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Pathogenicity of three type 2 porcine reproductive and respiratory syndrome virus strains in experimentally inoculated pregnant gilts

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ABSTRACT

Mechanisms of reproductive failure resulting from infection with porcine reproductive and respiratory syndrome virus (PRRSV) are still poorly understood. Presented herein are the results of a side-by-side evaluation of the pathogenicity of three type 2 PRRSV strains in a reproductive model, from a pilot study used to develop experimental conditions and laboratory methods for a larger experiment. Pregnant gilts were experimentally infected with PRRSV at gestation day 85 or served as uninfected negative controls. After 21 days, all gilts and fetuses were necropsied. Clinical signs, litter outcome, viral load, cytokine levels, and pathology were compared from samples collected among pigs exposed to the three PRRSV strains. Based on differences in histologic lesions, and fetal weights, and numeric differences in gilt serum cytokine levels, litter outcome and virus replication in fetal tissues KS06-483 appeared less virulent than NVSL 97-7895 and KS06-72109 isolates. Levels of chemokine ligand 2 (CCL2), interferon alpha (IFNα), and interferon gamma (IFNγ) were increased in PPRRSV-infected compared to non-infected gilts ($0.01 > P < 0.06$). Inoculation with NVSL 97-7895 induced higher levels of all three cytokines. All three PRRSV isolates were able to induce high mean viral load in individual litters, which was closely related to the proportion of PRRSV positive fetuses in the litter. Viral load in fetal samples was also positively associated with viral load at the maternal–fetal interface. All but one dead fetus were positive for PRRSV RNA, and higher concentrations of PRRSV RNA in fetal thymus increased the odds of fetal death. Our results suggest that virus replication in fetal tissues and the maternal–fetal interface, but not in other gilt tissues, are important for the outcome of reproductive PRRS. Additionally, our data indicate that umbilical lesions decreased corresponding to the use of pentobarbital sedation prior to euthanasia of pregnant gilts by captive bolt.

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1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most costly diseases affecting the global swine industry (Holtkamp et al., 2013; Nieuwenhuis et al., 2012). Although reproductive disease associated with PRRS virus (PRRSV) contributes to over $300 million in losses annually in the USA alone (Holtkamp et al., 2013), a proportionately small amount of research has focused on the reproductive form of the disease, and the underlying mechanisms of PRRSV-induced reproductive failure are still poorly understood. The outcome of infection in pregnant sows and gilts largely depends on the stage of gestation. In early gestation, PRRSV can cause embryonic death (Prieto et al., 1996, 1997), while in mid-gestation the virus does not readily cross transplacentally and does not induce reproductive failure (Christianson et al., 1993; Kranker et al., 1998). PRRSV infection in late gestation consistently

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results in transplacental infection of fetuses and clinical manifestation characterized by abortions, early farrowings, fetal death, and the birth of weak, congenitally infected piglets resulting in elevated pre-weaning mortality (Cheon and Chae, 2001; Kranker et al., 1998; Mengeling et al., 1994; Terpstra et al., 1991). The mechanisms of transplacental infection and fetal death remain unclear. It has been proposed that PRRSV-induced fetal death is not a direct result of PRRSV replication in the fetus, but is rather related to virus replication in the maternal–fetal interface resulting in apoptosis and necrosis of infected and surrounding cells leading to detachment and degeneration of the fetal placenta (Karniychuk et al., 2011, 2012).

PRRSV can be divided into two distinct genotypes: type 1 (European) and type 2 (North American). PRRSV strains show a high degree of genetic diversity, both between and within genotypes, leading to the sub-classification into at least 3 European subtypes (Stadejek et al., 2008) and multiple North American clades (Shi et al., 2010a,b). Different strains also display important biological differences, such as the ability and/or efficiency of propagation in different cell types (Benfield et al., 1992; de Abin et al., 2009), pathogenicity (Halbur et al., 1995, 1996; Han et al., 2013a; Martinez-Lobo et al., 2011; Morgan et al., 2013) and antigenic properties (Magar et al., 1997; Nelson et al., 1993; Wensvoort et al., 1992). Investigations of the pathogenicity of different PRRSV isolates were mainly performed in young pigs using respiratory models whereas relatively few studies have evaluated differences in pathogenicity of PRRSV isolates in a reproductive model (Cheon and Chae, 2004; Mengeling et al., 1996).

The present experiment was a pilot study to develop experimental conditions and laboratory methods for a larger experiment investigating phenotypic and genotypic predictors of PRRSV resistance in pregnant gilts (Ladinig et al., 2014b). Yet it is one of a select few experiments enabling side-by-side comparison of distinct strains in a reproductive model. Three type 2 PRRSV isolates were used to inoculate late-term pregnant gilts. The first and main objective of this study was to compare the pathogenicity of the three PRRSV isolates by investigating clinical signs, litter outcome, and levels of PRRSV RNA in fetal tissues. This information was used to select the virus isolate used in the main project. Given the differences we found amongst the three isolates in objective 1 and results of cytokine responses we subsequently obtained in our main project (Ladinig et al., 2014a), the second objective was to explore strain differences in histologic lesion severity in fetal tissues and the maternal–fetal interface, as well as cytokine profiles in gilt serum. This enabled us to gain insights into potential mechanisms of PRRSV reproductive pathogenicity.

2. Materials and methods

2.1. Animals

Purebred Landrace gilts from a high-health nucleus herd (free of PRRSV, *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae* based on absence of clinical signs and routine serologic monitoring) were selected at approximately 150 days of age in two experimental repetitions (rep one: $n = 7$; rep two: $n = 8$). Gilts were vaccinated against porcine parvovirus (PPV), erysipelas, *Leptospira* spp. (Farrowsure Gold B, Zoetis Animal Health, Canada, Kirkland, QC) twice prior to breeding, porcine circovirus type 2 (PCV2; Circoflex, Boehringer-Ingelheim (Canada) Ltd. Burlington, ON) at 3 weeks of age, and *Haemophilus parasuis* (Suvaxyn HPS, Zoetis) at 9 and 22 weeks of age. Estrus was stimulated by direct daily boar contact, and after each gilt had shown at least one estrus, they were moved into individual crates and synchronized by daily oral administration of 17.6 mg altrenogest (8 mL Regu-Mate 0.22% Solution, Merck Animal Health, Kirkland, QC) for a total of 18 days. Thirty-six hours after the last Regu-Mate dose, 800 international units (IU) pregnant mare serum gonadotropin (4 mL Folligon, Merck Animal Health) was administered intramuscularly (IM). Gilts were artificially inseminated using homospermic semen from Yorkshire boars. Pregnancy was confirmed ultrasonically, and pregnant gilts were housed in gestation stalls until gestation day 80 (±1) when they were transported to a biosafety level 2 (BSL2) animal care facility at the University of Saskatchewan. Prior to transportation, blood was drawn from the jugular vein to confirm PRRSV negativity by ELISA (IDEXX PRRS X3 Ab test, IDEXX laboratories, Inc., Maine, US) and PCR (Tetracore PRRSV real-time PCR kit, Tetracore, Inc., Rockville, USA). In BSL2, gilts were randomly allocated to one of the three PRRSV-challenged or the reference control group. Each of the four groups was placed in a separate room, with gilts housed in individual crates. Gilts had free access to water and were fed a standard wheat/barley-based gestation diet, 2.5 kg once daily, throughout gestation.

Table 1
Pairwise comparison of nucleotide identity in ORF5 among the three PRRSV isolates and VR2332.

	NVSL 97-7895	KS06-483	KS06-72109
KS06-483	89		
KS06-72109	89	88	
VR2332	91	94	89

2.2. PRRSV isolates

Three North American PRRSV isolates (generously provided by R. Rowland, Kansas State University) were used: NVSL 97-7895 (GenBank Accession No. AF325691), KS06-483 (GenBank Accession No. JX258843), and KS06-72109 (GenBank Accession No. KM035803). Based on the nucleotide sequence of ORF5, the three isolates were distinct from each other (Table 1, Fig. 1).

All three isolates were propagated in MARC-145 cells (generously provided by S. Carman, Animal Health Laboratory, University of Guelph) in minimum essential medium (MEM) supplemented with 1.6% Penicillin-streptomycin and 7% fetal bovine serum. Aliquots of stock virus were frozen at −80 °C. On each day of inoculation, one aliquot of each virus isolate was thawed and diluted to a final concentration of 1×10^5 $TCID_{50}$ in 4 mL MEM immediately prior to use.

2.3. Experimental procedures

The experiment was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use (permit #20110102).

After a five day acclimation period in BSL2, gilts were inoculated with 1×10^5 $TCID_{50}$ in 4 mL of their respective PRRSV isolate, split 2 mL IM and 1 mL into each nostril (experimental day 0). Control gilts (CTRL, $n = 3$) were similarly mock-inoculated with MEM. All gilts were observed twice daily for clinical signs and demeanor. Rectal temperatures and feed intake were assessed once daily. Rectal temperature exceeding 39.5 °C was considered a fever. Serum samples were collected on 0, 2, 7 and 19 days post inoculation (dpi), aliquoted and stored at −80 °C.

At 21 dpi (gestation day 106 ±1), the experiment was terminated and gilts were humanely euthanized by cranial captive bolt followed by pithing. In rep two, euthanasia technique was modified and gilts were sedated with intravenous barbiturate (30 mL Euthanyl Forte supplying 16,200 mg pentobarbital sodium, Vetoquinol, Lavaltrie, QC) immediately prior to euthanasia by

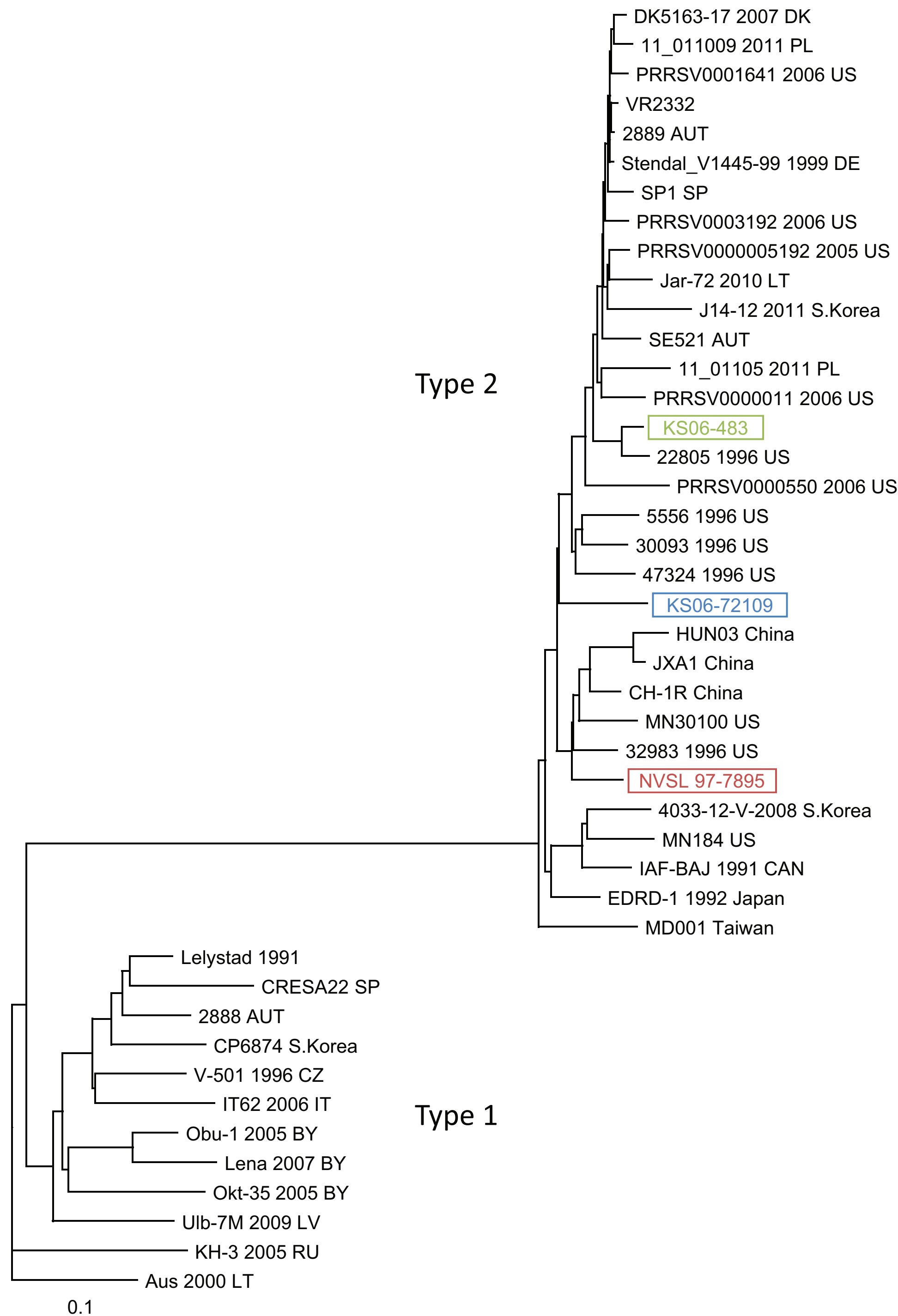

Fig. 1. Molecular phylogeny of Type 1 (EU genotype) and Type 2 (NA genotype) PRRSV. To generate the phylogenetic tree, multiple sequence alignments of ORF5 sequences from field isolates found in various countries (12 type 1 and 32 type 2 isolates downloaded from GenBank) and the three isolates used to inoculate gilts (NVSL 97-7895, KS06-483, and KS06-72109) were constructed with ClustalW (Thompson et al., 1997) with a gap opening/gap extension penalty of 50/5. Alignments were manually inspected and minor edits were made when necessary with GeneDoc (Nicholas et al., 1997) before trees were constructed using the neighbor-joining method in PHYLIP (Felsenstein, 1989).

captive bolt. The gravid reproductive tract was removed intact, placed in a trough and rinsed of maternal blood. The linearized uterus was carefully opened starting at the tip of each horn. Fetuses were numbered sequentially according to their position within each horn with “L1” and “R1” being the fetuses closest to the ovary on the left and right sides, respectively. Fetal preservation status was categorized as: viable (VIA), decomposed (DEC; dead with primarily white skin), autolyzed (AUT; dead with over 50% brown discolored skin), and mummified (MUM; <20 cm crown-rump-length) (Ladinig et al., 2014b). MUM were deemed to have died prior to inoculation and were excluded from further analyses. The weight of each fetus was recorded. Samples of lung, tonsil, and reproductive lymph node (*Lnn. uterini*) were collected from each gilt. Amniotic fluid was sampled from the stomach of each fetus using a sterile needle and syringe. Samples of thymus, lung, liver, heart, and umbilical cord were collected along with a sample of the

adjacent uterus (endometrium and myometrium), placenta and uterine artery from each fetus. Additionally, samples of mesenteric lymph node and kidney were collected from fetuses of rep 1 gilts, and cerebellum was collected from fetuses of rep 2 gilts. Fetal tissue samples were not collected from AUT fetuses in rep 1 gilts. Tissue samples were immediately frozen at −80 °C until further processing or fixed in 10% formaldehyde for microscopic evaluation. In VIA and DEC fetuses, blood was collected from the umbilical cord and serum subsequently separated and stored at −80 °C. To prevent cross contamination, instruments and necropsy surfaces were rinsed and disinfected with Synergize (Pro-AG products Ltd., Winnipeg, MB, Canada) for at least 10 min between animals (gilts and fetuses).

2.4. Quantification of PRRSV RNA

RNA was extracted from 140 μL of serum and amniotic fluid using the QIAamp Viral RNA mini kit (Qiagen Inc., Toronto, ON) or from 10 to 20 mg of tissue using the RNeasy extraction kit (Qiagen Inc., Toronto, ON) according to the manufacturer's instructions.

PRRSV RNA concentrations were measured in gilt serum (0, 2, 7, 19 dpi), gilt and fetal tissue samples, and fetal serum by quantitative reverse transcription polymerase chain reaction (qRT-PCR). An in-house SYBR assay was developed specifically for each virus strain used in the experiment. Primer sequences targeting a highly conserved region at the C-terminal end on ORF7 of each isolate were: NVSL 97-7895-F 5′-TAA TGG GCT GGC ATT CCT-3′; KS06-483-F 5′-TGA TGG GCT GGC ATT CTT-3′; KS06-72109-F 5′-TGA CGG GCT GGC ATT CTT-3′; all isolates-R 5′-ACA CGG TCG CCC TAA TTG-3′. For samples from CTRL gilts, a forward primer detecting all isolates was used: F 5′-TRA TGG GCT GGC ATT CYT-3′. Reactions contained 2 μL of sample or standard, 12.5 μL of Master mix (Brilliant II SYBR QRT-PCR Low ROX 1 Step Master Mix, Agilent technologies Canada Inc., Mississauga, ON), 5 pmol of forward primer, 5 pmol of reverse primer, 1 μL of reverse transcriptase/RNase block enzyme mixture (Brillant II qRT-PCR Master Mix, Agilent technologies Canada Inc., Mississauga, ON), and RNase-free water to a total volume of 25 μL. Reverse transcription was performed at 50 °C for 30 min followed by a PCR initial activation step at 95 °C for 10 min, and 38 cycles of a three-step denaturation (30 s at 95 °C), annealing/extension (30 s at 59 °C), and second annealing/extension (15 s at 74 °C). A dilution series (10^7–10^1 copies/μL) of *Hin*dIII linearized plasmid containing a 446 bp sequence specific for ORF7 of each isolate was used as a standard curve. Duplicate test samples and standards in triplicate were run on each PCR plate on a Stratagene MX3500P (Agilent Technologies, Mississauga, ON). Results were reported as logarithm base 10 ($\log_{10}$) target RNA concentration per mg or μL. The lower limit was <10 viral copies/μL and was noted as not detected (nd).

2.5. Serum cytokine testing

Gilt serum samples collected at 0, 2, 7 and 19 dpi were analyzed for a broad selection of cytokines/chemokines representative of the innate (interleukin [IL] 1β, IL8, chemokine ligand 2 [CCL2] and interferon alpha [IFNα]), T helper 1 (Th1; IL12, interferon gamma [IFNγ]), T helper 2 (Th2; IL4, IL13), and regulatory (IL10) immune responses, and selected based on the availability of antibody pairs suitable for the detection of swine cytokines. Interleukins 1β, IL4, IL8, IL10, IL12, CCL2 and IFNa were tested by Fluorescent Microsphere Immunoassays (FMIA) as previously described (Ladinig et al., 2014a; Lawson et al., 2010). An enzyme-linked immunosorbent assay (ELISA) was used to measure IFNγ and IL13. Methods for the IFNγ ELISA were previously described (Ladinig et al., 2014a). For IL13, ELISA plates were coated with IL13 anti-swine polyclonal capture antibody (Ab) (Kingfisher Biotech Inc., Saint Paul, MN, USA) diluted in Coating Buffer A (Life Technologies Inc., Burlington, ON). 5X Assay Buffer (Life Technologies Inc., Burlington, ON) diluted in ddH2O was used as a blocking solution and for dilution of biotinylated anti-swine polyclonal detection Ab (Kingfisher Biotech Inc., Saint Paul, MN, USA), Streptavidin-HRP Ab (Life Technologies Inc., Burlington, ON) and serum samples (1:5). Serum IL13 concentrations (pg/mL) were estimated by comparison with a 4-parameter standard curve generated by SoftMaxPro software v5.4 (Sunnyvale, CA, USA).

2.6. Microscopic assessment of PRRSV-associated lesions

Gilt and fetal samples collected in 10% buffered formalin were fixed for 24 h, routinely processed, paraffin embedded, and microsectioned for Hematoxylin and Eosin (H&E) staining. All tissues were examined for the presence or absence of characteristic lesions associated with *in-utero* PRRSV infection, primarily including: multicentric lymphoplasmacytic and histiocytic arteritis, panvasculitis, and perivasculitis (Lager and Halbur, 1996; Rossow, 1998; Rossow et al., 1996). Other previously reported lesions including lymphoplasmacytic endometritis/metritis (Rossow, 1998), placentitis (Karniychuk et al., 2012; Rossow, 1998), myocarditis (Lager and Halbur, 1996; Rossow et al., 1996), encephalitis (Lager and Halbur, 1996), hepatitis (Lager and Halbur, 1996; Rossow et al., 1996), focal hemorrhage and necrosis in the umbilical cord, kidney and lung (Lager and Ackermann, 1994; Lager and Halbur, 1996; Rossow et al., 1996), polykaryocytes in the lymph nodes (Lager and Halbur, 1996; Rossow et al., 1994), and focal placental detachment and trophoblast degeneration (Karniychuk et al., 2012; Rossow, 1998) were similarly categorized.

2.7. Statistical analysis

All statistical analyses were performed using Stata v13 (StataCorp, College Station, TX). A Kruskal–Wallis test was used to assess potential group differences in gilt-level clinical data (days with reduced feed intake, days with fever), litter size and litter outcome, PRRSV RNA concentrations (viral load) in gilt serum and tissues, mean PRRSV RNA concentrations in fetal thymus and fetal serum per litter, and cytokine levels in gilt serum over time (area under the curve (AUC) calculated using the formula: $AUC = (t_1 - t_0)(a_1 + a_0)/2 + (t_2 - t_1)(a_1 + a_2)/2 + \ldots + (t_n - t_{n-1})(a_{n-1} + a_n)/2$). If treatment groups differed significantly, a rank sum test was used to evaluate all pairwise comparisons. To account for multiple comparisons, $P < 0.01$ was considered significant and a trend reported if $0.01 < P < 0.1$.

Multilevel mixed-effects linear regression models (XTMIXED) controlling for litter of origin (gilt_id) as a random effect were used to assess group differences in viral load in fetal tissues and fetal weight. XTMIXED was also used to evaluate associations between PRRSV RNA concentrations in endometrium and different fetal compartments (fetal thymus, fetal serum, and amniotic fluid). The association between fetal death (used as dichotomous outcome) and viral load in fetal thymus was evaluated using multilevel mixed-effects logistic regression models (XTMELOGIT) controlling for litter of origin as a random effect. For all analyses of PRRSV viral load, only fetuses from inoculated gilts were used, and models controlled for fetal preservation as a fixed effect. All final models were evaluated to ensure normality and homoscedasticity of residuals.

Finally, a Fisher's exact test was used to compare the frequency of histologic lesions in various tissues of inoculated gilts and their fetuses among the three PRRSV inoculated groups, between live and dead fetuses, and between experimental replicate (rep) for fetal umbilical lesions.

3. Results

3.1. Clinical signs and litter outcomes

No gilt demonstrated respiratory signs including dyspnea or persistent paroxysmal coughing, or showed signs of lethargy or depression following infection with any of the three virus isolates. Reduced daily feed intake was observed in 4/4 gilts infected with NVSL 97-7895, and 2/4 gilts each with KS06-483 and KS06-72109. While 3/4 NVSL 97-7895 infected gilts and the two KS06-483 infected gilts had reduced feed intake for only one to three days, the two gilts infected with KS06-72109 were more severely affected with reduced feed intake for nine and 14 days. Fever, defined as a gilt with a rectal temperature exceeding 39.5 °C any day after PRRSV inoculation, was detected in 2/4 NVSL 97-7895 gilts (for two and three days), 1/4 KS06-483 gilts (for four days), and 2/4 KS06-72109 gilts (for one and three days). The number of days with reduced feed intake ($P = 0.47$) and the number of days with fever ($P = 0.59$) did not differ significantly among treatment groups.

The average litter size (excluding MUM) across all 15 gilts was 11.9 ± 3.7 fetuses per litter. MUM fetuses were found in 2 gilts (one mummy in one CTRL, three mummies in one KS06-72109 gilt) and were excluded from further analysis. The litter outcome from all 15 gilts, including number and location of fetuses and fetal preservation category, is illustrated in Figs. 2 and 3. The percentage of dead fetuses per litter, although numerically lower in KS06-483 gilts, did not differ significantly among groups ($P = 0.15$; Fig. 4A) due to the low number of gilts per treatment group.

Analyses of fetal weight only included data from VIA fetuses because autolysis precluded accurate measurement of weight in DEC and AUT fetuses. The effect of litter size on fetal weight was tested, but due to non-significance was not included in the statistical model comparing differences in fetal weight of VIA fetuses among treatment groups. Differences among treatment groups trended toward significance ($P = 0.1$), with fetuses from NVSL 97-7895 and KS06-72109 inoculated gilts being lighter than fetuses from CTRL gilts ($P = 0.04$) (Fig. 5).

3.2. Quantification of PRRSV RNA in gilt and fetal samples

PRRSV RNA was not detected by qRT-PCR in tissue or serum samples from CTRL, nor from sera of inoculated gilts collected prior to inoculation. All inoculated gilts were viremic on 2 and 7 dpi, and 3/4 gilts from each inoculated group remained viremic on 19 dpi. Peak levels of viremia occurred on 7 dpi for all except two gilts (one NVSL 97-7895, one KS06-483), which peaked on 2 dpi and had similar concentrations on 7 dpi (Fig. 6A). Lung tissue was PRRSV RNA positive in all except one gilt inoculated with KS06-72109 (Fig. 6B). All gilt tonsil and reproductive lymph nodes tested positive by qRT-PCR at termination (Fig. 6C and D). There were no significant differences in PRRSV RNA levels in tested tissues among the three PRRSV strains ($P \geq 0.22$ for all).

Fetal viral load is presented in Table 2. Although not statistically significant, the highest viral load within fetuses (thymus, serum and amniotic fluid) and the highest percentage of positive samples were found in fetuses from NVSL 97-7895 gilts (Table 2). The mean viral load in fetal thymus and fetal serum from all tested fetuses was calculated for each litter. All three PRRSV isolates were able to induce high mean viral load in individual litters (Fig. 4B and C) and mean viral load was closely related to the proportion of PRRSV positive fetuses in the litter. The mean percentage of fetuses in which PRRSV RNA was detected in either thymus, serum or amniotic fluid were 69.4 (±35), 56.0 (±24), and 79.4 (±25) for NVSL 97-7895, KS06-483, and KS06-72109, respectively. This did not differ across group ($P = 0.55$). PRRSV negativity in fetuses was clearly associated with fetal preservation status. While 43/97 VIA fetuses

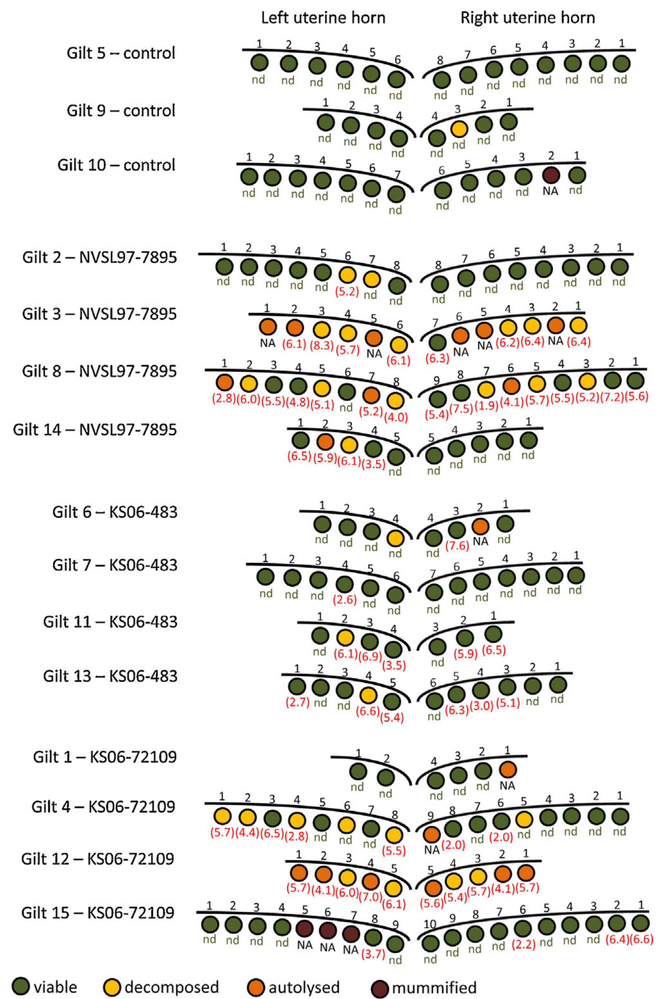

Fig. 2. Fetal preservation and position across PRRSV strain. Litter outcome is presented for all 15 gilts included in this study. Each fetus is represented by a colored circle. The relative location of each circle represents the position of each fetus within left and right uterine horn; the color represents the preservation categories: green = viable; yellow = decomposed; orange = autolyzed; red-brown = mummified. Mummified fetuses were those with crown rump length less than 20 cm and were deemed dead prior to inoculation, hence excluded from all analyses. PRRSV RNA concentrations in thymus ($\log_{10}$ copies/mg) are provided for each fetus below the circles; nd = not detected; NA = not analyzed.

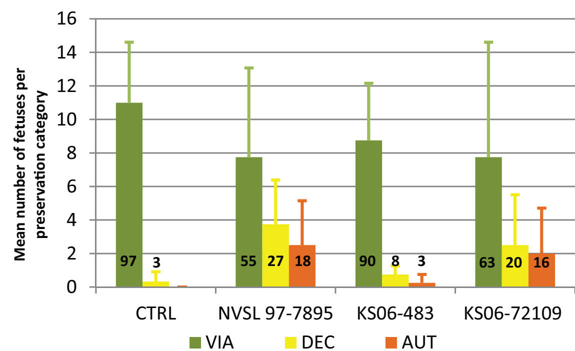

Fig. 3. Mean number of fetuses per preservation category. The mean numbers of viable (VIA), decomposed (DEC), and autolyzed (AUT) fetuses are presented for control (CTRL) and each PRRSV strain. Error bars indicate standard deviation; numbers indicate the percentage of fetuses in each preservation category. Due to the low number of gilts per treatment group, differences among groups were not significant.

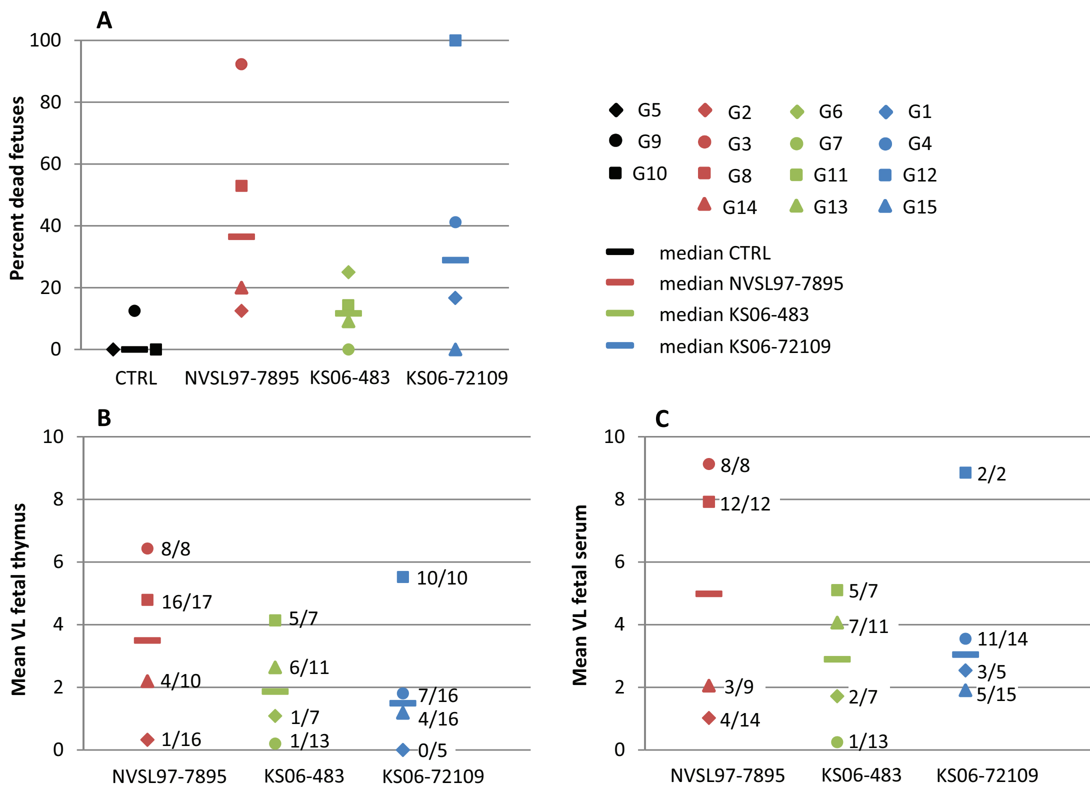

Fig. 4. Percent dead fetuses, mean viral load in fetal thymus and fetal serum per litter. (A) The percentage of dead fetuses is presented for control (CTRL), NVSL 97-7895, KS06-483, and KS06-72109 inoculated gilts. Symbols (diamond, circle, square, triangle) represent individual gilts, bars represent median values for each treatment group. The percentage of dead fetuses among treatment groups was not significantly different ($P = 0.15$), but numerically lower in fetuses from strain KS06-483 inoculated gilts as compared to those from NVSL 97-7895 and KS06-72109 inoculated gilts. (B and C) The mean viral load (VL) of all tested fetal thymus (B) and fetal serum (C) samples ($\log_{10}$ target copies/mg or μL) was calculated for each litter and is presented for NVSL 97-7895, KS06-483, and KS06-72109 inoculated gilts. Number of fetuses tested positive/number of fetuses tested is indicated for each gilt. Symbols (diamond, circle, square, triangle) represent individual gilts, bars represent median values for each treatment group. Differences among treatment groups were not statistically significant ($P > 0.5$).

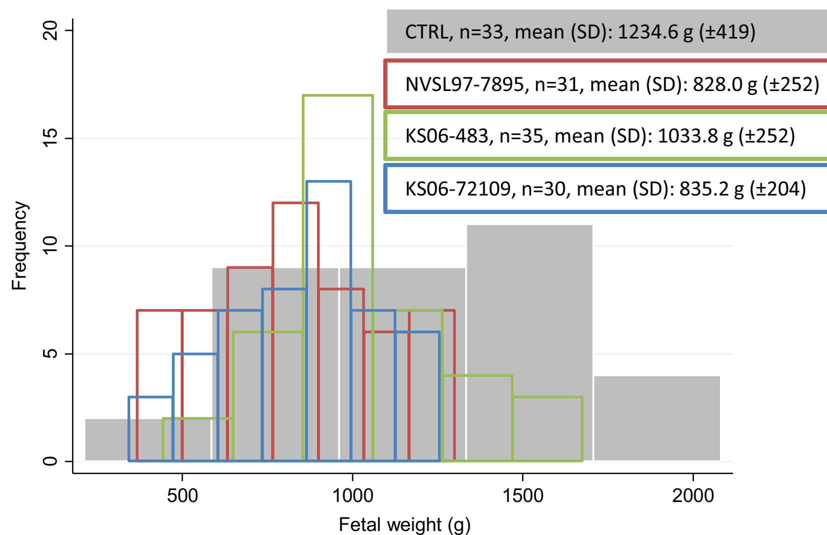

Fig. 5. Distribution of viable fetus body weights per treatment group. Weights were measured 21 days post inoculation at gestation day 106 (±1) in fetuses from NVSL 97-7895 inoculated gilts (red outline bars), KS06-483 inoculated gilts (green outline bars), KS06-72109 inoculated gilts (blue outline bars), and control gilts (CTRL, gray bars). Only weights from VIA fetuses were included since the autolytic process influenced fetal weight of dead (DEC and AUT) fetuses. Mean weights (±SD) are indicated for each treatment group. Overall differences among treatment groups trended toward significance ($P = 0.1$); CTRL × NVSL 97-7895: $P = 0.04$; CRTL × KS06-483: $P = 0.22$; CRTL × KS06-72109: $P = 0.04$; NVSL 97-7895 × KS06-483: $P = 0.34$; NVSL 97-7895 × KS06-72109: $P = 0.91$; KS06-483 × KS06-72109: $P = 0.31$.

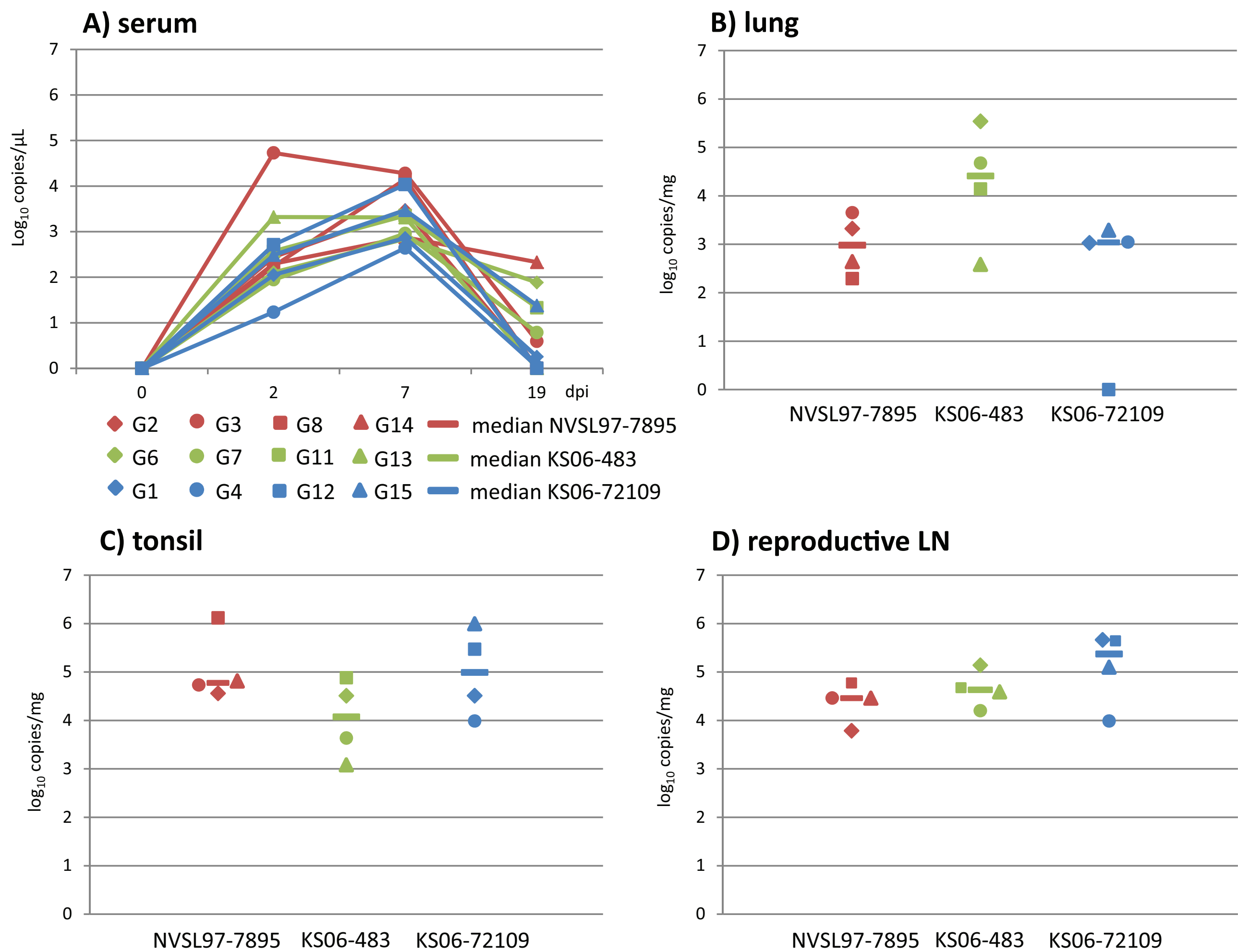

Fig. 6. PRRSV RNA concentration in gilt serum and tissues. PRRSV RNA concentration ($\log_{10}$ target copies/µL or mg) in sera over time (A) and in lung (B), tonsil (C), and reproductive lymph node (LN) (D) collected at necropsy (21 dpi) from gilts inoculated with one of three PRRSV isolates measured by qRT-PCR. Red: NVSL 97-789; green KS06-483; blue: KS06-72109 inoculated gilts. Symbols (diamond, circle, square, triangle) represent individual gilts, bars represent median values for each treatment group. No significant differences were found among treatment groups.

Table 2
Concentration of PRRSV RNA in fetal samples ($\log_{10}$ RNA copies/µL or mg).

Sample	Fetal preservation	NVSL 97-7895		KS06-483		KS06-72109		*P*-value
		n pos/*n* tested	Mean VL (SD)	*n* pos/*n* tested	Mean VL (SD)	*n* pos/*n* tested	Mean VL (SD)	
Fetal thymus	VIA	10/31	1.9 (2.8)	11/35	1.6 (2.6)	6/30	0.9 (2.0)	
	DEC	14/15	5.2 (2.0)	2/3	4.2 (3.7)	8/10	4.2 (2.4)	
	AUT	5/5	4.8 (1.4)	0		7/7	5.1 (1.2)	
	All	29/51	3.1 (2.9)	13/38	1.8 (2.7)	21/47	2.2 (2.7)	0.36+
Fetal serum	VIA	13/29	3.2 (4.0)	13/35	2.3 (3.3)	13/28	1.9 (2.4)	
	DEC	12/12	7.8 (2.3)	2/3	5.5 (4.8)	7/7	6.9 (2.4)	
	AUT	2/2	7.5 (0.8)	0		1/1	7.0	
	All	27/43	4.7 (4.1)	15/38	2.5 (3.5)	21/36	3.0 (3.1)	0.51+
Amniotic fluid	VIA	12/31	1.8 (2.4)	12/35	1.6 (2.3)	11/30	1.3 (2.0)	
	DEC	14/15	5.8 (1.9)	2/3	4.2 (3.6)	10/10	4.7 (1.2)	
	AUT	1/1	5.3	0		0/1		
	All	27/47	3.1 (2.9)	14/38	1.8 (2.5)	21/41	2.1 (2.3)	0.50+
Endometrium*	VIA	19/31	2.4 (2.2)	27/35	2.7 (1.7)	12/30	1.7 (2.2)	
	DEC	14/14	4.8 (1.3)	2/3	3.3 (2.9)	9/10	4.4 (2.0)	
	AUT	10/10	3.3 (0.9)	1/1	2.0	8/9	3.0 (0.6)	
	All	43/55	3.2 (2.0)	30/39	2.7 (1.8)	29/49	2.4 (2.2)	0.41+

PRRSV RNA concentrations are presented as mean (standard deviation) $\log_{10}$ copies/µL or mg for fetal samples.
+ *P*-values were obtained by linear mixed-effects regression models controlling for litter of origin as a random effect and fetal preservation as a fixed effect.
* Endometrium sample collected adjacent to the umbilical stump of each fetus and including adherent fetal placental layers in all but AUT fetuses.

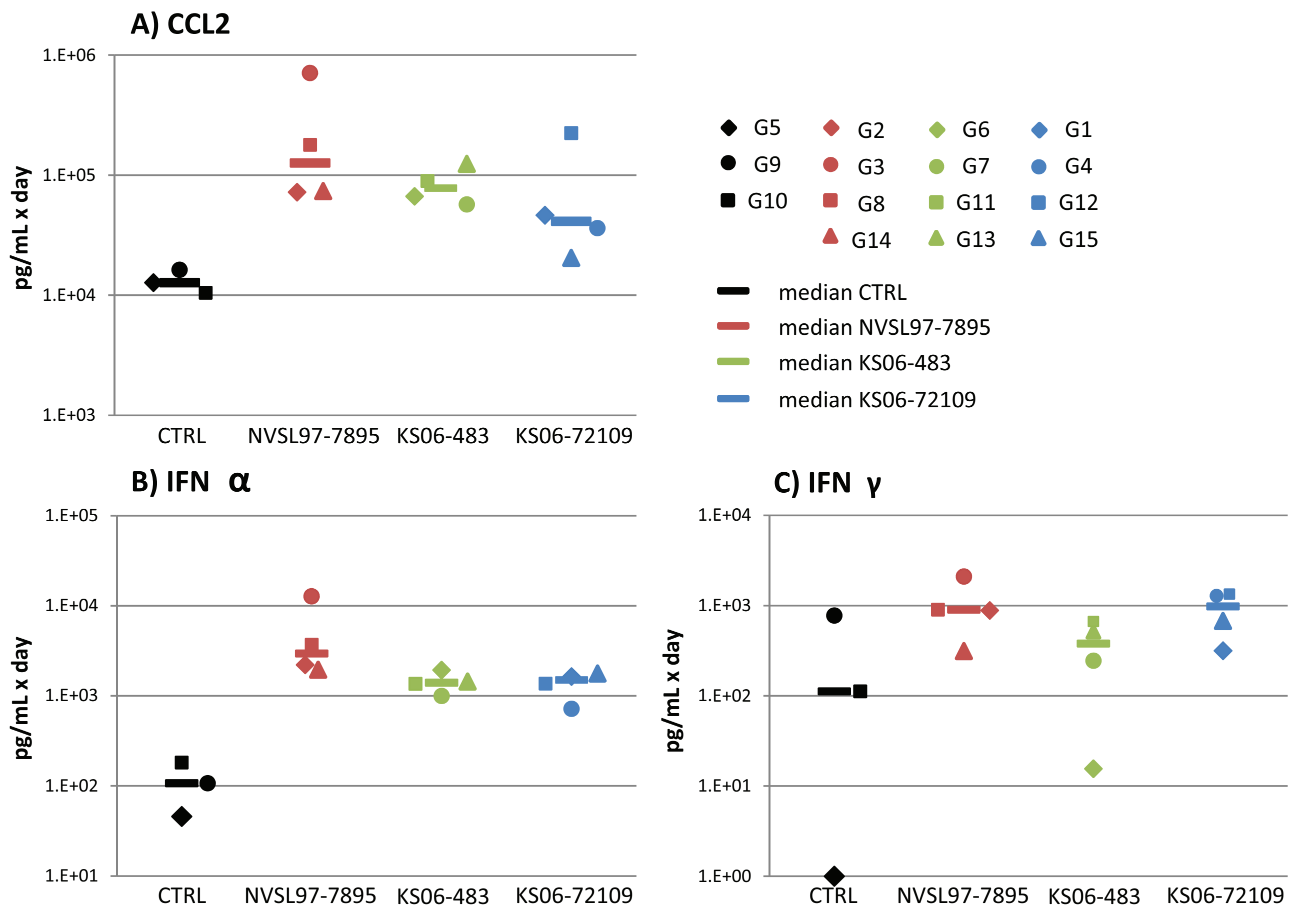

Fig. 7. CCL2, IFNα and IFNγ levels in gilt serum over time. CCL2 (A), IFNα (B) and IFNγ (C) levels (pg/mL × day) in serum from control (CTRL) and gilts inoculated with one of three PRRSV isolates over time (area under the curve (AUC) calculated using the formula AUC = $(t_1 - t_0)(a_1 + a_0)/2 + (t_2 - t_1)(a_1 + a_2)/2 + \ldots + (t_n - t_n - 1)(a_n - 1 + a_n)/2$). Red: NVSL 97-7895; green: KS06-483; blue: KS06-72109 inoculated gilts; back: control gilts. (A) CCL2: serum levels in gilts from all three inoculated groups over time trended higher than levels in control gilts ($P = 0.03$); levels did not differ between the three PRRSV isolates ($P > 0.1$). (B) IFNα: serum levels in gilts from all three inoculated groups trended higher than levels in control gilts ($P = 0.03$); levels in NVSL 97-7895 inoculated gilts trended higher than levels in KS06-483 ($P = 0.02$) and KS06-72109 ($P = 0.02$) inoculated gilts, while KS06-72109 did not differ from KS06-483 ($P = 1.0$). (C) IFNγ: serum levels in gilts inoculated with NVSL 97-7895 trended higher than levels in control gilts ($P = 0.08$), while levels in KS06-483 and KS06-72109 inoculated gilts did not ($P > 0.1$); levels in NVSL 97-7895 and KS06-72109 inoculated gilts trended higher than levels in KS06-483 inoculated gilts ($P = 0.08$) while KS06-72109 did not differ from NVSL 97-7895 ($P = 1$).

had no detectable PRRSV RNA in either thymus, serum or amniotic fluid, only 1/29 DEC fetuses and 0/18 AUT fetuses tested negative for PRRSV RNA in all fetal tissues ($P < 0.001$). Viral load in fetal samples (thymus, serum and amniotic fluid) was positively associated with viral load in the maternal–fetal interface; each one-$\log_{10}$ increase in PRRSV RNA concentration in maternal–fetal interface was associated with an increase of 0.69 $\log_{10}$ RNA copies/mg fetal thymus ($P < 0.001$), 0.93 $\log_{10}$ RNA copies/μL fetal serum ($P < 0.001$), and 0.64 $\log_{10}$ RNA copies/μL amniotic fluid ($P < 0.001$), respectively. Moreover, each $\log_{10}$ increase in fetal thymus PRRSV RNA concentration increased the odds of fetal death ($P < 0.001$; odds ratio (*OR*) 1.5, 95% CI 1.2–1.9). This association was significant in NVSL 97-7895 ($P < 0.001$; *OR* 1.6, 95% CI 1.2–2.1), trended toward significance in KS06-72109 ($P = 0.065$; *OR* 1.6, 95% CI 1.0–2.6), but was not significant in KS06-483 ($P = 0.14$; *OR* 1.4, 95% CI 0.9–2.1).

3.3. Cytokine levels in gilt serum

Cytokine concentrations were compared over time (AUC) among treatment groups. No significant differences were found in serum levels of IL1β, IL4, IL8, IL10, IL12, and IL13 among treatment groups (data not shown). Differences in serum levels of IFNα ($P = 0.01$), CCL2 ($P = 0.03$), and IFNγ ($P = 0.06$) over time trended toward significance among the treatment groups. For all three cytokines, levels increased in inoculated gilts after infection to varying degrees, with NVSL 97-7895 infected gilts showing the highest cytokine concentrations (Fig. 7).

3.4. Microscopic assessment of PRRSV-associated lesions

The predominant lesions of lymphoplasmacytic and histiocytic arteritis, panvasculitis, and perivasculitis were observed in most fetal and adjacent uterine tissues (Table 3). Although the lesions were not scored for severity, they subjectively varied from minimal to marked. The frequency of lesions in umbilical cords, placenta, fetal liver and fetal heart were significantly greater in dead (DEC + AUT) versus live (VIA) inoculated fetuses (Table 4). Lesions in uterine artery and myometrium showed a similar trend (Table 4). No lesions were observed in the kidney or thymus tissues of any fetuses. The previously reported lesion of polykaryocytes (Rossow et al., 1994) was observed in three of 56 fetuses from gilts inoculated with NVSL 97-7895. The most consistent lesion was the lymphoplasmacytic and histiocytic endometritis, observed in 142/145 fetuses (97%) of inoculated gilts (Table 3); the frequency did not differ across group. The predominant lesion in the umbilical cord was moderate to marked hemorrhage and edema most

Table 3
Relative frequency of histologic lesions in fetal and adjacent uterine tissues 21 days post inoculation with different PRRSV strains.

Tissue	Control	NVSL 97-7895	KS06-483	KS06-72109	Inoculated only (%)	P-value[*]
Umbilicus	11/33	36/53	18/39	29/46	83/138 (60)	0.102
Lung	0/34	12/46	13/38	9/40	34/124 (27)	0.483
Liver	0/34	11/45	9/38	13/41	33/124 (27)	0.693
Heart	0/34	8/46	3/38	12/41	23/125 (18)	0.049
Mesenteric LN	0/17	3/16	5/8	3/14	11/38 (29)	0.081
Cerebellum	0/20	2/22	0/18	4/19	6/59 (10)	0.120
Myometrium	0/34	47/56[a]	16/39[b]	40/49[a]	103/144 (72)	<0.001
Endometrium	0/34	54/55	37/39	49/49	140/143 (98)	0.271
Placenta	0/34	30/54	11/39	23/48	64/141 (45)	0.028
Uterine artery	0/34	34/49[a]	10/36[b]	27/47[c]	71/132 (54)	<0.001

Cumulative histopathologic lesions for the fetal and adjacent uterine tissues in control and inoculated gilts are presented: number of fetuses with lesions/number of fetuses examined. LN = lymph node.

[*] Fisher's exact test was used to compare the presence of lesions between the three inoculated groups; superscript letters indicate differences among PRRSV strains within row.

Differences in nominators can be explained by sampling errors: mesenteric LN was very small and difficult to locate and accurately dissect by staff and was therefore missed in some fetuses; cerebellum was only collected from rep 2 fetuses; for the remaining organs a few fetuses were inadvertently missed.

Table 4
Relative frequency of histologic lesions in fetal and adjacent uterine tissues 21 days post inoculation with different PRRSV strains according to live/dead status.

Tissue	Fetal preservation	CTRL	NVSL 97-7895	KS06 −483	KS06 −72109	Inoculated only (%)	P-value[a]
Umbilicus	LIVE	11/33	16/30	14/35	15/31	45/96 (47)	<0.001
	DEAD	0	20/23	4/4	14/15	38/42 (90)	
Lung	LIVE	0/33	6/31	13/35	5/31	24/97 (25)	ns
	DEAD	0/1	6/15	0/3	4/9	10/27 (37)	
Liver	LIVE	0/33	6/30	8/35	6/31	20/96 (21)	0.014
	DEAD	0/1	5/15	1/3	7/10	13/28 (46)	
Heart	LIVE	0/33	4/31	2/35	5/31	11/97 (11)	<0.001
	DEAD	0/1	4/15	1/3	7/10	12/28 (43)	
Mesenteric LN	LIVE	0/16	3/13	5/7	2/12	10/32 (31)	ns
	DEAD	0/1	0/3	0/1	1/2	1/6 (17)	
Cerebellum	LIVE	0/19	2/16	0/16	2/16	4/48 (8)	ns
	DEAD	0/1	0/6	0/2	2/3	2/11 (18)	
Myometrium	LIVE	0/33	26/31	15/35	23/31	64/97 (66)	0.048
	DEAD	0/1	21/25	1/4	17/18	39/47 (77)	
Endometrium	LIVE	0/33	31/31	33/35	31/31	95/97 (98)	ns
	DEAD	0/1	23/24	4/4	18/18	45/46 (98)	
Placenta	LIVE	0/33	9/31	8/35	7/31	24/97 (25)	<0.001
	DEAD	0/1	21/23	3/4	16/17	40/44 (91)	
Uterine artery	LIVE	0/33	18/29	10/32	16/31	44/92 (48)	0.057
	DEAD	0/1	16/20	0/4	11/16	27/40 (68)	

[a] Fisher's exact test was used to compare the presence of lesions between live and dead fetuses of PRRSV inoculated gilts (3 PRRSV strains combined). LIVE includes viable fetuses, DEAD includes decomposed and autolysed fetuses.

consistent with hypoxia. This lesion was most dramatic in the umbilical cords of the fetuses of rep 1 (57/87 fetuses) compared to rep 2 gilts (37/85 fetuses; $P = 0.006$). By contrast, lesions in fetal heart and liver were inflammatory in nature (primarily lymphoplasmacytic) and most likely related to PRRSV infection.

4. Discussion

Since the present pilot study was used to set up experimental conditions and laboratory methods for a larger experiment investigating phenotypic and genotypic predictors of PRRSV resistance in pregnant gilts (Ladinig et al., 2014b), the number of experimental animals per treatment group was small, but comparable with other reproductive experiments in pigs. Therefore, differences among treatment groups in some of the measured parameters, although often remarkable, were not statistically significant. To avoid any possible over-interpretation of our data, we set alpha conservatively at 0.01, because of the numerous comparisons performed. Levels of significance (*P* values) are provided for any results trending toward significance in order to assist the reader in judging potentially significant results.

In the present study, the animals were terminated 21 dpi. At this time point, fetal death had occurred in 32% of fetuses of PRRSV-inoculated gilts. In AUT fetuses, the fetal placenta had already separated from the maternal endometrium prohibiting the investigation of PRRSV RNA levels and histologic lesions within the fetal placenta. Therefore, one limitation of the present experiment was that the uterine sample from AUT fetuses used for qRT-PCR did not include fetal placenta. Another limitation was the sampling of a single piece of endometrium and fetal placenta as a combined sample; this sampling technique did not allow the evaluation of PRRSV RNA levels separately for endometrium and fetal placenta.

All three PRRSV isolates were able to cross the placental barrier and infect the majority of fetuses. However, our study provides evidence that KS06-483 is less virulent than NVSL 97-7895 and KS06-72109. This is based on significantly lower or trending lower histologic lesion frequency, cytokine production in gilt serum, and fetal weights, as well as numeric differences in litter outcome and levels of viral replication in fetal tissues. These findings match clinical data that describes the isolation of NVSL 97-7895 from cases of severe reproductive failure (also called "atypical PRRSV") in the mid-west of the US in the mid-1990s (Osorio et al., 2002). Similarly, KS06-72109 (GenBank Accession No. KM035803) was originally isolated from a sow herd with severe reproductive disease, while KS06-483 (GenBank Accession No. JX258843) was found to be less virulent (Feng et al., 2001).

Similar to growing pigs, for which the negative effects of PRRSV infection on growth performance and its economic outcome are

well known (Holtkamp et al., 2013), in utero PRRSV infection reduced the weight of VIA fetuses. This effect was more severe with the more virulent NVSL 97-7895 and KS06-72109 strains. However, the literature suggests that different virus isolates can produce different effects. Live born piglets from gilts experimentally inoculated with type 1 PRRSV three weeks before farrowing had shorter crown-rump-length compared to live born piglets from control gilts (Han et al., 2013b). On the other hand, Mengeling et al. (1996) found no evidence that infection of gilts with various PRRSV isolates late in gestation had any effect on birth weight of live born piglets.

The observed histologic lesions were similar to those described previously (Karniychuk et al., 2012; Lager and Ackermann, 1994; Lager and Halbur, 1996; Rossow, 1998; Rossow et al., 1996, 1994; Rowland et al., 2003), and were less severe in KS06-483 infected fetuses. Although sample collection was standardized among PRRSV isolates, collection at 21 dpi may have been too late for KS06-483 infected fetuses allowing the remission of some lesions. Characteristic polykaryocyte (Warthin-Finkeldey giant) cells were observed in three fetuses from gilts infected with NVSL 97-7895. These cells were originally described in the lymph nodes of congenitally PRRSV infected neonatal piglets (Rossow et al., 1994), experimentally PRRSV infected sows (Rossow, 1998) and gnotobiotic pigs (Rossow et al., 1994). They are associated with HIV and measles infections in humans and are likely to be of follicular dendritic cell origin (Orenstein, 1998; Wyplosz et al., 2013).

The method of euthanasia was changed between rep 1 and rep 2 by the addition of pentobarbital injection of the gilt prior to euthanasia by cranial captive bolt and pithing. This was due to a high number of fetal lesions (hemorrhage and edema) observed in rep 1 that may have been associated with fetal hypoxia at the time of death, which has been previously reported (Lager and Halbur, 1996). Although we acknowledge that other confounding factors may have been involved, the frequency of the umbilical lesions decreased significantly in rep 2, corresponding to the use of pentobarbital. Interestingly, this decrease in frequency was statistically significant only in control and the low virulent KS06-483 fetuses (compared to numerical decrease in KS-72109 and NVSL 97-7985), suggesting that virulent PRRSV strains induce similar lesions. Although the lesions were not scored for severity, it was easier to observe the sometimes subtle vasculitis lesions in the umbilical cord in the second experimental rep. Thus, we recommend the use of pentobarbital sedation prior to captive bolt in future experiments.

Levels of viremia and PRRSV RNA in tissues from inoculated gilts were comparable among all three PRRSV isolates. Similarly, PRRSV RNA concentrations in the maternal–fetal interface adjacent to each fetus were similar across strains even though the percentage of dead fetuses per litter was higher, although not statistically significant, in NVSL 97-7895 and KS06-72109 compared to KS06-483 gilts. This is an important finding as apoptosis associated with virus replication in the maternal–fetal interface was previously proposed as the most likely cause of fetal death in PRRSV infection (Karniychuk et al., 2011, 2012). However, the sampling method used in the present experiment did not allow the separate evaluation of PRRSV RNA levels in the endometrium from levels in the fetal placenta, which may have helped to elucidate the relative importance of endometrial and fetal pathophysiology. That being said, while this study was not designed to specifically investigate mechanism of fetal death, it does provide a number of important clues. Firstly, the fact that viral load in fetal thymus significantly increased the odds of fetal death, and PRRSV RNA was found in all but one dead fetus indicates that virus replication within fetal tissues is likely important in the pathogenesis of fetal death. Secondly, the frequency of lesions in various fetal and maternal tissues was significantly greater in dead compared to live inoculated fetuses suggesting that PRRSV may have a direct effect on fetal physiology as well as on the maternal–fetal interface. Importantly, the frequency of endometrial lesions did not differ between live and dead fetuses. Although it is not clear whether fetal death is related to the direct effects of the virus within fetal tissues or to high viral loads being carried back through umbilical blood to the maternal–fetal interface, the significantly higher prevalence of inflammatory lesions in the heart and liver of dead versus live fetuses indicates that PRRSV replication in fetal tissues may directly contribute to fetal death. This effect, however, may be strain dependent. Although all three PRRSV isolates efficiently crossed the placental barrier and infected fetuses, KS06-483 did not induce high fetal mortality rates in any of the four inoculated gilts (percent dead $\leq$25), even though two of the gilts had comparatively high mean viral loads within their litters.

Similar to the findings in this pilot experiment, the subsequent experiment, with 113 NVSL 97-7895 inoculated and 19 control gilts, found CCL2, IFNα, and IFNγ levels in gilt serum increased after PRRSV inoculation (Ladinig et al., 2014a). Interestingly, in the present (pilot) experiment, levels of those three cytokines differed across PRRSV strains. NVSL 97-7895 induced higher levels of all three cytokines and gilts inoculated with this isolate also had the highest percentage of dead fetuses per litter. Together with our previous findings (Ladinig et al., 2014a), this indicates that those three cytokines are important for the reproductive effects of PRRSV. This is noteworthy, as no other detailed reports on cytokine responses to PRRSV infection in pregnant sows or gilts exist. In contrast to our findings, Lowe et al. (2005) found that a strong cell-mediated immune response determined by use of an IFNγ ELISPOT was correlated with protection against reproductive failure in three of four farms during outbreaks of PRRS. However, direct comparison with the results of the present study are difficult because the Lowe et al. study measured IFNγ levels by ELISPOT in sows expressing clinical signs from chronically PRRSV-infected farms, 7 months to 5 years post introduction, in which the specific timing of infection or prior exposure status of individual animals was unknown. CCL2 responses of pregnant sows or gilts inoculated with various PRRSV isolates have not been previously described. In nursery piglets, CCL2 (a recruiter of monocytes) levels in serum increased within the first two weeks after PRRSV inoculation and were associated with serum PRRSV RNA concentration and growth performance (Souza et al., 2013). A PRRSV induced down-regulation of type I interferon production was previously described and was assumed to be one of the mechanisms involved in the immunomodulatory effects of PRRSV (Albina et al., 1998; Buddaert et al., 1998; Van Reeth et al., 1999). However, conflicting data on IFNα expression/production after PRRSV infection can be found in the literature. While some studies could not detect significant IFNα increases in serum of PRRSV inoculated pigs within one week of infection (Gomez-Laguna et al., 2009; Lawson et al., 2010), others did (Dwivedi et al., 2012; Guo et al., 2013). However, none of these experiments used pregnant sows or gilts. Therefore, our finding that IFNα has negative effects on the reproductive outcome of PRRSV infection needs further confirmation, but is in agreement with the results of the main experiment (Ladinig et al., 2014a). Moreover, the finding that IFNα up-regulated the expression of sialoadhesin and subsequently enhanced PRRSV infection of monocytes (Delputte et al., 2007) supports our results.

In conclusion, based on differences in histologic lesions and fetal weights, as well as other numeric differences in levels of CCL2, IFNα, and IFNγ in gilt serum, litter outcome and virus replication in fetal tissues, KS06-483 appeared less virulent than NVSL 97-7895 and KS06-72109. We found that IL1β, IL4, IL8, IL10, IL12, and IL13 were the least important cytokines in regards to reproductive PRRSV infection. Our data suggest that virus replication in fetal tissues and the maternal–fetal interface, but not in other gilt tissues, is important for the outcome of reproductive PRRS. The presence of virus

in the maternal–fetal interface does not appear to be sufficient by itself to induce fetal death. In the present study, all but one dead fetus were positive for PRRSV RNA suggesting that a certain level of virus at the maternal interface may be required to induce reproductive failure, that virus has to cross the placenta in order to induce fetal death, or both.

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