Abstract

The use of processing fluids to monitor the breeding herd's porcine reproductive and respiratory syndrome (PRRS) status has gained industry acceptance. However, little is known about PRRS virus RT-qPCR detection dynamics in processing fluids and factors that may contribute to maintain PRRS virus in the herd after an outbreak. This study aimed to describe weekly RT-qPCR processing fluid results in breeding herds after an outbreak and to evaluate the proportion of RT-qPCR positive results among parity groups. Processing tissues of 15 first parity (P1), 15 second parity (P2), and 15 third parity or higher (P3+) litters (i.e., parity groups) were collected weekly for between 19 and 46 weeks in nine breeding herds. Processing fluids were aggregated, and RT-qPCR tested by parity group weekly. Additionally, a subset of 743 processing fluid samples of litters that formed 50 parity groups, as previously described, were RT-qPCR tested individually at the litter level. The agreement between RT-qPCR results of processing fluid samples of parity groups (15 litters) and results based on individual litter testing was assessed using overall percent of agreement, Kappa statistic, and McNemar test. The association between RT-qPCR results and the parity group was evaluated using a generalized estimating equations model, after accounting for the effects of sampling week, breeding herd PRRS control strategy (i.e., open to replacements v/s closed) and herd. An autoregressive correlation structure was used to account for the repeated samplings within a herd in time. The overall agreement was 98%, and Kappa statistic 0.955 (McNemar p=1.0). Sensitivity of parity group processing fluid samples was estimated at 100% (95% CI 89% - 100%), while specificity was estimated at 94% (95% CI 71% – 100%). Although P1 aggregated litters had on average a higher proportion of RT-qPCR positive results from outbreak week 25 onwards, the proportion was not significantly different to the one observed for P2 and P3+ aggregated litters (p>0.13) Additionally, herds that interrupted gilt entry had lower odds of PRRS RT-qPCR positivity than herds that continued entering gilts (OR=0.35, 95% CI 0.16-0.78). PRRS virus persistence in processing fluids was not affected by the sow parity effect in most of the breeding herds studied. No evidence of disagreement between RT-qPCR results of an aggregated

25 sample of 15 litters and those of individual litters was observed. This level of litter aggregation testing
26 strategy may be of particular use at the last stages of an elimination program under low PRRS virus
27 prevalence.

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Introduction

Porcine reproductive and respiratory syndrome (PRRS) virus is a ubiquitous pathogen that causes extensive economic losses to the United States swine industry (Neumann et al., 2005; Holtkamp et al., 2013). The disease is endemic in most pork producing countries. In the United States, PRRS incidence in breeding herds has a marked seasonal pattern in breeding herds with peaks during autumn and winter and reduced incidence during spring and summer (Tousignant et al., 2015; Sanhueza et al., 2020).

In 2009, a PRRS classification of breeding herds was proposed (Holtkamp et al., 2011). Briefly, herds were classified as positive unstable (category I-A), as positive stable (category II), as provisionally negative (category III), and as negative (category IV). A common destination for breeding herds after a PRRS outbreak is category II (positive stable), which was achieved after four consecutive negative RT-qPCR tests in serum of at least 30 due-to-wean pigs sampled every 30 days.

Although slightly different definitions of time-to-stability have been generated, the time from PRRS outbreak to consistently weaning RT-qPCR negative pigs (time-to-last RT-qPCR negative result) had a median time of approximately 41-45 weeks in United States breeding herds (Linhares et al., 2014; Linhares et al., 2017; Sanhueza et al., 2019). However, time-to-stability may vary significantly according to the season when the outbreak occurred, the PRRS virus strain associated with the outbreak, and the intervention strategy used to begin the load-close-expose process (e.g. live virus inoculation, modified live vaccine or none). Nonetheless, monitoring PRRS virus status of the breeding herd using sera of 30 due-to-wean pigs may fail to detect the virus when the prevalence is below 10%. Hence, a herd may be mistakenly classified as stable when still weaning PRRS virus positive pigs at a low prevalence level leading to further viral recirculation upon gilt entry. Therefore, there was a need for sampling strategies that increase herd sensitivity of PRRS virus diagnosis and thus decrease the false herd stability classification.

Processing fluids (i.e. serosanguineous exudate originating from tails and testicles after castration and tail docking) are easy to collect and allows for RT-qPCR testing aggregated samples to monitor the PRRS virus status of the breeding herd (Lopez et al., 2018; Vilalta et al., 2018). At the litter level, processing fluids were observed to correlate with the RT-qPCR status of individual pigs with an overall sensitivity of 87%, a specificity of 94% and an overall agreement of 92.2% (Vilalta et al., 2018). Furthermore, it was observed that pigs in litters of first or second parity sows had a significantly higher proportion of PRRS RT-qPCR positive serum results than pigs in litters of third or higher parity sows (Vilalta et al., 2018), suggesting a potential role of young parity litters on virus persistence within the herd.

The effect of processing fluid aggregation on PRRS virus detection by RT-qPCR was assessed by Vilalta et al. (2019a). The study highlighted that 1) a large number of aggregated processing fluids samples could be used to monitor the PRRS virus status of the breeding herd and, 2) the ability to classify an aggregated/pooled processing fluid sample as positive when it included a PRRS virus RT-qPCR positive individual sample was largely dependent on its Ct-value (i.e. viral concentration). Other sample types as udder skin line wipe samples and wipe samples of crate surfaces did not perform as well as processing fluids in correlating with the true litter status as determined by individual serum samples (Vilalta et al., 2019b; Vilalta et al., 2021).

The use of processing fluids as tool to monitor the PRRS virus status of the breeding herd has been widely adopted in the US swine industry. Criteria to promote herds into a given category and to maintain the PRRS status category were proposed in 2021 updating the criteria proposed in 2011 (Holtkamp et al., 2011; Holtkamp et al., 2021). Briefly, the most significant changes in the classification of breeding herds were the splitting of the positive unstable category into a positive unstable with high and low PRRSV prevalence, the splitting of the positive stable category into a positive stable and positive stable with vaccination, the increase in the number of weaning-age-pigs sampled from 30 to 60 tested in pools of 10

74 instead of 5, and the inclusion of RT-qPCR results based on processing fluid testing as supporting
75 information to determine the herd PRRS status (Holtkamp et al., 2021).

76 Trevisan et al. (2019) estimated the time to stability, defined as the time to achieve two consecutive
77 negative results in herds under batch farrowing system, at 27 weeks. Nonetheless, there is scarce
78 information on processing fluid PRRS virus RT-qPCR weekly results dynamics after an outbreak in breeding
79 herds. In 2021, de Almeida et al. (2021) described RT-qPCR results of processing fluids in commercial
80 breeding herds in the US. They observed that the maximum time of consistently negative RT-qPCR results
81 between two positive RT-qPCR results was 11 weeks, highlighting the challenges producers face when
82 interpreting and making decisions based on processing fluids PRRS virus RT-qPCR results in breeding
83 herds. This investigation aimed to describe weekly processing fluid RT-qPCR result dynamics after a PRRS
84 outbreak in breeding herds, to assess the agreement between aggregated processing fluid RT-qPCR results
85 and RT-qPCR results of individual litters, and to evaluate the role of sow parity in maintaining PRRS virus
86 in the herd.

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Materials and Methods

Breeding herd selection and enrollment

Breeding herds participating of the Morrison Swine Health Monitoring Project and that had recent PRRS outbreak at the time of herd selection were eligible to participate of the study. Ten (A through J) breeding herds located in the Midwestern United States (e.g. Minnesota, Missouri, Nebraska and South Dakota) belonging to five pig production systems were initially enrolled. One breeding herd dropped the study after two weeks from enrolment. A system is here defined as farms under same ownership and similar management practices. The herds were purposely selected for participation in this longitudinal study. In these herds, the PRRS outbreak occurred in October 2017 (n=1), December 2017 (n=3), and January 2018 (n=6). Breeding herds were enrolled in the study between March and May of 2018 when they were between eight and 25 weeks after the PRRS onset in the herd. Breeding herds were located in the Midwestern United States (Minnesota, South Dakota, Nebraska, Missouri and Illinois).

Breeding herds A, B and C continued entering replacement gilts monthly during the follow-up period (i.e 19 to 46 weeks), whereas breeding herds D, E, F, G, H, I, and J did not enter replacement gilts (herd closure) during the study period. Five of the enrolled herds were PRRS virus negative when the outbreak occurred (PRRS virus category IV), while two were positive stable-vaccinated (PRRS virus category II), one was positive stable not vaccinated (PRRS virus category II), one provisionally negative (PRRS virus category III), and one positive unstable (PRRS virus category I). Breeding herd I left the study two weeks after enrollment because of the labor-intensive sampling required each week. Therefore, this herd was removed from further analyses. Table 1 summarizes the information recorded in each herd about the system, outbreak date, restriction fragment length polymorphism (RFLP) pattern of the PRRS virus associated with the outbreak, PRRS status when the outbreak occurred, whether the farm was closed during the follow-up period, enrollment date, and whether the farm was lost to follow-up.

Sample collection

Each herd agreed to submit weekly processing tissues of approximately three day-old-piglets. Each week, processing tissues were collected from 15 gilt litters (P1), 15 second parity sows (P2), and 15 third or higher parity sows (P3+). A total of 275 samples per parity group were required to detect an overall positive proportion difference of at least 10% with 95% confidence and 80% power and considering an intra-class correlation of 0.1. At processing, all tails and testicles of pigs from a single litter were placed into a Ziploc® bag (S.C. Johnson & Son, Inc. Racine, WI). Sampling was done under normal management conditions within each farm. Sow ID, parity number and date of collection were recorded for each litter at processing. The bag was properly closed and kept frozen at approximately -18°C for one or two weeks until laboratory submission. One or two weeks' worth of samples were sent overnight to the laboratory for processing and testing.

Sample processing and testing

At arrival to the laboratory, processing tissues from 15 litters in each herd and week were aggregated by parity (i.e P1, P2, and P3+ litters) in a one-gallon Ziploc® bag. A parity group was defined as the aggregate of processing fluids from 15 litters of a given parity (i.e. P1, P2, and P3+ litters) in a week in a herd. Aggregated samples were left to thaw at room temperature for approximately four hours. Once the aggregated sample was completely thawed, the contents were manually homogenized before 10 milliliters of processing fluid was extracted using a sterile disposable pipette and transferred into a 15-milliliter falcon tube. One point eight (1.8) milliliters of processing fluid were then transferred into a two-milliliter cryogenic vial that was kept frozen at -80°C as a backup sample. Three falcon tubes containing aggregated processing fluid samples of P1 litters, P2 litters, and P3+ litters were then submitted to Veterinary Diagnostic Laboratory (VDL) for RT-qPCR testing.

Wean-age pig sera

Wean-age pig sera RT-qPCR results were made available in three herds (E, F, and G). In these herds, blood samples of 30 wean-age pigs were collected and RT-qPCR tested in six pools of five samples. The RT-qPCR results of serum samples were described along with the results of processing fluids in these herds.

Individual litter samples

Every five weeks, litter processing fluid samples were stored individually before being aggregated into parity groups. For this, processing tissues were left to thaw at room temperature at arrival to the laboratory for approximately four hours. Once thawed, 0.5 milliliters of processing fluid present in the Ziploc® bag that contained a litter of processing tissues was pipetted and placed into a falcon tube for pooling and the rest of the exudate (maximum of 1.8 milliliters) placed into a two-milliliter cryogenic vial and stored at -80°C. A subset of individual litter processing fluid samples were RT-qPCR tested at the University of Minnesota -Veterinary Diagnostic Laboratory.

Individual litter testing

Processing fluids from 743 litters individually stored, that formed part of 50 parity group samples in six sow herds, were RT-qPCR tested individually. A total of 34 parity groups were selected based on a positive RT-qPCR result and 16 parity groups based on a negative RT-qPCR result. Overall, 226 individual litter samples belonged to parity 1 sows and formed part of 15 P1 parity groups, 232 litter samples belonged to parity 2 sows and formed part of 16 P2 parity groups, and 285 litter samples belonged to parity 3+ sows and formed part of 19 P3+ parity groups.

Comparison of parity group results based on aggregated or individual litter samples

Fifty group parity samples (34 rRT-Positive and 16 RT-qPCR negative) were purposely selected to evaluate the agreement between their RT-qPCR results and the RT-qPCR result of the same group based on

individual litter testing of processing fluids. For the latter, the group was considered as RT-qPCR positive when at least one litter that contributed to the group parity sample was RT-qPCR positive (Ct < 40).

Statistical analysis

Processing fluid RT-qPCR results were visualized over time by breeding herd and parity group using a mat plot.

The agreement was assessed using the overall percent of agreement, and Kappa statistic. Kappa values ≤0, >0-0.2, >0.2-0.4, >0.4-0.6, >0.6-0.8, >0.8 were considered as poor agreement, slight agreement, fair agreement, moderate agreement, substantial agreement, and almost perfect agreement, respectively (Dohoo et al., 2009). Additionally, sensitivity and specificity of RT-qPCR results of the 15 litters parity group sample compared to the results of individual litters that composed the parity group was estimated. Statistical significance was tested using the McNemar χ^2 test for paired proportions.

A generalized estimating equations (GEE) model was built to assess the association between aggregated RT-qPCR results (positive/negative) and parity groups (P1, P2, and P3+). Time in weeks after the outbreak was added to the model as fixed effect. The interaction between parity group and time was evaluated. Herd was incorporated in the model as a cluster variable to account for the repeated samplings carried out in the same herd. An autoregressive correlation structure (AR-1) was used to model the correlation between RT-qPCR results over time. Linearity of continuous variables against the log odds of the outcome was visually assessed. Whenever a nonlinear relationship was observed, the continuous variable was categorized into its quartiles.

Statistical analyses were done using R version 4.0.4 (R Core Team, 2021) and the R package geepack (Yan, 2002; Yan and Fine, 2004; Højsgaard et al., 2006).

Results

Overall, processing tissues from 11,834 litters were collected between March 2018 and February 2019. These litters were aggregated into 283 groups of P1 litters, 284 groups of P2 litters, and 285 groups of P3+ litters. Herd D did not send P1 samples one week and sent only P3+ samples during another week. From the 852 parity groups tested, 246 (28.4%, 95% CI 25.5%-31.5%) were RT-qPCR positive. Positive RT-qPCR results had Ct-values that ranged from 18.52 to 39.64, with a mean value of 29.7 (95% CI 29.1-30.3).

The unadjusted percentage of RT-qPCR positive P1 groups (31.1% [88/283], 95% CI 25.8% - 36.8%) was not significantly different from the percentage of RT-qPCR positive P2 groups (23.9% [68/284], 95% CI 19.1%-29.3%), or P3+ groups (30.2% [86/285], 95% CI 24.9%-35.9%). The mean Ct-value of positive P1, P2 and P3 groups was 30.1 (95% 29.1-31.1), 29.0 (95% CI 28.0-30.1), and 29.8 (95% CI 28.9-30.7), respectively. These differences were not statistically significant (p=0.31).

The nine herds provided processing tissues for 19 to 46 consecutive weeks. Four out of the nine farms had enough confidence that PRRS virus was not present in the herd after 10 consecutive negative weeks (herd E), 13 consecutive negative weeks (herd F and G), and 14 consecutive negative weeks (herd J). These herds proceeded to allow the entry of replacement gilts to the herd after this time. Herds G, F and E progressed into provisionally negative category (category III). Breeding herd J decided not to work towards elimination and used field virus to acclimate gilts. The latter herd had another outbreak on June 2019 with a virus which had an open reading frame 5 (ORF-5) sequence similarity below 98% compared to the previous one.

Figure 1 shows weekly processing fluids RT-qPCR results in the nine participating herds by parity group. The time from the PRRS outbreak to having at least 10 weeks of consecutive negative results was 33 weeks for herd J, 38 weeks for herd G, 39 weeks for herd F, and 48 weeks for herd E. However, herds A, B, C, D, and H were still working towards PRRS stability after at least 52 weeks from the PRRS outbreak. The

maximum number of consecutive RT-qPCR negative results between two RT-qPCR positive results was one week for herd G and J; two weeks for herd D; three weeks for herd B, C, and E; five weeks for herd F; eight weeks for herd H; and nine weeks for herd A.

Wean pig RT-qPCR sera results were available for herds E, F, and G. In herd G, blood samples of wean-age pigs started being collected at approximately six months (June 2018) after the start of the outbreak. The last sera RT-qPCR positive pool result occurred one week after the last RT-qPCR positive result of processing fluid samples. After that point, four monthly consecutive negative RT-qPCR tests in wean-age pig sera and 13 weeks of consecutive RT-qPCR negative processing fluid test results were observed. In herd F, wean-age pig blood samples started being taken at approximately six months (June 2018) after the initial outbreak. The last RT-qPCR positive result in wean-age pig sera occurred one week earlier than the last RT-qPCR positive result of processing fluids. After that point, three monthly consecutive negative RT-qPCR results were observed in wean-age pig sera and 13 weeks of consecutive negative RT-qPCR results weekly in processing fluids were achieved. Herd E had the last RT-qPCR positive result in wean-age pig sera in the first week of August 2018, while the last RT-qPCR positive result of processing fluids was obtained in the last week of September 2018. After that point, six bi-weekly consecutive negative RT-qPCR results in wean-age pig sera and 10 consecutive weeks of RT-qPCR negative processing fluid results were obtained.

Individual Litter RT-qPCR results

Overall, 123 out of 743 processing fluid samples individually tested at the litter level were RT-qPCR positive (16.6%, 95% CI 14.0% - 19.4%). RT-qPCR positive processing fluids at the litter level had a median Ct-value of 29.2 (1^{st} quartile 25.3, 3^{rd} quartile 33.1, minimum 17.3, and maximum 39.4). Among 50 parity groups, 34 groups tested RT-qPCR positive with a median Ct-value of 29.2 (1^{st} quartile 27.5, 3^{rd} quartile 31.7, minimum 20.8, and maximum 37.4). Out of these 34, 33 had at least one individual litter RT-qPCR positive,

223 and one group had no RT-qPCR positive litter (Figure 2, pooled parity sample number 18). Therefore, the
224 123 positive litters were distributed in 33 parity groups. Figure 2 shows Ct-values quantified in each of the
225 34 RT-qPCR positive group pooled processing fluid samples (red open crossed circles) and Ct-values
226 quantified in each RT-qPCR positive processing fluid samples from litters that formed the group sample
227 (blue dots).

228 Figure 3 shows the number of RT-qPCR positive litters within grouped processing fluid samples. Grouped
229 samples consisted of between 12 and 17 litters. Most positive parity groups (63.6% [21/33]) had three or
230 less RT-qPCR positive litters. Fifty percent (25/50) of parity samples RT-qPCR tested had none (n litters 17)
231 or one (n litters = 8) RT-qPCR positive litter.

232 Table 2 shows the agreement between RT-qPCR results of aggregated processing fluid samples and
233 individual litter processing fluid samples (at least one RT-qPCR positive litter for a positive aggregated
234 sample). The sensitivity and specificity were estimated at 100% (95% CI 89% - 100%) and 94% (95% CI 71%
235 – 100%), respectively. The total agreement was 98%, while the Kappa statistic was 0.955 indicating an
236 almost perfect agreement. The McNemar's χ^2 test was non-significant (continuity correction p = 1.00),
237 which indicates lack of statistical evidence for a difference between the paired RT-qPCR positive
238 proportions.

239 Multivariable model results using the GEE are presented in Table 3. A significant interaction was observed
240 between parity group and the week after the outbreak category in the association with PRRS virus RT-
241 qPCR status. At week category of <25 weeks from the outbreak, P3+ litters had 2.0 times (95% CI 1.29 –
242 3.16) the odds of yielding a positive RT-qPCR than P1 litters (p=0.002). However, at the week categories
243 of 25-34, 34-43, and >43 P1 litters had higher odds of RT-qPCR positivity than P3+ litters. Nonetheless,
244 these differences were not statistically significant. Nonetheless, in herd E, P1 aggregated litters had RT-
245 qPCR positive results for 11 weeks more than aggregated litters of P2 and P3+ sows.

Although the RT-qPCR positive proportion decreased in all parity groups through time, the reduction was greater in P3+ litters. The adjusted proportion of RT-qPCR positive P3+ litters decreased from 49.9% at <25 weeks after the outbreak, to 13.0% at ≥43 weeks after the outbreak. Similarly, the proportion of RT-qPCR positive P1 and P2 litters decreased from 33.0% and 38.5% at <25 weeks after the outbreak to 18.9% and 8.9% at ≥43 weeks after the outbreak, respectively (Figure 4).

Finally, herds that performed herd closure had 0.35 times the odds (95% CI 0.16 – 0.78) of RT-qPCR positivity than herds that kept introducing gilts during the study period; after accounting for the effects of herd, parity group and time after the outbreak. Overall, herds that adopted herd closure had a lower adjusted proportion of RT-qPCR positive results (16.5%, 95% CI 8.4% - 29.7%) compared to herds that did not close the herd (36.6%, 95% CI 28.6% - 44.1%).

Discussion

This study assessed the role of parity in maintaining PRRS virus in breeding herds. Although processing fluids of P1 litters had a numerical higher proportion of RT-qPCR positive results than processing fluids of P2 or P3+ litters after 25 weeks from the outbreak, the overall adjusted proportion of P1 RT-qPCR positive groups was not significantly different to the proportion of P2 or P3+ RT-qPCR positive groups at ≥25 - <34, ≥34 - <43, ≥43 weeks after the outbreak (Figure 4). A previous study in a single sow farm reported a significantly higher PRRS virus RT-qPCR positive proportion in P1 and P2 litters compared to that of third parity litters or higher (Vilalta et al., 2018). Similarly, one breeding herd in our study (herd E, Figure 1) followed a similar pattern in which P1 litters stayed positive for 11 weeks longer than P2 or P3+ litters. The herds used in this study were not selected at random from the population, and therefore it is unknown whether this proportion (11.1% [1/9], 95% CI 2.0% - 43.5%) is a good estimate of the proportion of herds in the population of United States breeding herds that will experience a prolonged PRRS virus positivity in P1 litters compared to P2 and P3+ litters.

A statistically significant interaction between sampling week category and parity group was observed in the data. The odds of RT-qPCR positivity were lower in P1 litters compared to that of P2 ($p=0.45$) and P3+ ($p=0.002$) litters at <25 weeks after the PRRS outbreak. However, the relationship changed after 25 weeks from the outbreak where the odds of RT-qPCR positivity tended to be higher in P1 litters compared to P2 and P3+ litters (Figure 2). Nonetheless, differences were not statistically significant among parity groups after 25 weeks from the PRRS outbreak ($p > 0.13$). However, in one out of the nine participating herds, P1 aggregated litters continued being RT-qPCR positive for 11 weeks after the last RT-qPCR positive result of P2 and P3+ aggregated litters. The sample size used in this study was enough to detect an overall difference among parity groups of at least 10% with 95% confidence and 80% power and considering an

intra-class correlation of 0.1. Nonetheless, the magnitude of the differences in the percentage of RT-qPCR positive litters among parity groups in our study was moderately low and most of the time below 10%.

Breeding herds that stopped introducing replacements during the stabilization period had 0.35 (95% CI 0.16 – 0.78) times the odds of having a RT-qPCR positive result as herds that kept entering gilts. Herd closure and rollover is a common strategy used to eliminate PRRS virus from a breeding herd. This strategy is based on the idea of reducing the susceptible population within the herd, which limits the spread of the virus, resulting in a decrease in the number of new infections and eventually the elimination of infection within the herd (Torremorell and Christianson, 2002; Corzo et al., 2010). Our results support the use of herd closure as a means of controlling and eliminating PRRS virus from breeding herds. By the end of the follow-up period, four out of the six herds that adopted herd closure were confident enough to classify themselves as PRRS virus stable and re-introduce gilts. In contrast, none of the three herds that kept entering gilts managed to reach stability during this time.

Cross-fostering is a common practice in breeding herds that aims to balance litter size, increase access to functional teats, reduce weight variation and increase piglet survivability (Calderón Díaz et al., 2018). In participating herds, the occurrence of this practice was limited and restricted mostly to the first 24 hours from birth between litters of the same parity group to ensure each pig had access to a functional teat. Size sorting and excessive movement of pigs was not allowed. Scarce data exist about the proportion of PRRS virus RT-qPCR positive pigs at processing age within a litter after a PRRS outbreak. Vilalta et al. (2019b) observed that after week 11 from the initial outbreak, the proportion of RT-qPCR positive pigs was 2.0% (2/102), 6.5% (8/124), 0.9% (1/112), 3.3% (4/121), and 1.6% (2/127) at weeks 11, 14, 17, 20, 23 after the outbreak, respectively. Therefore, if these data are representative of the frequency of RT-qPCR positive results in processing age pigs after a PRRS outbreak, then the likelihood of selecting a PRRS virus

positive pig and placing it into a PRRS virus negative litter may have been small, reducing the opportunities for litter RT-qPCR result misclassification.

In this study, 15 litters on average were RT-qPCR tested as an aggregated sample within a parity group. When RT-qPCR results of the grouped sample were compared with the group classification based on individual litter RT-qPCR results (Table 2), a substantial agreement was observed (Kappa 0.91), despite the moderate low number of RT-qPCR positive litters in a RT-qPCR positive grouped sample (20.7% or approximately a median of 3/15 litters). The disagreement was mainly due to one RT-qPCR positive parity group sample that had no RT-qPCR positive processing fluids in the litters that formed part of the group. Samples were retested obtaining the same results. We think that this observation may be attributed to cross-contamination at the laboratory when manually aggregating the processing fluid samples by parity group. If that's the true cause of the observed disagreement, then the specificity estimate would have been 100%.

Similarly, RT-qPCR results of litter-aggregated processing fluids were observed to correlate with RT-qPCR results of individual pigs within the litter (Vilalta et al., 2018), and despite the dilution effect that occurs when processing fluids of several litters are aggregated, a positive sample can still be detected in a wide range of dilutions depending mostly on the sample's initial Ct-value (Vilalta et al., 2019a).

The use of processing fluids has shown to be a reliable sample to monitor PRRS virus presence in breeding herds by means of RT-qPCR testing (Lopez et al., 2018; Vilalta et al., 2018; Trevisan et al., 2019; de Almeida et al., 2021). In our study, two breeding herds had a maximum of eight and nine weeks of consecutive negative RT-qPCR results followed by a RT-qPCR positive result. Similarly, de Almeida et al. (2021) observed a maximum of 11 weeks between two RT-qPCR positive processing fluid results in a herd. Having several weeks of RT-qPCR negative results followed by a RT-qPCR positive results in processing fluids seems to be a relatively common observation that should encourage swine veterinarians and producers

to keep testing the herd despite several weeks of negative RT-qPCR results in processing fluids to increase the confidence that at processing, piglets continue to be PRRS virus negative. Holtkamp et al. (2021) proposed a modification to the PRRS virus herd classification. It was suggested that the use of processing fluids can provide supporting evidence for PRRS virus herd stability, but testing wean-age pigs is also required. If processing fluids are used to provide evidence of PRRS virus stability, then one or more weekly pools of processing fluids should be RT-qPCR tested negative for 13 weeks. In addition, sera of 30 pigs should be RT-qPCR tested negative in pools of five monthly for 90 days.

The time from the onset of the PRRS outbreak to consistently negative processing fluids, defined here as having at least 10 weeks of consecutive negative RT-qPCR results in processing fluids was 33, 38, 39, and 48 weeks in four out of nine breeding herds (time to the 10^{th} RT-qPCR consecutive negative result). The remaining five herds were still not able to get 10 consecutive RT-qPCR negative results after 52 weeks from the start of the outbreak. Trevisan et al. (2019) estimated time to stability at 27 weeks in 29 batch-farrowing herds based on two consecutive negative processing fluid batches, negative RT-qPCR results in due-to-wean pig sera, and negative ELISA results in gilts after herd introduction. This estimation is shorter than what our data may suggest. However, the nine herds purposely included in our study were not batch-farrowing farms. It may be possible that the batch-farrowing system allows for a faster PRRS virus clearance from the farm. Further studies should evaluate the time to PRRS stability using processing fluids in a larger sample of herds to provide a more precise estimate and determine whether P1 sows have a role in maintaining PRRS virus in the herd.

Conclusion

The proportion of PRRS virus RT-qPCR processing fluid positive litters was not statistically higher in aggregated samples of first parity sows compared to that of second parity or third parity or higher sows after week 25 from the start of the outbreak. Therefore, the role of first parity sows and their litters in maintaining PRRS virus infection in breeding herds could not be confirmed in this investigation despite the consistent non-significant higher point estimate proportion observed from week 25 after the outbreak onwards. Processing fluid RT-qPCR result dynamics in nine herds showed that at least nine weeks of consecutive negative processing fluid RT-qPCR results may exist between two RT-qPCR positive weeks. This observation should encourage veterinarians and swine producers that use processing fluids to monitor PRRS virus circulation in breeding herds to continue testing the herd beyond this time span to avoid misclassification of herd PRRS virus status. The use of processing fluids continues to reaffirm their value for PRRS virus monitoring and surveillance activities in breeding herds. Monitoring the breeding herd PRRS virus status using aggregated processing fluid samples of 15 litters was a useful testing strategy throughout the outbreak in all herds assessed.

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Abstract

The use of processing fluids to monitor the breeding herd's porcine reproductive and respiratory syndrome (PRRS) status has gained industry acceptance. However, little is known about PRRS virus ~~rRT-PCR~~RT-qPCR detection dynamics in processing fluids and factors that may contribute to maintain PRRS virus in the herd after an outbreak. This study aimed to describe weekly ~~rRT-PCR~~RT-qPCR processing fluid results in breeding herds after an outbreak and to evaluate the proportion of ~~rRT-PCR~~RT-qPCR positive results among ~~sow~~ parity groups. Processing tissues of 15 first parity ~~one~~ (P1), 15 second parity ~~two~~ (P2), and 15 third parity ~~three~~ or higher (P3+) litters (i.e., parity groups) were collected weekly for between 19 and 46 weeks in nine breeding herds. Processing fluids were aggregated, and ~~rRT-PCR~~RT-qPCR tested by parity group weekly. Additionally, a subset of 743 processing fluid samples of litters that formed 50 parity groups, as previously described, were ~~rRT-PCR~~RT-qPCR tested individually at the litter level. The agreement between ~~rRT-PCR~~RT-qPCR results of processing fluid samples of parity groups (15 litters) ~~parity groups tested in aggregated fashion~~ and results based on individual litter testings~~or by litter~~ was assessed using overall percent of agreement, Kappa statistic, and McNemar test. The association between ~~aggregated rRT-PCR~~RT-qPCR results and the parity group was evaluated using a generalized estimating equations model, after accounting for the effects of sampling week, breeding herd PRRS control strategy (i.e., open to replacements v/s closed) and herd. ~~A generalized estimating equations model~~ An ~~with~~ autoregressive correlation structure was used to account for the repeated samplings within a herd in time. The overall agreement was 98%, and Kappa statistic 0.955 (McNemar p=1.0). Sensitivity of parity group processing fluid samples was estimated at 100% (95% CI 89% - 100%), while specificity was estimated at 94% (95% CI 71% – 100%). Although P1 aggregated litters had on average a higher proportion of ~~rRT-PCR~~RT-qPCR positive results from outbreak week 25 onwards, the proportion was not significantly different to the one observed for P2 and P3+ aggregated litters (p>0.13). ~~However, in one out of the nine participating herds, P1 aggregated litters continued being rRT-PCR positive for 11 weeks after the last rRT-~~

~~PCR positive result of P2 and P3+ aggregated litters.~~ Additionally, herds that interrupted gilt entry had lower odds of PRRS ~~rRT-PCR~~RT-qPCR positivity than herds that continued entering gilts (OR=0.35, 95% CI 0.16-0.78). PRRS virus persistence in processing fluids was not affected by the sow parity effect in most of the breeding herds studied. No evidence of disagreement between ~~rRT-PCR~~RT-qPCR results of an aggregated sample of 15 litters and those of individual litters was observed. This level of litter aggregation testing strategy may be of particular use at the last stages of an elimination program under low PRRS virus prevalence.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) virus is a ubiquitous pathogen that causes extensive economic losses to the United States swine industry (Neumann et al., 2005; Holtkamp et al., 2013). The disease is endemic in most pork producing countries. In the United States, PRRS incidence in breeding herds has a marked seasonal pattern in breeding herds with peaks during autumn and winter and reduced incidence during spring and summer (Tousignant et al., 2015; Sanhueza et al., 2020).

In 2009, a PRRS classification of breeding herds was proposed (Holtkamp et al., 2011). Briefly, herds were classified as positive unstable (category I-A), as positive stable (category II), as provisionally negative (category III), and as negative (category IV). A common destination for breeding herds after a PRRS outbreak is category II (positive stable), which was achieved after four consecutive negative ~~rRT-PCR~~RT-qPCR tests in serum of at least 30 due-to-wean pigs sampled every 30 days.

Although slightly different definitions of time-to-stability have been generated, the time from PRRS outbreak to consistently weaning ~~rRT-PCR~~RT-qPCR negative pigs (time-to-last ~~rRT-PCR~~RT-qPCR negative result) had a median time of approximately 41-45 weeks in United States breeding herds (Linhares et al., 2014; Linhares et al., 2017; Sanhueza et al., 2019). However, time-to-stability may vary significantly according to the season when the outbreak occurred, the PRRS virus strain associated with the outbreak, and the intervention strategy used to begin the load-close-expose process (e.g. live virus inoculation, modified live vaccine or none). Nonetheless, monitoring PRRS virus status of the breeding herd using sera of 30 due-to-wean pigs may fail to detect the virus when the prevalence is below 10%. Hence, a herd may be mistakenly classified as stable when still weaning PRRS virus positive pigs at a low prevalence level leading to further viral recirculation upon gilt entry. Therefore, there was a need for sampling strategies that increase herd sensitivity of PRRS virus diagnosis and thus decrease the false herd stability classification.

Processing fluids (i.e. serosanguineous exudate originating from tails and testicles after castration and tail docking) are easy to collect and allows for ~~rRT-PCR~~RT-qPCR testing aggregated samples to monitor the PRRS virus status of the breeding herd (Lopez et al., 2018; Vilalta et al., 2018). At the litter level, processing fluids were observed to correlate with the ~~rRT-PCR~~RT-qPCR status of individual pigs with an overall sensitivity of 87%, a specificity of 94% and an overall agreement of 92.2% (Vilalta et al., 2018). Furthermore, it was observed that pigs in litters of first or second parity ~~one or two~~ sows had a significantly higher proportion of PRRS ~~rRT-PCR~~RT-qPCR positive serum results than pigs in litters of third or higher parity ~~three or higher~~ sows (Vilalta et al., 2018), suggesting a potential role of young parity litters on virus persistence within the herd.

The effect of processing fluid aggregation on PRRS virus detection by ~~rRT-PCR~~RT-qPCR was assessed by Vilalta et al. (2019a). The study highlighted that 1) a large number of aggregated processing fluids samples could be used to monitor the PRRS virus status of the breeding herd and, 2) the ability to classify an aggregated/pooled processing fluid sample as positive when it included a PRRS virus ~~rRT-PCR~~RT-qPCR positive individual sample was largely dependent on its Ct-value (i.e. viral concentration). Other sample types as udder skin line wipe samples and wipe samples of crate surfaces did not perform as well as processing fluids in correlating with the true litter status as determined by individual serum samples (Vilalta et al., 2019b; Vilalta et al., 2021).

The use of processing fluids as tool to monitor the PRRS virus status of the breeding herd has been widely adopted in the US swine industry. Criteria to promote herds into a given category and to maintain the PRRS status category were proposed in 2021 updating the criteria proposed in 2011 (Holtkamp et al., 2011; Holtkamp et al., 2021). Briefly, the most significant changes in the classification of breeding herds were the splitting of the positive unstable category into a positive unstable with high and low PRRSV prevalence, the splitting of the positive stable category into a positive stable and positive stable with

vaccination, the increase in the number of weaning-age-pigs sampled from 30 to 60 tested in pools of 10 instead of 5, and the inclusion of ~~rRT-PCR~~RT-qPCR results based on processing fluid testing as supporting information to determine the herd PRRS status (Holtkamp et al., 2021).

Trevisan et al. (2019) estimated the time to stability, defined as the time to achieve two consecutive negative results in herds under batch farrowing system, at 27 weeks. Nonetheless, there is scarce information on processing fluid PRRS virus ~~rRT-PCR~~RT-qPCR weekly results dynamics after an outbreak in breeding herds. In 2021, de Almeida et al. (2021) described ~~rRT-PCR~~RT-qPCR results of processing fluids in commercial breeding herds in the US. They observed that the maximum time of consistently negative ~~rRT-PCR~~RT-qPCR results between two positive ~~rRT-PCR~~RT-qPCR results was 11 weeks, highlighting the challenges producers face when interpreting and making decisions based on processing fluids PRRS virus ~~rRT-PCR~~RT-qPCR results in breeding herds. This investigation aimed to describe weekly processing fluid ~~rRT-PCR~~RT-qPCR result dynamics after a PRRS outbreak in breeding herds, to assess the agreement between aggregated processing fluid ~~rRT-PCR~~RT-qPCR results and ~~rRT-PCR~~RT-qPCR results of individual litters, and to evaluate the role of sow parity in maintaining PRRS virus in the herd.

Materials and Methods

Breeding herd selection and enrollment

Breeding herds participating of the Morrison Swine Health Monitoring Project and that had recent PRRS outbreak at the time of herd selection were eligible to participate of the study. Ten (A through J) breeding herds located in the Midwestern United States (e.g. Minnesota, Missouri, Nebraska and South Dakota) belonging to five pig production systems were initially enrolled. One breeding herd dropped the study after two weeks from enrolment. A system is here defined as farms under same ownership and similar management practices. The herds were purposely selected for participation in this longitudinal study. In these herds, the PRRS outbreak occurred in October 2017 (n=1), December 2017 (n=3), and January 2018 (n=6). Breeding herds were enrolled in the study between March and May of 2018 when they were between eight and 25 weeks after the PRRS onset in the herd. Breeding herds were located in the Midwestern United States (Minnesota, South Dakota, Nebraska, Missouri and Illinois).

Breeding herds A, B and C continued entering replacement gilts monthly during the follow-up period (i.e 19 to 46 weeks), whereas breeding herds D, E, F, G, H, I, and J did not enter replacement gilts (herd closure) during the study period. Five of the enrolled herds were PRRS virus negative when the outbreak occurred (PRRS virus category IV), while two were positive stable-vaccinated (PRRS virus category II), one was positive stable not vaccinated (PRRS virus category II), one provisionally negative (PRRS virus category III), and one positive unstable (PRRS virus category I). Breeding herd I left the study two weeks after enrollment because of the labor-intensive sampling required each week. Therefore, this herd was removed from further analyses. Table 1 summarizes the information recorded in each herd about the system, outbreak date, restriction fragment length polymorphism (RFLP) pattern of the PRRS virus associated with the outbreak, PRRS status when the outbreak occurred, whether the farm was closed during the follow-up period, enrollment date, and whether the farm was lost to follow-up.

Sample collection

Each herd agreed to submit weekly processing tissues of approximately three day-old-piglets. Each week, processing tissues were collected from 15 gilt litters (P1), 15 second parity ~~two~~ sows (P2), and 15 third or higher parity ~~three~~ sows ~~or higher~~ (P3+). A total of 275 samples per parity group were required to detect an overall positive proportion difference of at least 10% with 95% confidence and 80% power and considering an intra-class correlation of 0.1. At processing, all tails and testicles of pigs from a single litter were placed into a Ziploc® bag (S.C. Johnson & Son, Inc. Racine, WI). Sampling was done under normal management conditions within each farm. Sow ID, parity number and date of collection were recorded for each litter at processing. The bag was properly closed and kept frozen at approximately -18°C for one or two weeks until laboratory submission. One or two weeks' worth of samples were sent overnight to the laboratory for processing and testing.

Sample processing and testing

At arrival to the laboratory, processing tissues from 15 litters in each herd and week were aggregated by parity (i.e P1, P2, and P3+ litters) in a one-gallon Ziploc® bag. A parity group was defined as the aggregate of processing fluids from 15 litters of a given parity (i.e. P1, P2, and P3+ litters) in a week in a herd. Aggregated samples were left to thaw at room temperature for approximately four hours. Once the aggregated sample was completely thawed, the contents were manually homogenized before 10 milliliters of processing fluid was extracted using a sterile disposable pipette and transferred into a 15-milliliter falcon tube. One point eight (1.8) milliliters of processing fluid were then transferred into a two-milliliter cryogenic vial that was kept frozen at -80°C as a backup sample. Three falcon tubes containing aggregated processing fluid samples of P1 litters, P2 litters, and P3+ litters were then submitted to Veterinary Diagnostic Laboratory (VDL) for ~~rRT-PCR~~RT-qPCR testing.

Wean-age pig sera

Wean-age pig sera ~~rRT-PCR~~RT-qPCR results were made available in three herds (E, F, and G). In these herds, blood samples of 30 wean-age pigs were collected and ~~rRT-PCR~~RT-qPCR tested in six pools of five samples. The ~~rRT-PCR~~RT-qPCR results of serum samples were described along with the results of processing fluids in these herds.

Individual litter samples

Every five weeks, litter processing fluid samples were stored individually before being aggregated into parity groups. For this, processing tissues were left to thaw at room temperature at arrival to the laboratory for approximately four hours. Once thawed, 0.5 milliliters of processing fluid present in the Ziploc® bag that contained a litter of processing tissues was pipetted and placed into a falcon tube for pooling and the rest of the exudate (maximum of 1.8 milliliters) placed into a two-milliliter cryogenic vial and stored at -80°C. A subset of individual litter processing fluid samples were ~~rRT-PCR~~RT-qPCR tested at the University of Minnesota -Veterinary Diagnostic Laboratory.

Individual litter testing

Processing fluids from 743 litters individually stored, that formed part of 50 ~~sow~~ parity group samples in six sow herds, were ~~rRT-PCR~~RT-qPCR tested individually. A total of 34 parity groups were selected based on a positive ~~rRT-PCR~~RT-qPCR result and 16 parity groups based on a negative ~~rRT-PCR~~RT-qPCR result. Overall, 226 individual litter samples belonged to parity 1 sows and formed part of 15 P1 parity groups, 232 litter samples belonged to parity 2 sows and formed part of 16 P2 parity groups, and 285 litter samples belonged to parity 3+ sows and formed part of 19 P3+ parity groups.

Comparison of parity group results based on aggregated or individual litter samples

Fifty group parity samples (34 rRT-Positive and 16 ~~rRT-PCR~~RT-qPCR negative) were purposely selected to evaluate the agreement between their ~~rRT-PCR~~RT-qPCR results and the ~~rRT-PCR~~RT-qPCR result of the same group based on individual litter testing of processing fluids. For the latter, the group was considered as ~~rRT-PCR~~RT-qPCR positive when at least one litter that contributed to the group parity sample was ~~rRT-PCR~~RT-qPCR positive (Ct < 40).

Statistical analysis

Processing fluid ~~rRT-PCR~~RT-qPCR results were visualized over time by breeding herd and ~~aggregated~~ parity group using a mat plot. ~~Locally estimated scatterplot smoothing (LOESS) was used to visualize the proportion of rRT-PCR positive results through time (weeks from the initial PRRS outbreak) in each parity group.~~

The agreement was assessed using the overall percent of agreement, and Kappa statistic. Kappa values ~~of~~ ~~of~~ ≤0, >0-0.2, >0.2-0.4, >0.4-0.6, >0.6-0.8, >0.8 were considered as poor agreement, slight agreement, fair agreement, moderate agreement, substantial agreement, and almost perfect agreement, respectively (Dohoo et al., 2009). Additionally, sensitivity and specificity of ~~rRT-PCR~~RT-qPCR results of the 15 litters ~~aggregated~~ parity group sample compared to the results of individual litters that composed the parity group was estimated. Statistical significance was tested using the McNemar χ^2 test for paired proportions.

A generalized estimating equations (GEE) model was built to assess the association between aggregated ~~rRT-PCR~~RT-qPCR results (positive/negative) and parity groups (P1, P2, and P3+). Time in weeks after the outbreak was added to the model as fixed effect. The interaction between parity group and time was evaluated. Herd was incorporated in the model as a cluster variable to account for the repeated samplings carried out in the same herd. An autoregressive correlation structure (AR-1) was used to model the correlation between ~~rRT-PCR~~RT-qPCR results over time. Linearity of continuous variables against the log

odds of the outcome was visually assessed. Whenever a nonlinear relationship was observed, the continuous variable was categorized into its quartiles.

Statistical analyses were done using R version 4.0.4 (R Core Team, 2021) and the R package geepack (Yan, 2002; Yan and Fine, 2004; Højsgaard et al., 2006).

Results

Overall, processing tissues from 11,834 litters were collected between March 2018 and February 2019. These litters were aggregated into 283 groups of P1 litters, 284 groups of P2 litters, and 285 groups of P3+ litters. Herd D did not send P1 samples one week and sent only P3+ samples during another week. From the 852 parity groups tested, 246 (28.4%, 95% CI 25.5%-31.5%) were ~~rRT-PCR~~RT-qPCR positive. Positive ~~rRT-PCR~~RT-qPCR results had Ct-values that ranged from 18.52 to 39.64, with a mean value of 29.7 (95% CI 29.1-30.3).

The unadjusted percentage of ~~rRT-PCR~~RT-qPCR positive P1 groups (31.1% [88/283], 95% CI 25.8% - 36.8%) was not significantly different from the percentage of ~~rRT-PCR~~RT-qPCR positive P2 groups (23.9% [68/284], 95% CI 19.1%-29.3%), or P3+ groups (30.2% [86/285], 95% CI 24.9%-35.9%). The mean Ct-value of positive P1, P2 and P3 groups was 30.1 (95% 29.1-31.1), 29.0 (95% CI 28.0-30.1), and 29.8 (95% CI 28.9-30.7), respectively. These differences were not statistically significant (p=0.31).

The nine herds provided processing tissues for 19 to 46 consecutive weeks. Four out of the nine farms had enough confidence that PRRS virus was not present in the herd after 10 consecutive negative weeks (herd E), 13 consecutive negative weeks (herd F and G), and 14 consecutive negative weeks (herd J). These herds proceeded to allow the entry of replacement gilts to the herd after this time. Herds G, F and E progressed into provisionally negative category (category III). Breeding herd J decided not to work towards elimination and used field virus to acclimate gilts. The latter herd had another outbreak on June 2019 with a virus which had an open reading frame 5 (ORF-5) sequence similarity below 98% compared to the previous one.

Figure 1 shows weekly processing fluids ~~rRT-PCR~~RT-qPCR results in the nine participating herds by parity group. The time from the PRRS outbreak to having at least 10 weeks of consecutive negative results was 33 weeks for herd J, 38 weeks for herd G, 39 weeks for herd F, and 48 weeks for herd E. However, herds

A, B, C, D, and H were still working towards PRRS stability after at least 52 weeks from the PRRS outbreak. The maximum number of consecutive ~~rRT-PCR~~RT-qPCR negative results between two ~~rRT-PCR~~RT-qPCR positive results was one week for herd G and J; two weeks for herd D; three weeks for herd B, C, and E; five weeks for herd F; eight weeks for herd H; and nine weeks for herd A.

Wean pig ~~rRT-PCR~~RT-qPCR sera results were available for herds E, F, and G. In herd G, blood samples of wean-age pigs started being ~~to be~~ collected at approximately six months (June 2018) after the start of the outbreak. The last sera ~~rRT-PCR~~RT-qPCR positive pool result occurred one week after the last ~~rRT-PCR~~RT-qPCR positive result of processing fluid samples. After that point, four monthly consecutive negative ~~rRT-PCR~~RT-qPCR tests in wean-age pig sera and 13 weeks of consecutive ~~rRT-PCR~~RT-qPCR negative processing fluid test results were observed. In herd F, wean-age pig blood samples started being~~to be~~ taken at approximately six months (June 2018) after the initial outbreak. The last ~~rRT-PCR~~RT-qPCR positive result in wean-age pig sera occurred one week earlier than the last ~~rRT-PCR~~RT-qPCR positive result of processing fluids. After that point, three monthly consecutive negative ~~rRT-PCR~~RT-qPCR results were observed in wean-age pig sera and 13 weeks of consecutive negative ~~rRT-PCR~~RT-qPCR results weekly in processing fluids were achieved. Herd E had the last ~~rRT-PCR~~RT-qPCR positive result in wean-age pig sera in the first week of August 2018, while the last ~~rRT-PCR~~RT-qPCR positive result of processing fluids was obtained in the last week of September 2018. After that point, six bi-weekly consecutive negative ~~rRT-PCR~~RT-qPCR results in wean-age pig sera and 10 consecutive weeks of ~~rRT-PCR~~RT-qPCR negative processing fluid results were obtained.

Individual Litter ~~rRT-PCR~~RT-qPCR results

Overall, 123 out of 743 processing fluid samples individually tested at the litter level ~~s~~ were ~~rRT-PCR~~RT-qPCR positive (16.6%, 95% CI 14.0% - 19.4%). ~~rRT-PCR~~RT-qPCR positive processing fluids at the litter level ~~litters~~ had a median Ct-value of 29.2 (1st quartile 25.3, 3rd quartile 33.1, minimum 17.3, and maximum

39.4). Among 50 parity groups, ~~The~~ 34 ~~positive~~ groups tested RT-qPCR positive with ~~had~~ a median Ct-value of 29.2 (1st quartile 27.5, 3rd quartile 31.7, minimum 20.8, and maximum 37.4). Out of these 34, 33 had at least one individual litter ~~rRT-PCR~~RT-qPCR positive, and one group had no ~~rRT-PCR~~RT-qPCR positive litter (Figure 2, pooled parity sample number 18). Therefore, the 123 positive litters were distributed in 33 parity groups. Figure 2 shows Ct-values quantified in each of the 34 ~~rRT-PCR~~RT-qPCR positive group pooled processing fluid samples (red open crossed circles) and Ct-values quantified in each ~~rRT-PCR~~RT-qPCR positive processing fluid samples from litters that formed the group sample (blue dots).

Figure 3 shows the number of ~~rRT-PCR~~RT-qPCR positive litters within grouped processing fluid samples. Grouped samples consisted of between 12 and 17 litters. Most positive parity groups (63.6% [21/33]) had three or less ~~rRT-PCR~~RT-qPCR positive litters. Fifty percent (25/50) of parity samples ~~rRT-PCR~~RT-qPCR tested had none (n litters 17) or one (n litters = 8) ~~rRT-PCR~~RT-qPCR positive litter.

Table 2 shows the agreement between ~~rRT-PCR~~RT-qPCR results of aggregated processing fluid samples and individual litter processing fluid samples (at least one ~~rRT-PCR~~RT-qPCR positive litter for a positive aggregated sample). The sensitivity and specificity were estimated at 100% (95% CI 89% - 100%) and 94% (95% CI 71% – 100%), respectively. The total agreement was 98%, while the Kappa statistic was 0.955 indicating an almost perfect agreement. The McNemar's χ^2 test was non-significant (continuity correction p = 1.00), which indicates lack of statistical evidence for a difference between the paired ~~rRT-PCR~~RT-qPCR positive proportions.

Multivariable model results using the GEE are presented in Table 3. A significant interaction was observed between parity group and the week after the outbreak category in the association with PRRS virus ~~rRT-PCR~~RT-qPCR status. At week category of <25 weeks from the outbreak, P3+ litters had 2.0 times (95% CI 1.29 – 3.16) the ~~higher~~ odds of yielding a positive ~~rRT-PCR~~RT-qPCR than P1 litters (p=0.002). However, at the week categories of 25-34, 34-43, and >43 P1 litters had higher odds of ~~rRT-PCR~~RT-qPCR positivity than

P3+ litters. Nonetheless, these differences were not statistically significant. Nonetheless, in herd E, P1 aggregated litters had RT-qPCR positive results for 11 weeks more than aggregated litters of P2 and P3+ sows.

Although the ~~rRT-PCR~~RT-qPCR positive proportion decreased in all parity groups through time, the reduction was greater in P3+ litters. The adjusted proportion of ~~rRT-PCR~~RT-qPCR positive P3+ litters decreased from 49.9% at <25 weeks after the outbreak, to 13.0% at ≥43 weeks after the outbreak. Similarly, the proportion of ~~rRT-PCR~~RT-qPCR positive P1 and P2 litters decreased from 33.0% and 38.5% at <25 weeks after the outbreak to 18.9% and 8.9% at ≥43 weeks after the outbreak, respectively (Figure 4).

Finally, herds that performed herd closure had 0.35 times the odds (95% CI 0.16 – 0.78) of ~~rRT-PCR~~RT-qPCR positivity than herds that kept introducing gilts during the study period; after accounting for the effects of herd, parity group and time after the outbreak. Overall, herds that adopted herd closure had a lower adjusted proportion of RT-qPCR positive results (16.5%, 95% CI 8.4% - 29.7%) compared to herds that did not close the herd (36.6%, 95% CI 28.6% - 44.1%).

Discussion

This study assessed the role of parity in maintaining PRRS virus in breeding herds. Although processing fluids of P1 litters had a numerical higher proportion of ~~rRT-PCR~~RT-qPCR positive results than processing fluids of P2 or P3+ litters after 25 weeks from the outbreak, the overall adjusted proportion of P1 ~~rRT-PCR~~RT-qPCR positive groups was not significantly different to the proportion of P2 or P3+ ~~rRT-PCR~~RT-qPCR positive groups at ≥25 - <34, ≥34 - <43, ≥43 weeks after the outbreak (Figure 4). A previous study in a single sow farm reported a significantly higher PRRS virus ~~rRT-PCR~~RT-qPCR positive proportion in P1 and P2 litters compared to that of third parity ~~three~~ litters or higher ~~or higher~~ (Vilalta et al., 2018). Similarly, one breeding herd in our study (herd E, Figure 1) followed a similar pattern in which P1 litters stayed positive for 11 weeks longer than P2 or P3+ litters. The herds used in this study were not selected at random from the population, and therefore it is unknown whether this proportion (11.1% [1/9], 95% CI 2.0% - 43.5%) is a good estimate of the proportion of herds in the population of United States breeding herds that will experience a prolonged PRRS virus positivity in P1 litters compared to P2 and P3+ litters.

A statistically significant interaction between sampling week category and parity group was observed in the data. The odds of ~~rRT-PCR~~RT-qPCR positivity were lower in P1 litters compared to that of P2 (p=0.45) and P3+ (p=0.002) litters at <25 weeks after the PRRS outbreak. However, the relationship changed after 25 weeks from the outbreak where the odds of ~~rRT-PCR~~RT-qPCR positivity tended to be higher in P1 litters compared to P2 and P3+ litters (Figure 2). Nonetheless, differences were not statistically significant among parity groups after 25 weeks from the PRRS outbreak (p > 0.13). However, in one out of the nine participating herds, P1 aggregated litters continued being RT-qPCR positive for 11 weeks after the last RT-qPCR positive result of P2 and P3+ aggregated litters. The sample size used in this study was enough to detect an overall difference among parity groups of at least 10% with 95% confidence and 80% power and considering an intra-class correlation of 0.1. Nonetheless, the magnitude of the differences in the

percentage of ~~rRT-PCR~~RT-qPCR positive litters among parity groups in our study was moderately low and most of the time below 10%.

Breeding herds that stopped introducing replacements during the stabilization period had 0.35 (95% CI 0.16 – 0.78) times the odds of having a ~~rRT-PCR~~RT-qPCR positive result as herds that kept entering gilts. Herd closure and rollover is a common strategy used to eliminate PRRS virus from a breeding herd. This strategy is based on the idea of reducing the susceptible population within the herd, which limits the spread of the virus, resulting in a decrease in the number of new infections and eventually the elimination of infection within the herd (Torremorell and Christianson, 2002; Corzo et al., 2010). Our results support the use of herd closure as a means of controlling and eliminating PRRS virus from breeding herds. ~~Overall, herds that adopted herd closure had a lower adjusted proportion of rRT-PCR positive results (16.5%, 95% CI 8.4% - 29.7%) compared to herds that did not close the herd (36.6%, 95% CI 28.6% - 44.1%), after accounting for the effects of parity group, week category after the PRRS outbreak and herd of origin. Furthermore, b~~By the end of the follow-up period, four out of the six herds that adopted herd closure were confident enough to classify themselves as PRRS virus stable and re-introduce gilts. In contrast, none of the three herds that kept entering gilts managed to reach stability during this time.

Cross-fostering is a common practice in breeding herds that aims to balance litter size, increase access to functional teats, reduce weight variation and increase piglet survivability (Calderón Díaz et al., 2018). In participating herds, the occurrence of this practice was limited and restricted mostly to the first 24 hours from birth between litters of the same parity group to ensure each pig had access to a functional teat. Size sorting and excessive movement of pigs was not allowed. Scarce data exist about the proportion of PRRS virus ~~rRT-PCR~~RT-qPCR positive pigs at processing age within a litter after a PRRS outbreak. Vilalta et al. (2019b) observed that after week 11 from the initial outbreak, the proportion of ~~rRT-PCR~~RT-qPCR positive pigs was 2.0% (2/102), 6.5% (8/124), 0.9% (1/112), 3.3% (4/121), and 1.6% (2/127) at weeks 11,

14, 17, 20, 23 after the outbreak, respectively. Therefore, if these data are representative of the frequency of ~~rRT-PCR~~RT-qPCR positive results in processing age pigs after a PRRS outbreak, then the likelihood of selecting a PRRS virus positive pig and placing it into a PRRS virus negative litter may have been small, ~~and further~~ reduc~~inged~~ the opportunities for litter ~~rRT-PCR~~RT-qPCR result misclassification.

In this study, 15 litters on average were ~~rRT-PCR~~RT-qPCR tested as an aggregated sample within a parity group. When ~~rRT-PCR~~RT-qPCR results of the grouped sample were compared with the group classification based on individual litter ~~rRT-PCR~~RT-qPCR results (Table 2), a substantial agreement was observed (Kappa 0.91), despite the moderate low number of ~~rRT-PCR~~RT-qPCR positive litters in a ~~rRT-PCR~~RT-qPCR positive grouped sample (20.7% or approximately a median of 3/15 litters). The disagreement was mainly due to one RT-qPCR positive parity group sample that had no RT-qPCR positive processing fluids in the litters that formed part of the group. Samples were retested obtaining the same results. We think that this observation may be attributed to cross-contamination at the laboratory when manually aggregating the processing fluid samples by parity group. If that's the true cause of the observed disagreement, then the specificity estimate would have been 100%.

Similarly, ~~rRT-PCR~~RT-qPCR results of litter-aggregated processing fluids were observed to correlate with ~~rRT-PCR~~RT-qPCR results of individual pigs within the litter (Vilalta et al., 2018), and despite the dilution effect that occurs when processing fluids of several litters are aggregated, a positive sample can still be detected in a wide range of dilutions depending mostly on the sample's initial Ct-value (Vilalta et al., 2019a).

The use of processing fluids has shown to be a reliable sample to monitor PRRS virus presence in breeding herds by means of ~~rRT-PCR~~RT-qPCR testing (Lopez et al., 2018; Vilalta et al., 2018; Trevisan et al., 2019; de Almeida et al., 2021). In our study, two breeding herds had a maximum of eight and nine weeks of consecutive negative ~~rRT-PCR~~RT-qPCR results followed by a ~~rRT-PCR~~RT-qPCR positive result. Similarly, de

Almeida et al. (2021) observed a maximum of 11 weeks between two ~~rRT-PCR~~RT-qPCR positive processing fluid results in a herd. Having several weeks of ~~rRT-PCR~~RT-qPCR negative results followed by a ~~rRT-PCR~~RT-qPCR positive results in processing fluids seems to be a relatively common observation that should encourage swine veterinarians and producers to keep testing the herd despite several weeks of negative ~~rRT-PCR~~RT-qPCR results in processing fluids to increase the confidence that at processing, piglets continue to be PRRS virus negative. Holtkamp et al. (2021) proposed a modification to the PRRS virus herd classification. It was suggested that the use of processing fluids can provide supporting evidence for PRRS virus herd stability, but testing wean-age pigs is also required. If processing fluids are used to provide evidence of PRRS virus stability, then one or more weekly pools of processing fluids should be ~~rRT-PCR~~RT-qPCR tested negative for 13 weeks. In addition, sera of 30 pigs should be ~~rRT-PCR~~RT-qPCR tested negative in pools of five monthly for 90 days.

The time from the onset of the PRRS outbreak to consistently negative processing fluids, defined here as having at least 10 weeks of consecutive negative ~~rRT-PCR~~RT-qPCR results in processing fluids was 33, 38, 39, and 48 weeks in four out of nine breeding herds (time to the 10th ~~rRT-PCR~~RT-qPCR consecutive negative result). The remaining five herds were still not able to get 10 consecutive ~~rRT-PCR~~RT-qPCR negative results after 52 weeks from the start of the outbreak. Trevisan et al. (2019) estimated time to stability at 27 weeks in 29 batch-farrowing herds based on two consecutive negative processing fluid batches, negative ~~rRT-PCR~~RT-qPCR results in due-to-wean pig sera, and negative ELISA results in gilts after herd introduction. This estimation is shorter than what our data may suggest. However, the nine herds purposely included in our study were not batch-farrowing farms. It may be possible that the batch-farrowing system allows for a faster PRRS virus clearance from the farm. Further studies should evaluate the time to PRRS stability using processing fluids in a larger sample of herds to provide a more precise estimate and determine whether P1 sows have a role in maintaining PRRS virus in the herd. ~~However, the~~

~~limited number and the purposive selection of herds in our study does not allow to provide a precise and accurate estimate of time to stability based on processing fluids.~~

Conclusion

The proportion of PRRS virus ~~rRT-PCR~~RT-qPCR processing fluid positive litters was not statistically higher in aggregated samples of first parity ~~one~~ sows compared to that of second parity ~~two~~ or third parity ~~three~~ or higher sows after week 25 from the start of the outbreak. Therefore, the role of first parity ~~one~~ sows and their litters in maintaining PRRS virus infection in breeding herds could not be confirmed in this investigation despite the consistent non-significant higher point estimate proportion observed from week 25 after the outbreak onwards. Processing fluid ~~rRT-PCR~~RT-qPCR result dynamics in nine herds showed that at least nine weeks of ~~negative weekly~~consecutive negative processing fluid ~~rRT-PCR~~RT-qPCR results may exist between two ~~rRT-PCR~~RT-qPCR positive weeks. This observation should encourage veterinarians and swine producers that use processing fluids to monitor PRRS virus circulation in breeding herds to continue testing the herd beyond this time span to avoid misclassification of herd PRRS virus status. The use of processing fluids continues to reaffirm their value for PRRS virus monitoring and surveillance activities in breeding herds. Monitoring the breeding herd PRRS virus status using aggregated processing fluid samples of 15 litters was a useful testing strategy throughout the outbreak in all herds assessed.

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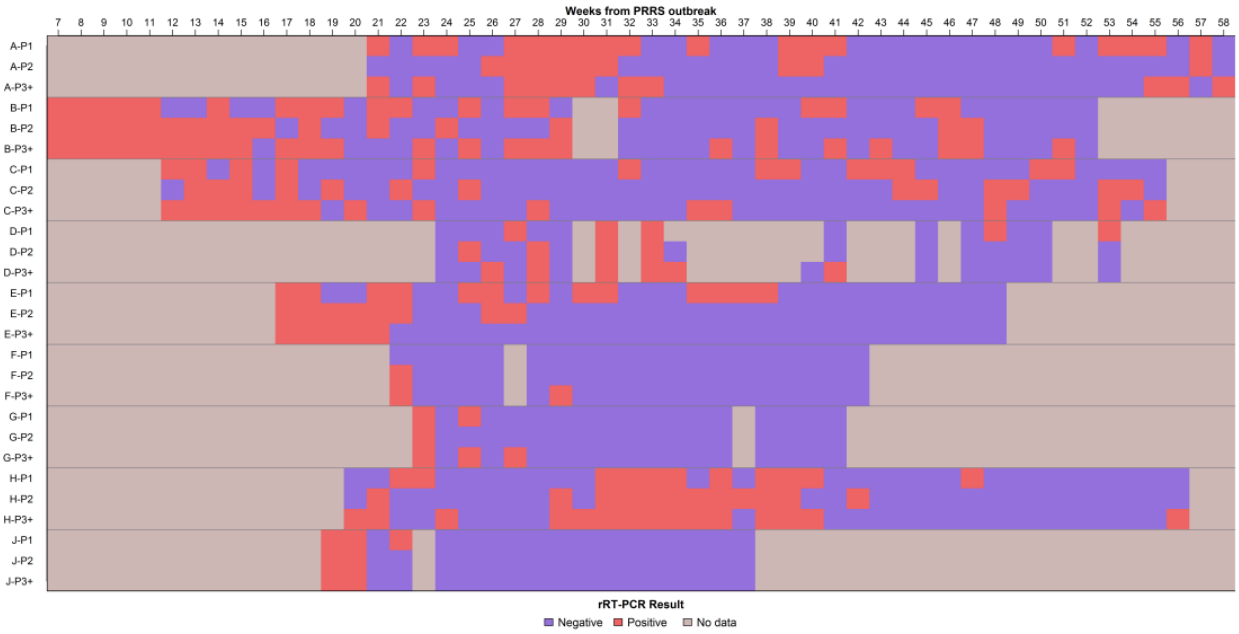
Figure 1: PRRS virus rRT-PCR processing fluid results in nine ~~Midwestern United States~~ breeding herds by parity group.



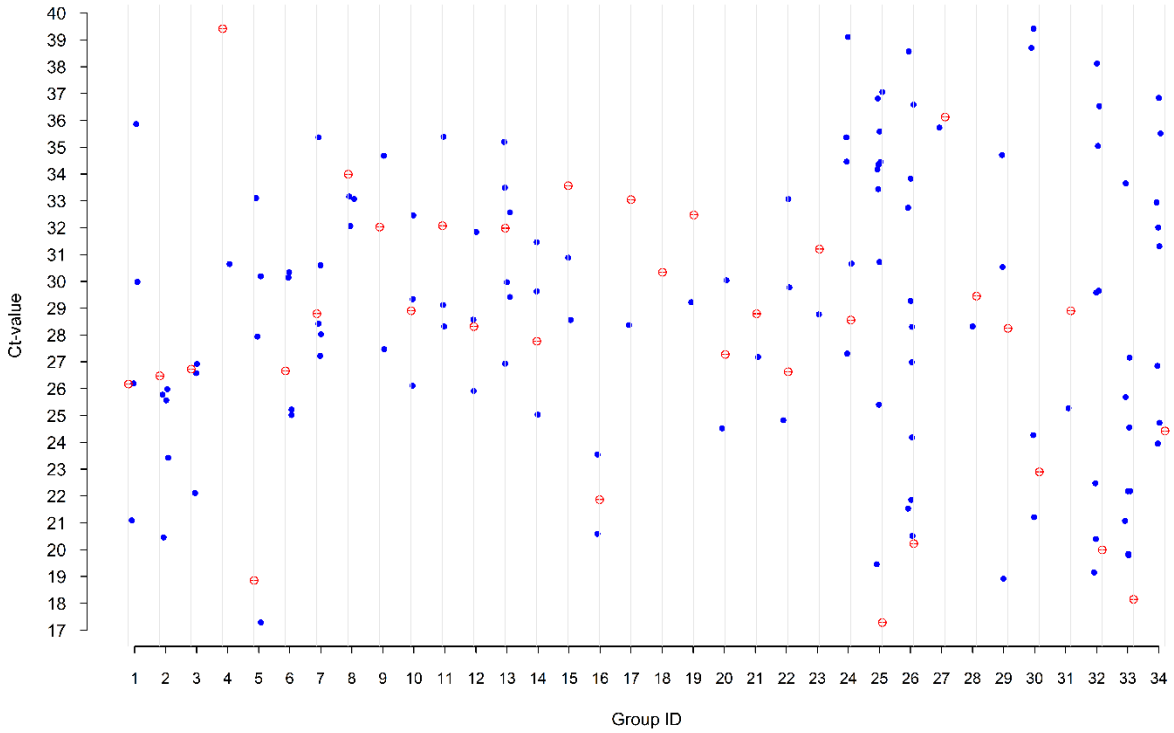


Figure 2: Ct-values in 34 rRT-PCR positive (Ct-value < 40) parity aggregated processing fluid groups (red open crossed circles)~~rRT-PCR positive (Ct-value < 40)~~ and Ct-values of rRT-PCR positive litters that formed the aggregated sample (blue dots).

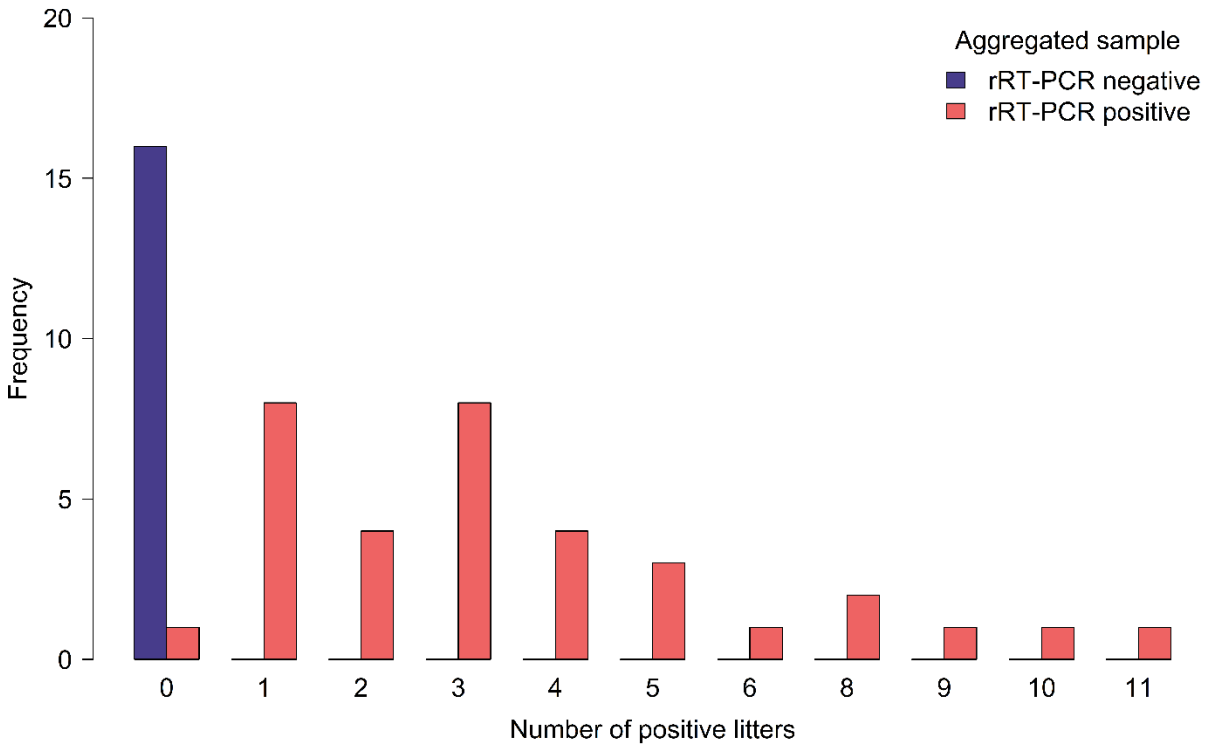


Figure 3: Number of rRT-PCR positive litters in rRT-PCR positive and negative grouped processing fluid samples.

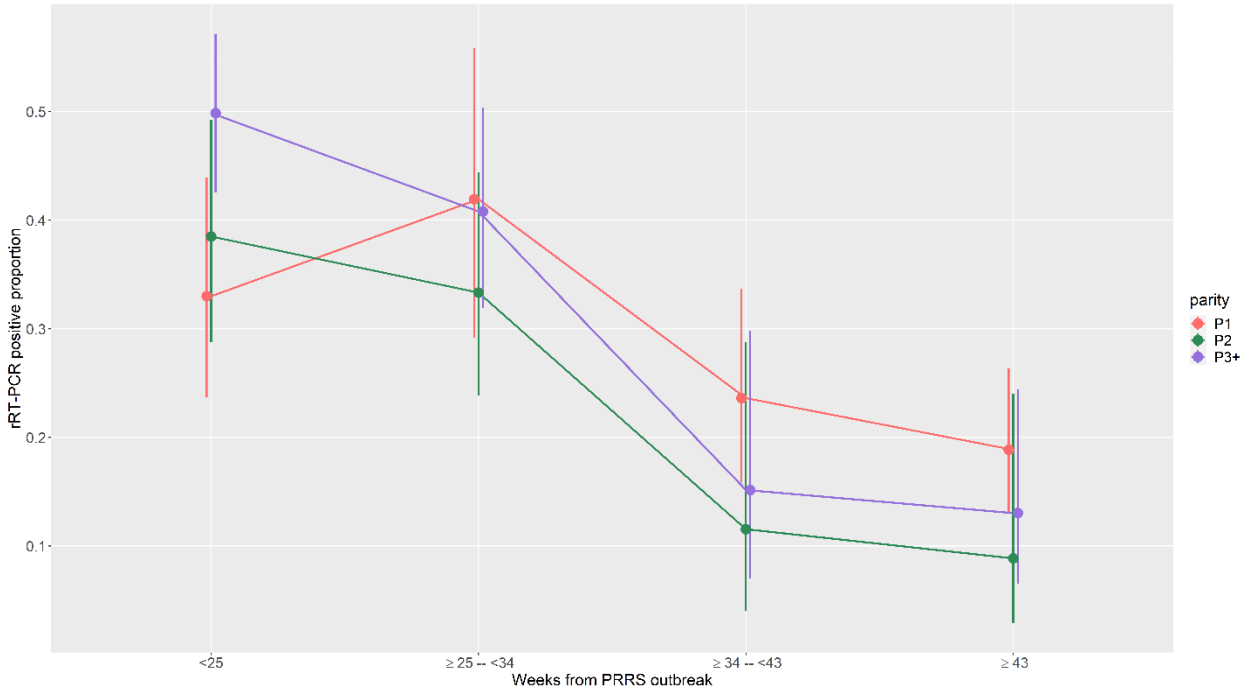


Figure 4: Adjusted proportion and 95% confidence interval (vertical line) of rRT-PCR positive results among the three parity groups in nine breeding herds across four categories of time after the PRRSv outbreak.

Figure

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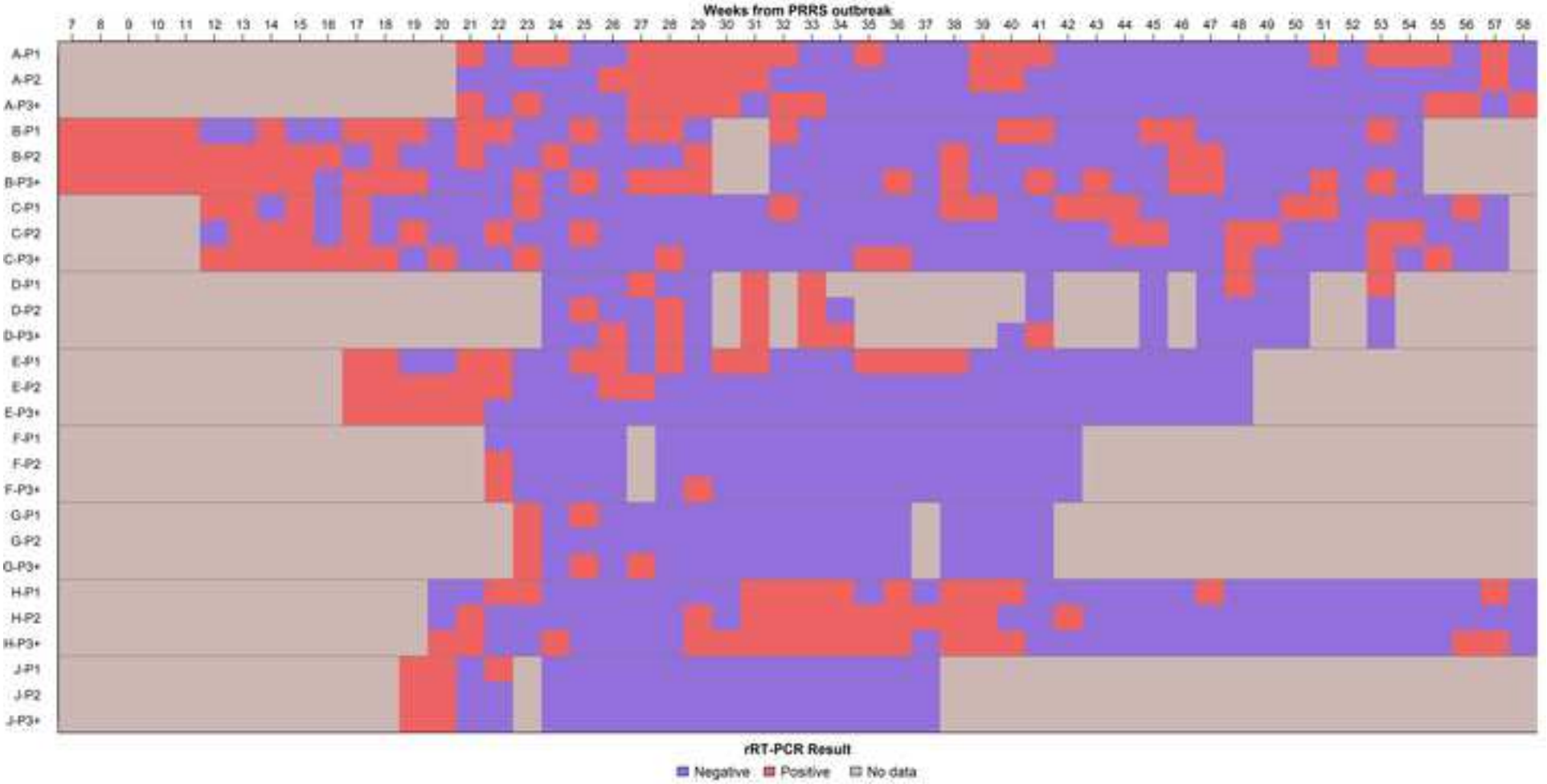

Figure

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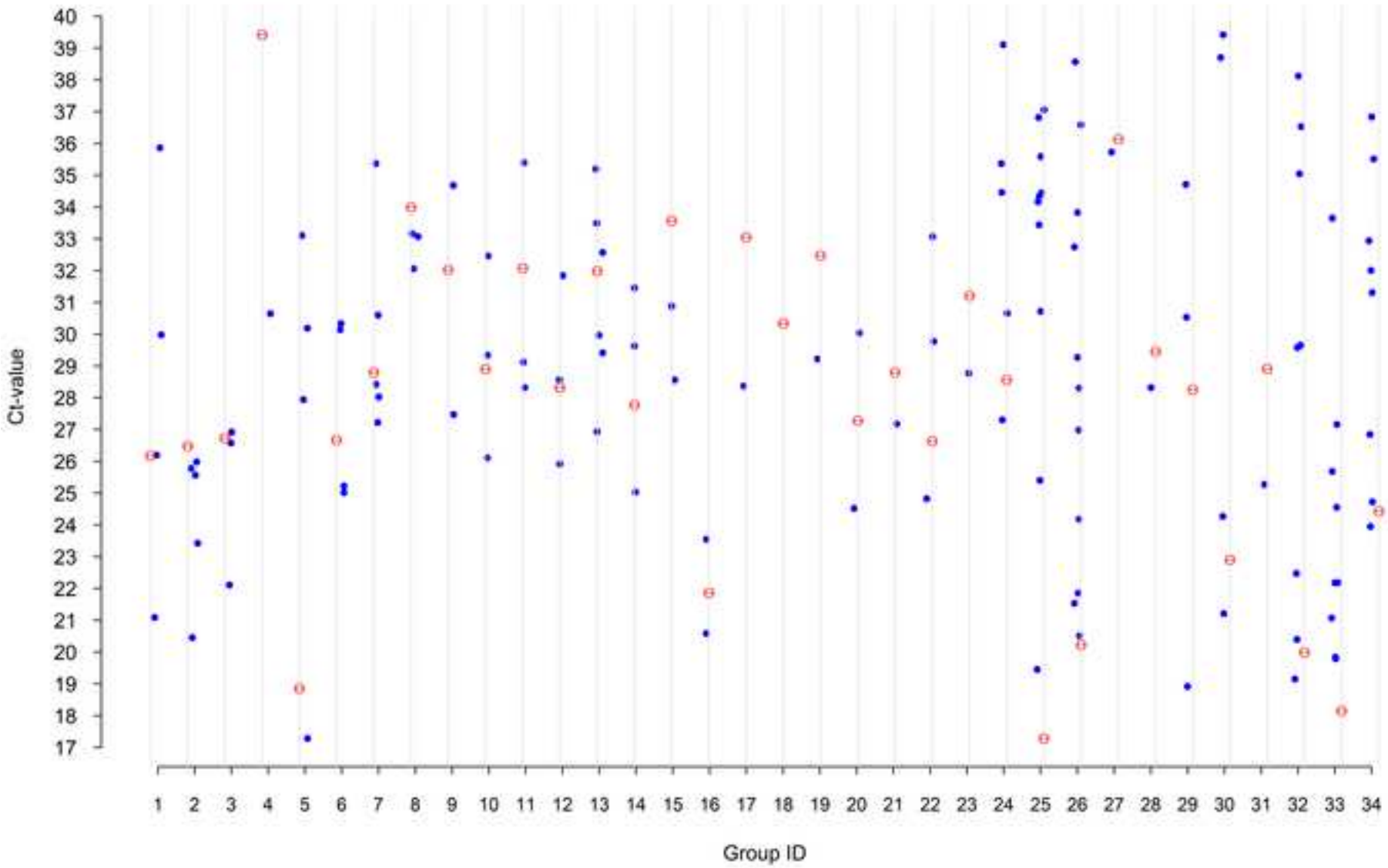

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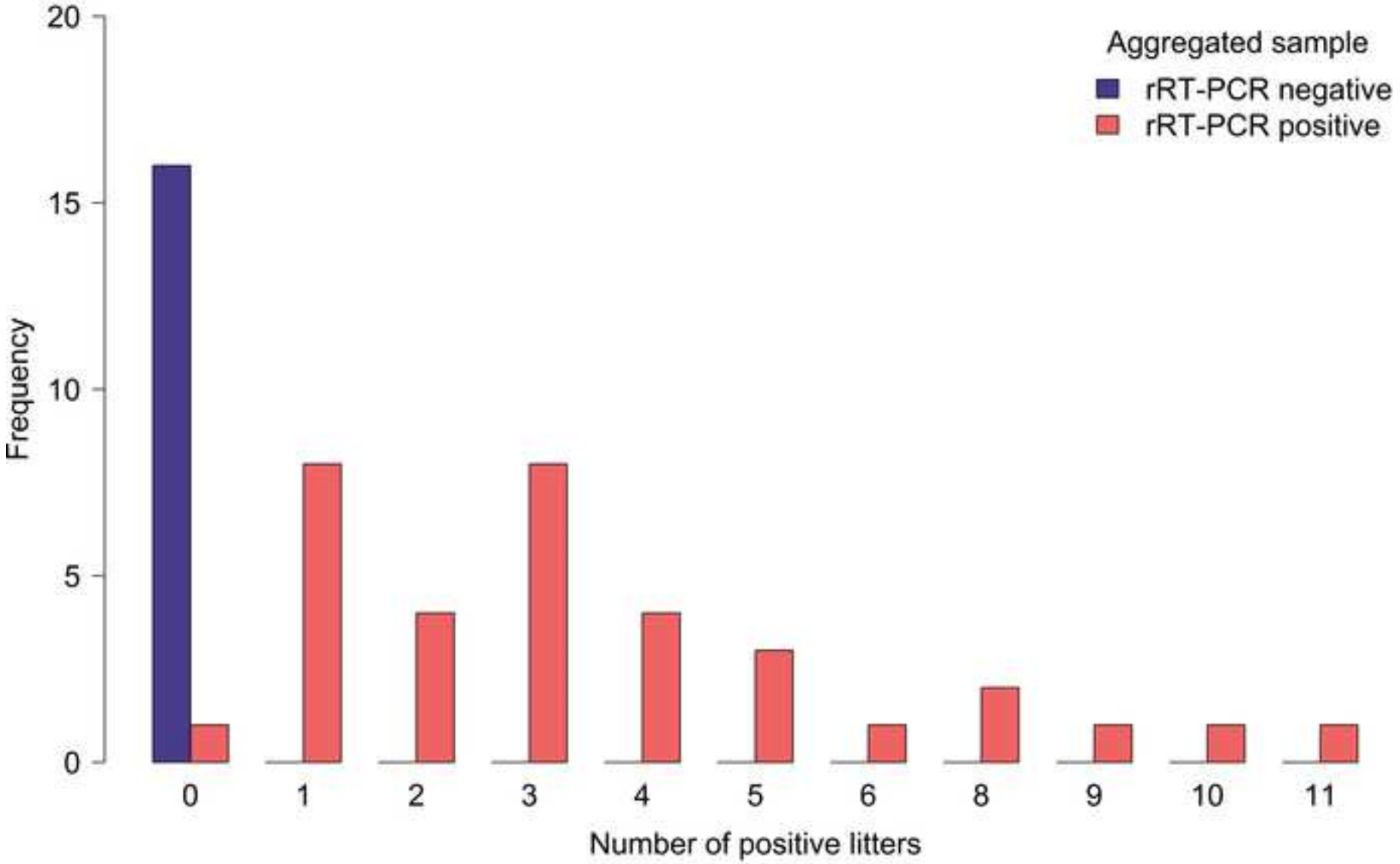

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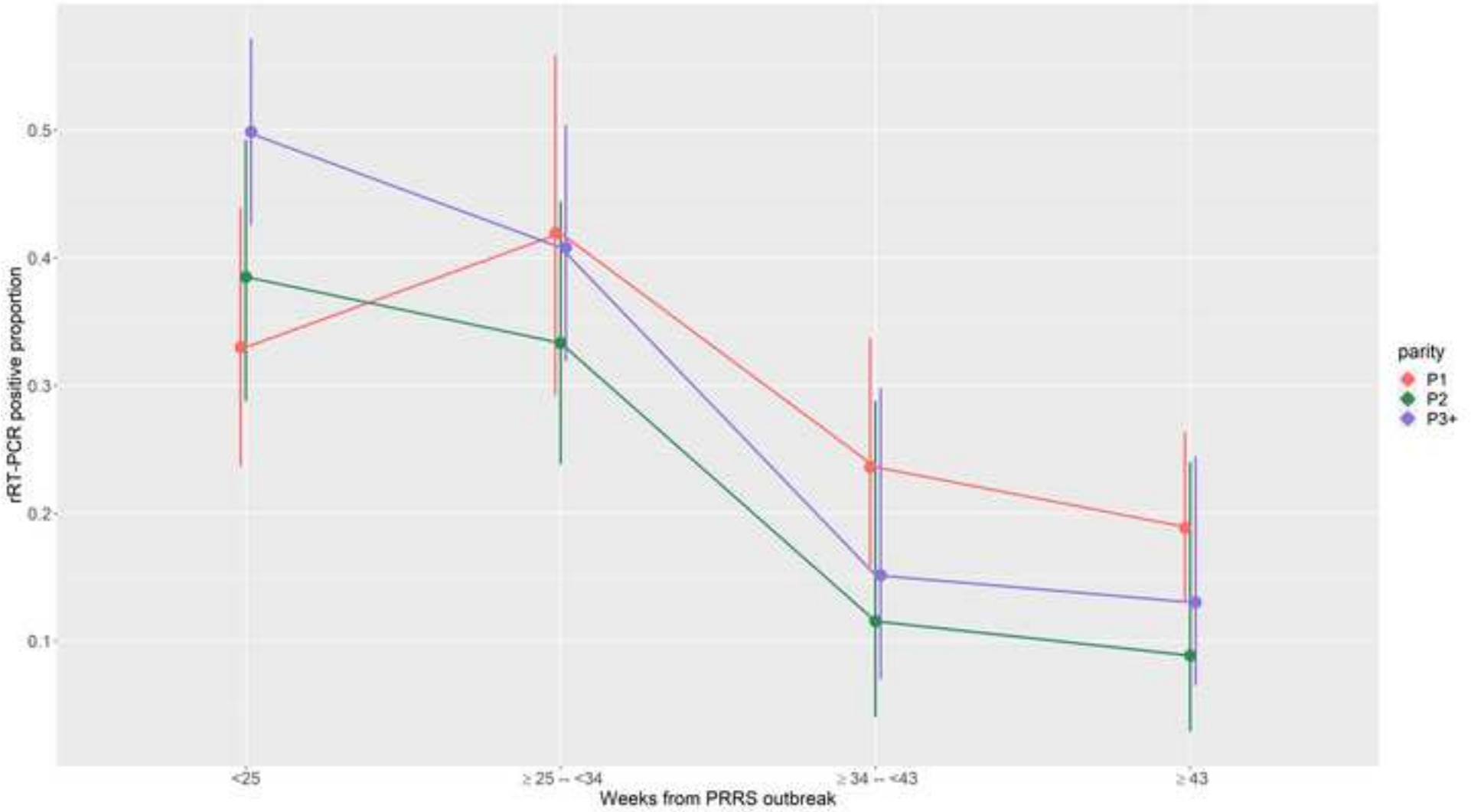

Table 1: Summary of PRRS outbreak information in the 10 study participant herds.

Herd	System	Herd size	Outbreak	RFLP	Status at break	Closure	Enrolled
A	1	5200	Oct, 2017	184	4	No	Mar, 2018
B	1	2500	Jan, 2018	142	2-vaccinated	No	Mar, 2018
C	1	5200	Jan, 2018	134	2	No	Mar, 2018
D	2	5400	Nov, 2017	144	1	Yes	Mar, 2018
E	3	2900	Jan, 2018	132	4	Yes	May, 2018
F	4	2500	Dec, 2017	184	4	Yes	May, 2018
G	4	4000	Dec, 2017	184	4	Yes	May, 2018
H	5	6000	Jan, 2018	184	4	Yes	May, 2018
I[1]	4	2800	Dec, 2017	Unknown	3	Yes	Jun, 2018
J	3	2400	Jan, 2018	Unknown	2-vaccinated	Yes	Jun, 2018

[1]Lost to follow-up

Table 2: Group classification agreement between rRT-PCR results of an aggregated sample of 15 litters and the rRT-PCR results of testing individual litters (at least one litter positive to classify the group as positive).

	Group based on individual litter +	Group based on individual litter -	Total
Group based on aggregated litter +	33	1	34
Group based on aggregated litter -	0	16	16
Total	33	17	50

Table 3: Generalized estimating equations multivariable model results of the association between rRT-PCR results and parity group, week category after the outbreak, and herd closure in nine breeding herds.

Variable	Levels	OR (95% CI)	p-value
Parity x Week category	Parity 2 / Week <25	1.27 (0.69 - 2.36)	0.446
	Parity 3+ / Week <25	2.02 (1.29 - 3.16)	0.002
	Parity 1 / Week <25	Reference	

	Parity 2 / Week ≥25 < 34	0.69 (0.43 - 1.11)	0.128
	Parity 3+ / Week ≥25 < 34	0.95 (0.52 - 1.74)	0.879
	Parity 1 / Week ≥25 <34	Reference	
	Parity 2 / Week ≥34 < 43	0.42 (0.1 - 1.85)	0.253
	Parity 3+ / Week ≥34 < 43	0.58 (0.17 - 2.02)	0.390
	Parity 1 / Week ≥34 < 43	Reference	
	Parity 2 / Week ≥43	0.42 (0.13 - 1.36)	0.146
	Parity 3+ / Week ≥ 43	0.65 (0.31 - 1.36)	0.248
	Parity 1 / Week ≥43	Reference	
Closure	Yes	0.35 (0.16 - 0.78)	0.011
	No		

Conflict of Interest

Declarations of interest: none.

Title page (with author details)

Assessing the role of sow parity on PRRSv detection by rRT-PCR through weekly processing fluids monitoring in breeding herds

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