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1 **Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)**
2 **infection in wild boars**

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12
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14

15 **Abstract**

16 The Porcine Reproductive and Respiratory Syndrome (PRRS) is one of the economically
17 most important swine diseases worldwide. The virus can be spread by viraemic and
18 persistent infected pigs. Spread and infection are advanced in areas with high herd and
19 population densities. As wild boars have been found seropositive for PRRSV in
20 Germany, France, and the USA, exchange between wild and domestic pig populations
21 may exist. However, comprehensive information on PRRSV infection in wild boars is
22 presently not available. The aim of the current study was thus, to systematically study
23 *PRRSV* infection in wild boars to provide information on spatiotemporal, host and viral
24 effects. The study was based on 531 wild boars from 52 hunts in Germany (2004 - 2007).
25 PRRSV-infection was determined and strains (US/EU) were classified by PCR. A total of
26 15.9% of the wild boars were PRRSV-positive (US: 14.2%; EU: 6.2%), with remarkable
27 effects of state (US: 5.1 - 46.2%; EU: 0 - 17.6%), season (0 - 36.5%) and tissue (lungs:
28 89%; tonsils: 11%). Prevalences did neither correlate with age or weight, nor with density
29 of production units, domestic pigs or wild boars. ORF 1-sequences within EU and US
30 strains did not differ among wild boar samples. Homologies between EU-
31 samples/Lelystad-Virus and US-samples/PRRSV-MLV virus were 99.3% and 97%,
32 respectively. This is the first comprehensive evidence of PRRSV infection in wild boars.
33 We conclude that there is only a weak relation between wild boar and domestic PRRSV
34 infection.

35

36 **Keywords:** Porcine Reproductive and Respiratory Syndrome, PRRS, Epidemiology,
37 Wild boars

38

39 **1. Introduction**

40

41 The Porcine Reproductive and Respiratory Syndrome (PRRS) is one of the economically
42 most important diseases of swine (Cho and Dee, 2006). Clinical outbreaks have been
43 reported since the late 1980's in the USA and were widespread throughout Europe by
44 1991. The *PRRS-Virus* (*PRRSV*) is an enveloped single-stranded positive-sense RNA
45 virus, classified in the family *Arteriviridae* of the order *Nidovirales* (Benfield et al.,
46 1992). The 15 kb *PRRSV* genome consists of 7 open reading frames (ORFs), with distinct
47 genetic diversity between (35-50%) and within (20%) the major prototypes: the European
48 isolate (Lelystad virus) and the North American isolate (VR-2332).

49 The virus can be found in and spread by all secretions and excretions of infected pigs
50 (Wills et al., 1997b; Senn et al., 1998). Spread can be realised by vectors or aerosols
51 (Terpstra et al., 1991), but it is best facilitated by direct contact of sensitive pigs with
52 viremic or persistent infected individuals (Albina et al., 1994; Wills et al., 1997a,
53 Torremorell et al., 1997). A further source of infection may be the spread of attenuated
54 live vaccine virus (Botner et al., 1997; Storgaard et al., 1999; Nielsen et al., 2002).

55 Rates of spread and infection are advanced in areas with high herd and population
56 densities (Geue, 1995; Mortensen et al., 2002). Aerosols and insects are able to bridge up
57 to 3 km (Komijn et al., 1991; Blaha and Bükér, 1995; Lager and Mengeling, 2000; Dee et
58 al., 2004). The virus may survive in porcine environment and slurry up to 11 and 7 days,
59 respectively (Pirtle and Beran, 1996; Dee et al., 2005).

60 Wild boars can act as a reservoir for infectious diseases of domestic pigs (Elbers et al.,
61 2000; Laddomada, 2000; Al Dahouk et al., 2005). Exchange and interaction between

62 infectious agents of wild boar and domestic pigs are best understood regarding *CSF* and
63 AD (Albina et al., 2000). The outlined ways of spread and infection between domestic
64 pig herds might also potentially affect wild boars, and vice versa, albeit data about
65 prevalence and distribution of *PRRSV* infection in wild boar populations are currently
66 unavailable. Merely antibodies against *PRRSV* have been found in German (Oslage et al.,
67 1994), French (Albina et al., 2000) and North-American wild boar populations (Saliki et
68 al., 1998).

69 The aim of the present study was to systematically study *PRRSV* infection in wild boars
70 to provide information on spatiotemporal, host and viral effects.

71

72 **2. Materials and methods**

73 *2.1 Wild boars*

74 Samples from lungs and tonsils of 531 wild boars were collected during 52 hunts from 46
75 hunting grounds situated in fourteen federal states of Germany (Table 1). Samples were
76 drawn from November to March of the hunting seasons 2004/2005 (n=186), 2005/2006
77 (n=175) and 2006/2007 (n=170).

78 Pigs were collected after the hunting at central places. They were numbered, rated
79 according to age (dentition) and weight, and samples were taken half an hour to 2 hours
80 after death. Tissues were stored at -20 °C until further usage.

81

82 *2.2 Isolation of RNA from tissue samples*

83 RNA was extracted separately from lung and tonsil samples with the QIAamp Viral RNA
84 Mini kit (Qiagen, Hilden, Germany). For each extraction, 25-50 mg of tissue sample were

85 added to 560 μ l of AVL lysis buffer. Tissue cells were disrupted in a ball mill with 5 mm
86 grinding balls at a frequency of 25 Hz for 2 min. The suspension was incubated for at
87 least 10 min at room temperature. After centrifugation (3 min., 16,000 \times g), the
88 supernatants were applied to spin columns. Further steps were performed as described by
89 the manufacturer. Resulting RNA was eluted in 60 μ l of elution buffer and stored at -
90 20°C.

91

92 *2.3 Multiplex nested PCR assay to detect PRRSV in tissue samples*

93 Three PCR assays using three different primer sets were conducted to detect PRRSV
94 specific sequences in tissue samples of wild boars. Primer set 1 was as described by
95 Gilbert et al. (1997) with minor modifications (Tab. 2) and primer set 2 was taken from
96 Pesch (2003) amplifying ORF7 sequences. For both PCR reactions One-Step RT-PCR
97 was performed with common primers amplifying both North American and European
98 PRRSV sequences. Subsequently a Nested Multiplex PCR was carried out to differentiate
99 between North American and European PRRSV isolates. Primer set 3 was from Goldberg
100 et al. (2000) amplifying ORF5 sequences of North American PRRSV isolates.

101 RT-PCR was performed in a 20 μ l PCR reaction consisting of 1x RT-PCR buffer, 1.0 μ M
102 primermix, 400 μ M of each dNTP, 2.0 mM MgCl₂, 5 mM dithiothreitol, 0.2 U/ μ l RNase
103 inhibitor, 0.05 U/ μ l AmpliTaq Gold DNA polymerase, 0.3 U/ μ l MuLV Reverse
104 Transcriptase (all enzymes from Applied Biosystems, Darmstadt, Germany) and 2 μ l of
105 template RNA. RNA was transcribed at 48°C for 30 min, followed by activation of
106 AmpliTaq Gold DNA polymerase at 95°C for 10 min. Amplification of cDNA was
107 performed in 20 (30, 35) cycles of denaturation at 94°C for 30 seconds, annealing at 55°C

108 (58°C, 61°C) for 30 (45, 30) seconds and extension at 72°C for 60 (45, 90) seconds,
109 followed by a final extension at 72°C for 7 min. One microlitre of RT-PCR product was
110 used as template for the nested PCR assay (nPCR). Multiplex nPCR was carried out in a
111 total volume of 10 µl, containing 5 µl of 2x Multiplex PCR Mastermix (Qiagen, Hilden,
112 Germany), 0.4 µM primermix specific for European and 0.6 (0.4) µM primermix specific
113 for American PRRSV isolates. Cycling conditions were as follows: activation of DNA
114 polymerase at 95°C for 15 min, 35 (30) cycles of denaturation at 94°C for 30 seconds,
115 annealing at 55°C (58°C) for 90 seconds and extension at 72°C for 1 min, followed by a
116 final extension at 72°C for 10 min. Cycling conditions for RT-PCR for primer set 2 and 3
117 and for Multiplex nPCR for primer set 2 are bracketed. PCR products were analyzed on
118 1.5% agarose gels stained with ethidium bromide.

119

120 *2.4 Verification of PCR products and phylogenetic analysis*

121 PCR products were verified by sequencing (Qiagen sequencing service, Hilden,
122 Germany) of 15 and 3 PRRSV-US and PRRSV-EU positive amplicons, respectively.
123 PRRSV-US and PRRSV-EU sequences included ORF 1 from nucleotide (nt) 9070 to nt
124 9119 and nt 8662 to nt 8809, respectively. PRRSV-US positive samples came from states
125 throughout Germany: Baden-Wurttemberg, Bavaria, Thuringia, Saxony, Northrhine-
126 Westphalia, Saxony Anhalt and Mecklenburg-West Pomerania. One sample of the
127 commercially available Ingelvac[®] PRRSV MLV vaccine was sequenced in the same
128 manner. Homology to sequences deposited in the Genbank database was established by
129 BLAST. Homologous sequences were extracted from the database and aligned with
130 ClustalW (1.81). Phylograms were constructed with Treeview (1.6.6).

131

132 *2.5 Domestic and wild boar reports*

133 Statistical reports on numbers of wild boars hunted, numbers of domestic pigs and herds,
134 and on state areas were available for each of the seasons at the Federal Statistical Agency,
135 Wiesbaden (<http://www-ec.destatis.de>). Statistics on domestic pig production have been
136 reported by Gatzka et al. (2007).

137

138 *2.6 Statistical analysis*

139 Statistical analysis was done with the "Statistical Package for Social Sciences" (SPSS,
140 version 15.0). Significances of differences in frequencies were tested with Chi²-test.

141 Estimated ages and weights were transformed into 9 and 16 groups, respectively, to
142 reduce estimation errors. Correlations and regressions between state specific numbers of
143 domestic and wild boars and the state specific *PRRSV* prevalences were calculated from a
144 data set of 14 cases (one for each state).

145

146

147 **3. Results**

148

149 Of 531 wild boars sampled from all over Germany, 15.9% were tested *PRRSV*-positive
150 only by Multiplex nPCR (14.2% positive with US-strain, 6.2% positive with EU-strain)
151 using primer set 1 but not by PCR amplifying ORF5 and ORF7 sequences, respectively.
152 4.5% of wild boars were tested positive with both *PRRSV*-strains. There were remarkable
153 differences in prevalence between states, spanning 5.1 to 46.2% for total *PRRSV* and the

154 US-strain, and 0 to 17.6% for the EU-strain virus. EU-strain virus was not detected in
155 wild boars of five states, while none of the states were free from US-strain virus during
156 the whole time span of the study. Wild boars of seven states were infected with just one
157 *PRRSV*-strain. In the other states, RNAs of both *PRRSV*-strains together were found in
158 2.6 to 14.7% of the wild boars.

159 *PRRSV* RNA was significantly more frequently isolated from lung tissue than from
160 tonsils. From pigs positive for the *PRRSV*-EU strain, 89% were positive with lungs and
161 11% with tonsils. None of the pigs were positive with both tissues. The respective
162 numbers for pigs positive for the US-strain of *PRRSV* were 97% (lungs) and 3%
163 (tonsils). All tonsils positive with *PRRSV*-US were also tested positive in lung tissues
164 (data not shown).

165 Figure 1 shows the spatiotemporal distribution of hunting grounds and the respective
166 prevalences of *PRRSV*-US and -EU positive pigs. The state effect was significant on
167 *PRRSV*-prevalence (Figure 2). Seven, three and four states had a prevalence of *PRRSV*
168 positive wild boars of 5 to 10%, 10 to 25%, and over 25%, respectively. Highest
169 prevalences (30 to over 45%) were found in Lower Saxony, Saxony, Brandenburg, and
170 Berlin.

171 Prevalences of *PRRSV* varied significantly with season. Average prevalences for the
172 seasons 2004/2005, 2005/2006 and 2006/2007 are shown in Fig. 3. During the season of
173 2005/2006, no *PRRSV*-RNA positive samples have been detected, but detection rates
174 have been high during 2006/2007, especially the US-strain. During this season, 36.5% of
175 wild boars were found positive with *PRRSV*-US RNA. RNA was not detected in just two

176 of 19 hunting grounds, involved. Prevalences of the other hunting grounds varied
177 between 14.3 and 100% (Fig. 1).

178 Prevalence of PRRSV did neither correlate with age, nor with weight of the wild boars.
179 There were no correlations between state-specific domestic and wild boar parameters
180 (e.g. density of domestic pigs and wild boars per state, density and size of production
181 units) and PRRSV prevalences.

182 Differences in prevalences between neighboured hunting grounds were as large as those
183 between far distanced grounds.

184 Amplicons of PRRSV (EU and US strain) of wild boar samples have been verified by
185 sequence analysis (Table 3). Fifteen PRRSV-US and 3 PRRSV-EU samples did not show
186 any variation within this part of ORF1 within each of the PRRSV-strains. The
187 phylogenetic association with already published RNA sequences of domestic pig PRRSV
188 strains shows Fig. 4. Homologies between the wild boar PRRSV-US strain(s) and the
189 MLV vaccine virus (PRU87392; Ingelvac[®] PRRSV) was 97%. The corresponding
190 homology between the wild boar PRRV-EU samples and the Lelystad-PRRSV (Genbank
191 M96262) was 99.3%. The sequences of PRU87392 did not differ from those of a
192 commercially available Ingelvac[®] PRRSV MLV vaccine sample.

193

194

195

196 **4. Discussion**

197

198 PRRS is a serious and worldwide-spread infectious disease in swine (Cho and Dee,
199 2006). Depending on the virulence of the virus, infectious pressure and co-infections,
200 hygiene, stress factors, breed and immunity, the clinical outcome of PRRS can vary
201 significantly (Van der Linden et al., 2003). Viremic and persistent infected pigs play a
202 decisive role in transmission of PRRSV. The virus can remain infective within lymphatic
203 tissues for more than 200 days (Benfield et al., 1997; Loving et al., 2006). PRRSV
204 remained infective in tonsillar tissue from infected pigs for more than 150 days (Wills et
205 al., 1997c). Viremia, persistence and shedding are badly correlated with antibody titres
206 and cellular immunity. Thus, they cannot be prevented by immunisation. Besides
207 transmission via direct contact between pigs, distribution of the agent via aerosols is the
208 second route of infection within and between breeds. Although prevalences in domestic
209 pig herds are significantly higher in regions with high swine density, the aerogen route
210 seems to play a minor role in the spread of the disease.

211 Furthermore, wild boars might play an important role in the spread and as a reservoir of
212 PRRSV. The significance of this problem has been shown with respect to CSF (Albina et
213 al., 2000), but data on the prevalence of PRRSV infection in wild boars are not available
214 from the literature.

215 Antibodies against PRRSV have been reported for wild boars in Germany (Oslage et al.,
216 1994), France (Albina et al., 2000) and the USA (Saliki et al., 1998), with
217 seroprevalences of 0.4, 1.3 and 1.7%, respectively. No sero-positive wild boars have been
218 detected by other studies in Germany (Lutz and Wurm, 1996), Kansas/USA (Gipson et

219 al., 1999), Poland (Zupancic et al., 2002), Spain (Vicente et al., 2002; Ruiz-Fons et al.,
220 2006), and Slovenia (Vengust et al., 2006). One case of a PRRSV-EU positive wild boar,
221 killed by a traffic accident, was reported from Italy in 2005 (Bonilauri et al., 2006). There
222 are no reports on PRRS in wild boar (Albina et al., 1997).

223 The present study demonstrates for the first time the distribution of PRRSV-RNA among
224 wild boar populations. Rates of PRRSV infected wild boars vary drastically with
225 location, season, and PRRSV-strain. The effect of season that exists within the whole
226 population and within smaller regions and even within the same hunting area, might serve
227 as an indicator for epidemiological instability within the wild boar populations.

228 Exchange of the virus between domestic pigs and wild boars can be expected because of
229 high prevalences of PRRSV in domestic pigs in Germany. These prevalences are not yet
230 available from literature, but can be estimated to 80% (Große Beilage, pers. comm.).
231 Approximately 50% of domestic sows and 15% of piglets have been vaccinated against
232 PRRSV during the time interval of the study (Schagemann, pers. comm.). The effect of
233 the federal state discriminates the northern and eastern parts of Germany from the middle
234 and southern parts (Fig. 1, Fig. 2). The reason for this difference remains unclear, as no
235 correlations with production parameters or wild boar population data have been found by
236 the present study. Astonishingly, numbers of PRRSV-infected wild boars were not
237 correlated with the numbers of wild boars per state, a value that can be roughly assumed
238 by the numbers of hunted pigs per year (Federal Statistical Office, 2006). We can thus
239 underline the conclusion of Oslage et al. (1994) whereby regional epidemiological factors
240 like PRRS in domestic pigs, density of wild boar populations, proximity of production
241 units and the application of live vaccines do not seem to increase the risk of infection and

242 spread in wild boar populations. Serological tests done between 1992 and 1996 were all
243 negative (Lutz and Wurm, 1996) confirming that, instead of the growing relevance of this
244 virus in domestic pig production, wild boars are marginally involved. The present results
245 show that prevalences of closely neighboured populations do not have to be more related
246 than those of more distant populations. Taken together, these aspects point towards the
247 concept that PRRSV-infection in wild boars in Germany is a more sporadic instead of a
248 systematic event.

249 Prevalences of PRRSV-US-strain were significantly higher than those of the EU-strain.
250 This was the least expected result of the present study. As vaccination of domestic pigs
251 with an attenuated US-strain PRRSV vaccine is a common source of PRRSV-US positive
252 PCR in Europe (Botner et al., 1997; Storgaard et al., 1999; Nielsen et al., 2002), and as
253 wild boars are not vaccinated against PRRSV, it might be speculated that the vaccine
254 virus has spread from domestic pig to wild boar populations, as an explanation of the
255 positive PCR results of the present study. If this were the case, we would expect sequence
256 homology between the vaccine virus and the wild boar strain(s) or, if not, sequence
257 variation from the vaccine sequence to the least homologue wild boar sequence. But the
258 finding of a two-base difference between wild boar samples and the vaccine virus within
259 the relatively high conserved ORF1, together with the discovery that all PRRSV-US wild
260 boar samples, although collected from isolated populations from all over Germany, were
261 unique within this sequence, argues against this hypothesis. A possible explanation might
262 be the hypothesis of Plagemann (2003), who postulates that a mutant of a closely related
263 *Arterivirus* of mice (lactate dehydrogenase-elevating virus) might have infected wild
264 boars in central Europe, and that the virus came to the USA by imported, infected wild

265 boars in 1912. If this hypothesis applies, a PRRSV strain like that of the present study,
266 with homology to the US strain, but not identical to the MLV virus, should be found
267 among wild boars in Europe. This critical relation might have not been detected with
268 other, higher variable ORFs.

269 Although ORF1 sequences doubtlessly document the existence of PRRSV within the
270 samples, we failed in amplifying ORF5 as well as ORF7 sequences from any of the wild
271 boars with three PCR-systems that are applied in daily routine diagnostic on domestic pig
272 samples in our lab and in others. A possible explanation for this finding might be a very
273 low number of RNA copies within the feral pig samples that could only be detected by
274 the ORF1 nPCR.

275 Based on prevalences and distribution of both PRRSV strains in wild boar samples, we
276 conclude that only weak relations between wild boar and domestic PRRSV do exist and
277 that further research is needed to explain the existence of PRRSV-US strains in wild-boar
278 populations in Germany.

279

280

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282

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286

287 **7. References**

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- 408
- 409

410 Table 1: Distribution of hunting grounds and wild boars with states.

State	hunting grounds	wild boars	wild boars during seasons		
			2004/2005	2005/2006	2006/2007
Baden-Wurtemberg	5	42	22	8	12
Bavaria	4	43	15	15	13
Saarland	3	47	19	11	17
Rhineland Palatinate	4	46	19	11	16
Hesse	5	40	17	13	10
Thuringia	4	39	6	20	13
Saxony	3	35	10	11	14
North Rhine Westphalia	6	40	15	15	10
Saxony-Anhalt	3	38	8	21	9
Lower Saxony	3	42	18	12	12
Brandenburg	3	34	15	10	9
Berlin	2	13	4	0	9
Meckl. West Pomerania	4	40	10	17	13
Schleswig-Holstein	3	32	8	11	13
Total	52	531	186	175	170
Mean	3.7	37.9	13.3	12.5	12.1
SD	1.07	8.32	5.57	5.21	2.54

411

412

413

414 Table 2: Primers used for the amplification of *PRRSV*-specific sequences

415	Primer name	Primer sequence (5' to 3')	Amplicon size [bp]	genotype
416	PRRSV1*	CAA CCT CCT GTA TGA ACT TGC	258	common
417	PRRSV2*	AGG TCC TCG AAC TTG AGC TG		
418				
419	PRRSV3*	CTG TAT GAA CTT GCA GGA TG	186	European
420	PRRSV4*	CGA CAA TAC CAT GTG CTG		
421				
422	PRRSV_NA1*	GGC GCA GTG ACT AAG AGA	108	North American
423	PRRSV_NA2*	GTA ACT GAA CAC CAT ATG CTG		
424				
425	PLR**	TCG CCC TAA TTG AAT AGG TG	432	common
426	PLS**	ATG GCC AGC CAG TCA ATC A		
427				
428	US-7-s**	AGT CCA GAG GCA AGG GAC CG	336	North American
429	US-7-as**	TCA ATC AGT GCC ATT CAC CAC		
430				
431	EU-7-s**	ATG ATA AAG TCC CAG CGC CAG	219	European
432	EU-7-as**	CTG TAT GAG CAA CCG GCA GCA T		
433				
434	3F***	GAG ACC ATG AGG TGG GCA AC	720	North American
435	5R***	CGC CAA AAG CAC CTT TTG T		

437 * from Gilbert et al. with minor modifications (1997), three and two additional bases were added at the 5'
438 end to primer PRRSV1 and PRRSV3, respectively; two bases were removed from primer PRRSV4 at the 5'
439 end

440 ** from Pesch (2003)

441 *** from Goldberg et al. (2000)

442

443

444 Table 3: Sequence alignment of wild boar derived (W) PRRSV (US-strain; ORF1) and
445 Ingelvac[®] PRRSV MLV sequences (V).

446

447 (W) ACACCATTTATAGTTTGGTGATCTATGCACAGCATATGGTGTTTCAGTTAC

448 (V) ACACCATTTATAGTTTGGTGATCTATGCACAGCATATGGTGCTTAGTTAC

449 ***** * *****

450 nt 9070

9119

451

452

453

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454 **Figure captions:**

455

456 **Fig. 1:** Spatiotemporal distribution of *PRRSV* (US- and EU-strain) infected pigs in
457 Germany. Numbers show prevalence by hunting grounds. Circles: season
458 2004/2005; triangles: season 2005/2006; quadrates: season 2006/2007. Bold
459 letters: B, Berlin; BA, Bavaria; BB, Brandenburg; BW, Baden-Wurtemberg; HE,
460 Hesse; MVP, Mecklenburg West Pomerania; NRW, North-Rhine Westphalia; NS,
461 Lower Saxony; RP, Rhineland-Palatinate; SA, Saxony; SAH, Saxony Anhalt; SH,
462 Schleswig-Holstein; SL, Saarland; TH, Thuringia.

463

464 **Fig. 2:** Percented shares of wild boars by states infected with *PRRSV* (total, US-, and
465 EU-strain).

466

467 **Fig. 3:** *PRRSV*-strain combinations in wild boars by seasons.

468

469 **Fig. 4:** Phylogenetic tree based on RNA sequence data of ORF1 (US: nt 9070 to nt 9119;
470 EU: nt 8662 to nt 8809) of 15 wild boar *PRRSV*-US and 3 wild boar *PRRSV*-EU
471 samples from German hunting grounds and on already published RNA sequences,
472 exclusively from domestic pig strains.

473

474

Figure 1

PRRSV-US

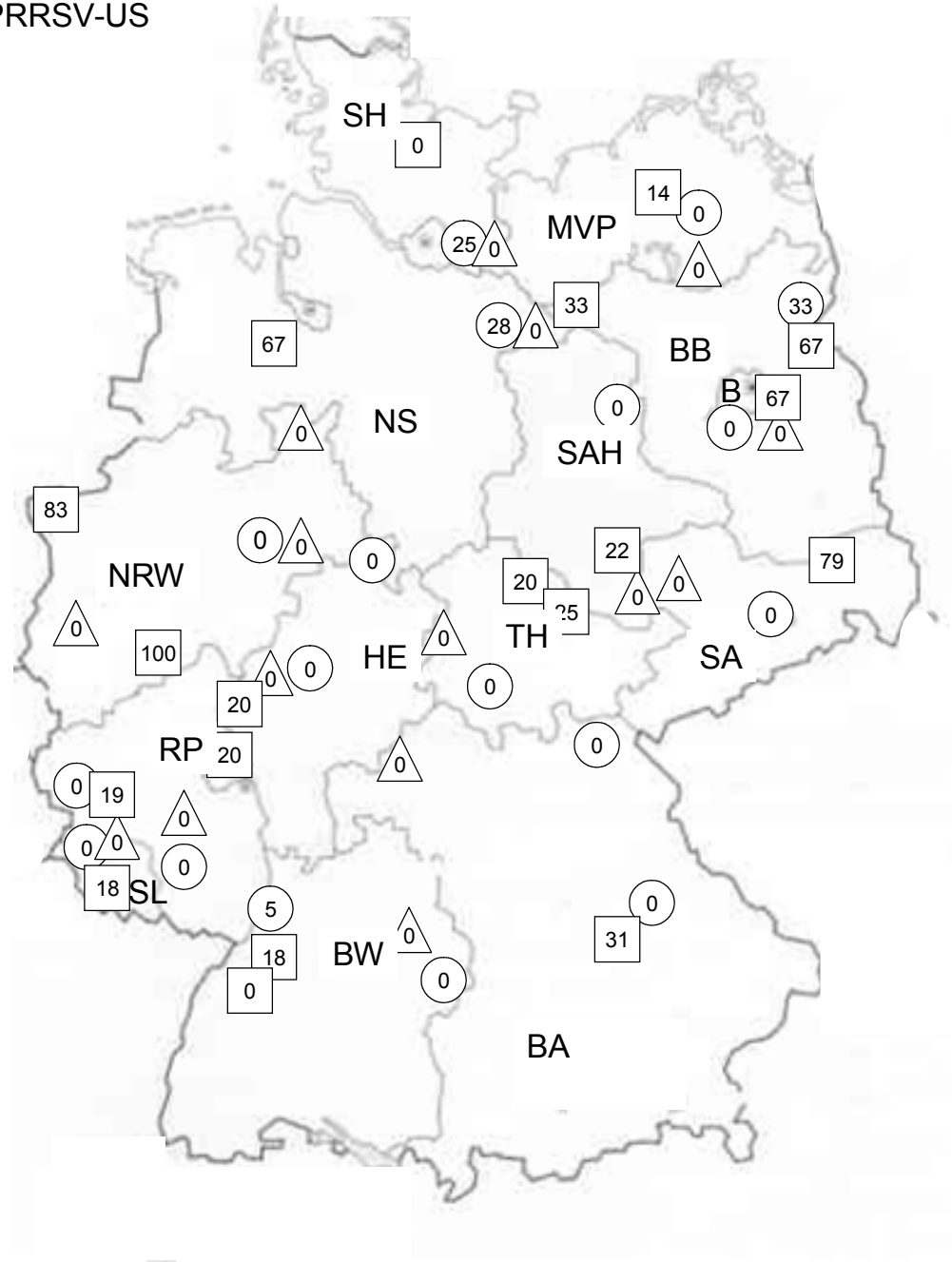


Figure 1 continued

PRRSV-EU

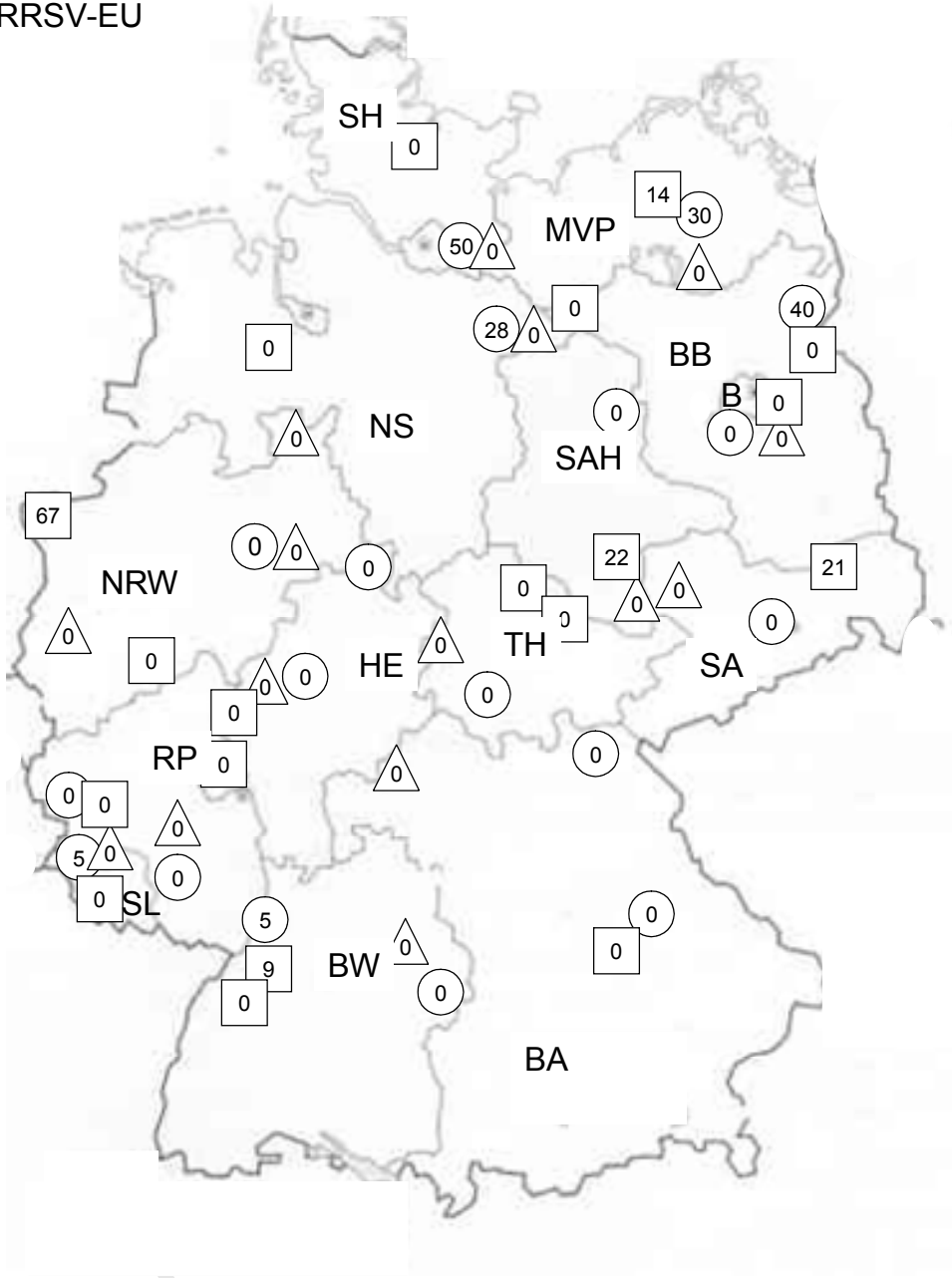


Figure 2

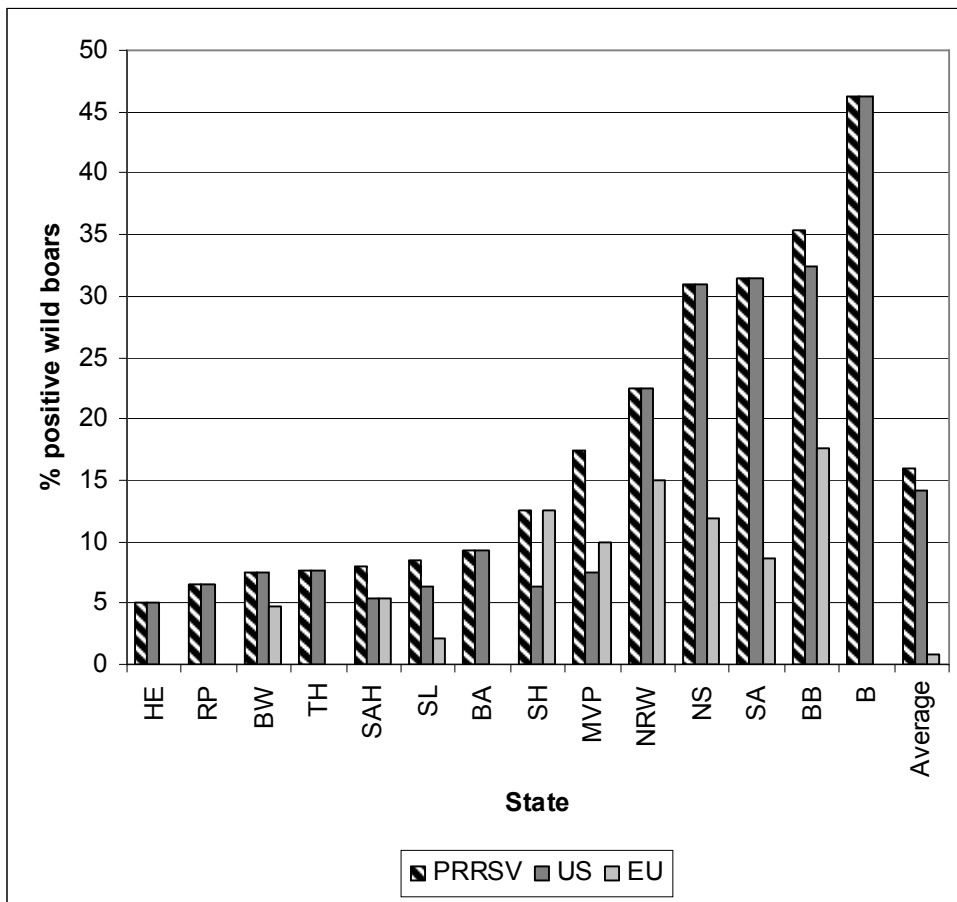


Figure 3

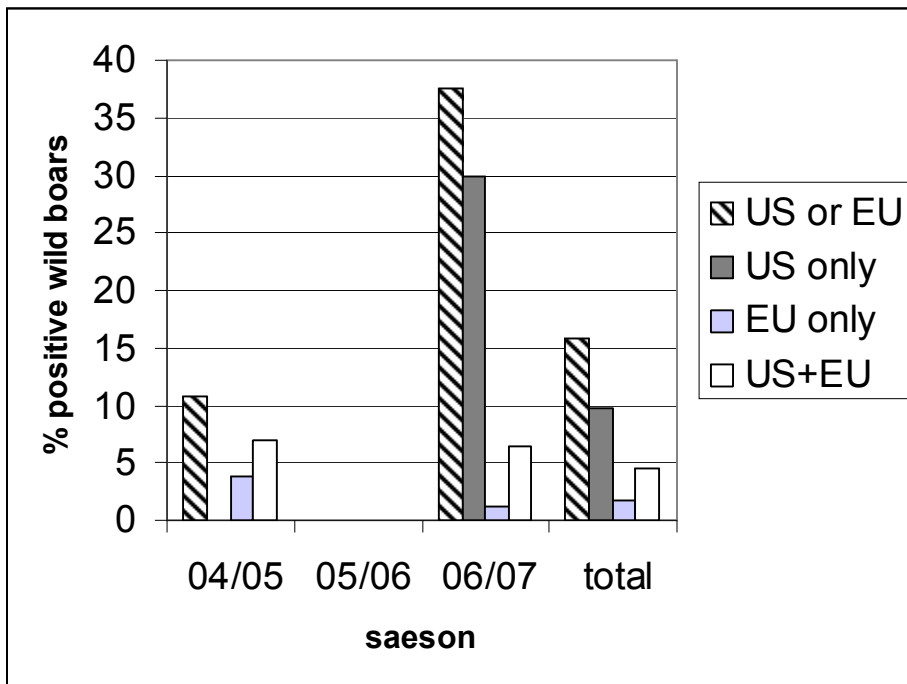


Figure 4

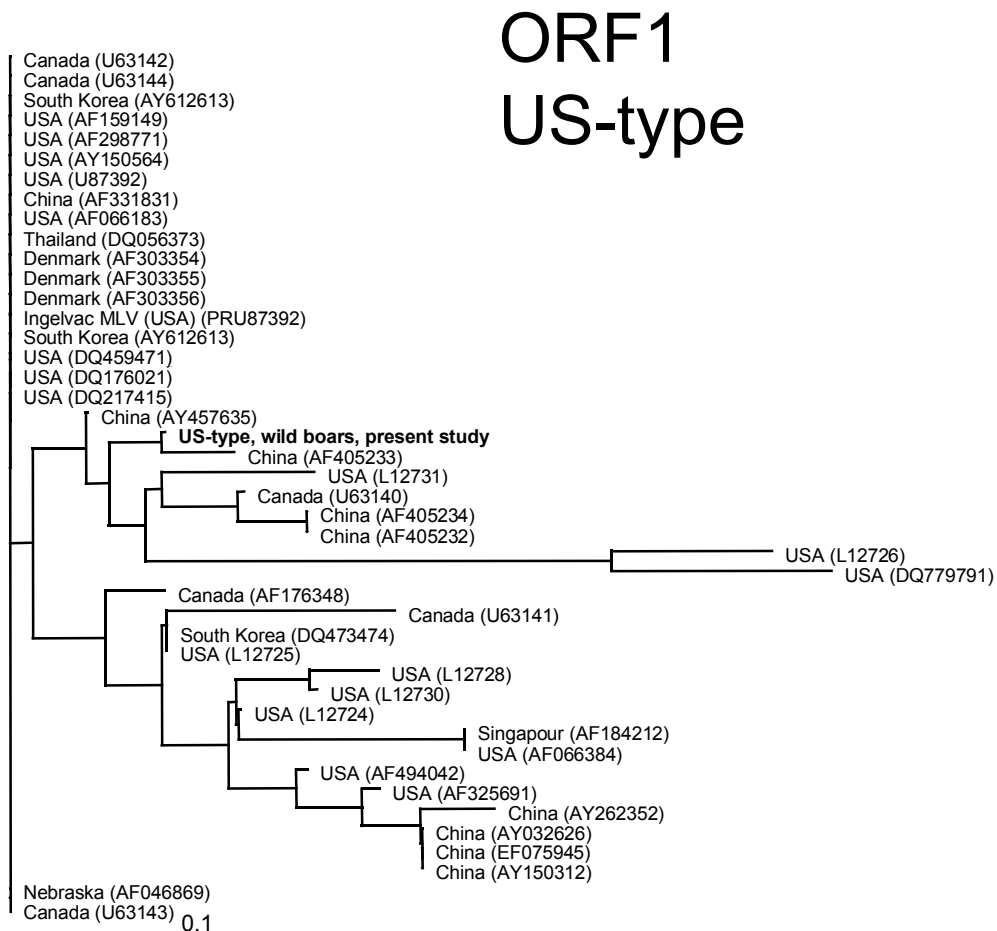
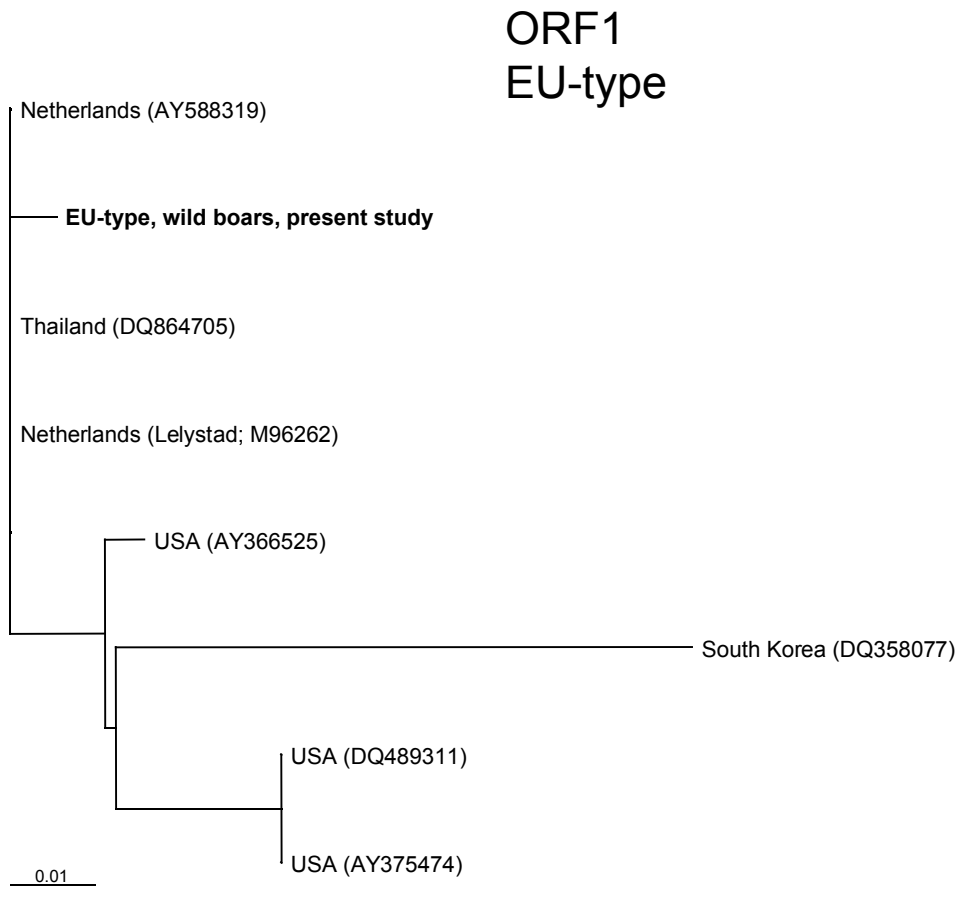


Figure 4 continued



Accet