by randomly assigning the ECqs in PRV and NC groups to one of two strata, each with the same size as the number of "true positive" and "true negative" samples. The diagnostic sensitivity and specificity for specific cutoffs were then calculated for *Xi*. Confidence interval lower and upper bounds were computed as the 5th and 95th percentiles of the sensitivities or specificities derived from 10,000 iterations (Carpenter and Bithell, 2000).

3. Results

No clinical signs were observed in the NC, MLV, and MLV-PRV treatment groups. In the PRV group, 3 of 10 pigs exhibited lethargy, ataxia, and tremors on 7 dpi, but recovered by 14 dpi. In the NC group, all serum ($n = 120$), nasal swab ($n = 80$), and oral fluid ($n = 80$) samples were negative on antibody and DNA tests, i.e., the group remained free of PRV.

The PRV qPCR testing of oral fluid samples required on 11 PCR plates with overall mean (standard deviation; SD) PCR amplification efficiency estimated at 1.69 (0.22). The qPCR testing of nasal swab samples was done on 6 plates with an estimated mean (SD) PCR amplification efficiency of 1.66 (0.11). As shown in Fig. 1, gB PCR-positive nasal swab and oral fluid samples were initially detected in the PRV group at 2 dpi (study day = 30). Nasal swab positivity peaked (9 of 10 pigs) on 2 dpi whereas oral fluid positivity peaked (9 of 10 pigs) on 9 dpi. A significant dependency between pig-matched nasal swab and oral fluid samples by study day was observed (Fisher's exact test, $p = 0.015$). That is, a pig with a gB PCR positive nasal swab was also more likely to be oral fluid gB positive and *vice versa*. As shown in Fig. 2, similar ECqs were observed in oral fluid and nasal swab samples (clustered paired Wilcoxon signed-

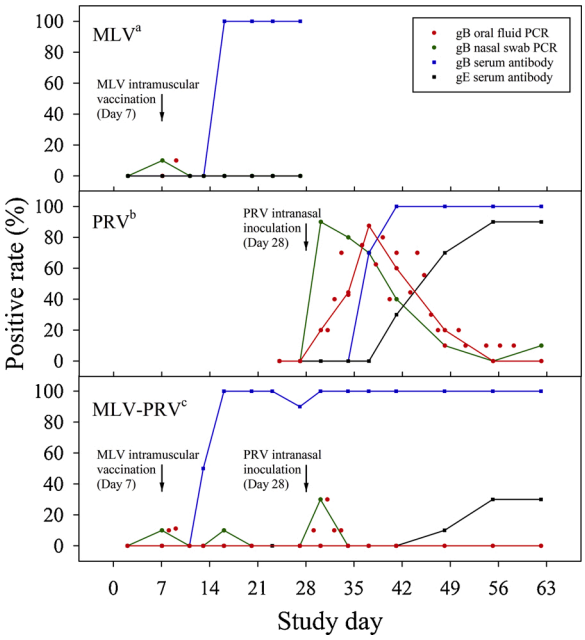

Fig. 1. Positive rate (%) in serum, nasal swab, and oral fluid specimens by test, treatment group, and study day. All serum ($n = 120$), nasal swab ($n = 80$), and oral fluid ($n = 80$) samples from the Negative Control group were negative on all tests (data not shown).
[a]MLV treatment group. Pigs ($n = 10$) intramuscularly vaccinated with a modified live virus vaccine (Ingelvac® Aujeszky MLV, Boehringer Ingelheim, Ridgefield, CT) on Day 7 and humanely euthanized on Day 27.
[b]PRV treatment group. Pigs ($n = 10$) intranasally inoculated with PRV isolate 3CR Ossabaw ($1 \times 10^{3.5}$ $TCID_{50}$ per pig) on Day 28.
[c]MLV-PRV treatment group. Pigs ($n = 10$) vaccinated on Day 7 and challenged on Day 28.

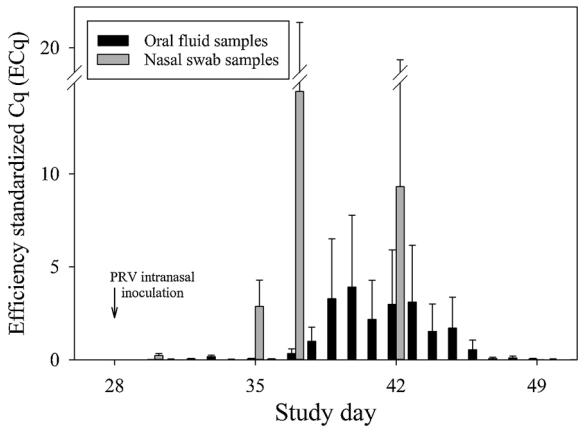

Fig. 2. PRV gB efficiency standardized Cq detection ($\bar{x}$, standard error) by specimen and study day from the PRV group (refer to Fig. 1). Efficiency standardized Cq (ECq) represents the fold-change of the quantity of PRV gB DNA in a sample relative to the plate positive amplification control (PAC). ECq = $(E_{target})^{-\Delta Ct\ target\ (sample\ -\ PAC)}$, where E_{target} is the PCR amplification efficiency and $\Delta Ct_{target\ (sample\ -\ PAC)}$ the difference in raw Cq values between test samples and plate positive amplification controls (PACs).

ranked test, $p = 0.155$). gB serum antibody was detected in 7 of 10 pigs on 9 dpi, with 10 of 10 animals positive by 11 dpi. As shown in Fig. 1, the appearance of gE serum antibody was delayed compared to gB. Despite temporal differences in kinetics, a significant correlation between serum gB and gE blocking ELISAs was observed (Fisher's exact test, $p < 0.001$).

In the MLV-PRV group, gB DNA testing produced one false positive nasal swab collected on the day of vaccination (study day 7). Otherwise, PRV gB DNA was sporadically detected in nasal swab and oral fluid samples for 9 days post vaccination (dpv) and 4 days post challenge. The maximum nasal swab positivity occurred on 9 dpv (1 of 10 pigs) and 2 dpi (3 of 10 pigs). Similarly, oral fluid positivity peaked (3 of 10 pigs) on 2 dpi. gB serum antibody was detected on 6 dpv and remained detectable in all pigs until the end of the study. In contrast, gE antibody was not detected until day 20 post challenge and was detected in 3 of 10 pigs. Statistical analysis showed no dependence between the two serum ELISAs (Fisher's exact test, $p > 0.05$). The effect of vaccination on gB DNA detection was further evaluated by comparing post-inoculation qPCR positivity rates (oral fluid and nasal swabs) between unvaccinated (PRV group) and vaccinated animals (MLV-PRV group) using generalized linear mixed effects models. For the post wild-type virus exposure period (study day 29–49), the odds of gB positivity were significantly lower for vaccinated pigs (MLV-PRV group) than unvaccinated pigs (PRV group) in both nasal swab (odds ratio (OR) = 0.016, 95 % confidence interval (CI): 0.003, 0.097) and oral fluid (OR = 0.053, 95 % CI: 0.022, 0.125).

In the MLV group, among 80 nasal swab and 213 oral fluid samples, two positive gB DNA testing results were observed: one false positive nasal swab sample collected on the day of vaccination (study day 7) and one oral fluid gB DNA positive in one pig 1 dpv (Fig. 1). gB antibody was detected starting day 9 post MLV vaccination and persisted through the termination of the observation period; gE antibody was not detected because the vaccine was a gE-deleted MLV.

The ROC curve analyses for the PRV gB PCR were based on ECq results for nasal swabs ($n = 140$) and oral fluids ($n = 239$), with true infection status assigned as specified in the Material and Methods. Samples with no Cq measured by the PCR instrument were denoted ECq = 0, i.e., the PCR target was absent. The performance of the PRV gB qPCR was similar for both nasal swab (AUC = 85 %, 95 % CI: 78 %, 92 %) and oral fluid (AUC = 83 %, 95 % CI: 79 %, 88 %) specimens, i.e., no difference was detected between the two AUCs (DeLong method, $p = 0.6979$). Diagnostic sensitivity and specificity by ECq cut-off are given in Table 3.

Table 3
PRV gB PCR diagnostic sensitivity (%) and specificity (%) by specimen and cutoff *.

	Nasal swab		Oral fluid	
Cutoff (ECq)	Diagnostic sensitivity (95 % CI)	Diagnostic specificity (95 % CI)	Diagnostic sensitivity (95 % CI)	Diagnostic specificity (95 % CI)
0.01	70.0 (55.0, 82.5)	100.0 (NA, NA)	52.5 (43.2, 61.9)	100.0 (NA, NA)
0.02	67.5 (52.5, 80.0)	100.0 (NA, NA)	43.2 (33.9, 52.5)	100.0 (NA, NA)
0.03	62.5 (47.5, 77.5)	100.0 (NA, NA)	38.1 (29.7, 46.6)	100.0 (NA, NA)
0.04	60.0 (45.0, 75.0)	100.0 (NA, NA)	28.0 (20.3, 36.4)	100.0 (NA, NA)
0.05	60.0 (45.0, 75.0)	100.0 (NA, NA)	25.4 (17.8, 33.1)	100.0 (NA, NA)
0.06	47.5 (32.5, 62.5)	100.0 (NA, NA)	23.7 (16.1, 31.4)	100.0 (NA, NA)

NA: not applicable.
ECq: efficiency standardized Cq represents the fold change of PCR target quantity in test samples relative to which in positive amplification controls.
* Point estimates of diagnostic sensitivities/specificities derived from ROC analyses.

4. Discussion

In this study, the patterns of PRV qPCR detection in nasal swabs agreed with prior reports based on virus isolation and PRV gB/gE PCRs (Arellano et al., 1992; Mengeling et al., 1992; Zanella et al., 2012). Likewise, using a PCR protocol similar to that used in the current study, Panyasing et al. (2018a), 2018b reported the detection of PRV gB DNA in oral fluid specimens from 3 to 21 dpi, the termination of the study. The present study expanded upon prior reports by providing gB qPCR results on nasal swab and oral fluid samples from PRV, MLV, MLV-PRV, and NC treatment groups for up to 34 dpi (Fig. 1) in the context of gB and gE serum antibody responses. Consistent with previous reports, PRV gB DNA was detected in the PRV group between 2 and 34 dpi in nasal swabs and 2 and 30 dpi in oral fluid samples. In contrast, PRV gB DNA detection in samples from vaccinated animals (MLV and MLV-PRV treatments) was both less frequent and shorter.

This study proposed a solution to two issues associated with PCR-based testing. The first issue is the effect of amplification efficiencies on PCR results. Current methods for the relative quantitation of nucleic acids in biological specimens assume 100 % amplification efficiency for all PCR reactions and/or equal efficiency among PCR targets under all circumstances, whereas the actual amplification efficiency in real-time PCR experiments can vary from 70 % to 100 % (Gibson et al., 1996; Livak and Schmittgen, 2001; Liu and Saint, 2002; Wong and Medrano, 2005; Yuan et al., 2008; Ruijter et al., 2009; Svec et al., 2015). Importantly, even a small change in amplification efficiency leads to major differences in the PCR results (Arezi et al., 2003; Caraguel et al., 2011). For example, using Eq. 4 (Tichopad et al., 2003), it can be shown that a PCR with 100 % amplification efficiency ($E = 2.0$) will produce 102,400 copies/mL of PCR product ($P = 102{,}400$) after 10-cycles of amplification ($n = 10$) from a test sample with 100 copies/mL of input PCR target ($I = 100$). Under the same test parameters, a PCR with 90 % amplification efficiency ($E = 1.9$) would produce 61,311 copies/mL ($P = 61{,}311$). That is, a 0.1 reduction in E would result in ~40 % reduction in the number of copies per mL resulting from the reaction.

$$P = I \times E^{n} \quad (4)$$

The second issue is the selection of PCR cutoffs. The positive/negative status of PCR test samples is commonly based on the number of PCR amplification cycles (Cqs) completed at the point that the fluorescence intensity crosses a fixed threshold (Heid et al., 1996). Samples with Cq values lower than a defined cutoff are classified as positive and samples

with Cq values higher than the cutoff are negative. Although the World Organization of Animal Health (OIE) guidelines recommend establishing assay cutoff as a function of diagnostic performance, e.g., diagnostic sensitivity and specificity, the selection of Cq cutoff more often relies on the developer's experience or on analytical sensitivity (limit of detection) studies (Bustin and Nolan, 2004; Burns and Valdivia, 2008; Bustin et al., 2009; OIE, 2019; Vettraino et al., 2010). The fundamental obstacle to establishing PCR cutoffs and assessing diagnostic performance using a statistical approach is the fact that PCR-negative samples are not assigned a specific Cq value (Burns and Valdivia, 2008; Bustin et al., 2009). Reporting negative samples as the number of PCR cycles programmed to a run, e.g., 40 or 45, is statistically invalid because a truncated Cq value neither represents the absence of target nor a measurement (Elfving et al., 2014; Shipley, 2020). In terms of performance evaluation, neither the exclusion of negative results nor the use of truncated Cqs can produce valid analyses and inferences (Caraguel et al., 2011; Trang et al., 2015). The current study has provided a solution by calculating ECq, the fold-change of PCR targets in a sample compared to a calibrator (PAC). Negative samples with no Cq assigned by PCR instruments can be ECq of 0 which represents the quantity of target is 0 (0 times any value is 0). Thus, the use of ECq allows these negative samples to be included for statistical analyses and diagnostic evaluation.

Working in gene expression research, Pfaffl (2001) used the quantitative detection of endogenous reference genes to normalize target expression and remove non-biological variation, e.g., variability introduced by DNA/RNA purification, PCR inhibitors, and differences in reverse transcription efficiencies (Pfaffl, 2001; Huggett et al., 2005; Chervoneva et al., 2010). Thus, R in Eq. 5 represents the ratio of the target gene in a test sample relative to a standardized control where E_{target} is the PCR amplification efficiency of the target gene, E_{ref} the amplification efficiency of the reference gene; $\Delta Ct_{\text{target (control – sample)}}$, and $\Delta Ct_{\text{ref (control - sample)}}$ the difference of Cq values between the standardized control and a test sample of the target and reference genes.

$$R = \frac{\left(\mathrm{E}_{\text{target}}\right)^{\Delta \mathrm{Ct}_{\text{target (control-sample)}}}}{\left(\mathrm{E}_{\text{ref}}\right)^{\Delta \mathrm{Ct}_{\text{ref (control-sample)}}}} \quad (5)$$

Distinct from gene expression research conducted under experimental conditions, diagnostic PCRs for pathogen detection must function reliably with various biological specimens and the diverse collection, handling, and storage conditions to which they are exposed prior to reaching the diagnostic laboratory. Under these circumstances, host endogenous reference genes expressed uniformly across specimens and animals for diagnostic data normalization have not been identified. Adding an exogenous reference gene to the sample prior to DNA/RNA extraction and PCR is a common approach for monitoring the testing process (Chittick et al., 2011; Schroeder et al., 2013; Kittawornrat et al., 2014; Pepin et al., 2015; Panyasing et al., 2016), but exogenous reference genes cannot be used for data normalization because they do not reflect the total initial DNA/RNA in the test sample matrix (Pfaffl, 2001). In this study, the ECq method (Eq. 2) was used to standardize PCR responses in a diagnostic testing environment. Eq. 2 excluded the reference gene-based normalization (denominator in Eq. 4) and used the PCR amplification efficiency for each plate estimated from the second derivative maximum point of the sigmoidal model that best fit the amplification plot (Luu-The et al., 2005; Ritz and Spiess, 2008; Ruijter et al., 2009). Alternatively, PCR amplification efficiency could be estimated from the standard curve.

The net effect of this approach is standardization of sample test results relative to the PAC in terms of "fold change". For example, given a valid PCR run with 80 % PCR amplification efficiency ($E_{\text{target}} = 1.8$), a sample (S_1) with a Cq of 31, and a plate PAC with a Cq of 30, the ECq of S_1 can be calculated as 0.55 ($\text{ECq} = 1.8^{-(31-30)} = 0.55$). That is, the initial quantity of the PCR target in S_1 was 0.55 times the quantity of the PCR target in the PAC. Under the same test parameters, the ECq of sample (S_2) with a Cq of 27 may be calculated as 5.83 ($\text{ECq} = 1.8^{-(27-30)} = 5.83$), meaning that the initial quantity of PCR target in S_2 was 5.83 times that of the PAC and 10.6 times (5.83/0.55 = 10.6) that of S_1. Correction for PCR amplification is crucial to the standardization process. Thus, if the PCR amplification efficiency were 70 % ($E_{\text{target}} = 1.7$), a sample (S_3) with a Cq of 27 (i.e., identical to S_2) run on a plate with a PAC with a Cq of 32 would have a ECq of 14.20 ($\text{ECq} = 1.7^{-(27-32)} = 14.20$). That is, the initial quantity of PCR target in S_3 was 2.44 times S_2 (14.20/5.83 = 2.44), despite the fact that S_2 and S_3 had the same raw Cq.

Overall, this study described the temporal detection of PRV gB DNA in oral fluid specimens from animals of known infection and/or vaccination status and provided estimates of the diagnostic sensitivity and specificity of the gB qPCR assay. The primary limitation of the study is the inclusion of a relatively small number of animals under experimental conditions. Conversely, these conditions allowed for the precise determination of the true PRV status of the animal. Regardless, under the testing conditions described in this study, an ECq of 0.01 obtained the best diagnostic performance (Table 1) and was recommended as the optimal diagnostic cutoff. This approach could also be used for other qPCRs routinely performed in diagnostic laboratories, but would require standardization of the PAC preparation.

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Declaration of Competing Interest

The authors declare no conflicts of interest with respect to their authorship and/or the publication of this manuscript, with the exception that J. Zimmerman serves as a consultant to IDEXX Laboratories, Inc. on areas of diagnostic medicine independent of this study. The terms of the consulting arrangement have been reviewed and approved by Iowa State University in accordance with its conflict of interest policies.

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