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## 1 **Abstract**

2 The use of processing fluids to monitor the breeding herd's porcine reproductive and respiratory  
3 syndrome (PRRS) status has gained industry acceptance. However, little is known about PRRS virus RT-  
4 qPCR detection dynamics in processing fluids and factors that may contribute to maintain PRRS virus in  
5 the herd after an outbreak. This study aimed to describe weekly RT-qPCR processing fluid results in  
6 breeding herds after an outbreak and to evaluate the proportion of RT-qPCR positive results among parity  
7 groups. Processing tissues of 15 first parity (P1), 15 second parity (P2), and 15 third parity or higher (P3+)  
8 litters (i.e., parity groups) were collected weekly for between 19 and 46 weeks in nine breeding herds.  
9 Processing fluids were aggregated, and RT-qPCR tested by parity group weekly. Additionally, a subset of  
10 743 processing fluid samples of litters that formed 50 parity groups, as previously described, were RT-  
11 qPCR tested individually at the litter level. The agreement between RT-qPCR results of processing fluid  
12 samples of parity groups (15 litters) and results based on individual litter testing was assessed using overall  
13 percent of agreement, Kappa statistic, and McNemar test. The association between RT-qPCR results and  
14 the parity group was evaluated using a generalized estimating equations model, after accounting for the  
15 effects of sampling week, breeding herd PRRS control strategy (i.e., open to replacements v/s closed) and  
16 herd. An autoregressive correlation structure was used to account for the repeated samplings within a  
17 herd in time. The overall agreement was 98%, and Kappa statistic 0.955 (McNemar  $p=1.0$ ). Sensitivity of  
18 parity group processing fluid samples was estimated at 100% (95% CI 89% - 100%), while specificity was  
19 estimated at 94% (95% CI 71% – 100%). Although P1 aggregated litters had on average a higher proportion  
20 of RT-qPCR positive results from outbreak week 25 onwards, the proportion was not significantly different  
21 to the one observed for P2 and P3+ aggregated litters ( $p>0.13$ ). Additionally, herds that interrupted gilt  
22 entry had lower odds of PRRS RT-qPCR positivity than herds that continued entering gilts (OR=0.35, 95%  
23 CI 0.16-0.78). PRRS virus persistence in processing fluids was not affected by the sow parity effect in most  
24 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.

28

## 29 **Introduction**

30 Porcine reproductive and respiratory syndrome (PRRS) virus is a ubiquitous pathogen that causes  
31 extensive economic losses to the United States swine industry (Neumann et al., 2005; Holtkamp et al.,  
32 2013). The disease is endemic in most pork producing countries. In the United States, PRRS incidence in  
33 breeding herds has a marked seasonal pattern in breeding herds with peaks during autumn and winter  
34 and reduced incidence during spring and summer (Tousignant et al., 2015; Sanhueza et al., 2020).

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

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

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

59 The effect of processing fluid aggregation on PRRS virus detection by RT-qPCR was assessed by Vilalta et  
60 al. (2019a). The study highlighted that 1) a large number of aggregated processing fluids samples could be  
61 used to monitor the PRRS virus status of the breeding herd and, 2) the ability to classify an  
62 aggregated/pooled processing fluid sample as positive when it included a PRRS virus RT-qPCR positive  
63 individual sample was largely dependent on its Ct-value (i.e. viral concentration). Other sample types as  
64 udder skin line wipe samples and wipe samples of crate surfaces did not perform as well as processing  
65 fluids in correlating with the true litter status as determined by individual serum samples (Vilalta et al.,  
66 2019b; Vilalta et al., 2021).

67 The use of processing fluids as tool to monitor the PRRS virus status of the breeding herd has been widely  
68 adopted in the US swine industry. Criteria to promote herds into a given category and to maintain the  
69 PRRS status category were proposed in 2021 updating the criteria proposed in 2011 (Holtkamp et al.,  
70 2011; Holtkamp et al., 2021). Briefly, the most significant changes in the classification of breeding herds  
71 were the splitting of the positive unstable category into a positive unstable with high and low PRRSV  
72 prevalence, the splitting of the positive stable category into a positive stable and positive stable with  
73 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.

87

## 88 **Materials and Methods**

### 89 **Breeding herd selection and enrollment**

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

100 Breeding herds A, B and C continued entering replacement gilts monthly during the follow-up period (i.e  
101 19 to 46 weeks), whereas breeding herds D, E, F, G, H, I, and J did not enter replacement gilts (herd closure)  
102 during the study period. Five of the enrolled herds were PRRS virus negative when the outbreak occurred  
103 (PRRS virus category IV), while two were positive stable-vaccinated (PRRS virus category II), one was  
104 positive stable not vaccinated (PRRS virus category II), one provisionally negative (PRRS virus category III),  
105 and one positive unstable (PRRS virus category I). Breeding herd I left the study two weeks after  
106 enrollment because of the labor-intensive sampling required each week. Therefore, this herd was  
107 removed from further analyses. Table 1 summarizes the information recorded in each herd about the  
108 system, outbreak date, restriction fragment length polymorphism (RFLP) pattern of the PRRS virus  
109 associated with the outbreak, PRRS status when the outbreak occurred, whether the farm was closed  
110 during the follow-up period, enrollment date, and whether the farm was lost to follow-up.

111

112 **Sample collection**

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

123 **Sample processing and testing**

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



134 **Wean-age pig sera**

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

138 **Individual litter samples**

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

146 **Individual litter testing**

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

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

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

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

## 158 **Statistical analysis**

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

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

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

175 Statistical analyses were done using R version 4.0.4 (R Core Team, 2021) and the R package geePack (Yan,  
176 2002; Yan and Fine, 2004; Højsgaard et al., 2006).

177 **Results**

178 Overall, processing tissues from 11,834 litters were collected between March 2018 and February 2019.  
179 These litters were aggregated into 283 groups of P1 litters, 284 groups of P2 litters, and 285 groups of P3+  
180 litters. Herd D did not send P1 samples one week and sent only P3+ samples during another week. From  
181 the 852 parity groups tested, 246 (28.4%, 95% CI 25.5%-31.5%) were RT-qPCR positive. Positive RT-qPCR  
182 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).

183 The unadjusted percentage of RT-qPCR positive P1 groups (31.1% [88/283], 95% CI 25.8% - 36.8%) was  
184 not significantly different from the percentage of RT-qPCR positive P2 groups (23.9% [68/284], 95% CI  
185 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  
186 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.  
187 These differences were not statistically significant ( $p=0.31$ ).

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

196 Figure 1 shows weekly processing fluids RT-qPCR results in the nine participating herds by parity group.  
197 The time from the PRRS outbreak to having at least 10 weeks of consecutive negative results was 33 weeks  
198 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,  
199 and H were still working towards PRRS stability after at least 52 weeks from the PRRS outbreak. The

200 maximum number of consecutive RT-qPCR negative results between two RT-qPCR positive results was  
201 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;  
202 eight weeks for herd H; and nine weeks for herd A.

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

### 217 **Individual Litter RT-qPCR results**

218 Overall, 123 out of 743 processing fluid samples individually tested at the litter level were RT-qPCR positive  
219 (16.6%, 95% CI 14.0% - 19.4%). RT-qPCR positive processing fluids at the litter level had a median Ct-value  
220 of 29.2 (1<sup>st</sup> quartile 25.3, 3<sup>rd</sup> quartile 33.1, minimum 17.3, and maximum 39.4). Among 50 parity groups,  
221 34 groups tested RT-qPCR positive with a median Ct-value of 29.2 (1<sup>st</sup> quartile 27.5, 3<sup>rd</sup> quartile 31.7,  
222 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  $\chi^2$  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.

246 Although the RT-qPCR positive proportion decreased in all parity groups through time, the reduction was  
247 greater in P3+ litters. The adjusted proportion of RT-qPCR positive P3+ litters decreased from 49.9% at  
248 <25 weeks after the outbreak, to 13.0% at ≥43 weeks after the outbreak. Similarly, the proportion of RT-  
249 qPCR positive P1 and P2 litters decreased from 33.0% and 38.5% at <25 weeks after the outbreak to 18.9%  
250 and 8.9% at ≥43 weeks after the outbreak, respectively (Figure 4).

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

256

## 257 Discussion

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

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

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

281 Breeding herds that stopped introducing replacements during the stabilization period had 0.35 (95% CI  
282 0.16 – 0.78) times the odds of having a RT-qPCR positive result as herds that kept entering gilts. Herd  
283 closure and rollover is a common strategy used to eliminate PRRS virus from a breeding herd. This strategy  
284 is based on the idea of reducing the susceptible population within the herd, which limits the spread of the  
285 virus, resulting in a decrease in the number of new infections and eventually the elimination of infection  
286 within the herd (Torremorell and Christianson, 2002; Corzo et al., 2010). Our results support the use of  
287 herd closure as a means of controlling and eliminating PRRS virus from breeding herds. By the end of the  
288 follow-up period, four out of the six herds that adopted herd closure were confident enough to classify  
289 themselves as PRRS virus stable and re-introduce gilts. In contrast, none of the three herds that kept  
290 entering gilts managed to reach stability during this time.

291 Cross-fostering is a common practice in breeding herds that aims to balance litter size, increase access to  
292 functional teats, reduce weight variation and increase piglet survivability (Calderón Díaz et al., 2018). In  
293 participating herds, the occurrence of this practice was limited and restricted mostly to the first 24 hours  
294 from birth between litters of the same parity group to ensure each pig had access to a functional teat.  
295 Size sorting and excessive movement of pigs was not allowed. Scarce data exist about the proportion of  
296 PRRS virus RT-qPCR positive pigs at processing age within a litter after a PRRS outbreak. Vilalta et al.  
297 (2019b) observed that after week 11 from the initial outbreak, the proportion of RT-qPCR positive pigs  
298 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  
299 after the outbreak, respectively. Therefore, if these data are representative of the frequency of RT-qPCR  
300 positive results in processing age pigs after a PRRS outbreak, then the likelihood of selecting a PRRS virus



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

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

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

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

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

331 The time from the onset of the PRRS outbreak to consistently negative processing fluids, defined here as  
332 having at least 10 weeks of consecutive negative RT-qPCR results in processing fluids was 33, 38, 39, and  
333 48 weeks in four out of nine breeding herds (time to the 10<sup>th</sup> RT-qPCR consecutive negative result). The  
334 remaining five herds were still not able to get 10 consecutive RT-qPCR negative results after 52 weeks  
335 from the start of the outbreak. Trevisan et al. (2019) estimated time to stability at 27 weeks in 29 batch-  
336 farrowing herds based on two consecutive negative processing fluid batches, negative RT-qPCR results in  
337 due-to-wean pig sera, and negative ELISA results in gilts after herd introduction. This estimation is shorter  
338 than what our data may suggest. However, the nine herds purposely included in our study were not batch-  
339 farrowing farms. It may be possible that the batch-farrowing system allows for a faster PRRS virus  
340 clearance from the farm. Further studies should evaluate the time to PRRS stability using processing fluids  
341 in a larger sample of herds to provide a more precise estimate and determine whether P1 sows have a  
342 role in maintaining PRRS virus in the herd.

343

344 **Conclusion**

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

358

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422

## 1 Abstract

2 The use of processing fluids to monitor the breeding herd's porcine reproductive and respiratory  
3 syndrome (PRRS) status has gained industry acceptance. However, little is known about PRRS virus ~~rRT-~~  
4 ~~PCRRT-qPCR~~ detection dynamics in processing fluids and factors that may contribute to maintain PRRS  
5 virus in the herd after an outbreak. This study aimed to describe weekly ~~rRT-PCRRT-qPCR~~ processing fluid  
6 results in breeding herds after an outbreak and to evaluate the proportion of ~~rRT-PCRRT-qPCR~~ positive  
7 results among ~~sow~~ parity groups. Processing tissues of 15 first parity ~~one~~ (P1), 15 second parity ~~two~~ (P2),  
8 and 15 third parity ~~three~~ or higher (P3+) litters (i.e., parity groups) were collected weekly for between 19  
9 and 46 weeks in nine breeding herds. Processing fluids were aggregated, and ~~rRT-PCRRT-qPCR~~ tested by  
10 parity group weekly. Additionally, a subset of 743 processing fluid samples of litters that formed 50 parity  
11 groups, as previously described, were ~~rRT-PCRRT-qPCR~~ tested individually at the litter level. The  
12 agreement between ~~rRT-PCRRT-qPCR~~ results of processing fluid samples of parity groups (15 litters) parity  
13 groups tested in aggregated fashion and results based on individual litter testings~~or by litter~~ was assessed  
14 using overall percent of agreement, Kappa statistic, and McNemar test. The association between  
15 aggregated rRT-PCRRT-qPCR results and the parity group was evaluated using a generalized estimating  
16 equations model, after accounting for the effects of sampling week, breeding herd PRRS control strategy  
17 (i.e., open to replacements v/s closed) and herd. ~~A generalized estimating equations model~~ An with  
18 autoregressive correlation structure was used to account for the repeated samplings within a herd in time.  
19 The overall agreement was 98%, and Kappa statistic 0.955 (McNemar p=1.0). Sensitivity of parity group  
20 processing fluid samples was estimated at 100% (95% CI 89% - 100%), while specificity was estimated at  
21 94% (95% CI 71% - 100%). ~~Although P1 aggregated litters had on average a higher proportion of rRT-~~  
22 ~~PCRRT-qPCR~~ positive results from outbreak week 25 onwards, the proportion was not significantly  
23 different to the one observed for P2 and P3+ aggregated litters (p>0.13). ~~However, in one out of the nine~~  
24 ~~participating herds, P1 aggregated litters continued being rRT-PCR positive for 11 weeks after the last rRT-~~

25 ~~PCR positive result of P2 and P3+ aggregated litters.~~ Additionally, herds that interrupted gilt entry had  
26 lower odds of PRRS ~~rRT-PCR~~rRT-qPCR positivity than herds that continued entering gilts (OR=0.35, 95% CI  
27 0.16-0.78). PRRS virus persistence in processing fluids was not affected by the sow parity effect in most of  
28 the breeding herds studied. No evidence of disagreement between ~~rRT-PCR~~rRT-qPCR results of an  
29 aggregated sample of 15 litters and those of individual litters was observed. This level of litter aggregation  
30 testing strategy may be of particular use at the last stages of an elimination program under low PRRS virus  
31 prevalence.

32

### 33 Introduction

34 Porcine reproductive and respiratory syndrome (PRRS) virus is a ubiquitous pathogen that causes  
35 extensive economic losses to the United States swine industry (Neumann et al., 2005; Holtkamp et al.,  
36 2013). The disease is endemic in most pork producing countries. In the United States, PRRS incidence in  
37 breeding herds has a marked seasonal pattern in breeding herds with peaks during autumn and winter  
38 and reduced incidence during spring and summer (Tousignant et al., 2015; Sanhueza et al., 2020).

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

44 Although slightly different definitions of time-to-stability have been generated, the time from PRRS  
45 outbreak to consistently weaning ~~rRT-PCR~~RT-qPCR negative pigs (time-to-last ~~rRT-PCR~~RT-qPCR negative  
46 result) had a median time of approximately 41-45 weeks in United States breeding herds (Linhares et al.,  
47 2014; Linhares et al., 2017; Sanhueza et al., 2019). However, time-to-stability may vary significantly  
48 according to the season when the outbreak occurred, the PRRS virus strain associated with the outbreak,  
49 and the intervention strategy used to begin the load-close-expose process (e.g. live virus inoculation,  
50 modified live vaccine or none). Nonetheless, monitoring PRRS virus status of the breeding herd using sera  
51 of 30 due-to-wean pigs may fail to detect the virus when the prevalence is below 10%. Hence, a herd may  
52 be mistakenly classified as stable when still weaning PRRS virus positive pigs at a low prevalence level  
53 leading to further viral recirculation upon gilt entry. Therefore, there was a need for sampling strategies  
54 that increase herd sensitivity of PRRS virus diagnosis and thus decrease the false herd stability  
55 classification.



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

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

73 The use of processing fluids as tool to monitor the PRRS virus status of the breeding herd has been widely  
74 adopted in the US swine industry. Criteria to promote herds into a given category and to maintain the  
75 PRRS status category were proposed in 2021 updating the criteria proposed in 2011 (Holtkamp et al.,  
76 2011; Holtkamp et al., 2021). Briefly, the most significant changes in the classification of breeding herds  
77 were the splitting of the positive unstable category into a positive unstable with high and low PRRSV  
78 prevalence, the splitting of the positive stable category into a positive stable and positive stable with

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

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

93

## 94 **Materials and Methods**

### 95 **Breeding herd selection and enrollment**

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

106 Breeding herds A, B and C continued entering replacement gilts monthly during the follow-up period (i.e  
107 19 to 46 weeks), whereas breeding herds D, E, F, G, H, I, and J did not enter replacement gilts (herd closure)  
108 during the study period. Five of the enrolled herds were PRRS virus negative when the outbreak occurred  
109 (PRRS virus category IV), while two were positive stable-vaccinated (PRRS virus category II), one was  
110 positive stable not vaccinated (PRRS virus category II), one provisionally negative (PRRS virus category III),  
111 and one positive unstable (PRRS virus category I). Breeding herd I left the study two weeks after  
112 enrollment because of the labor-intensive sampling required each week. Therefore, this herd was  
113 removed from further analyses. Table 1 summarizes the information recorded in each herd about the  
114 system, outbreak date, restriction fragment length polymorphism (RFLP) pattern of the PRRS virus  
115 associated with the outbreak, PRRS status when the outbreak occurred, whether the farm was closed  
116 during the follow-up period, enrollment date, and whether the farm was lost to follow-up.

117

118 **Sample collection**

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

129 **Sample processing and testing**

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

#### 140 **Wean-age pig sera**

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

#### 145 **Individual litter samples**

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

#### 153 **Individual litter testing**

154 Processing fluids from 743 litters individually stored, that formed part of 50 ~~sow~~-parity group samples in  
155 six sow herds, were ~~rRT-PCR~~rRT-qPCR tested individually. A total of 34 parity groups were selected based  
156 on a positive ~~rRT-PCR~~rRT-qPCR result and 16 parity groups based on a negative ~~rRT-PCR~~rRT-qPCR result.  
157 Overall, 226 individual litter samples belonged to parity 1 sows and formed part of 15 P1 parity groups,  
158 232 litter samples belonged to parity 2 sows and formed part of 16 P2 parity groups, and 285 litter samples  
159 belonged to parity 3+ sows and formed part of 19 P3+ parity groups.

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

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

## 166 **Statistical analysis**

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

171 The agreement was assessed using the overall percent of agreement, and Kappa statistic. Kappa values ~~of~~  
172 ~~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  
173 agreement, moderate agreement, substantial agreement, and almost perfect agreement, respectively  
174 (Dohoo et al., 2009). Additionally, sensitivity and specificity of ~~rRT-PCRRT-qPCR~~ results of the 15 litters  
175 ~~aggregated~~ parity group sample compared to the results of individual litters that composed the parity  
176 group was estimated. Statistical significance was tested using the McNemar  $\chi^2$  test for paired proportions.

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

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

185 Statistical analyses were done using R version 4.0.4 (R Core Team, 2021) [and the R package geePack](#) (Yan,  
186 2002; Yan and Fine, 2004; Højsgaard et al., 2006).

187 **Results**

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

194 The unadjusted percentage of ~~rRT-PCR~~rRT-qPCR positive P1 groups (31.1% [88/283], 95% CI 25.8% - 36.8%)  
195 was not significantly different from the percentage of ~~rRT-PCR~~rRT-qPCR positive P2 groups (23.9%  
196 [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  
197 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-  
198 30.7), respectively. These differences were not statistically significant ( $p=0.31$ ).

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

207 Figure 1 shows weekly processing fluids ~~rRT-PCR~~rRT-qPCR results in the nine participating herds by parity  
208 group. The time from the PRRS outbreak to having at least 10 weeks of consecutive negative results was  
209 33 weeks for herd J, 38 weeks for herd G, 39 weeks for herd F, and 48 weeks for herd E. However, herds



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

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

### 229 **Individual Litter ~~rRT-PCRRT-qPCR~~ results**

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

233 39.4). Among 50 parity groups, The 34 positive groups tested RT-qPCR positive with had a median Ct-value  
234 of 29.2 (1<sup>st</sup> quartile 27.5, 3<sup>rd</sup> quartile 31.7, minimum 20.8, and maximum 37.4). Out of these 34, 33 had at  
235 least one individual litter ~~rRT-PCRRT-qPCR~~ positive, and one group had no ~~rRT-PCRRT-qPCR~~ positive litter  
236 (Figure 2, pooled parity sample number 18). Therefore, the 123 positive litters were distributed in 33  
237 parity groups. Figure 2 shows Ct-values quantified in each of the 34 ~~rRT-PCRRT-qPCR~~ positive group pooled  
238 processing fluid samples (red open crossed circles) and Ct-values quantified in each ~~rRT-PCRRT-qPCR~~  
239 positive processing fluid samples from litters that formed the group sample (blue dots).  
240 Figure 3 shows the number of ~~rRT-PCRRT-qPCR~~ positive litters within grouped processing fluid samples.  
241 Grouped samples consisted of between 12 and 17 litters. Most positive parity groups (63.6% [21/33]) had  
242 three or less ~~rRT-PCRRT-qPCR~~ positive litters. Fifty percent (25/50) of parity samples ~~rRT-PCRRT-qPCR~~  
243 tested had none (n litters 17) or one (n litters = 8) ~~rRT-PCRRT-qPCR~~ positive litter.  
244 Table 2 shows the agreement between ~~rRT-PCRRT-qPCR~~ results of aggregated processing fluid samples  
245 and individual litter processing fluid samples (at least one ~~rRT-PCRRT-qPCR~~ positive litter for a positive  
246 aggregated sample). The sensitivity and specificity were estimated at 100% (95% CI 89% - 100%) and 94%  
247 (95% CI 71% – 100%), respectively. The total agreement was 98%, while the Kappa statistic was 0.955  
248 indicating an almost perfect agreement. The McNemar's  $\chi^2$  test was non-significant (continuity correction  
249  $p = 1.00$ ), which indicates lack of statistical evidence for a difference between the paired ~~rRT-PCRRT-qPCR~~  
250 positive proportions.

251 Multivariable model results using the GEE are presented in Table 3. A significant interaction was observed  
252 between parity group and the week after the outbreak category in the association with PRRS virus ~~rRT-~~  
253 ~~PCRRT-qPCR~~ status. At week category of <25 weeks from the outbreak, P3+ litters had 2.0 times (95% CI  
254 1.29 – 3.16) the higher odds of yielding a positive ~~rRT-PCRRT-qPCR~~ than P1 litters ( $p=0.002$ ). However, at  
255 the week categories of 25-34, 34-43, and >43 P1 litters had higher odds of ~~rRT-PCRRT-qPCR~~ positivity than

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

259 Although the ~~rRT-PCR~~RT-qPCR positive proportion decreased in all parity groups through time, the  
260 reduction was greater in P3+ litters. The adjusted proportion of ~~rRT-PCR~~RT-qPCR positive P3+ litters  
261 decreased from 49.9% at <25 weeks after the outbreak, to 13.0% at ≥43 weeks after the outbreak.  
262 Similarly, the proportion of ~~rRT-PCR~~RT-qPCR positive P1 and P2 litters decreased from 33.0% and 38.5%  
263 at <25 weeks after the outbreak to 18.9% and 8.9% at ≥43 weeks after the outbreak, respectively (Figure  
264 4).

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

270

## 271 Discussion

272 This study assessed the role of parity in maintaining PRRS virus in breeding herds. Although processing  
273 fluids of P1 litters had a numerical higher proportion of ~~rRT-PCR~~RT-qPCR positive results than processing  
274 fluids of P2 or P3+ litters after 25 weeks from the outbreak, the overall adjusted proportion of P1 ~~rRT-~~  
275 PCR~~RT-qPCR~~ positive groups was not significantly different to the proportion of P2 or P3+ ~~rRT-PCR~~RT-qPCR  
276 positive groups at  $\geq 25 - < 34$ ,  $\geq 34 - < 43$ ,  $\geq 43$  weeks after the outbreak (Figure 4). A previous study in a  
277 single sow farm reported a significantly higher PRRS virus ~~rRT-PCR~~RT-qPCR positive proportion in P1 and  
278 P2 litters compared to that of third parity ~~three\_~~litters or higher or higher (Vilalta et al., 2018). Similarly,  
279 one breeding herd in our study (herd E, Figure 1) followed a similar pattern in which P1 litters stayed  
280 positive for 11 weeks longer than P2 or P3+ litters. The herds used in this study were not selected at  
281 random from the population, and therefore it is unknown whether this proportion (11.1% [1/9], 95% CI  
282 2.0% - 43.5%) is a good estimate of the proportion of herds in the population of United States breeding  
283 herds that will experience a prolonged PRRS virus positivity in P1 litters compared to P2 and P3+ litters.

284 A statistically significant interaction between sampling week category and parity group was observed in  
285 the data. The odds of ~~rRT-PCR~~RT-qPCR positivity were lower in P1 litters compared to that of P2 ( $p=0.45$ )  
286 and P3+ ( $p=0.002$ ) litters at  $< 25$  weeks after the PRRS outbreak. However, the relationship changed after  
287 25 weeks from the outbreak where the odds of ~~rRT-PCR~~RT-qPCR positivity tended to be higher in P1 litters  
288 compared to P2 and P3+ litters (Figure 2). Nonetheless, differences were not statistically significant among  
289 parity groups after 25 weeks from the PRRS outbreak ( $p > 0.13$ ). However, in one out of the nine  
290 participating herds, P1 aggregated litters continued being RT-qPCR positive for 11 weeks after the last RT-  
291 qPCR positive result of P2 and P3+ aggregated litters. The sample size used in this study was enough to  
292 detect an overall difference among parity groups of at least 10% with 95% confidence and 80% power and  
293 considering an intra-class correlation of 0.1. Nonetheless, the magnitude of the differences in the

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

296 Breeding herds that stopped introducing replacements during the stabilization period had 0.35 (95% CI  
297 0.16 – 0.78) times the odds of having a ~~rRT-PCRRT-qPCR~~ positive result as herds that kept entering gilts.

298 Herd closure and rolover is a common strategy used to eliminate PRRS virus from a breeding herd. This  
299 strategy is based on the idea of reducing the susceptible population within the herd, which limits the  
300 spread of the virus, resulting in a decrease in the number of new infections and eventually the elimination  
301 of infection within the herd (Torremorell and Christianson, 2002; Corzo et al., 2010). Our results support

302 the use of herd closure as a means of controlling and eliminating PRRS virus from breeding herds. ~~Overall,~~  
303 ~~herds that adopted herd closure had a lower adjusted proportion of rRT-PCR positive results (16.5%, 95%~~  
304 ~~CI 8.4% – 29.7%) compared to herds that did not close the herd (36.6%, 95% CI 28.6% – 44.1%), after~~  
305 ~~accounting for the effects of parity group, week category after the PRRS outbreak and herd of origin.~~

306 ~~Furthermore,~~ ~~b~~By the end of the follow-up period, four out of the six herds that adopted herd closure  
307 were confident enough to classify themselves as PRRS virus stable and re-introduce gilts. In contrast, none  
308 of the three herds that kept entering gilts managed to reach stability during this time.

309 Cross-fostering is a common practice in breeding herds that aims to balance litter size, increase access to  
310 functional teats, reduce weight variation and increase piglet survivability (Calderón Díaz et al., 2018). In  
311 participating herds, the occurrence of this practice was limited and restricted mostly to the first 24 hours  
312 from birth between litters of the same parity group to ensure each pig had access to a functional teat.

313 Size sorting and excessive movement of pigs was not allowed. Scarce data exist about the proportion of  
314 PRRS virus ~~rRT-PCRRT-qPCR~~ positive pigs at processing age within a litter after a PRRS outbreak. Vilalta et  
315 al. (2019b) observed that after week 11 from the initial outbreak, the proportion of ~~rRT-PCRRT-qPCR~~  
316 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,

317 14, 17, 20, 23 after the outbreak, respectively. Therefore, if these data are representative of the frequency  
318 of ~~rRT-PCR~~rRT-qPCR positive results in processing age pigs after a PRRS outbreak, then the likelihood of  
319 selecting a PRRS virus positive pig and placing it into a PRRS virus negative litter may have been small, ~~and~~  
320 ~~further-reduced~~reduced the opportunities for litter ~~rRT-PCR~~rRT-qPCR result misclassification.

321 In this study, 15 litters on average were ~~rRT-PCR~~rRT-qPCR tested as an aggregated sample within a parity  
322 group. When ~~rRT-PCR~~rRT-qPCR results of the grouped sample were compared with the group classification  
323 based on individual litter ~~rRT-PCR~~rRT-qPCR results (Table 2), a substantial agreement was observed (Kappa  
324 0.91), despite the moderate low number of ~~rRT-PCR~~rRT-qPCR positive litters in a ~~rRT-PCR~~rRT-qPCR positive  
325 grouped sample (20.7% or approximately a median of 3/15 litters). The disagreement was mainly due to  
326 one RT-qPCR positive parity group sample that had no RT-qPCR positive processing fluids in the litters that  
327 formed part of the group. Samples were retested obtaining the same results. We think that this  
328 observation may be attributed to cross-contamination at the laboratory when manually aggregating the  
329 processing fluid samples by parity group. If that's the true cause of the observed disagreement, then the  
330 specificity estimate would have been 100%.

331 Similarly, ~~rRT-PCR~~rRT-qPCR results of litter-aggregated processing fluids were observed to correlate with  
332 ~~rRT-PCR~~rRT-qPCR results of individual pigs within the litter (Vilalta et al., 2018), and despite the dilution  
333 effect that occurs when processing fluids of several litters are aggregated, a positive sample can still be  
334 detected in a wide range of dilutions depending mostly on the sample's initial Ct-value (Vilalta et al.,  
335 2019a).

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

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

351 The time from the onset of the PRRS outbreak to consistently negative processing fluids, defined here as  
352 having at least 10 weeks of consecutive negative ~~rRT-PCRRT-qPCR~~ results in processing fluids was 33, 38,  
353 39, and 48 weeks in four out of nine breeding herds (time to the 10<sup>th</sup> ~~rRT-PCRRT-qPCR~~ consecutive  
354 negative result). The remaining five herds were still not able to get 10 consecutive ~~rRT-PCRRT-qPCR~~  
355 negative results after 52 weeks from the start of the outbreak. Trevisan et al. (2019) estimated time to  
356 stability at 27 weeks in 29 batch-farrowing herds based on two consecutive negative processing fluid  
357 batches, negative ~~rRT-PCRRT-qPCR~~ results in due-to-wean pig sera, and negative ELISA results in gilts after  
358 herd introduction. This estimation is shorter than what our data may suggest. However, the nine herds  
359 purposely included in our study were not batch-farrowing farms. It may be possible that the batch-  
360 farrowing system allows for a faster PRRS virus clearance from the farm. Further studies should evaluate  
361 the time to PRRS stability using processing fluids in a larger sample of herds to provide a more precise  
362 estimate and determine whether P1 sows have a role in maintaining PRRS virus in the herd. However, the

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

365



366 **Conclusion**

367 The proportion of PRRS virus ~~rRT-PCR~~RT-qPCR processing fluid positive litters was not statistically higher  
368 in aggregated samples of first parity ~~one~~ sows compared to that of second parity ~~two~~ or third parity ~~three~~  
369 or higher sows after week 25 from the start of the outbreak. Therefore, the role of first parity ~~one~~ sows  
370 and their litters in maintaining PRRS virus infection in breeding herds could not be confirmed in this  
371 investigation despite the consistent non-significant higher point estimate proportion observed from week  
372 25 after the outbreak onwards. Processing fluid ~~rRT-PCR~~RT-qPCR result dynamics in nine herds showed  
373 that at least nine weeks of ~~negative weekly~~consecutive negative processing fluid ~~rRT-PCR~~RT-qPCR results  
374 may exist between two ~~rRT-PCR~~RT-qPCR positive weeks. This observation should encourage veterinarians  
375 and swine producers that use processing fluids to monitor PRRS virus circulation in breeding herds to  
376 continue testing the herd beyond this time span to avoid misclassification of herd PRRS virus status. The  
377 use of processing fluids continues to reaffirm their value for PRRS virus monitoring and surveillance  
378 activities in breeding herds. Monitoring the breeding herd PRRS virus status using aggregated processing  
379 fluid samples of 15 litters was a useful testing strategy throughout the outbreak in all herds assessed.

380

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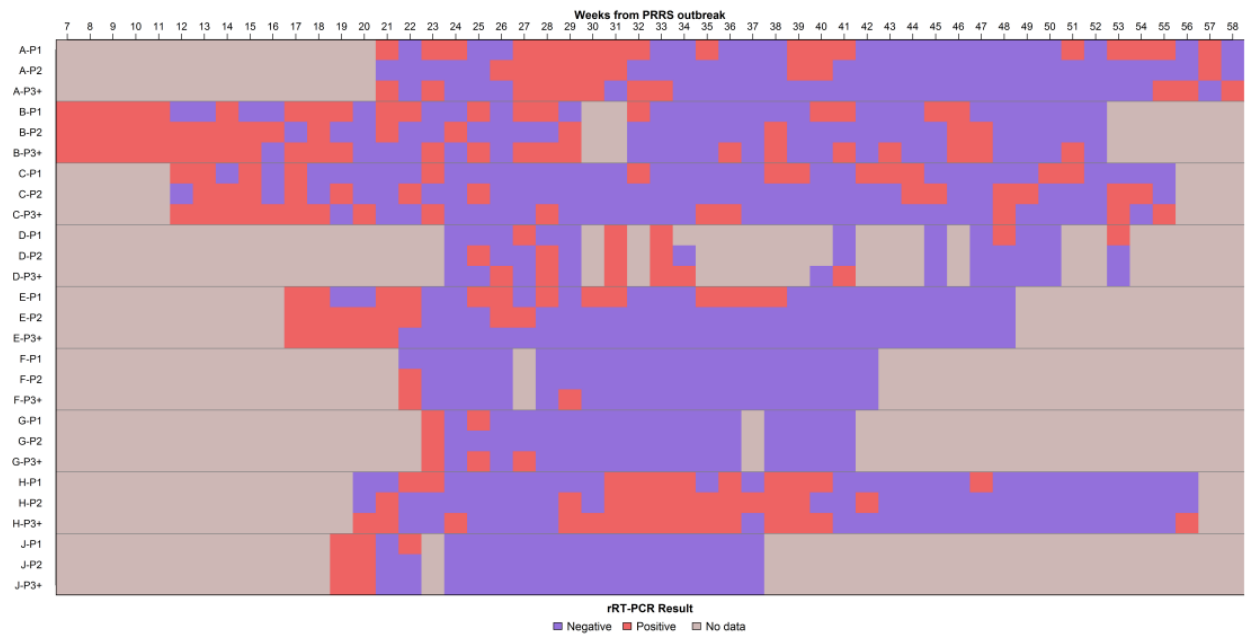
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444

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



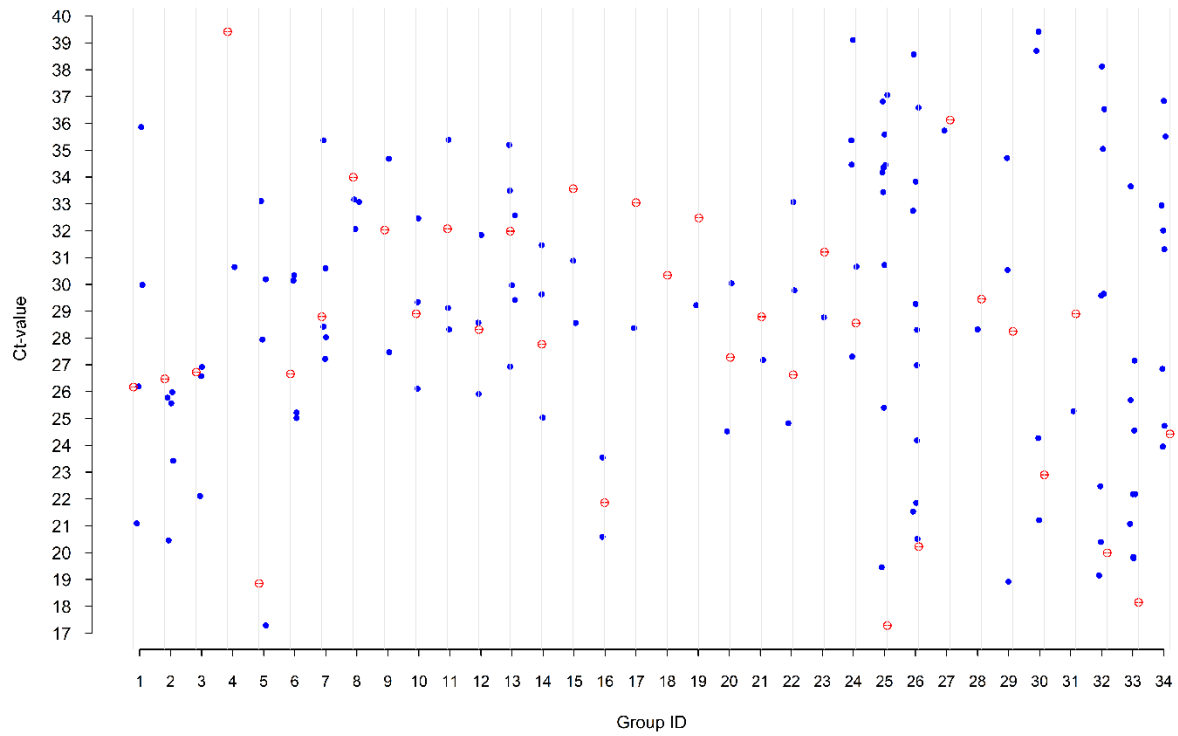


Figure 2: Ct-values in 34 rRT-PCR positive (Ct-value < 40) parity aggregated processing fluid groups (red open crossed circles) rRT-PCR positive (Ct-value < 40) and Ct-values of rRT-PCR positive litters that formed the aggregated sample (blue dots).

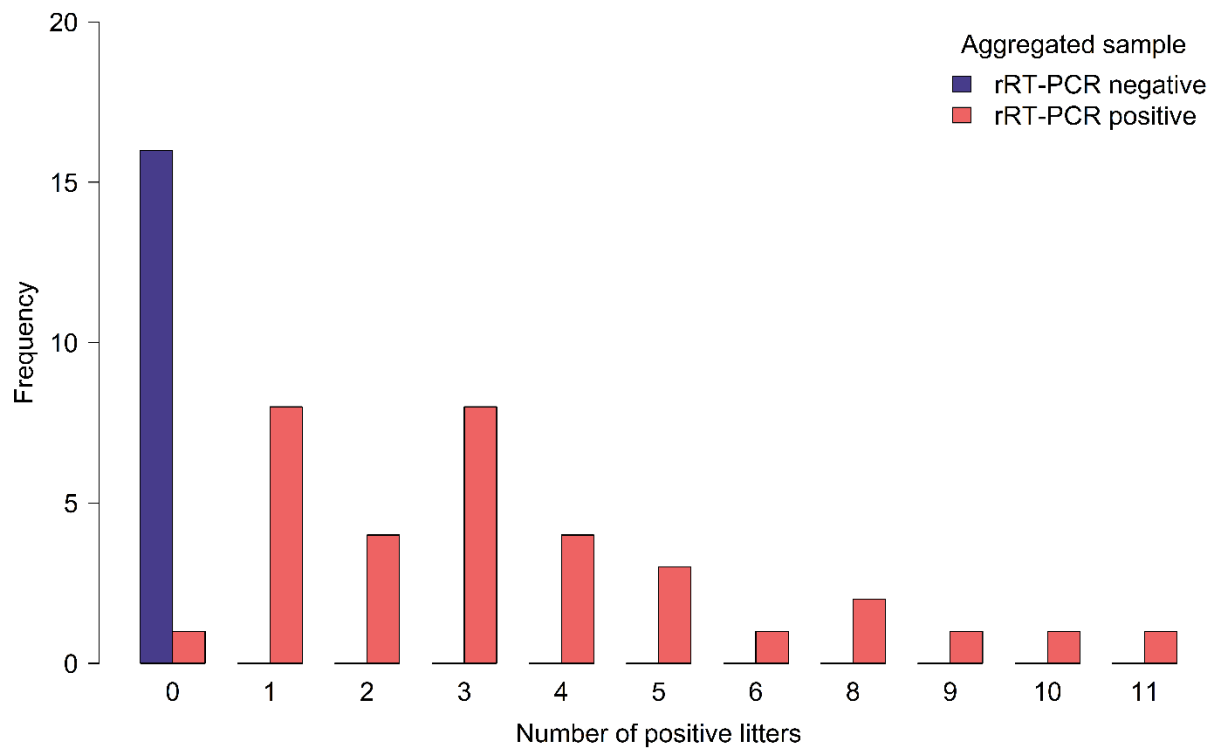


Figure 3: Number of rRT-PCR positive litters in rRT-PCR positive and negative grouped processing fluid samples.

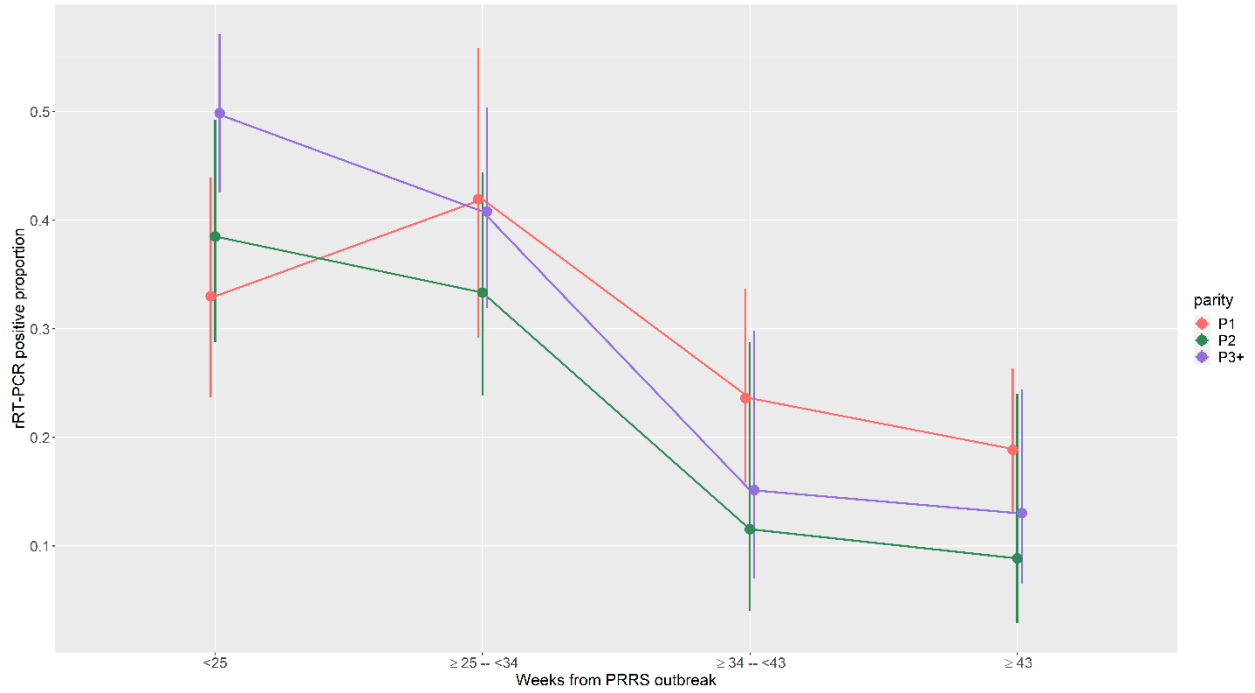
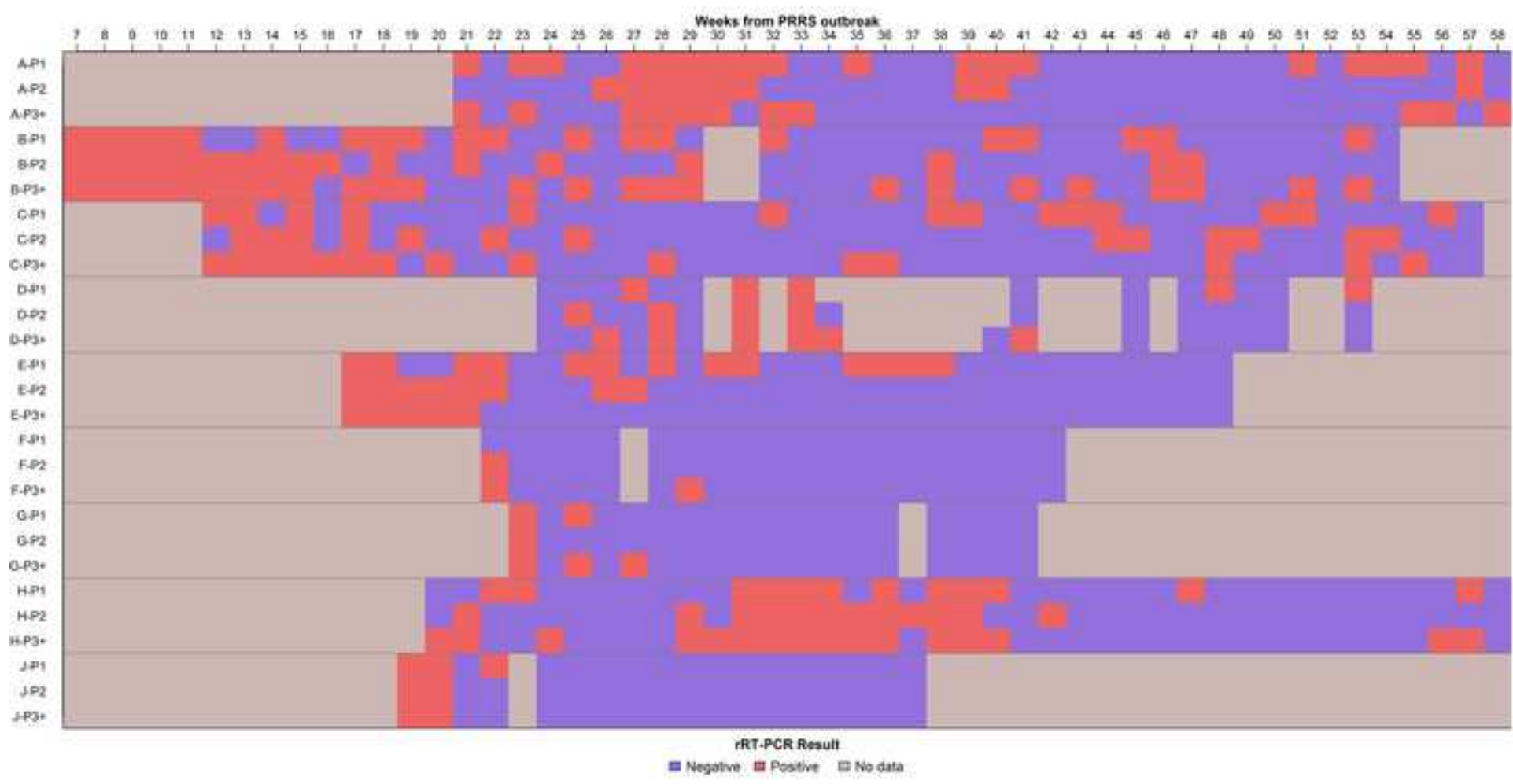
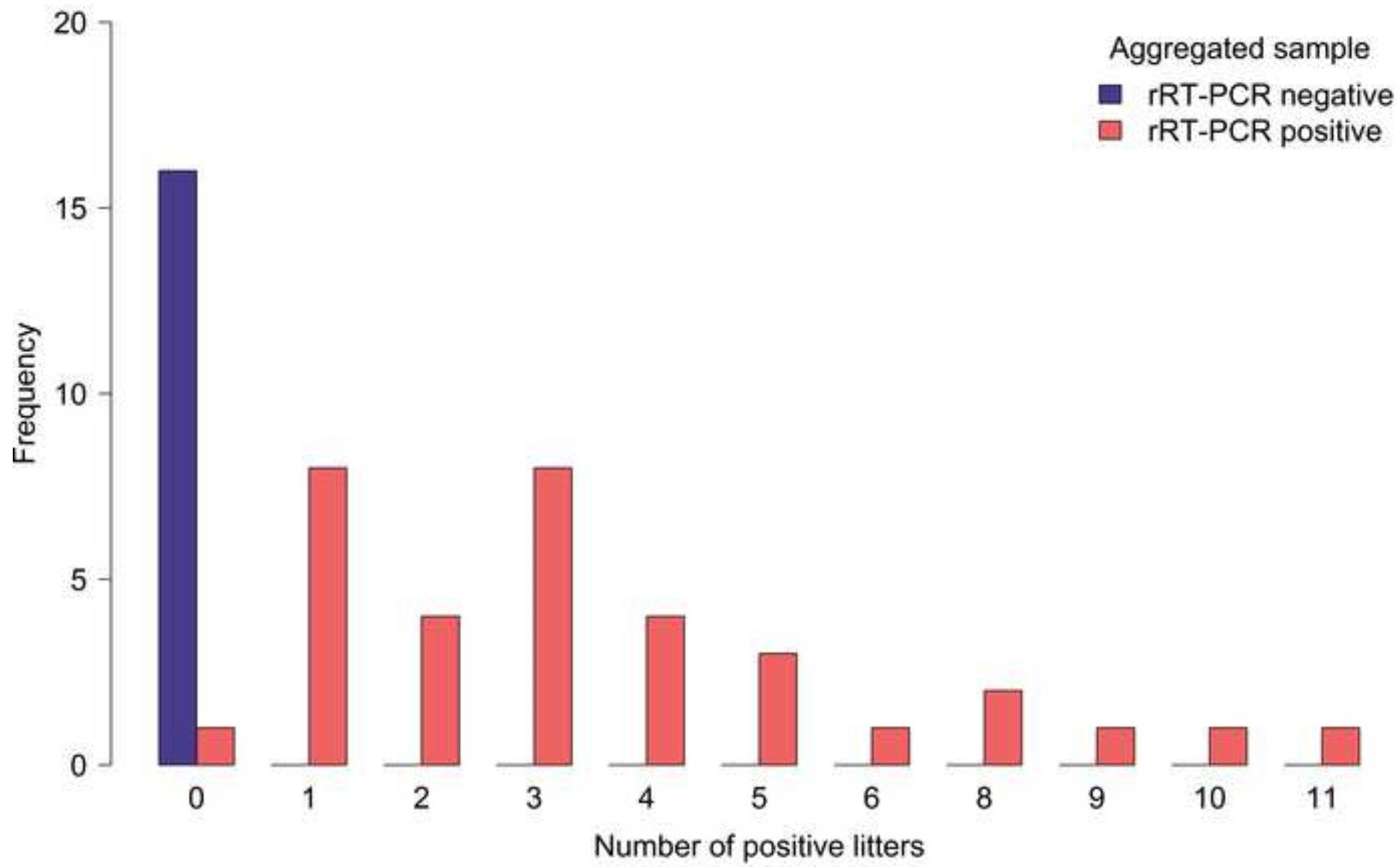


Figure 4: Adjusted proportion and 95% confidence interval (vertical line) of rRT-PCR positive results among the three parity groups in nine breeding herds across four categories of time after the PRRSv outbreak.









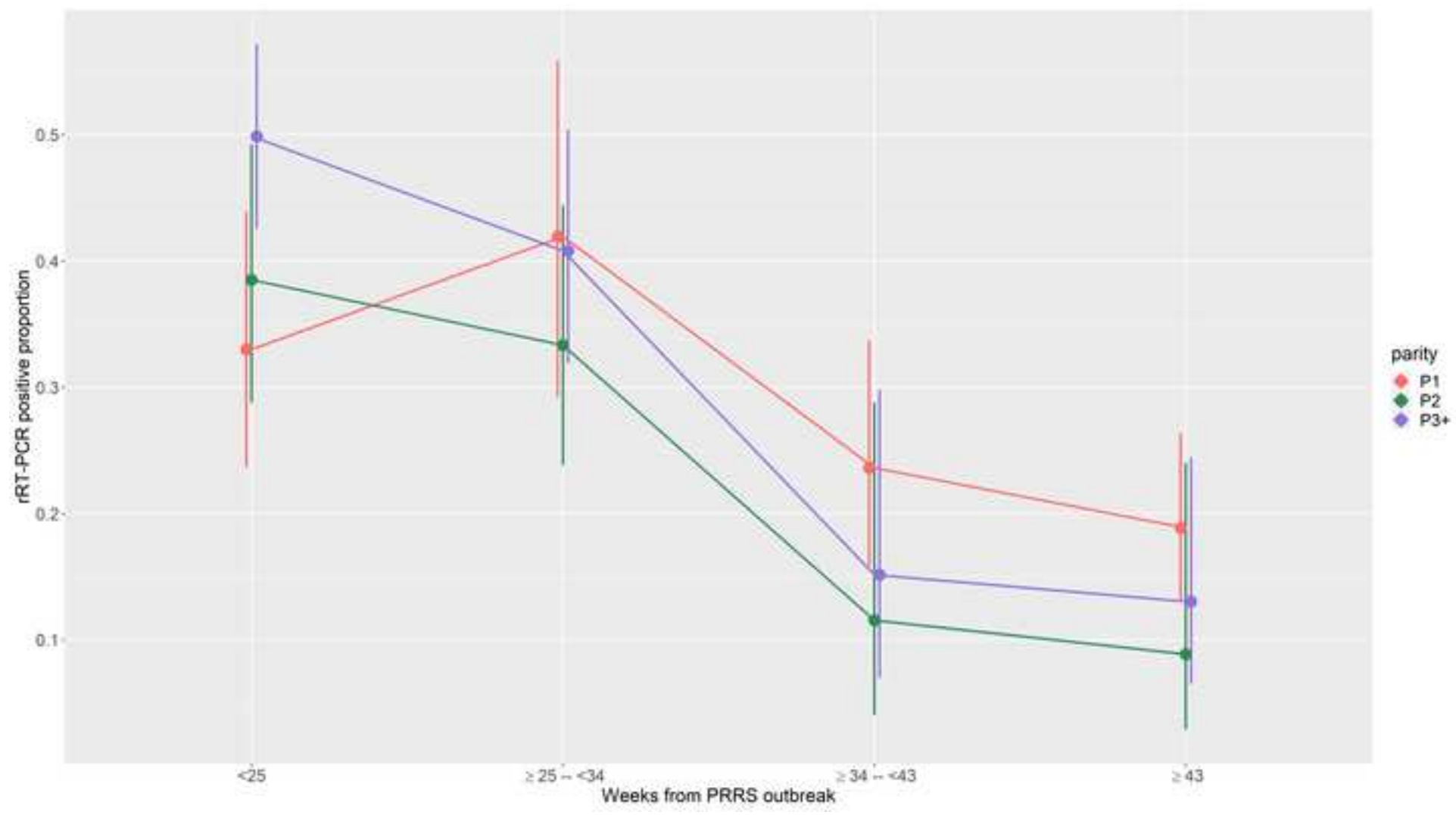


Table 1: Summary of PRRS outbreak information in the 10 study participant herds.

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

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

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Declarations of interest: none.

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

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