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1 **Detection of PRRSV circulation in herds without clinical signs of PRRS:**

2 **Comparison of five age groups to assess the preferred age group and sample size**

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26 Key words: PRRSV, porcine reproductive and respiratory syndrome virus, rt-PCR, virus circulation,
27 seroprevalence.

29 **Abstract**

30 A cross-sectional study was conducted to find the most effective diagnostic approach to detect
31 circulation of porcine reproductive and respiratory syndrome virus (PRRSV). The study was
32 performed in 10 Dutch swine herds, with sows and fattening pigs or breeding stock. Herds did not
33 experience clinical signs of PRRS during the last 6 months before sampling, but a PRRSV infection
34 was confirmed at most 2 years before sampling. Blood samples were collected from 5 age groups:
35 sows during early and late gestation, weaners at 9 weeks of age, fatteners or breeding stock at 16 and
36 22 weeks of age. For each category, 20 serum samples were examined; in total 100 serum samples per
37 herd. Samples were analysed for PRRSV antibodies with ELISA (n=1002), and rt-PCR when ELISA
38 S/P-ratios were above 1.5 (n = 307) or below 0.4 (n=187; random selection from each age group). A
39 logistic regression analysis was used to obtain factors associated with the probability of virus detection
40 in a pig (PCR positive test result). Herd, ELISA-result, and age group were included as explanatory
41 variables. Variables remained in the model when statistically significant. ELISA results showed that
42 none of the herds could be considered to be free of PRRSV infection. Mean PRRSV seroprevalence in
43 unvaccinated animals varied between 18% to 82%, and mean PRRS-virus prevalence varied between
44 0% and 41%. In only one of the 10 herds, no PRRS-virus could be detected. The odds of finding
45 PRRS-virus in blood samples were 8.6 (95% CI, 5.3-13.9) in pigs of 9 weeks of age and 4.6 (95% CI,
46 3.0-7.0) in pigs of 16 weeks of age, compared with fatteners of 22 weeks of age. This result indicates
47 that 9- to 16-week-old pigs are the preferred age group to detect PRRS-virus, in herds without clinical
48 signs of PRRS. We concluded that PRRS-virus circulation could be detected in 8 out of 9 of the study-
49 herds, with a relatively low number of blood samples. Testing 12 blood samples in both rt-PCR and
50 ELISA, with 6 samples in pigs 9 weeks of age and 6 samples in pigs 16 weeks of age, will lead to a
51 cost-efficient first evaluation of the PRRSV infection-status in herds without clinical signs of PRRS.

52

53

54 **1. Introduction**

55 Porcine reproductive and respiratory syndrome virus (PRRSV) is known to play an important role in
56 reproductive problems in sows, as well as in respiratory problems in weaners, fatteners and rearing
57 pigs. The economic impact of PRRS has been quantified in North America (Neumann et al., 2005),
58 and figures on the economic impact of PRRS in the Netherlands are similar (Brouwer et al., 1994).
59 Although the economic impact of PRRS in the Netherlands was not recently re-examined, the costs of
60 respiratory and reproductive diseases initiated or enhanced by PRRSV are still considerable, and one
61 of the reasons for Dutch farmers to consider the control or eradication of this virus.
62 Often, diagnostic tests are used to assess the PRRSV infection status of herds. Several serological tests
63 are available for the diagnosis of PRRSV-infections (Mattheu et al. 2006; Wellenberg, 2006.). For
64 routine settings, both ELISA test methods and reversed transcriptase PCR are mostly used. In herds
65 with no clinical signs of PRRS, these diagnostic tests can be used to determine whether virus
66 circulation is still present. The farmer can use this information to focus on the control or eradication of
67 PRRSV. The decision of farmers to control or eliminate PRRSV can either be made shortly after an
68 outbreak of PRRS, or in endemic situations without clinical signs of PRRS. In both cases, more
69 information on the actual PRRSV circulation on herd level is required to support decisions of farmers
70 to implement management measures. The absence of virus circulation in herds could be the starting
71 point to control or eliminate PRRSV from the herd. However, information is lacking on the most
72 effective method, regarding age groups and sample size, to examine PRRS-virus circulation within
73 herds without clinical signs of PRRS. The objective of this study was to determine the preferred age
74 group for sampling, and the required sample size, in order to detect PRRS-virus circulation in herds
75 without clinical signs.

76

77 **2. Materials and methods**

78 *2.1 Herds and animals*

79 Ten herds were selected on the absence of clinical signs of PRRS for a period of at least six months.
80 This selection was based on information of the herd veterinarian and the farmer. All herds were
81 selected on the basis of a confirmed circulation of PRRSV, and consisted of a combination of sows

82 and fatteners or breeding stock. Most of these herds used a vaccination scheme for PRRSV, mainly in
83 the sows, only one farm vaccinated piglets (Table 2). On average, these herds consisted of 330 (150 –
84 750) sows and 1400 (850 – 2800) fatteners and/or breedingstock. In all ten herds, a cross-sectional
85 sampling of one hundred blood samples was carried out from January to April 2005. Twenty samples
86 were collected from each of the following five age groups; sows in early gestation, sows in late
87 gestation, piglets at 9 weeks of age, and young fattening or breeding pigs at 16 weeks and 22 weeks of
88 age.

89

90 *2.2 Test systems and analyses*

91 Sera were tested for the presence of PRRSV antibodies by ELISA (IDEXX HerdCheck PRRS 2XR
92 ELISA, Maine, USA). All sera with an ELISA S/P ratio of > 1.5 ($n= 307$) were analysed for the
93 presence of PRRS-virus by conventional reverse-transcriptase-PCR (rt-PCR) (Van Maanen et al.,
94 2006) to monitor prolonged virus circulation in infected animals. In addition, 20 sero-negative serum
95 samples per herd (S/P ratio < 0.4) were chosen at random, and tested by rt-PCR ($n= 187$) to look for
96 virus circulation in animals expected to be negative for PRRSV or infected recently.

97

98 *2.3 Statistical analysis*

99 A logistic regression analysis was carried out in SPSS 15.0 (SPSS Inc., 1989-2006) to obtain factors
100 associated with the probability of virus detection in a pig (PCR positive test result). Herd, ELISA
101 result and age group were included as explanatory variables. Variables remained in the model when
102 statistically significant at $P \leq 0.05$. In order to find manageable and predictable sample sizes for
103 veterinary practice, stratification by means of sampling on age group level was examined. Based on
104 the predicted prevalence of the final logistic regression model for each age group, the required sample
105 size was calculated to detect at least one PRRSV PCR or antibody positive animal with 95%
106 confidence, assuming a random spread of virus and antibodies in the animals present in the different
107 age groups. This calculation was done to obtain the age group with the highest prevalence and the
108 smallest required sample-size.

109 The antibody prevalence was only based on the unvaccinated pigs: weaners, fatteners and in one herd
110 also the sows. One herd vaccinated all pigs; this herd was not included for calculation. The ELISA
111 was assumed to have a sensitivity of 97.6% and a specificity of 98.6 % (Wellenberg, 2006), and the rt-
112 PCR a 100% sensitivity and specificity (van Maanen et al., 2006).

113

114 **3. Results**

115

116 *3.1 Seroprevalence, virus prevalence and estimated sample size*

117 In total, 1002 blood samples were screened for the presence of PRRSV antibodies. In all herds
118 antibodies against PRRSV were found. The mean seroprevalence in unvaccinated pigs on herd level
119 was 53%, and between herds it varied between 18% and 82%. The seroprevalence (in unvaccinated
120 pigs) was the highest in fatteners and/or breeding stock of 16 and 22 weeks old. Based on herd level
121 prevalence, the calculated sample size to detect seropositive pigs on herd level varied between 3 and
122 16 samples. The calculated samples sizes to detect PRRSV circulation by antibody detection in the
123 different age groups, varied between 3 and 15 (Table 1). In 9 of 10 examined herds, the presence of
124 PRRS-virus was detected by rt-PCR. The virus prevalence on herd level varied between 0% and 41%.
125 Based on herd level prevalence, the calculated sample size to detect virus on herd level, varied
126 between 6 and 237 samples. To estimate the required sample-size on age group level, the virus
127 prevalence on age group level was assessed (Table 1). The highest virus prevalence was found in the
128 weaners at 9 weeks of age and fatteners at 16 weeks of age. In these groups, the calculated samples
129 sizes were 9 and 10 samples. For each of the 10 study herds, more detailed results on the virus
130 prevalences for the age groups with the highest prevalences, 9 and 16 weeks, are presented in Table 2.
131 The range in virus prevalence is not further examined for the sows and fatteners of 22 weeks of age, as
132 the needed sample size for virus-detection was too high for practical use, considering the low virus
133 prevalence in these age groups. Virus prevalences in the 10 examined herds ranged from 0 to 100% in
134 9 week old pigs and from 0 to 85% in 16 week old pigs (Table 2).

135

136 *3.2 Results of rt-PCR-testing on ELISA-negative samples or samples with S/P-ratio > 1,5*

137 The sera tested for PRRSV antibodies with ELISA were divided in three classes based on S/P-ratio's;
138 S/P-ratio < 0.4 (N = 364); S/P-ratio higher than 0.4 but lower than 1.5 (N = 331); S/P-ratio higher than
139 1.5 (N = 307). The rt-PCR was performed on a random selection of samples with ELISA S/P-ratio
140 below 0.4 (n=187) and all samples with ELISA S/P-ratio higher than 1.5. The results were: 23 of 187
141 (12.2 %) serum samples with S/P-ratio <0.4, and 46 of 307 (15.3 %) serum samples with S/P ratio
142 >1,5 were positive by rt-PCR. In total, 69 (13.9%) of the 494 tested serum samples were rt-PCR
143 positive.

144

145 *3.3: Logistic regression analysis for the presence of virus.*

146 In Table 3, the results of the logistic regression analysis for the presence of virus are summarized. The
147 logistic regression model for detection of virus circulation included serostatus as an independent
148 variable. Weaners of 9 weeks of age had 8.6 (95%CI 5.3-13.9) times higher odds for virus presence
149 than pigs of 22 weeks of age (= reference group in this study). Fatteners of 16 weeks of age had 4.6
150 (95% CI 3.0-7.0) times higher odds compared to pigs of 22 weeks of age. Sows were the age group
151 with the lowest odds ratio (0.06 95%CI 0.02-0.18) for detection of virus. Seropositive pigs were 3
152 times (95%CI 2.1-4.1) more likely to be virus positive than seronegative pigs.

153

154 *3.4. Sampling scenarios in age groups of 9 and 16 weeks*

155 Based on the prevalences in our study (Table 2), we made a comparison between sampling scenarios
156 in order to detect higher (at least 40%) or lower levels (at least 22%) of virus circulation in the
157 preferred (non-vaccinated) age groups of 9 and 16 weeks of age. The highest number of farms was
158 detected by sampling 6 or 12 pigs from both of these two age groups, both in rt-PCR and in ELISA (8
159 of 9 herds; 88%). However, no difference was found in the number of PRRSV-positive herds in case 6
160 or 12 blood samples were collected from both age groups (12 or 24 samples in total). Sampling of only
161 one of these two age groups, by taking 6 or 12 blood samples, resulted in the detection of 7 of 9 (77%)
162 infected farms with PRRSV circulation

163

164 **Discussion**

165 The objective of this study was to determine the preferred age group, and the required sample size in 5
166 selected age groups, in order to detect PRRS-virus circulation in herds without clinical signs of PRRS.
167 The selected 5 age groups did not include piglets of 3-6 weeks of age. Although virus circulation in
168 this agegroup can be present, the comparison of PCR-results with ELISA-results would not be
169 possible due to the presence of maternal antibodies in this age group.

170 In this study, PCR and ELISA were used as diagnostic methods. The actual virus circulation can be
171 determined by using PCR-technique (Van Maanen et al., 2006; Wellenberg, 2006). Although the
172 period of viraemia, the time frame to detect the virus by PCR in blood samples, can persist for several
173 weeks in young pigs (Albina et al. 1998), the time window to detect PRRSV infections by ELISA test
174 methods may be longer than by PCR (Fano et al., 2007). However, detection of antibodies does not
175 provide information on the actual circulation of virus. Compared to serological tests, the advantage of
176 using PCR methods is that this test method is independent of, and not influenced by the presence of
177 either maternal antibodies, or antibodies induced by vaccination. In addition, PCR can be used to
178 demonstrate early infections. Ten Dutch swine herds without clinical signs of PRRS but with histories
179 of PRRSV infections were included in the study. Although the 10 examined herds are not
180 representative for all Dutch farming systems, they were representative for the 218 herds with both
181 sows and fatteners or breeding stock of an average size (330 sows and 1400 fatteners or breeding
182 stock). Although no clinical signs of PRRS were recorded during the last 6 months, none of these 10
183 herds could be classified as a “PRRSV-free herd”. Antibodies against PRRSV were detected in
184 unvaccinated pigs in all herds. Because all pigs were 9 weeks or older, the detected antibodies against
185 PRRSV are considered not to be of maternal origin (Nodelijk et al., 2003). To detect PRRS-virus
186 circulation in low prevalent herds, large sample sizes are needed. In order to use smaller sample sizes,
187 and reduce costs, we examined not only the virus prevalences and seroprevalences on herd level but
188 also within different age groups (Table 1). Based on the virus prevalences and seroprevalences found
189 in this study, the sample size could be calculated for each age group. For detection of virus, the highest
190 odds ratio's, were found for the age groups of 9 and 16 weeks. The overlapping confidence intervals
191 for the odds ratios of these two groups indicate that both age groups are preferred for the detection of
192 PRRSV circulation by rt-PCR. The narrower confidence interval of the odds ratio in the age group of

193 16 weeks shows that this group gives a more reliable outcome. The virus prevalences in the age
194 groups of 9 and 16 weeks of age were the highest, with estimated mean virus prevalences of 30% and
195 27% (Table 1). As stated in Materials and Methods, paragraph 2.2, PCR-testing was not done in
196 animals with ELISA S/P-ratios from 0.4 to 1.5, which may have influenced the virus prevalence.
197 However, because viraemia in young pigs is known to last for longer periods (Zimmermann et al.
198 (2006a; Albina et al. 1998), comparable virus prevalences are to be expected, when pigs with S/P-
199 ratios from 0.4 to 1.5 are tested with rt-PCR. The range in virus prevalence between herds ranged from
200 0 to 100% in 9 week old pigs and ranged from 0 to 85% in 16 week old pigs (Table 2). This pattern is
201 in accordance with those described earlier by Evans et al. (2008) and Zimmermann et al. (2006b).
202 They characterised the spread of PRRSV within herds as two different patterns: either at least 90% of
203 weaners are infected at 8.5 weeks of age, or 20% to 40% were infected at 10-12 weeks of age. These
204 findings support the use of a virus prevalence of 20% as a threshold-value in distinguishing highly
205 infected groups of pigs from groups where virus circulation is less prominent. Therefore, a sample-size
206 of 12 samples seems appropriate to detect virus circulation in over 22% of the pigs with 95%
207 confidence when monitoring for active virus circulation. Given the above mentioned percentages of
208 infected pigs, and the reproduction ratio R estimated to be 3 (Nodelijk et al., 2000) this number of
209 samples will be sufficient when monitoring for new outbreaks too. When comparing sampling-
210 scenario's, it appears that the use of 12 or even 6 samples from each of the non-vaccinated age groups
211 of 9 and 16 weeks (24 or 12 in total), will result in a high detection rate of virus circulation in the
212 farms examined in this study. For a first evaluation of the PRRSV infection-status of herds with sows
213 and fatteners or breeding stock, samples should be taken from both age groups for both rt-PCR and
214 ELISA. In contrast, PRRSV infection in farm 4 will not be detected by using these sampling scenarios.
215 A higher number of samples would be required to detect a seroprevalence of 15% or lower in the age
216 groups of 9 weeks, or to detect a virus prevalence of 20% and lower in the age group of 16 weeks
217 (Table 2). As 1:4 pooling of samples for rt-PCR-testing was established to be feasible (van Maanen et
218 al. 2006), costs for examination of PRRS-virus circulation within herds can be reduced (Van Maanen,
219 personal communication). In case of vaccination of piglets, PCR is the only option to detect virus
220 circulation in herds by examining blood samples from weaners and young fatteners or breedingstock.

221 The results of this study indicate that the use of antibody tests alone, was not enough to determine
222 PRRS-virus circulation. In a random selection of seronegative samples, 12,2 % of these samples were
223 PRRS-virus positive by rt-PCR. This finding supports the use of the rt-PCR test method as additional
224 test to detect virus circulation. Based on the information received from the study farms, we
225 hypothesized that the positive rt-PCR reactions were the result of circulating field virus and not
226 vaccine virus. The rt-PCR positive age groups were not vaccinated with PRRS-MLV vaccines or at
227 least 6 weeks before sampling (one farm).

228 We conclude that for a first evaluation of PRRSV-circulation on herds with sows and fatteners or
229 breeding stock, a total of 12 samples is sufficient. The collection of 6 samples from both 9 and 16
230 week old pigs, and analysing them using rt-PCR and ELISA, is the most efficient method and leads to
231 the detection of 8 out of 9 of farms with virus circulation.

232

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236

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Table 1: Sample size (with 95% confidence intervals) per age group based on the estimated virus- and seroprevalence to detect PRRS virus and antibodies with 95% confidence in a group of 1000 pigs by PCR with 100% sensitivity and specificity, and by ELISA with 97.6% sensitivity and 98.6% specificity. The seroprevalence is based on unvaccinated weaners and fatteners (or breeding stock) in 9 herds, and in sows from one herd.

Age groups	Virus prevalence					Sero-prevalence				
	N	Mean (%)	95% confidence interval (%)	Sample size (n)	95% confidence interval sample size (n)	N	Mean (%)	95% confidence interval (%)	Sample size (n)	95% confidence interval sample size (n)
Sows	185	0,5	0-1.6	450	170-1000	40	53	37-69	7	3-11
Piglets 9 weeks	92	30	21-40	9	6-13	178	29	23-35	15	12-19
Fatteners/ breedingstock 16 weeks	120	27	19-35	10	7-15	180	61	54-68	6	3-7
Fatteners/ breedingstock 22 weeks	97	8	3-14	36	20-94	180	69	62-76	3	3-6

Table 2: Virus prevalence and seroprevalence in weaners of 9 weeks of age and fatteners or breeding stock of 16 weeks of age for each of the 10 herds. Except for herd 10, all weaners and fatteners or breeding stock were unvaccinated.

Included are the used vaccinationschemes in each herd. S = vaccination of sows; G = vaccination of gilts; P = vaccination of piglets

Herd nr	Weaners 9 weeks of age			Fatteners/breeding stock 16 weeks of age										
	Used Vaccination Type	Used Scheme	Virus prevalence	Sero-prevalence	Virus prevalence	Sero-prevalence								
	Type	Used Scheme	Number of samples tested	Virus prevalence %	Standard error %	Number of samples tested	Sero-prevalence %	Standard error %	Number of samples tested	Virus prevalence %	Standard error %	Number of samples tested	Sero-prevalence %	Standard error %
1	None	-	6	100	0	20	50	11	13	85	10	20	70	10
2	EU-MLV	S + G	10	0	0	20	5	5	17	0	0	20	90	7
3	EU-MLV	S	4	0	0	20	40	11	10	0	0	20	5	5
4	EU-KV	S	9	0	0	20	15	8	10	20	13	20	0	0
5	EU-KV	G	5	0	0	20	50	11	14	7	7	20	95	5
6	EU-KV	S	14	7	7	18	0	0	13	31	13	20	75	10
7	EU-KV	S	14	71	12	20	90	7	12	0	0	20	100	0
8	EU-MLV	S	8	100	0	20	5	5	10	50	16	20	20	9
9	US-MLV	S	7	0	0	20	0	0	7	14	13	20	95	5
10	EU-MLV	S + P	15	20	10	20*	45*	11	17	47	12	20*	90*	7
Total	-	-	92	30	5	178	29	3	120	27	4	180	61	4

*Herd 10 vaccinated the piglets; these results have been excluded in the calculation of sero-prevalence.

Table 3: Results of the logistic regression analyses on virus presence in 10 Dutch swine herds.

Variable	Category of variable	Regression coefficient	Standard Error	P-value	Odds ratio	95% confidence interval
Intercept		-2.54	0.29	<0.01	-	-
ELISA result	Seronegative	Ref.*	-	-	1.0	-
	Seropositive	1.09	0.33	<0.01	2.9	2.1-4.1
Age Group	Pigs 22 weeks of age	Ref.*	-	-	1.0	-
	Sows (all groups together)	-2.76	1.07	0.01	0.06	0.02-0.18
	Piglets 9 weeks of age	2.15	0.48	0.00	8.6	5.3-13.9
	Pigs 16 weeks of age	1.52	0.43	0.00	4.6	3.0-7.0

Ref*: in this study used as the reference group.