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1 **Seroprevalence and risk factors for the presence of ruminant**
2 **pestiviruses in the Dutch swine population**

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28 **ABSTRACT**

29 Swine can be infected with classical swine fever (CSF) virus, as well as ruminant
30 pestiviruses: bovine viral diarrhoea (BVD) virus, and Border disease (BD) virus. Cross-
31 reactions between pestiviruses occur, both regarding protective immunity and in diagnostic
32 tests. The presence of BVDV and BDV in a swine population may thus affect the
33 transmission of CSFV, but also the diagnosis of a CSFV infection. In this study, the
34 seroprevalence against BVDV and BDV in two categories of swine, sows and finishing pigs,
35 in the Netherlands was determined. Furthermore, several risk factors, associated with the
36 presence of swine and ruminants on the same farm or in the immediate surroundings, were
37 evaluated. In sows, the seroprevalence against BVDV was 2.5% on the animal level, and
38 11.0% on herd level. In finishing pigs these prevalences were 0.42% and 3.2% respectively.
39 Antibodies against BDV were found in 3 sows only. Risk factors, associated with a BVDV-
40 seropositive status in breeding pigs, were the presence of cattle on the same premises and
41 a high density of sheep and/or goats herds in a radius of 3 kilometres. While BVDV and BDV
42 hardly pose any threat to the swine population themselves, knowledge, and therefore regular
43 monitoring, on the presence of these viruses in the swine population is important with
44 respect to CSF eradication. It will allow for a better interpretation of diagnostic test results,
45 both in terms of possible false positives and false negatives, but may also bring about
46 additional measures or surveillance protocols in times of CSF outbreaks to avoid surprises
47 caused by cross-reactivity with ruminant pestiviruses.

48

49 **Key words:** swine, seroprevalence, classical swine fever, bovine viral diarrhoea, border disease

50

51 **INTRODUCTION**

52 Ruminant pestiviruses (bovine viral diarrhoea (BVD) virus and Border disease (BD) virus)
53 are closely related to classical swine fever (CSF) virus and all belong to the genus of
54 pestiviruses (Van Rijn et al., 1997; Becher et al., 2003). Natural hosts for BVDV and BDV
55 are cattle and small ruminants respectively, but both are able to infect swine as well
56 (Wrathall et al., 1978; Terpstra and Wensvoort, 1988; Walz et al., 2004; Wieringa-Jelsma et
57 al., 2006). In swine, only CSF is notifiable to the OIE and can cause massive economic
58 damages as was shown in recent outbreaks in Western Europe, in the Netherlands in 1997-
59 1998 (Elbers et al., 1999), the UK in 2000 (Sandvik et al., 2000), and Germany in 2006.

60 Cross-reactions between pestiviruses occur, both regarding protective immunity and in
61 diagnostic tests (Liess and Moennig, 1990; Dahle et al., 1993b; Wieringa-Jelsma et al.,
62 2006). The presence of ruminant pestiviruses in a swine population may thus affect the
63 transmission of CSFV, but also the diagnosis of a CSFV infection. During the 1997/98
64 outbreak of CSF in the Netherlands, the presence of BVDV and BDV in the swine population
65 caused considerable problems in the serological diagnosis of CSF (De Smit et al., 1999).

66 A new development in the past decade has been the introduction of the first generation
67 of marker vaccines against CSF. These marker vaccines are E2-subunit vaccines,
68 generating only antibodies against E2 after vaccination (Van Rijn et al., 1999). Detection of
69 infected animals in a vaccinated population is based on detection of antibodies against E^{ms} .
70 In 2003 a serological test to detect antibodies against E^{ms} was evaluated by 15 national
71 reference laboratories for CSF in the EU, under the guidance of the community reference
72 laboratory in Hanover, Germany. This test was approved, thus allowing for the first time the
73 use of marker vaccines in Europe (Blome et al., 2006). A setback of this test is, however,
74 that it also detects antibodies against E^{ms} from ruminant pestiviruses. New generations of
75 marker vaccines may still rely on the detection of E^{ms} -antibodies and will therefore face the
76 same problem of cross-reactivity in diagnostic tests (Van Gennip et al., 2000; Koenig et al.,
77 2007).

78 Within the framework of surveillance and eradication of CSF, including the possible use
79 of the above mentioned marker vaccines, it is important to have information on the
80 prevalence of ruminant pestiviruses in a country or region where it is applied.

81 In this study, the seroprevalence of ruminant pestiviruses in sows and finishing pigs in
82 the Netherlands was determined. Furthermore, several risk factors, associated with the
83 presence of swine and ruminants on the same farm or in the immediate surroundings, were
84 evaluated.

85 **MATERIALS AND METHODS**

86 *Sampling*

87 In order to estimate the seroprevalence against BVDV and BDV in sows and finishing
88 pigs in the Netherlands with reasonable precision, the following preconditions were used: an
89 a priori expected herd prevalence of 2% (finishing pigs) to 5% (sows), a maximum allowable
90 error in the prevalence estimate of approximately 2%, a 95% confidence in the estimate, and
91 a population size of approximately 3000 finishing herds and 6000 sow herds. A random
92 sample size of at least 178 finishing herds and 424 sow herds are required to meet these
93 criteria (Thrusfield et al., 2001). Samples for the prevalence study were collected in the
94 framework of the Dutch swine vesicular disease (SVD) surveillance program. This
95 surveillance program, part of the Animal Disease Farm Inspection Regulation (ADFIR), ran
96 under government supervision between 1993 and 2004 and after that under the supervision
97 of farmers organisations. Every farmer with more than four pigs was obliged to submit
98 between 5 and 12 samples every four months, depending on herd size (Hunneman et al.,
99 1996). Blood sampling was carried out by the general practitioner. A total of 6020 serum
100 samples taken from 616 sow herds and 1890 serum samples from 189 finishing herds were
101 selected by a stratified (by region) random process out of this daily pool of samples and
102 used for detection of antibodies against ruminant pestiviruses.

103 *Serological tests*

104 Blood samples were pre-screened in a pan-pesti NS3-ELISA (PrioCHECK[®] BVDV AB,
105 Prionics). Positive samples in the NS3-ELISA were tested in an E^{ms}-ELISA (Idexx Chekit[®]
106 CSF-Marker), and VNT's against one CSFV strain (Brescia), two BVDV strains (NADL, a
107 BVDV-1a strain, and Osloss, a BVDV-1b strain) and two BDV strains (Frijters and Moredun).

108 The NS3-ELISA contains two monoclonal antibodies directed against different highly
109 conserved epitopes on the non-structural protein NS3 of pestiviruses. The test has a relative
110 sensitivity and specificity of approximately 98%, respectively 99%, compared to a virus
111 neutralisation test (VNT) (Kramps et al., 1999). The test was considered positive if the
112 inhibition percentage was above 50%.

113 The E^{ms}-ELISA was originally developed as a companion test to E2-subunit vaccines
114 against classical swine fever, but also detects antibodies directed against E^{ms} from BVDV
115 and BDV (Floegel-Niesmann, 2001). The test was considered positive if the inhibition
116 percentage was above 40%.

117 The VNT was carried out as described by Terpstra et al (1984). VNT's were validated by
118 back-titration of the virus stock used in the test and titration of two control sera per virus
119 species of known titre and variation. Results of the test were only valid if all were within
120 range. If there was a response against more than one strain in the VNT, the sample was
121 categorised as having antibodies against the strain with the highest antibody titre. The other
122 titres were regarded as a result of cross-reaction and therefore neglected. For BVDV only
123 BVDV-1-strains of two different subgroups were included in the VNT, as BVDV-2 appears to
124 be highly prevalent only in North America (Fulton et al., 2000; Evermann and Ridpath, 2002)
125 and seems relatively rare in other continents (Wolfmeyer et al., 1997; Sakoda et al., 1999).
126 Within each species and subgroup, the strains should be representative enough to detect
127 antibodies against most or all strains within that subgroup, be it that the height of titres may
128 vary (Wensvoort et al., 1989; Dekker et al., 1995; Patel et al., 2005).

129 *Risk factors*

130 From the herds in the study population, the following information was obtained:

131 - herd size of the pig herd;

132 - number of goats, sheep and cattle housed on the same premises as the pig herd

133 - number of goats, sheep and cattle housed within a radius of 3 kilometres from the pig
134 herd;

135 - number of herds with pigs, goats, sheep or cattle within a radius of 3 kilometres of the
136 pig farm;

137 - nearest distance to herds with pigs, goats, sheep or cattle in the neighbourhood.

138 *Statistical analysis*

139 95% confidence intervals for proportions were calculated according to the efficient-score
140 method (corrected for continuity) described by Newcombe (1998).

141 Statistical Analysis System (SAS/STAT, version 8, 1999) was used for frequency counts
142 and univariate analysis. Differences in presence of risk factors between pig herds with or
143 without antibodies against BVDV were analyzed with multivariate linear logistic regression
144 (PROC LOGISTIC, SAS 1999). Summary Odds Ratios (sOR), adjusted for other risk factors
145 in the model, with accessory 95% confidence limits (CI) were calculated to measure the
146 strength of the association. In terms of disease causation, an OR=1 indicates no
147 relationship, whereas an OR>1 or OR<1 are indicative of increased or reduced risk
148 (protection) risk, respectively (Dohoo et al., 2003).

149 **RESULTS**

150 In sows, 309 out of 6020 samples were positive in the NS3-ELISA. From these 309
151 samples, 126 were positive in the Erns-ELISA and 152 in at least one of the VNT's, mainly
152 those samples that has inhibition percentages of >80% in the NS3-ELISA (table 1, figure 1).
153 From these 152 positive samples in the VNT, 151 had titres against strain Osloss, 124

154 against NADL, 60 against Moredun, 67 against Frijters and 32 against Brescia. In 142 of
155 these cases the titres against Osloss were the highest (ranging from 10 to 15360) and in 7
156 samples titres against NADL were the highest (ranging from 15 to 1280). All the other titres
157 against NADL (ranging from 10 to 2560) were lower than the titre against Osloss in the same
158 sample (on average almost 7 times as low). Except for 2 highest titres against strain
159 Moredun (titres of 15 and 20) and 1 against Frijters (titre of 10), all titres against these
160 strains and against strain Brescia were considered the result of cross-reaction. Titres against
161 Osloss were on average 50, 61 and 130 times higher than against strains Frijters, Moredun
162 and Brescia respectively. In sows, the seroprevalence against BVDV was therefore
163 estimated to be 2.5% on the animal level, and 11% on the herd level (table 2), mainly against
164 BVDV-1b. In only 3 of the samples low titres against BDV were found that could not be
165 attributed to cross-reaction with BVDV.

166 In finishing pigs only 8 out of 1890 samples were positive in the NS3-ELISA. All 8 tested
167 positive in the VNT, with titres against strain Osloss being consistently the highest (ranging
168 from 15 to 3840). Some cross-reactivity was seen in all other VNT's (5 positive against
169 NADL, range 30-160; 4 positive against Frijters, range 10-15; 2 positive against Moredun,
170 both 10; and 1 positive against Brescia, titre of 10). The seroprevalence against BVDV was
171 estimated at 0.42% on the animal level, and 3.2% on the herd level (table 2), all against
172 BVDV-1b. It was concluded that none of the samples contained BDV specific antibodies.

173 Univariate analysis revealed several risk factors to be associated ($P < 0.20$) with a
174 BVDV-seropositive status of sow herds and finishing herds (table 3). In the final multivariate
175 logistic regression model two risk factors were significantly ($P < 0.05$) associated with a
176 BVDV-seropositive status of sow herds (table 4), but no risk factors were significantly ($P <$
177 0.05) associated with a BVDV-seropositive status of finishing herds. Sow herds with cattle
178 present on the premises had a 3.4 higher odds of having a BVDV-seropositive status
179 compared to sow herds without cattle present on the premises. Furthermore, if the number
180 of sheep and/or goats herds in a radius of 3 kilometre of the sow herds was higher than 60,

181 the odds of having a BVDV-seropositive status was 2.1 to 2.6 times higher than for sow
182 herds with less than 60 sheep and/or goat herds in the neighbourhood.

183 **DISCUSSION**

184 The seroprevalence of ruminant pestiviruses in swine is currently very low in the
185 Netherlands, especially in finishing pigs. Because on average only 10 samples per herd
186 were tested, herds with a low intra-herd prevalence may be missed. Thus, the
187 seroprevalence on a herd level may be somewhat higher in reality. Risk factors suggest that
188 direct introduction from mainly cattle, but possibly also small ruminants occurs. Further
189 transmission among pigs and spread through live pig transports between pig farms can not
190 be ruled out based on our results. However, a recent transmission experiment in pigs
191 (Wieringa-Jelsma et al., 2006), using a recent Dutch BVDV isolate originating from a pig,
192 showed a very limited transmission of BVDV among pigs. The results from that transmission
193 experiment suggested that BVDV would become extinct from the pig population if no
194 repeated introductions from outside the pig population would occur.

195 The NS3-ELISA seemed to have a somewhat lower specificity in sows than what was
196 previously determined for cattle (Kramps et al., 1999). With a cut-of value of 50% inhibition,
197 we ultimately ended up with 157 false positive results, or a specificity of 97.3% compared to
198 the VNT. Especially in the range of 50-70% inhibition, few NS3-positives could be confirmed
199 by VNT. Neither could they by an E^{ms}-ELISA, that, in itself, was able to detect 126 out of 149
200 sows seropositive against BVDV in the VNT. Because of this, it is not expected that a
201 relevant number of BVDV-infected sows was missed in the VNT because of the selection of
202 strains that were used.

203 Prevalence studies of ruminant pestiviruses in swine are relatively rare and difficult to
204 compare because of different sampling schemes, different age categories of tested animals,
205 and different tests being used. In wild boar seroprevalences of 0% to 8% against ruminant
206 pestiviruses were found in several countries over the last few decades (Dahle et al., 1993a;
207 New et al., 1994; Schmitt, 1999; Zupancic et al., 2002; Roic et al., 2007). In domestic pigs

208 seroprevalences were reported of 2.2% (BVDV strain NADL) in Norway (Loken et al., 1991),
209 6.4% (BVDV strain Ug59) in Denmark (Holm Jensen, 1985) and 3.2% (BVDV strain NADL),
210 0.2% (BVDV strain NADL) and 0% (BDV strain 137/4) in Ireland (O'Connor et al., 1991;
211 Graham et al., 2001). Seroprevalences of up to 40% in breeding pigs are however
212 mentioned as well (Terpstra and Wensvoort, 1988; Liess and Moennig, 1990).

213 BVDV- and BDV-seroprevalences in the Netherlands used to be much higher in the past
214 than the ones found in the current study. In the late 1980's a seroprevalence of 20% (BVDV-
215 strain Oregon) was found in Dutch sows and boars, testing more than 700 samples from the
216 slaughterhouse (Terpstra and Wensvoort, 1991). Ten years later, BDV/BVDV antibody
217 seroprevalence of 11% was found when testing 12,000 sows (De Smit et al., 1999). This
218 seroprevalence was determined by ELISA, and it was estimated that approximately two-third
219 of these were BDV-seropositive and one-third BVDV-seropositive.

220 The presence of cattle on the same premises as a risk factor for BVDV infections in
221 swine was reported before (Liess and Moennig, 1990). With a high BVDV-seroprevalence in
222 cattle in the Netherlands, results of 82% (Kramps et al., 1999) and 46% (Mars and Van
223 Maanen, 2005) of seropositive herds were published, this is not unexpected. The current low
224 seroprevalence in swine in the Netherlands is likely caused by a continued specialisation of
225 farms. In former times, farms used to have all kind of production animals on the same
226 premises: cattle, poultry, pigs, small ruminants, etcetera. In the past few decades however,
227 more and more farms specialized in only one type of production animal. Furthermore, in
228 those cases where more than one species is present on one farm, housing became such
229 that the possibilities for direct contact became less and less. Mixed farms, where different
230 species live in close contact with each other, exchanging infectious agents, have become
231 rare.

232 The presence of small ruminants in the neighbourhood of swine herds has not been
233 reported before as a possible risk factor for BVDV infections in swine. Knowledge of
234 seroprevalences against BVDV in sheep is relatively scarce, but infections do occur.
235 Seroprevalences of 6.7% (Bechmann, 1997) and 4.5% (Loken et al., 1991) were reported in

236 Germany and Norway respectively. As such, they may therefore be a possible source for
237 BVDV infections in swine as well and represent a risk factor if many herds are present in the
238 neighbourhood. Whether small ruminants also pose a possible risk factor for BDV in swine,
239 could not be determined, as almost no titres against BDV were found. This means that either
240 BDV is not present in the small ruminant populations either or that transmission of BDV from
241 small ruminants to swine is more difficult than transmission of BVDV. For both hypotheses
242 there is currently insufficient evidence.

243 Another reason for the current low BVDV-seroprevalences may be that BVDV
244 contaminations of vaccines, which was also considered an important cause for BVDV
245 infections in swine (Vannier et al., 1988; Wensvoort and Terpstra, 1988; Liess and Moennig,
246 1990), is becoming less of an issue due to increased quality control systems. This is,
247 however, still not a route of introduction to be fully neglected, as shown by an incident in the
248 Netherlands several years ago with a bovine herpes virus vaccine in cattle (Barkema et al.,
249 2001).

250 The presence of BVDV and BDV antibodies in pigs at the time of a CSFV infection may
251 affect the transmission of CSFV in and between infected herds (Wieringa-Jelsma et al.,
252 2006). Transmission of CSFV within a herd may be reduced, to a level that an introduction of
253 CSFV in a swine herd may result in a very small and self-limiting outbreak. Such an outbreak
254 may go unnoticed and doesn't necessarily pose a threat for any other herd. However, CSFV
255 infections will also result in less noticeable clinical symptoms, and in case transmission of
256 CSFV still occurs, such herds would be detected with a certain delay, in the mean time
257 posing a threat for other herds in direct or indirect contact.

258 More important is probably how ruminant pestiviruses may affect serological CSF
259 surveillance. Currently the serological diagnosis of CSF relies on a parallel interpretation of
260 up to three virus neutralisation tests (EU Commission Decision 2002/106/EG). A CSFV-
261 infection in an animal with antibodies against BVDV may however result in higher titres
262 against BVDV than against CSFV (Wieringa-Jelsma et al., 2006), thus giving a false
263 negative result for CSF. False positive results on the other hand will occur in situations

264 where the E^{ms}-test is being used, which is primarily after vaccination with a marker vaccine.
265 Serological confirmation of the CSF-result, and differentiation with ruminant pestiviruses is
266 so far not possible. The presence of antibodies against ruminant pestiviruses in a population
267 may therefore seriously hamper any eradication strategy that involves vaccination with
268 marker vaccines, especially during the phase where freedom of disease needs to be proven
269 again.

270 Based on this study, diagnostic procedures for the serological surveillance of CSF were
271 adapted in the Netherlands. BDV is no longer routinely used in the virus neutralisation test,
272 lowering the costs and increasing the efficiency of the diagnostic procedure. Furthermore,
273 emergency vaccination plans for CSF in the Netherlands were adapted. In the current plan
274 sows, who have a much higher seroprevalence than finishing pigs, will not be vaccinated
275 against CSF, amongst others to avoid diagnostic problems due to the presence of antibodies
276 against BVDV in breeding pigs. As such, these are good examples on how knowledge of
277 seroprevalence of ruminant pestiviruses affects the diagnostic procedure and may affect
278 intervention measures during a future CSF outbreak.

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409 **Figure caption**

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411 **Figure 1:** Highest titre against any of the 5 strains in the VNT, as a function against the
412 percentage inhibition in the NS3-ELISA. Negative VNT-results were given an artificial titre of
413 5 to be able to show them in the figure.

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Table 1: NS3-ELISA results of 6020 blood samples from sows, grouped in categories according to inhibition percentage and compared to results in an E^{ms}-ELISA and a VNT against 5 different pestivirus strains (samples are considered positive if they are positive in at least one of these VNT's).

NS3-ELISA		E ^{ms} -ELISA		VNT	
% Inhibition	n	Positive	%	Positive	%
≤50	5711	nd	nd	nd	nd
>50	309	126	40.8	152	49.2
50-60	57	1	1.8	4	7.0
60-70	69	2	2.9	6	9.5
70-80	41	5	12.2	10	24.4
80-90	16	4	25.0	8	50.0
>90	126	114	90.5	124	98.4

Table 2: Seroprevalence against BVDV and BDV in Dutch swine herds, on animal level and herd level (with 95% confidence interval).

	n	BVD	BD
Sows			
Herd level	616	11% (8.7-14)	0.49% (0.13-1.5)
Animal level	6020	2.5% (2.1-2.9)	0.050% (0.013-0.16)
Finishing pigs			
Herd level	189	3.2% (1.3-7.1)	0% (0-2.5)
Animal level	1890	0.42% (0.20-0.87)	0% (0-0.25)

Table 3: Univariate analysis of risk factors associated ($p < 0.20$) with a positive BVDV-status of sow herds (with 95% range or percentage within brackets).

Variable	Sows			Finishing pigs		
	BVDV+ n=68	BVDV- n=548	P-value	BVDV+ n=6	BVDV- n=183	P-value
Median number of total pigs on farm	223 (45-1724)	406 (8-8600)	0.0006	275 (80-500)	384 (12-4000)	0.14
≥ 1 bovine on same farm	39 (57%)	145 (26%)	<0.0001			ns
Median # of pig farms in 3 km radius	37 (0-194)	37 (0-168)	0.10			ns
Median distance to closest cattle farm	191 (38-703)	226 (27-1659)	0.03			ns
Median # of cattle farms in 3 km radius	87 (0-351)	73 (0-313)	0.002	115 (67-166)	81 (0-321)	0.11
Median # of bovines <1 year old in 3 km radius	847 (0-1625)	787 (0-2193)	0.13			ns
Median # of bovines 1-2 years old in 3 km radius	1827 (0-30554)	1340 (0-24870)	0.02	2069 (1253-2951)	1592 (0-2357)	0.10
Median # of bovines >2 year old in 3 km radius	2219 (0-3848)	2057 (0-5626)	0.17	3055 (1360-4249)	2374 (0-4661)	0.14
Median # of small ruminant farms in 3 km radius	60 (0-127)	46 (0-217)	0.001	73 (43-97)	49 (0-212)	0.15
Median # of goats in 3 km radius			ns	467 (76-4131)	110 (0-3898)	0.05
Median # of other goats in 3 km radius			ns	17 (4-26)	7 (0-828)	0.15
Median # of ewes in 3 km radius	538 (0-1661)	401 (0-9180)	0.03			ns
Median # of ewes with lam in 3 km radius	102 (0-549)	76 (0-3377)	0.07			ns
Median # of other ewes in 3 km radius	39 (0-2009)	31 (0-8078)	0.18			ns

ns = not significant

Table 4: Summary of odds ratios (sOR) and accessory 95% confidence intervals of sow herds at risk for infection with BVDV in the Netherlands.

Risk factor	Value	N	sOR	95% CI	P-value
Presence of cattle (1 or more animals) on premises	Yes	184	3.4	2.0 - 5.7	<0.0001
	No	432	1 [#]		
Number of goat and/or sheep herds within a radius of 3000 m of sow herd	≥80	79	2.6	1.3 - 5.1	0.006
	60-80	120	2.1	1.1 - 3.8	0.02
	<60	417	1 [#]		

: Reference category