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Samples sizes required to accurately quantify viral load and histologic lesion severity at the maternal–fetal interface of PRRSV-inoculated pregnant gilts

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Abstract. Porcine reproductive and respiratory syndrome virus (PRRSV) is transmitted vertically, causing fetal death in late gestation. Spatiotemporal distribution of virus at the maternal–fetal interface (MFI) is variable, and accurate assessment of viral concentration and lesions is thus subject to sampling error. Our objectives were: 1) to assess whether viral load and lesion severity in a single sample of endometrium (END) and placenta (PLC), collected near the base of the umbilical cord (the current standard), are representative of the entire organ; and 2) to compare sampling strategies and evaluate if spatial variation in viral load can be overcome by pooling of like-tissues. Spatially distinct pieces of END and PLC of 24 fetuses from PRRSV-2–infected dams were collected. PRRSV RNA quantified by RT-qPCR was compared in 5 individual pieces per fetus and in respective pools of tissue and extracted RNA. Three distinct pieces of MFI were assessed for histologic severity. Concordance correlation and kappa inter-rater agreement were used to characterize agreement among individual samples and pools. The viral load of individual samples and pools of END had greater concordance to a referent standard than did samples of PLC. Larger pool sizes had greater concordance than smaller pool sizes. Average viral load and lesion severity did not differ by location sampled, and no technical advantages of pooling tissues versus RNA extracts were found. We conclude that multiple pieces of MFI tissues must be evaluated to accurately assess lesion severity and viral load. Three pieces per fetus provided a reasonable balance of cost and logistic feasibility.

Key words: concordance; endometrium; maternal–fetal interface; placenta; PRRSV; swine.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an economically devastating disease affecting the pork industry.[5] Discovered in 1991,[2,24] the causative viruses, PRRSV-1 and -2 (*Betaarterivirus suid 1* and *2*), have worldwide distribution, but are separate species[1] with origins in the European Union and United States, respectively. A feature of the *Arteriviridae* family is its genome plasticity; there exist many different strains across a spectrum of virulence including a highly pathogenic lineage of PRRSV-2 that emerged with devastating effects in southeast Asia since the mid-2000s.[7] Neither species of virus is zoonotic nor infects other livestock or mammals. In naive infected pigs, disease is characterized by interstitial pneumonia accompanied by immune suppression in post-natal animals and reproductive losses (abortion, fetal death, congenital infection, weak neonates, higher preweaning mortality) in pregnant animals infected during the third trimester.[8] The virus replicates in macrophages expressing the CD163 cell surface receptor,[9] and following infection of late-gestation females, viremia and endometritis rapidly develop whereas placentitis and placental detachment develop more slowly.[19] Although the mechanism of in-utero transmission is not understood completely, the virus crosses the placenta within a few days,[23] progressively infecting fetal placenta, umbilical cord, and the fetus.[16]

Swine have diffuse epitheliochorial placentation with each fetus compartmentalized in its own placental unit. The chorion and allantois fuse and vascularize in early gestation forming the chorioallantois (allantochorion); the tips of the elongated chorion that fail to fuse become necrotic.[25] The latter may be a mechanism by which PRRSV may transmit

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between fetuses.[10] The allantochorion superficially adheres to the endometrium and together forms an expansive network of interdigitating primary and secondary folds with underlying respective maternal and placental vasculatures.[3]

Not all fetuses within a given litter are equally susceptible to PRRSV, and infected viable, dead, and uninfected fetuses are known to coexist in the same litter.[12] However, infection and fetal death cluster within a litter,[11] either by coincidence or possibly related to regional structural or physiologic feature of the porcine maternal–fetal interface (MFI; defined as endometrium with adherent fetal allantochorion). The distribution of PRRSV particles[9] and endometrial lesions[13] (Novakovic P, unpublished) vary within and between fetuses, posing challenges for diagnostic and research sampling protocols, particularly those requiring accurate quantification of viral load and lesion severity of the MFI.

Although viral RNA concentration in MFI and the severity of endometrial lesions[19,21] potentially contribute to fetal demise, there is no assessment to our knowledge showing that a single sample per fetus, either randomly or systematically collected, is representative of the entire MFI. Given the size of each placental unit and heterogenicity of fetal outcome following PRRSV infection, it is necessary to examine each fetus to assess viability, lesion severity, and viral load. With litter sizes averaging ~15 total born in contemporary sows, sample numbers can quickly become overwhelming in a large experiment, therefore judicious and efficacious sampling is required. Sampling a single site or tissue per fetus is practical and efficient at the time of collection and also requires fewer resources during the laboratory analyses, but, if accuracy is paramount, it is important to determine the optimal number of tissue pieces per fetus that should be assessed. Our objectives were to determine: 1) optimal sample numbers required to accurately quantify PRRSV RNA concentration and histologic lesion severity across the entire endometrial and placental surface per fetus; 2) if viral load and lesion severity differ by location of MFI sampled; and 3) if pooling of collected MFI tissues or RNA extracts prior to reverse-transcription quantitative PCR (RT-qPCR) testing improves the accuracy of quantification compared to testing multiple tissue pieces individually.

Materials and methods

Animal experiment and sample collection

We used tissues collected from a previously described PRRSV challenge experiment,[16,19,23] adhering to principles established by the Canadian Council on Animal Care and approved by the Animal Research Ethics Board at the University of Saskatchewan (Certification 20160023). Briefly, 15 purebred Landrace gilts from a PRRSV-naive farm were experimentally inoculated intranasally and intramuscularly (total dose 1×10^5 $TCID_{50}$) with PRRSV-2 strain NVSL 97-7895 at 86 ± 0.4 d of gestation. Five control gilts were mock-inoculated. On each of 5 post-inoculation days (2, 5, 8, 12, and 14 dpi), 1 control and 3 PRRSV-inoculated gilts were euthanized using barbiturate overdose followed by cranial captive bolt stunning and exsanguination. Fetuses were removed from the gravid reproductive tract and their placement in the left or right uterine horn recorded. The preservation status of each fetus was assessed as viable, meconium-stained, or decomposed, according to pre-established guidelines.[12] The respective proportion of MFI supporting each fetus was removed while maintaining its identity with the fetus. We used tissues from 4 to 6 randomly selected fetuses per litter that had been subjected to more intensive sampling. From each of these intensively sampled fetuses, 3 spatially distinct pieces (Pc1–3) of MFI tissue were collected into 10% neutral-buffered formalin, then processed routinely for hematoxylin and eosin–stained tissue sections. Five unique pieces (Pc1–5) were collected for quantification of viral load by RT-qPCR (Suppl Fig. 1).

PRRSV RNA extraction and sample pooling

After manually separating the placenta (PLC) from endometrium (END), the PRRSV RNA concentration was quantified in both tissues as described previously, using an in-house probe-based assay specifically designed for the inoculum strain, NVSL 97-7895.[16] As a preliminary step, the central MFI piece adjacent to the umbilical stump (Pc1) was tested for all intensively sampled fetuses ($n = 68$) across 15 PRRSV-infected litters. These results were used to select a subset of 24 fetuses (11 female, 13 male) in which viral load was assessed in all 5 pieces of END and PLC (240 pieces in total). These intensively sampled fetuses were categorized into 3 groups (8 per group) based on their fetal preservation score and RNA concentration of Pc1: (a) meconium-stained or decomposed fetuses, (b) viable fetuses with consistent viral load in PLC and END, and (c) viable fetuses with inconsistent viral load in PLC and END. In this context, "consistent" referred to fetuses in which the viral loads in END and PLC were similar (i.e., both had high or low viral load), whereas "inconsistent" referred to fetuses in which the viral load differed between these 2 tissues (i.e., one had high and the other had low viral load). The purpose of categorizing fetuses was to ensure that the subset was representative of the entire fetal population. High viral load was considered as ≥ 4 $\log_{10}$ viral particles/mg of tissue, and low viral load was < 4 $\log_{10}$.

Individual pieces of END and PLC (30 mg) were homogenized by shaking with lysis buffer in a vial containing a steel ball (30 Hz for 4 min). Thereafter, 600 µL of each homogenate was robotically extracted using a commercial kit as per the manufacturer's instructions (RNeasy mini kit; Qiagen).

Two pools of pieces were also generated. A "tissue pool" was made by combining 120 µL of each END or PLC homogenate from the 5 individual pieces per fetus into a 600-µL

pool that was thoroughly mixed and extracted as described above. A "RNA pool" was made by combining 5 μL of extracted RNA from each of the 5 individually extracted pieces per fetus. These were thoroughly mixed and the PRRSV RNA quantified using RT-qPCR. RNA concentration and purity (A260/A280 ratio ≥ 2) were assessed using 2 μL of the sample by spectrophotometry (NanoDrop spectrophotometer; Thermo Fisher).

Quantitative PCR

We used a probe-based PCR protocol specific to PRRSV strain NVSL 97-7895 that amplified a highly conserved region of open reading frame 7 (ORF7). The PCR primers and protocol has been described previously.[12] Briefly, plasmid HindIII pCR2.1TOPO-NVSL containing a 446-bp sequence of ORF7 was used for the 5-point standard curve (10^7, 10^5, 10^3, 10^2, 10^1) run in triplicate on each plate. Samples were run in duplicate on 96-well plates (35 samples/plate) that also contained appropriate no template, positive control (pool of PRRSV-positive tissues), and RT-qPCR controls. A statistical process control (SPC) chart was used to monitor the positive control sample across plates. Individual samples were re-run if the cycle quantification (Cq) standard deviation between the duplicates was > 1.5 or if 1 of the 2 duplicates had no cycle threshold (Ct). Negative results were re-run to confirm the absence of viral RNA. Results were reported as $\log_{10}$ target RNA concentration per mg of tissue. The limits of quantification were defined by the least and most concentrated standards. Samples were recorded as negative if target RNA was not detected, or DNQ (detected, but not quantifiable) if target RNA was detected but the concentration was below the least concentrated standard.

Histopathology

Lesions were assessed in each of 3 pieces of MFI (Suppl. Fig. 1) collected from 87 intensively sampled fetuses (42 female, 45 male) independently by 2 pathologists (P Novakovic, J Zarate) blinded to group identity, fetal preservation status, and times of infection. Eight-mm long tissue cross-sections were cut to fit inside a standard 4-well tissue cassette in the same order each time. Both PRRSV-infected and control litters were included (range: 3–6 per litter). A score was generated for characteristic lesions produced by PRRSV, specifically endometritis, placentitis, endometrial vasculitis, and placental detachments, as described previously.[19,20] The lymphocytic-to-lymphohistiocytic inflammatory cell infiltrate in END and PLC was predominantly composed of lymphocytes admixed with small numbers of histiocytes and rare plasma cells. This infiltrate was scored: normal (0) no inflammatory cell infiltrate, minimal (1) < 10% of section contains inflammatory cell infiltrate, mild (2) 10–25% of tissue contains inflammatory cell infiltrate, moderate (3) 25–50% of section contains inflammatory cell infiltrate, and severe (4) > 50% of section contains inflammatory cell infiltrate. Vasculitis, with and without perivasculitis, was defined by the presence of ≥ 3 inflammatory cells within these locations. Accompanying changes of edema, vascular or endothelial degeneration, and vacuolation were very slight in most cases and not considered. The distribution of vasculitis was evaluated separately in the endometrium and fetal PLC as: normal (0) no blood vessels inflamed, focal (1) < 30% of blood vessels inflamed, multifocal (2) 30–70% of blood vessels inflamed, or diffuse (3) > 70% of blood vessels inflamed. The severity of placental detachment was scored: normal (0) no detachment of PLC along the interdigitation area, mild (1) < 25% of interdigitation area was detached, moderate (2) 25–50% of interdigitation area was detached, and severe (3) > 50% of interdigitation area was detached.

Data analysis

PRRSV RNA quantification. To determine the optimal number of pieces per fetus needed to accurately quantify viral load in END and PLC (analyzed separately), the average of the 5 individual pieces per fetus was used as the referent quantification (gold standard for this experiment) to which the viral load of individual and pooled pieces were compared. Pooled samples included the RNA and homogenized tissue pools described above as well as the viral load of virtual pools of 2, 3, and 4 individual pieces created in silico by averaging the viral load of the pairwise combinations of 5 individual pieces. Twenty-four virtual pools were created: 10 virtual pools of 2 pieces (Pc1+2, 1+3, 1+4. . .4+5), 6 virtual pools of 3 (1+2+3, 1+2+4, 1+2+5. . .3+4+5), and 5 virtual pools of 4 pieces (1+2+3+4, 1+2+3+5. . .2+3+4+5). Lin concordance coefficients (r_c) were calculated to compare the level of agreement in viral RNA concentration between individual pieces and pools to the referent sample, with higher values reflecting a stronger association between the pool and gold standard. A Dunn test was used to compare viral load across the 5 sampling locations. To compare variation in viral load across the 5 sampling locations, a Levene robust test was used.

PRRSV histologic lesion scores. Average lesion scores were compared between PRRSV-inoculated and control groups using a Mann–Whitney U test. To evaluate the level of agreement in lesion scores across the 3 pieces (PLC and END evaluated separately) and between the pathologists, a kappa statistic of inter-rater agreement was calculated. Qualitative terminology describing the level of agreement was defined[14]: fair (κ = 0.21–0.4), moderate (κ = 0.41–0.6), substantial (κ = 0.61–0.8), almost perfect (κ = 0.81–1.0). To determine the number of pieces required to accurately assess lesion severity, Lin concordance coefficients (r_c) were calculated to assess agreement between individual pieces (2 pathologists each

Table 1. PRRSV RNA concentration, coefficients of variation, and levels of agreement for endometrial and placental samples and pools.

	Single pieces	Virtual pools of 2	Virtual pools of 3	Virtual pools of 4	Virtual pools of 5	Pool of extracted RNA	Pool of homogenized tissues
Endometrium							
Viral load* (mean)	6.37	6.37	6.38	6.37	6.37	6.29	6.54
Viral load* (median)	5.10	5.26	5.28	5.31	5.33	5.39	5.49
Viral load (CV%)	108.5	107.1	106.5	106.1	106.0	105.2	106.3
r_c median	0.68	0.83	0.89	0.99	1.00	0.81	0.88
Precision	0.78	0.88	0.93	0.96	1.00	0.85	0.90
Accuracy	0.86	0.95	0.98	0.99	1.00	0.94	0.98
Placenta							
Viral load* (mean)	8.25	8.27	8.23	8.27	8.27	6.19	6.43
Viral load* (median)	2.16	2.37	2.49	2.66	4.8	2.59	2.59
Viral load (CV%)	112.3	110.4	109.5	108.5	128.2	108.1	107.4
r_c median	0.44	0.62	0.88	0.97	1.00	0.67	0.62
Precision	0.62	0.73	0.82	0.92	1.00	0.70	0.64
Accuracy	0.83	0.92	0.96	0.99	1.00	0.96	0.97

Accuracy = bias correction factor (c_b); precision = Pearson correlation coefficient (r); r_c = Lin robust concordance coefficient; product of $r \times$ c_b.
* $\log_{10}$ copies PRRSV RNA per mg tissue.

scoring 3 pieces) to the referent sample (average score of all 3 tissue pieces) for each PRRSV-related lesion. Pairwise combinations of pieces, representative of 1 pathologist scoring 2 or 3 pieces, and 2 pathologists scoring 1 or 2 pieces, were also assessed. Accuracy and precision were calculated using the bias-correction factor (c_b) and Pearson correlation coefficient (r), respectively.[15] A Dunn test was used to determine if lesion scores differed by the location in the MFI. All statistical analyses were performed with Stata v.15 (StataCorp).

Results

PRRSV RNA quantification

Median RNA concentrations for the individual pieces across the 5 locations were 5.1 (range: 0.0–7.9) and 2.2 (0.0–10.3) $\log_{10}$ target copies per mg for END and PLC, respectively. PRRSV RNA was not detected in 22 of 120 (18%) and 34 of 120 (28%) individual pieces of END and PLC, respectively. Median viral load and within-location variation in viral load did not differ by sampling location in either END and PLC (Suppl. Fig. 2) suggesting that multiple pieces collected randomly or systematically would yield similar results.

For END and PLC, the viral load estimates for the virtual pools of all sizes were equivalent to the estimate for the 5 pieces tested individually (Table 1). The viral load estimates of larger pools had greater concordance with the viral load estimates of individual pieces than did smaller pools, with the most dramatic gains in concordance noted when 2 or more pieces of END, or 3 or more placental pieces were pooled for a single PCR test (Table 1, Fig. 1). Virtual pools of 4 pieces had near perfect agreement to the individual pieces, indicating no advantage of collecting more than 4 pieces per fetus for assessment of viral load. Concordance coefficients were greater for virtual pools of END than virtual pools of PLC, particularly in smaller pool sizes, indicating greater heterogeneity of virus distribution in the PLC.

For END, the mean viral load estimates of the tissue homogenate and RNA pools were within 0.25 $\log_{10}$/mg of the individual pieces and virtual pool averages. By contrast, the viral load estimates of the placental pools of homogenized tissues and RNA were ~2 $\log_{10}$/mg lower than the individual pieces and virtual pools (Table 1). Pooling homogenized END had higher concordance than pooling the extracted RNA, but the opposite trend was evident for PLC (Fig. 1). This suggests that there is no clear benefit to pooling either before or after extraction for MFI tissues. Expectedly, both the tissue homogenate and RNA pools had lower concordance with individual pieces than did the virtual pools. This is explained by the virtual pools being created in silico by averaging different combinations of the pieces tested individually, whereas the tissue and RNA pools were created by resampling the tissue pieces collected at postmortem examination and therefore were different technical replicates.

Accuracy and precision were greater in END than PLC, and both increased with virtual pool size. Across all pool sizes, viral load estimates had greater accuracy than precision, although the gap between the 2 measures narrowed as virtual pool size increased. This result also suggests that viral load is heterogeneous in the MFI, especially in PLC.

Histopathology

The severity of endometritis and placentitis was greater in PRRSV-infected compared to control fetuses (Table 2). Endometrial vasculitis was only present in PRRSV-infected

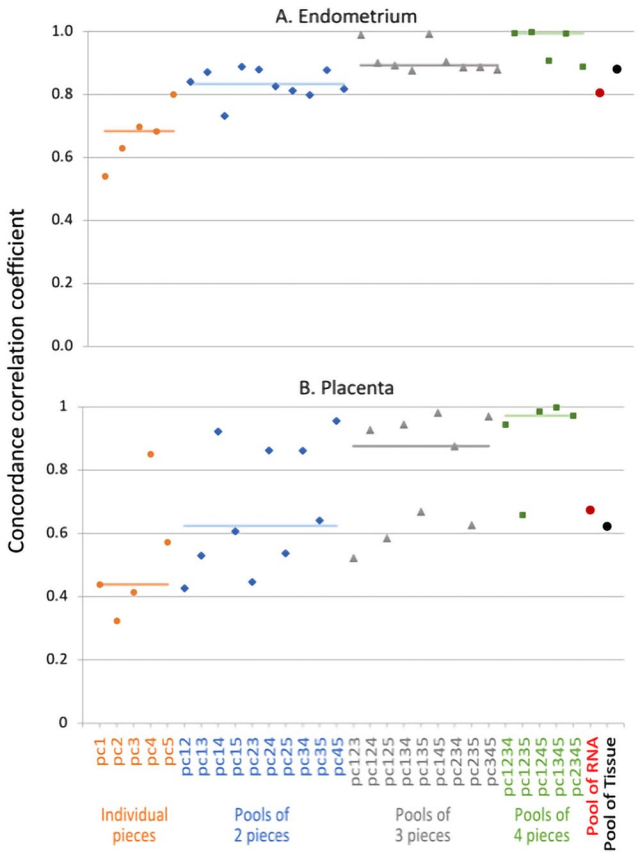

Figure 1. Viral load concordance. Dot plot depicting the level of agreement (y-axis, Lin concordance correlation) in viral RNA concentration among individual pieces and pools (x-axis, color-coded by pool size and type) to a referent standard for endometrium (**A**) and placenta (**B**). Horizontal lines represent the median concordance correlation coefficient value for that group of samples, with larger pool sizes showing greater agreement.

samples, except for one control with a focal lesion. Vasculitis was not observed in placentas of either group. Mild placental detachments were observed in roughly one-third of fetuses (24 of 62 for control; 72 of 223 for PRRSV-infected) but median scores did not differ between PRRSV-infected and the non-infected group. However, pregnancies were terminated 2–14 dpi in our experiment, likely too early for placental detachment to occur in infected fetuses.

The distribution of lesion scores at the umbilical stump (Pc1, Table 2) helps to provide some context for interpreting the inter-rater agreement among the 3 pieces of MFI assessed for each fetus. Lesion severity did not significantly differ among the 3 locations sampled. Lesion scores were not equally distributed across all severity scores (0–4), thus the kappa values presented below should be interpreted cautiously. There was substantial agreement (κ = 0.61–0.8) between the 2 pathologists for all lesion types (Table 3). There was lower agreement (κ < 0.50) with respect to the lesion scores each pathologist assigned across location within the same fetus (Table 4). For endometritis and vasculitis distribution, the lesion scores of unaffected (score 0) and severely affected (scores 3–4) pieces were in moderate agreement, whereas lesion scores of the mildly and moderately (scores 1–2) affected pieces were in fair agreement. This result suggests that both pathologists were more consistent at scoring the extremes (normal, severe), or that endometrial lesion severity is heterogeneous across the MFI. For placental lesions (inflammation, detachment), there was poor-to-slight agreement in the pathologists' lesion scores across location, likely associated with great heterogeneity across the PLC.

Regarding the optimal number of pieces and pathologists required to accurately assess lesion severity, the endometrial lesion scores of all individual pieces and pairwise combinations had high concordance with the referent sample (average lesion scores of 3 pieces by 2 pathologists; Fig. 2A, 2B) and were both highly accurate and precise (Table 2). By contrast, placental lesion scores had lower concordance than endometrial lesions overall and improved when more pieces and pathologists were included (Fig. 3A, 3B). Lesion scores were reasonably accurate but much less precise (Table 2). Interestingly, the evaluation of 3 pieces by either pathologist had superior concordance than if either pathologist evaluated 2 pieces. These results indicate that accurate assessment of placental lesions requires multiple pieces from multiple locations (the more the better, dependent on feasibility) but there is no measurable advantage of assessment by multiple pathologists. Unlike PLC, the assessment of a single piece of END, however, is sufficient to accurately assess the severity of endometritis and vasculitis distribution. This was likely the result of the diffuseness of the endometrial lesions compared to the heterogeneity and lower occurrence of placental lesions.

Discussion

Contemporary sows are highly prolific, bearing litter sizes averaging ~14.9 (± 1.0) piglets, and the top 10% of farms averaged > 16 piglets per litter in North America in 2019.[22] Thus, diseases such as PRRS that can drastically reduce litter sizes can be financially devastating. It is estimated that pigs on ~45–70% of U.S. farms are infected with PRRSV,[18] with ~20–25% of farms classified as positive "unstable,"[17] characterized by active circulation and shedding of PRRSV within the population of breeding females.[6] PRRSV is one of several causes of reproductive failure in swine, and diagnosis in individual animals, for instance following abortion, can be challenging, particularly in farms where the virus is known to preexist. Examination of the reproductive tract in cases of reproductive failure, including placentae and conceptae, may provide useful information regarding underlying causes of late-gestation reproductive failure; however, with PRRS, lesions in fetuses are sparse[13,20] and not all fetuses within the litter are infected with virus.[9,12]

Table 2. Distribution of histologic lesion scores in endometrium and placenta in tissues used to assess inter-rater agreement.

	Distribution of lesion scores (number of total cases)*					Group averages† (median score [IQR])			
	0	1	2	3	4	Control (n = 62–63)	PRRSV (n = 198–209)	Accuracy (c_b)	Precision (r)
Endometritis (n = 293)	Normal 38 (13%)	Minimal 93 (32%)	Mild 50 (17%)	Moderate 49 (17%)	Severe 63 (22%)	0.7 (0.4)[a]	2.7 (2.4)[b]	0.999 (0.002)	0.978 (0.019)
Placentitis (n = 279)	139 (50%)	123 (44%)	15 (5%)	2 (0.7%)	0 (0%)	0.3 (0.3)[a]	0.7 (0.7)[b]	0.966 (0.036)	0.819 (0.113)
Endometrial vasculitis distribution (n = 275)	None 138 (50%)	Focal 29 (10%)	Multifocal 53 (19%)	Diffuse 55 (20%)		0.0 (0.0)[a]	1.7 (2.3)[b]	0.998 (0.003)	0.969 (0.025)
Placental detachment (n = 285)	None 189 (66%)	Mild 96 (34%)	Moderate 0 (0%)	Severe 0 (0%)		(0.0)	0.0 (0.0)	0.943 (0.060)	0.784 (0.137)

* Refer to Materials and methods for full description of lesion scoring.
† Superscripts ([a, b]) signify statistically significant differences in average lesion scores within row (Mann–Whitney U test; $p < 0.05$).

Table 3. Levels of agreement in lesion severity scores between pathologists as determined by the kappa inter-rater statistic.

	Pairs of pieces			
	Pc1+4	Pc2+5	Pc3+6	p value
Endometritis	0.70	0.82	0.69	< 0.001
Endometrial vasculitis	0.63	0.81	0.78	< 0.001
Placentitis	0.64	0.59	0.68	< 0.001
Placental detachment	0.77	0.77	0.66	< 0.001

Two pathologists scored the same 3 pieces of H&E-stained maternal–fetal interface for each fetus. Pathologist 1 scores are represented by pieces (Pc)1–3. Pathologist 2 scores are represented by Pc4–6. Kappa values for the same tissue piece assessed independently by both pathologists are in columns Pc1+4, Pc2+5, and Pc3+6.

Table 4. Levels of agreement in lesion severity scores within pathologists as determined by the kappa inter-rater statistic.

	Lesion score						
	0	1	2	3	4	All	p value
Pathologist 1							
Endometritis	0.53	0.33	0.40	0.24	0.53	0.40	< 0.001
Endometrial vasculitis	0.72	0.20	0.30	0.51		0.48	< 0.001
Placentitis	0.25	0.15	0.0			0.19	0.001
Placental detachment	0.01	0.0	0.0	0.32		0.00	NS
Pathologist 2							
Endometritis	0.51	0.51	0.34	0.23	0.53	0.44	< 0.001
Endometrial vasculitis	0.78	0.05	0.31	0.48		0.50	< 0.001
Placentitis	0.18	0.05	0.14	0.0		0.11	0.023
Placental detachment	0.02	0.03	0.0			0.03	NS

Each of 3 pieces of maternal fetal interface was scored using ordinal scales by 2 pathologists blinded to group identity. Kappa values represent the level of agreement for each individual score (0–4) and overall scores (All). NS = not significant.

PRRSV-induced lymphohistiocytic endometritis is highly prevalent and develops rapidly following infection, but the severity of inflammatory infiltrate and vasculitis varies widely across the entire MFI.[19,20] However, ~20% of endometrial samples may test negative for PRRSV RNA 2–3 wk post-inoculation.[12,16] In a diagnostic setting, PLC is the only available sample, unless the sow is submitted with fetuses still in-utero. Therefore, testing at least 3–5, randomly located pieces of PLC is likely sufficient for diagnosis. In a research setting in which accurate quantification of lesion severity and viral load at the MFI is required, it is relevant to understand how many pieces of MFI per fetus are required

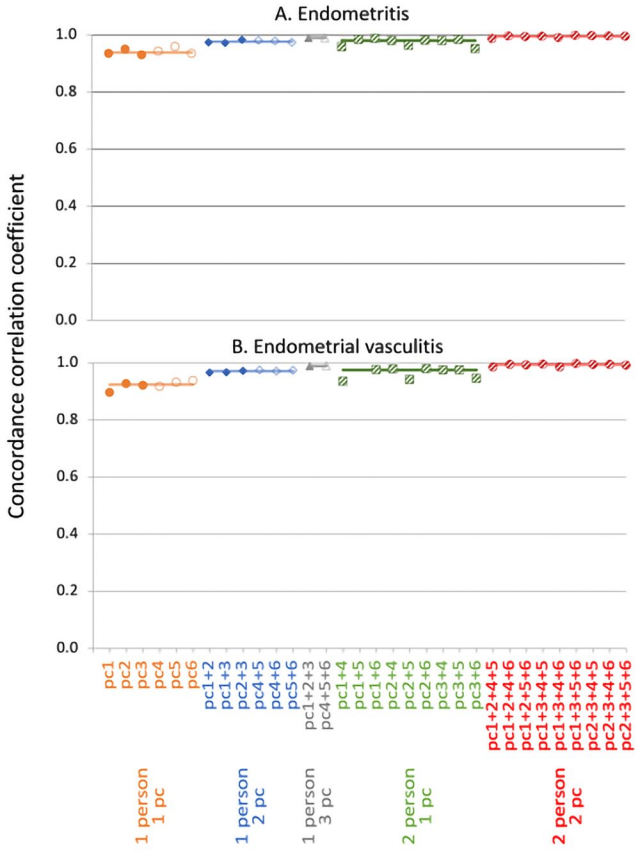

Figure 2. Endometrial lesion concordance. Dot plot depicting the level of agreement (y-axis, Lin concordance correlation) in ordinal lesion scores among individual pieces and their pairwise combinations (x-axis, color-coded by number of pieces examined and pathologists involved) to the referent standard for endometritis (**A**) and endometrial vasculitis distribution (**B**). The combinations of pieces and pathologists (x-axis) were selected to mimic 1 or 2 pathologists requested to evaluate 1, 2, or 3 pieces each. Horizontal lines represent the median concordance correlation coefficient value for that group of samples. Pieces 1–3 (solid markers) and 4–6 (open markers) were assessed by pathologist 1 and 2, respectively. Hatched markers represent combinations of pieces assessed by both pathologists. Endometrial lesion scores have high accuracy and precision regardless of the number of pieces and pathologists involved.

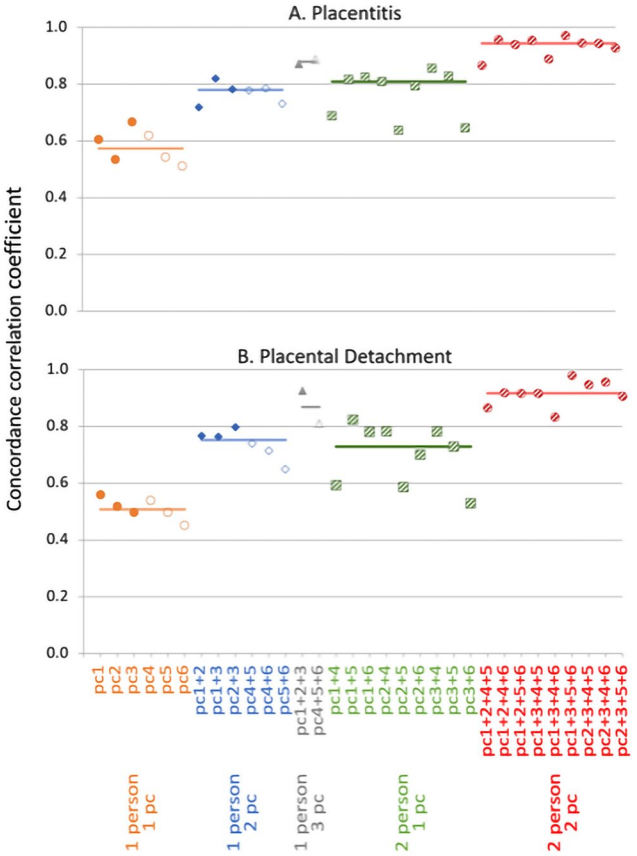

Figure 3. Placental lesion concordance. Dot plot depicting the level of agreement (y-axis, Lin concordance correlation) in ordinal lesion scores among individual pieces and their pairwise combinations (x-axis, color-coded by number of pieces examined and pathologists involved) to the referent standard for placentitis (**A**) and placental detachment (**B**). The combinations of pieces and pathologists (x-axis) were selected to mimic 1 or 2 pathologists requested to evaluate 1, 2, or 3 pieces each. Horizontal lines represent the median concordance correlation coefficient value for that group of samples. Pieces 1–3 (solid markers) and 4–6 (open markers) were assessed by pathologist 1 and 2, respectively. Hatched markers represent combinations of pieces assessed by both pathologists. Placental lesion scores have reasonably high accuracy but low precision.

knowing that costs can increase substantially with increasing litter size. In our past research, a single piece of MFI collected adjacent to the umbilical stump of each fetus was collected and tested given the large number of animals (n = 1,400) included in the evaluation.[4] Although this approach may be appropriate for large-scale screening, our present results indicate that when feasible, additional samples should be collected to improve accuracy and precision, but the number of pieces remains unclear.

Lin concordance correlation is a combined measure of the accuracy and precision of data to a referent standard.[15] Accuracy refers to how close estimates are to the true value, whereas precision refers to how close estimates are to each other regardless of how close they are to the true value. Although the concordance correlation is the most appropriate test for the present research, relevant questions including "what is an appropriate reference standard" and "what is a sufficient correlation" are outstanding. Without a preexisting gold standard (for viral load and lesion severity across tissue), the "average of all" was chosen as the best option.

Although greater concordance coefficient values are preferred, the optimum or target value largely depends on the research question, resources available to the project, and accuracy and precision required. Although arbitrary, our conclusions are based on our desire to achieve a concordance correlation ≥ 0.90, with roughly equal measures of precision and accuracy. Our finding of greater accuracy and precision

in larger pool sizes was expected. That accuracy was greater than precision in both END and PLC is indicative of the heterogeneity in viral load throughout the MFI, and supports the need for examining multiple pieces. In the absence of locational bias, the samples collected could be systematically or randomly collected if the selected number are representative of the entire organ.

Samples were collected at different days post-infection (2, 5, 8, 12, and 14 dpi), and some of the variability might be the result of differences in viral load at these times. As the infection progresses and the virus establishes its presence in the host, viral load increases on average but also becomes more heterogeneous within an animal. Unfortunately, the number of fetuses we included at each time was not balanced and was too small to accurately characterize changes in viral load heterogeneity by day. The exact day of infection is rarely known in diagnostic cases, but is likely to be > 7 dpi after clinical signs, fetal compromise, or abortion are first observed. This implies that diagnosticians should anticipate great viral load heterogeneity in diagnostic cases and err on the side of caution by collecting more, rather than fewer, samples of PLC or END, then pool within an animal.

Although PRRSV infection clusters in litters, the clustering is unpredictable, and the heterogeneity of the placental viral load and lesions adds additional factors to consider. It is essential to sample as many fetuses as is practical to fully understand the impact of PRRSV across the entire litter. This is particularly important when examining placentae because the placenta is more heterogeneous in terms of viral load and lesion severity than the maternal uterine layers, based on the results of our research. In terms of pathology assessments, the number of pathologists recruited is less important than the number of pieces examined per fetus. Our results indicate good agreement in semi-subjective lesion scores between 2 pathologists with reasonable expertise in examining PRRSV-affected reproductive tissues. As our results indicate, at least 3 pieces of placenta would be necessary for an accurate assessment of placental lesions by 1 pathologist. If a pathologist lacks expertise or in cases in which a robust scoring methodology is unavailable, inter-pathologist agreement may be lower.

If it is necessary to collect multiple samples from all or many fetuses, the task of accurately assessing viral load and/or lesion severity quickly becomes unfeasible on a large scale. Thus, pooling becomes a necessity. Although there is no alternative but to evaluate pieces individually for histopathology, pooling of tissues is an alternative for assessment of viral load by RT-qPCR. Although the simplest way of pooling is to combine and homogenize small portions of tissue, this technique is prone to quantification error because the portions will invariably differ in weight and the viral load estimate will be biased towards the viral load of the largest pieces. We evaluated 2 methods of pooling for viral load quantification in our study. The most cost-effective method was by combining equal volumes of homogenized tissue, each with a roughly equivalent starting weight. The alternative, pooling of tissue pieces after extraction, was costlier in terms of reagent costs and time. Given that neither method provided contingently superior results, pooling of tissue homogenates is the logical recommendation.

The fetal PLC used for our study originated from dams 2–14 dpi and encompassed a wide range of viral load as well as PRRSV-negative samples. Although the heterogeneity of samples used may be perceived as a limitation, we believe this is reflective of the range of caseload a diagnostic laboratory may experience, which supports the external validity of our results.

The optimal number of samples to pool ultimately depends on the tissue, level of accuracy and precision required, and any logistical limitations associated with the task. Fewer pieces of END are required compared to PLC for assessment of viral load and lesion severity; however, in smaller pools, individual outliers have greater impact. For our subsequent research, we chose tissue homogenates of 3 pieces as a reasonable balance of cost, feasibility, accuracy, and precision. With this strategy, the anticipated concordance correlation will be close to our target of 0.90. Although larger pools would have provided concordance > 0.9, larger pools would only provide a practical benefit for placentae in terms of precision while adding substantially to the laboratory cost and workload. In spite of these results, past studies in which single pieces of tissue were assessed remain valid but should be interpreted with some caution. Overall, our results offer insights on sampling techniques for future research projects and diagnostic investigations related to PRRSV and possibly other transplacental reproductive viruses.

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplementary material

Supplementary material for this article is available online.

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