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Genetic diversity of *Porcine reproductive and respiratory syndrome virus* (PRRSV) in selected herds in a pig dense region of North-Western Germany

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34 **Abstract**

35 The *Porcine reproductive and respiratory syndrome virus* (PRRSV) can persist
36 for several weeks in infected pigs, and readily spreads from viraemic pigs to other
37 pigs as well as to other herds. In regions with dense pig populations such as the
38 North-Western part of Germany the virus is endemic. The aim of this study was to
39 analyse the genetic diversity of PRRSV found in 18 herds in this region, which
40 had pigs with respiratory or reproductive failures, and where PRRSV had been
41 demonstrated by RT-PCR. Phylogenetic trees were calculated using the complete
42 nucleotide sequences of the ORF5. Of the 65 samples sequenced, five PRRSV
43 from four herds were of the North American (NA; Type 2) genotype, and 60 of
44 the European (EU; Type 1) genotype. To ascertain if the field PRRSV varied with
45 time and to monitor the health condition of the herds they were revisited two years
46 later. Although only two herds still reported clinical signs, PRRSV was found by
47 RT-PCR in 10 of the 18 herds. Phylogenetic analysis showed that of the 23
48 PRRSV sequenced, 15 were of the European (EU) genotype. The EU genotype
49 isolates from both samplings could be assigned to one of 12 clusters. There was
50 no indication for the existence of herd specific clusters. ORF5 sequence identities
51 between PRRSV from one herd in one cluster were either 100%, or had single
52 base exchanges. These data indicate that the mutation rates for the European field
53 isolates are similar to that found for the NA genotype vaccine strain used in
54 Germany.

55

56 1. Introduction

57 Porcine reproductive and respiratory syndrome (PRRS) is an economically
 58 important viral disease caused by the PRRS virus (PRRSV), a member of the
 59 genus *Arterivirus* within the order *Nidovirales* (Cavanagh, 1997). The disease
 60 was first reported in the United States in 1987 (Keffaber, 1989), and now occurs
 61 almost world wide. The virus was first isolated 1991 in the Netherlands (strain
 62 Lelystad; LV) (Wensvoort et al., 1991), and then in the USA (strain VR-2332)
 63 (Collins et al., 1992). Genetic typing using the nucleotide sequences of the ORF5,
 64 which codes for the main surface glycoprotein of the virus, allows to distinguish
 65 between the European (EU, Type 1) and the North American (NA, Type 2)
 66 genotype. Within the EU genotype, three new Eastern European subtypes have
 67 been found (Stadejek et al., 2006; Stadejek et al., 2008). Both NA (Goldberg et
 68 al., 2000; Kapur et al., 1996; Key et al., 2001) and EU (Forsberg et al., 2002;
 69 Indik et al., 2000; Oleksiewicz et al., 2000) genotype virus isolates are
 70 antigenically and genetically highly diverse. This heterogeneity will likely pose a
 71 major obstacle for effective prevention and control of PRRS (Meng, 2000). It will
 72 definitely perturb diagnosis, as primers for RT-PCR used for routine diagnosis
 73 might not bind any more if the target fragment is mutated.

74 The clinical signs observed in PRRSV infected animals are reproductive disorders
 75 in breeding pigs and respiratory malfunction primarily apparent in growing and
 76 fattening pigs (Prieto and Castro, 2005; Zimmerman et al., 2006). The severity of
 77 PRRSV infections can vary widely, ranging from an almost complete lack of
 78 clinical signs to a severe outbreak of disease (Benfield et al., 1999; Mengeling et

79 al., 1996). The variation may be due to the properties of the PRRSV isolates,
80 which can be apathogenic to highly pathogenic (Osorio et al., 1998; Zuckermann
81 et al., 2007) and is also influenced by the age of the pigs and co-infections with
82 other pathogens (Cho et al., 2006; Thanawongnuwech et al., 2000; Van Reeth et
83 al., 1996).

84 The control of the disease is complicated by several problems. One of them is the
85 fact that the virus induces a prolonged viremia, and virus or viral RNA can be
86 detected for several months Batista (Batista et al., 2002). In spite of inducing
87 neutralising antibodies it can persist for an extended period in a single pig. The
88 period the pigs remain potentially infectious is still not conclusively determined.

89 Transmission of PRRSV to susceptible contact pigs has been detected for times
90 ranging between 56 and 99 days after experimental infection Bierk (Bierk et al.,
91 2001; Terpstra et al., 1992). Due to the long persistence of the virus in the pigs,
92 herds usually remain endemically infected Allende (Allende et al., 2000b; Batista
93 et al., 2002; Prieto and Castro, 2005; Wills et al., 2003). In addition, transmission
94 of the virus from pig to pig and from herd to herd is very effective. This seems to
95 proceed mainly by direct contact. Despite extensive replication of PRRSV in the
96 respiratory tract, it is not readily disseminated by aerosols (Cho and Dee, 2006;
97 Cho et al., 2007; Desrosiers, 2005; Fano et al., 2005).

98 Fundamental to the control of PRRS are adequate management practices, which
99 should aim to minimize virus circulation in the herd, for instance by not
100 introducing naïve gilts and by keeping a constant level of immunity. Control is
101 also attempted by vaccination. Although field PRRSV can not readily be

102 eliminated by vaccination with the currently available modified live vaccines
 103 (MLV), viral shedding can be reduced. In addition, vaccination seems to reduce
 104 clinical signs, but does not prevent infection. Vaccination is applied to prevent
 105 economic losses (Cano et al., 2007; Mengeling et al., 2003). The use of attenuated
 106 vaccine strains (Dewey et al., 2004) is complicated by their potential to revert to
 107 virulence by as yet not clearly understood mutations throughout the genome
 108 (Allende et al., 2000a; Grebennikova et al., 2004; Kwon et al., 2008; Li et al.,
 109 2007; Nielsen et al., 2001; Storgaard et al., 1999).
 110 In Germany, two MLV and an inactivated vaccine are in use. The latter one is
 111 available under two different brands and consists of an inactivated EU genotype
 112 virus strain (Ingelvac[®]PRRS KV and Merial Progressis[®]). The Ingelvac[®] PRRS
 113 modified live vaccine (MLV; Boehringer Ingelheim Vetmedica, Germany)
 114 contains an attenuated derivative of the NA genotype strain VR-2332 (Mengeling
 115 et al., 1999) . It was introduced in Germany in April 1996. The second MLV
 116 vaccine, Porcilis[®] PRRS (Intervet, The Netherlands) is based on the avirulent EU
 117 genotype strain DV belonging to the LV cluster (Intervet, personal
 118 communication), and is in use in Germany since February 2001.
 119 The aim of this study was to analyse diversity and clustering of the PRRSV field
 120 isolates circulating in 18 herds with a history of reproductive and/or respiratory
 121 disorders. The herds are located in the North Western part of Germany, which is
 122 one of the regions with the highest pig densities in Europe. After two years, the
 123 herds were resampled and tested again for the presence of PRRSV by RT-PCR.
 124 Herd specific information was gathered, focussing on vaccine use. To obtain an

insight into the molecular epidemiology, the ORF5 amplicons were sequenced and used for genotyping.

2. Materials and Methods

2.1. Samples. The samples originated from 18 herds (Table 1) that had a history of respiratory symptoms and/or reproductive disorders. The samples had been found PRRSV positive by RT-PCR in a diagnostic laboratory between December 2004 and March 2005. They consisted of lung tissue from aborted fetuses or from pigs with respiratory disease submitted for diagnostics. The herds were located in the pig-dense regions of the North-Western German states of Lower Saxony and North Rhine-Westphalia (Figure 1). The samples were from nine farrow-to-finish herds (1-site production), eight herds keeping the sow herd and nursery pigs at the same location (2-site production) and from one sow herd with a 3-site production system. The herd sizes ranged between 80 and 3000 sows (average 390). A second sampling of the 18 herds was performed between February 2006 and March 2007. Ten serum samples each from 4, 7 and 9 weeks old nursery pigs were obtained for analysis. Nomenclature of the isolates was as follows: Herd number, first or second sampling and lower case letters for each isolate; e.g. H-01-1a: herd No. 01, first sampling; isolate a.

2.2. Acquisition of herd specific data. A questionnaire was prepared to be completed by the pig producers and the attending veterinarians. Information concerning clinical signs, duration of clinical signs, the type of production system (1-, 2-, 3-site production), herd size, and the vaccination policy was surveyed.

148 **2.3. RNA isolation and cDNA synthesis.** The total RNA from lung tissue was
 149 isolated using the RNeasy Lipid Tissue Kit (Qiagen, Germany). RNA from serum
 150 was isolated with the High Pure Viral RNA Kit (Roche, Germany). For reverse
 151 transcription, 8 µl of RNA were added to 18 µl of a mixture containing 1 x RT
 152 buffer and 2.5 mM of each dNTP (Roche, Mannheim, Germany), and heated to 80
 153 °C for 5 min. Immediately thereafter the mixture was transferred to an ice bath.
 154 Then 25 µl of a master mix consisting of 0.1 mM DTT, 100 µM random hexamers
 155 (Roche, Germany), 2.5 mM MgCl₂, and 10,000 U of Superscript III Reverse
 156 Transcriptase (Invitrogen, USA) were added. The samples were incubated for 5
 157 min at 25 °C, 60 min at 55 °C, and 5 min at 99 °C. After cooling to 4 °C, they
 158 were either used directly for PCR or stored at -20 °C.

159 **2.4. RT-PCR: primers and assay conditions.** The primers used for
 160 amplification of the ORF5 and ORF7 are listed in Table 2. The PCR reaction was
 161 performed in a volume of 50 µl. The PCR mix consisted of 1 x PCR reaction
 162 buffer, 200 µM of each dNTP (Roche, Germany), 2.5 mM MgCl₂, 10 pmol of each
 163 primer and 1 IU of Taq polymerase (Invitrogen, USA), and 4 µl of cDNA.
 164 Amplification was performed for 39 cycles: denaturation at 94 °C for 45 sec,
 165 annealing at the temperatures indicated in Table 2 for 45 sec, and elongation at
 166 72 °C for 1 min. After this, the samples were incubated for additional 10 min at
 167 72 °C and then cooled to 4 °C until further processing.

168 **2.5. Sequencing.** After verifying the success of RT-PCR by electrophoresis in
 169 1.5 % agarose gels followed by ethidium bromide staining, the amplicons were
 170 purified using the QIAquick PCR Purification Kit (Qiagen, Hilden) according to

the manufacturer's instructions. Purified products were cycle-sequenced in both directions by MWG (Ebersberg, Germany) using the corresponding PCR primers (Table 2).

2.6. Genetic typing. Nucleotide sequences were edited and analysed using BioEdit 7.0.9 (Hall, 1999), and aligned in ClustalX 2.08 (Larkin et al., 2007). Phylogenetic trees were calculated with the Neighbor-joining method as implemented in ClustalX or in MEGA 4.0 (Tamura et al., 2007). The sequences of the EU-genotype strains Lelystad (M96262) and Porcilis[®]PRRS (DQ324710), and of the NA-genotype strains VR-2332 (AY150564) and Ingelvac[®]PRRS MLV (AF535152) were included in the phylogenetic analyses. Sequences from the Eastern European subgroups EU-2, EU-3 and EU-4 (Stadejek et al., 2006) were downloaded from GenBank (DQ324677; DQ324686; DQ324696; DQ324694; DQ324671; DQ324682; DQ324667). Up to date, PRRSV of these subgroups were found in Belarus and Lithuania.

3. Results

3.1. Herd specific data: structure, clinical signs and vaccination status

Evaluation of the questionnaire showed that of the 18 herds, nine had an anamnesis for reproductive disorders, and from the nine remaining herds respiratory symptoms were reported. The first samples were submitted between December 2004 and March 2005. About two years later (February 2006 to March 2007), the evaluation of the second questionnaire revealed that 16 herds no longer

193 had clinical signs. Only two herds continued to have ongoing problems, one with
194 respiratory diseases, and one with reproductive failures (Table 1).

195 In many of the herds, vaccination practice differed between the first and the
196 second sampling. At the beginning of the study, both sows and piglets in five
197 herds were vaccinated with the attenuated NA-genotype vaccine (Ingelvac®
198 PRRS MLV). In five more herds only the sows were vaccinated with this vaccine,
199 while the piglets were not vaccinated. The sows in two herds were vaccinated
200 using an inactivated vaccine while the piglets remained unvaccinated. Sows and
201 piglets in three herds were vaccinated using the EU-genotype vaccine (Porcilis®
202 PRRS). In two more herds, only the sows were vaccinated using this vaccine,
203 while the piglets were not vaccinated. Sows and piglets in the remaining one herd
204 were not vaccinated.

205 Two years later, in several cases the owners had switched the vaccine from the
206 Ingelvac® PRRS MLV to the Porcilis® PRRS vaccine. In eight herds the
207 vaccination of sows and piglets was done using the Porcilis® PRRS vaccine, and
208 in one herd using the Ingelvac® PRRS MLV. In five herds vaccination of sows
209 was performed with the Porcilis® PRRS MLV and in one with the
210 Ingelvac® PRRS MLV, while the piglets were not vaccinated. In one herd,
211 vaccination of sows only using the inactivated vaccine was continued (Table 1)
212 and two herds were unvaccinated.

213

214

215 **3.2. Amplification and genotyping using the PRRSV ORF5 nucleotide** 216 **sequences**

217 All samples obtained from the diagnostic laboratory (first period) and the sera
 218 obtained during the second period were first tested for the presence of PRRSV by
 219 amplifying the ORF7, using the primer pair 307/308 (Oleksiewicz et al., 1998).
 220 This primer pair amplifies a fragment comprising flanking sequences of the ORF6
 221 and the 3' NTR, generating amplicons with 659 bp from NA genotype, and 636
 222 bp from EU genotype PRRSV (Table 2). Due to the amplicon size, these primers
 223 allow the differentiation between the NA- and the EU-genotypes in agarose gels.
 224 Sensitivity of this RT-PCR and of the reactions amplifying the ORF5 was
 225 significantly increased by performing the cDNA synthesis using random primers
 226 and Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, USA), instead of
 227 using specific primers as in the original protocol (Oleksiewicz et al., 1998). From
 228 all samples the ORF5 was successfully amplified using at least one of the primer
 229 pairs listed in Table 2. The amplicons were purified and cycle sequenced. The
 230 sequences were aligned and the Neighbor-joining trees were calculated. To
 231 examine clustering of the German isolates from both sampling periods, the
 232 phylogram was calculated using the complete ORF5 sequences (Figure 2). As
 233 expected, the NA- and the EU-genotype isolates were clearly separated, and the
 234 German field isolates generated several clusters within the EU genotype. In
 235 addition to the EU-LV cluster, 11 clusters were found, and assigned letters from
 236 a-k. Many of the bootstrapping values were low, but ranging from 40 to 100%.
 237 The relatively low values are due to the fact that although the number and length

238 of the sequences included in the calculations are large enough, sequence
 239 divergence in the individual clusters is small. In these cases, low bootstrapping
 240 values are not significant (Nei and Kumar, 2009). To find out if any of the isolates
 241 belonged to one of the Eastern European subtypes, the sequences were trimmed to
 242 the corresponding fragment of the published ORF5 sequences of the EU-2, EU-3
 243 and EU-4 subtype isolates, with 553 nucleotides (Stadejek et al., 2006). Here, a
 244 radial tree without the names of the single isolates was chosen instead of a
 245 phylogram, because visualization is clearer and there is no need for outgrouping.
 246 Besides showing clear clustering of the German isolates, it was found that none of
 247 them belonged to the Eastern European subtypes (Figure 2, insert). Confirming
 248 previous results (Dee et al., 2001; Larochelle et al., 2003; Mateu et al., 2003), in
 249 five herds both NA- and EU genotype PRRSV were detected (Table 3).

250

251 **3.3. NA genotype isolates**

252 Samples available from the first period showed that NA-genotype virus was
 253 detectable in four of the 18 herds. While animals in two of these herds had been
 254 vaccinated with the corresponding vaccine, this vaccine had not been used in the
 255 remaining two herds. Prior use of this vaccine in these herds is unknown. After
 256 about two years, this virus was found in samples from three herds, although they
 257 all had stopped the use of the NA-genotype vaccine or had not used it at all
 258 (Table 1).

259 The phylogram (Figure 2) indicates that among the NA genotype isolates, one
 260 isolate obtained in the second sampling period from one herd (H-16-2c) clustered

separately from the Ingelvac®PRRS MLV /VR2332 isolates. The nucleotide identity of the ORF5 from the Ingelvac®PRRS MLV vaccine virus with this isolate was 96.6%. Alignment of the ORF7 sequences showed that nucleotide identities of the vaccine strain and the H-16-2c isolate was of 98.1% (not shown). The deduced amino acid identities were 95.5% (ORF5) and 96.7% (ORF7), respectively. In contrast, the amino acid identities of the remaining NA-genotype isolates - including the other isolates from herd 16 - were $\geq 98\%$ (not shown), thus indicating that they most probably all originated from the Ingelvac®PRRS MLV.

3.4. EU genotype isolates

The phylogram shows that the EU genotype virus isolates from the herds were genetically diverse (Figure 2). They were tentatively assigned to 9 clusters and three branches with single isolates (EU-1a through EU-1k, plus the LV cluster). Nucleotide identities of the ORF5 sequences within individual clusters were between 94.8 and 100%, and between clusters between 83.3 and 95.8% (Table 4). Porcilis® PRRS-like virus (LV cluster) was detected in samples from one herd from each sampling period, respectively (Table 3). On both occasions, the corresponding vaccines had been used only few weeks before, thus implying that the virus originated from the vaccine. The isolates had single nucleotide substitutions, but nucleotide sequence identities with the Porcilis® PRRS vaccine virus strain was $\geq 98\%$ (Table 4). The field isolates had between 86.3 and 93.2% nucleotide identity with the Porcilis® PRRS vaccine strain. Identities of the field

isolates with the Ingelvac® PRRS vaccine strain were between 59 and 62% (Table 4).

3.5. Molecular epidemiology

First sampling: different numbers of samples from individual pigs in each of the 18 herds were available. To assess heterogeneity of the isolates within one herd, the ORF5 from the cDNA of all positive samples that were available was sequenced. For example from herd H-03, the 14 isolates sequenced belonged to a single cluster, namely EU-1a (Figure 2). Several isolates had identical sequences, others had single nucleotide exchanges, some of which led to amino acid substitutions (Figure 3). One of these isolates had a N46K mutation. This glycosylation site was shown to be important for virion production and infectivity (Wissink et al., 2004). Interestingly, all isolates in cluster EU-1a had an additional potential glycosylation site at position 37 (Mateu et al., 2003; Stadejek et al., 2002). This glycosylation site may compensate functionality of the one at position 46. The isolates from pigs in herds H-01 and H-06 gave similar results. Remarkably, it seems that most clusters are not herd specific. Besides the isolates from herd H-03-1, isolates H-10-1e, H-26-1a and H-29-1cd from the first sampling, and H-26-2b and H-30-2b (second sampling) belong to cluster EU-1a (Figures 2 and 3). In samples of pigs in herd H-29, isolates belonging to two different clusters (EU-1a and EU-1h) were found (Figure 2). In addition with respect to the ORF5 sequences, identical isolates were present in different herds (e.g. H-03-1fhln, H-10-1e and H29-1d in cluster EU-1a, or H-13-1a and H-21-1a in cluster EU-1c; Figure 2).

306

307 *Second sampling:* After about two years new samples were obtained, and PRRSV
 308 field isolates were found in 8 of the 18 herds. Because of the limited number of
 309 herds analysed, no evident correlation between the persistence of clinical signs,
 310 the detection of PRRSV, or the vaccination strategy were found. Three isolates
 311 related to the Porcilis® PRRS vaccine strain DV, differing in single base
 312 exchanges, were found in herd H-26 (Figure 2), where this vaccine had been used
 313 for the sows shortly before sampling. As for the field isolates, the ORF5 of isolate
 314 H-26-2b had 100% nucleotide identity with the isolate obtained about one year
 315 earlier (H-26-1a); both were in cluster EU-1a. In contrast, in samples from herd
 316 H-30 three different field isolates were detected, belonging to clusters EU-1a, EU-
 317 1h and EU-1j, respectively. At the first sampling, one isolate was a vaccine-
 318 related NA-genotype virus (H-30-1a), and the second one clustered in EU-1j (H-
 319 30-1b). This last isolate was still present one year later (H-30-2d; 100% identical
 320 ORF5 nucleotide sequence). Similarly, isolates in herds H-05, H-16, H-20 and
 321 H-26 also seem to have persisted, and in some cases new isolates appeared. For
 322 example herd H-16: pigs in this herd were initially vaccinated with the Ingelvac®
 323 PRRS vaccine, and three field isolates (H-16-1abd; cluster EU-1d) were found in
 324 samples from the first period. By the time the herd was re-sampled, pigs were
 325 being vaccinated with Porcilis® PRRS. Besides finding the same field isolate in
 326 two samples (H-16-2ad, cluster EU-1d), three isolates were of the NA-genotype.
 327 Interestingly, the ORF5 of one of them showed only 96.6% nucleotide and 95.5%
 328 deduced amino acid identity with the Ingelvac® PRRS MLV, respectively, while

the other NA-genotype isolates had > 98% nucleotide identity with the NA-vaccine strain. The nucleotide identity of the ORF7 sequences from both viruses was 98.1% (Table 4).

4. Discussion

The states of Lower Saxony and North Rhine-Westphalia (NRW) are located in the northern part of Germany, bordering with the Netherlands (Figure 1). It is one of the regions with the highest pig density in Europe. In Lower Saxony and in NRW the pig population actually (November 2008) amounts to 8.0 and 6.2 million, respectively (http://www.statistikportal.de/Statistik-Portal/de_jb11_jahrtab22.asp). Lower Saxony has an area of about 48,000 km², of which 61% are used for agriculture. In this area, the average pig density amounts to > 270 pigs/km². NRW has an area of about 34,000 km², of which about 50% are used for agriculture. Here, the average pig density is of > 350 pigs/km². The aim of this study was to analyse the genetic diversity of the field PRRSV in herds in a pig-dense region with a clinical history of reproductive and/or respiratory disorders that were PRRSV positive by RT-PCR. We wanted to determine if there is clustering and if herd specific clusters exist, and to ascertain if and how the field isolates might have changed in one to two years after the first assessment.

It is well established that EU-genotype PRRSV isolates are genetically diverse (Indik et al., 2000; Stadejek et al., 2006; Stadejek et al., 2002). Whether there is a geographical distribution of isolates of the different genetic clusters is as yet

352 ambiguous. It has been reported that based on geographical distribution three
353 different European clusters exist, geographically skewed within Europe (Forsberg
354 et al., 2002). In the same manner, Spanish PRRSV isolates were grouped into two
355 well-defined clusters and a group of unrelated sequences (Prieto et al., 2008), and
356 Austrian EU-genotype isolates were grouped into three clusters (Indik et al.,
357 2005). Although an evolutionary influence of the geographical location or the
358 time of sample collection, and of PRRSV vaccination on strain development have
359 been questioned (Pesch et al., 2005), these items remain to be unraveled and
360 conclusively analyzed.

361 In the present study, we selected 18 herds in Northern Germany (Figure 1)
362 showing respiratory and/or reproductive failure. All herds harbored field isolates.
363 The same herds were visited two years later, and new samples were obtained and
364 analyzed. From both samplings, the ORF5 of 86 PRRSV isolates was sequenced
365 in both directions. To assess clustering, a radial tree was calculated including the
366 ORF5 sequences available from the GenBank database from isolates belonging to
367 the new subgenotypes EU-2, EU-3 and EU-4 (Stadejek et al., 2006). None of the
368 field isolates analyzed belonged to one of these last genotypes (Figure 2, insert).
369 The nucleotide sequences of the ORF5 of the PRRSV from both sampling periods
370 allowed the assignment of the field isolates to 8 different clusters and the LV-
371 cluster within the EU-1 genotype, and three single isolates (Figure 2). Most of the
372 clusters contained PRRSV from more than one herd, indicating that there seem to
373 be no herd-specific viruses. This is in concert with the high pig density in
374 Northern Germany, especially as transmission of the virus from herd to herd by

different routes is a well established fact (Batista et al., 2002; Cho and Dee, 2006; Indik et al., 2005). As has been found before (Dee et al., 2001; Larochelle et al., 2003; Mateu et al., 2003), in some cases herds can harbor more than one isolate at the same time (two field isolates: H29-1cd – EU-1a, and H29 be – EU-1h; the NA-genotype vaccine virus and a field isolate: H16-2bce and H16-2ad – EU-1ad; or the EU- and NA-genotype vaccine viruses and a field isolate, H10-1e, H10-1ab and H10-1cd, respectively). Alignment of the ORF5 sequences from EU genotype isolates obtained from one herd showed that some of them had single nucleotide substitutions, many of them leading to amino acid exchanges (Figure 3, cluster EU-1a). Nucleotide identities remained $\geq 98\%$, implying that they were closely related (Collins, 1998), with mutations within the population (Allende et al., 2000a; Goldberg et al., 2003). This is also in concert with the sequence variation of the NA-genotype isolates found in Western Europe, which are most probably derived from the Ingelvac® PRRS vaccine (Greiser-Wilke et al., 2008; Indik et al., 2005). As this genotype was not present in Western Europe and was introduced with the vaccine in 1996, genetic drift can be nicely tracked. One variant with less than 98% nucleotide identity was found in one herd (H16-2c), but also in Austria (Indik et al., 2005; Prieto et al., 2008). Ingelvac® PRRS MLV related isolates were found in herds that had not been using this vaccine for longer periods, and in the meantime this strain has become endemic in Lower Saxony and North Rhine-Westphalia (Greiser-Wilke et al., 2008). The next years will show if new populations arise, and give new insights into the evolution of this virus.

398 When the herds were visited for the second time, only two of them reported
399 clinical signs. From pigs in 10 herds PRRSV was isolated again (Table 1). Among
400 these, in five herds field isolates with the identical ORF5 sequence as the isolates
401 from two years before were found (herds H-05, H-16, H-20, H-26 and H-30). For
402 a virus which is known to have a high mutation rate this finding is unusual, and up
403 to date there is no plausible explanation. Porcilis® PRRS derived vaccine virus
404 was isolated from three samples from one of the herds (herd H-26). Sows were
405 being vaccinated with this compound. Re-isolation of the vaccine strain is an
406 expected consequence, as the strain replicates and persists in vaccinated animals
407 for a certain period of time. During that period the strain can be transmitted to
408 naïve animals and to other herds. This has been demonstrated for example for the
409 NA vaccine strain (Allende et al., 2000b; Botner et al., 1997; Storgaard et al.,
410 1999). During the next years, more samples will have to be analyzed to determine
411 whether the Porcilis-like isolates can also spread and evolve as has been found for
412 the Ingelvac® PRRS vaccine virus-like isolates (Allende et al., 2000b; Botner et
413 al., 1997; Storgaard et al., 1999).

414 At both sampling times herd specific data were collected with the help of a
415 questionnaire. Probably due to the small number of herds included in the study,
416 there was no evident correlation between vaccination strategy, vaccine used,
417 report of clinical signs, and the presence of PRRSV field isolates. From samples
418 in some of the herds, the same or a related isolate as the year before was detected.
419 Other herds had acquired new isolates (Figure 2). The first finding is in concert
420 with the long persistence of PRRSV in pig populations (Christopher-Hennings et

421 al., 2001; Goldberg et al., 2003; Storgaard et al., 1999). The second finding
 422 highlights the risk for the introduction of new virus isolates by various
 423 mechanisms (i.e. replacement gilts, vehicles, personnel, nearest neighbourhood
 424 spread). In addition, at the second visit, only two herds reported clinical signs, but
 425 PRRSV was still detectable in animals from 10 herds. As only the ORF5
 426 sequences were analysed, no statement concerning the pathogenicity of the
 427 isolates can be inferred.

428 As expected the EU genotype field isolates that were obtained from herds located
 429 in a region with a high pig density were genetically diverse, thus confirming
 430 previous reports from many European countries, including Germany (Balka et al.,
 431 2008; Forsberg et al., 2002; Mateu et al., 2006; Pesch et al., 2005; Stadejek et al.,
 432 2002). Phylogenetic analysis of the ORF5 sequences using the Neighbor-joining
 433 algorithm showed that they could be assigned to discrete clusters. This diversity
 434 may be a consequence of the extensive trade with pigs especially within the
 435 European Union. If at all, the appearance of isolates of the newly identified
 436 subgroups EU2-4 in Western Europe may be just a matter of time. In some herds,
 437 isolates with identical or almost identical ($\geq 98\%$) ORF5 sequences were found in
 438 samples from both periods. In addition, several herds seemed to be infected with
 439 the same isolates, and other herds had two different isolates at the same time. The
 440 fact that isolates with identical nucleotide sequences of the ORF5 were found in
 441 different herds and two years apart indicates that the high mutation frequency is
 442 only the first step in the emergence of new PRRSV populations. Selection of
 443 populations with better growth characteristics is probably one of several strategies

444 (Rowland et al., 1999). This would also explain the relatively slow genetic drift of
445 the virus isolates in the herds analysed. There are strong indications that
446 recombination will also play a role (Forsberg et al., 2002; Murtaugh et al., 2001;
447 van Vugt et al., 2001; Yuan et al., 1999).
448

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681

682 **Legends to Figures**

683 **Figure 1:** Map of Germany and the surrounding countries. The 18 herds were
684 located in the counties of Lower Saxony and North Rhine-Westphalia (dotted in
685 the map).

686
687 **Figure 2.** The phylogenetic tree using the ORF5 nucleotide sequences of isolates
688 from both sampling periods (Table 1) was inferred using the Neighbor-joining
689 method as implemented in MEGA4, and displayed as a phylogram. The
690 evolutionary distances were computed using the Jukes-Cantor method. Bootstrap
691 analysis was performed with 500 replicates (numbers next to the branches in %).
692 There were a total of 606 positions in the final dataset. Nomenclature of the
693 isolates was as follows: Herd number, first or second sampling and lower case
694 letters for each isolate; e.g. H-01-1a: herd No. 01; first sampling; isolate a.
695 Bold: isolates with 100% identical sequences (ORF5) from one herd and both
696 periods

697 Bar: number of base substitutions per site

698 **Figure 2, insert.** Radial tree inferred using the Neighbor-joining method as
699 implemented in ClustalX. Sequences were trimmed to match the published ORF5
700 sequences of genotypes EU-2-4. Bootstrap analysis was performed with 1000
701 replicates. There were a total of 561 positions in the final dataset. Cluster
702 nomenclature is as shown in Figure 2.

703 Bar: number of base substitutions per site

704

705 **Figure 3:** Alignment of the deduced amino acid sequences of the GP5 of the field
706 isolates in cluster EU-1a. Identical amino acids are displayed as dots. The signal
707 peptide, the ectodomain and the glycosylation sites (arrows) are indicated
708 (Wissink et al., 2004; Wissink et al., 2003).

Table 1: Herds, herd types and PRRS virus isolates obtained from the 18 herds during the two samplings; vaccines used and clinical signs recorded in each sampling period

First sampling (2004/2005)						Second sampling (2006/2007)			
Herd No.	Herd structure ¹	Clinical signs ²	Vaccine sows ³	Vaccine piglets ³	PRRSV isolates	Clinical signs ²	Vaccine sows ³	Vaccine piglets ³	PRRSV isolates
H-01	2	RD	NA	NA	H-01-1adefgijk ⁴	none	EU	EU	H-01-2ab
H-02	3	RF	NA	none	H-02-1abdefgk	none	EU	none	H-02-2a
H-03	2	RF	EU	EU	H-03-1abdefghijklmnop	none	EU	EU	H-03-2abde
H-05	1	RF	NA	NA	H-05-1abcd	none	EU	EU	H-05-2ab
H-06	1	RF	NA	none	H-06-1abcdefg	none	EU	none	-
H-10	1	RF	NA	none	H-10-1cde	RD	EU	none	-
H-13	1	RD	NA	none	H-13-1a	none	NA	none	-
H-16	2	RD	NA	none	H-16-1abd	none	EU	EU	H-16-2abcde
H-20	1	RD	EU	none	H-20-1a	none	EU	none	H-20-2a
H-21	2	RF	EU	EU	H-21-1a	none	EU	EU	-
H-23	2	RD	EU	none	H-23-1a	none	EU	EU	-
H-25	2	RD	Inact.	none	H-25-1a	none	Inact.	none	-
H-26	1	RF	NA	NA	H-26-1a	RF	EU	none	H-26-2abcd
H-29	1	RF	NA	NA	H-29-1bcde	none	NA	NA	-
H-30	2	RD	EU	EU	H-30-1ab	none	EU	EU	H-30-2abd
H-45	1	RF	Inact.	none	H-45-1a	none	none	none	H-45-2a
H-46	2	RD	none	none	H-46-1a	none	none	none	-
H-47	1	RD	NA	NA	H-47-1a	none	EU	EU	H-47-2a

¹ 1 = one-site production, 2 = two-site production, 3 = three-site production system

² RD: respiratory diseases; RF: reproductive failure; none: no clinical signs reported

³ EU = Porcilis®PRRS, NA = Ingelvac®PRRS MLV, inact. = KV/Progressis inactivated vaccines;

⁴ Herd number (H-01, H-47)-sampling time (1 or 2), Letters for each isolate (abc...)

Table 2: List of primers used for amplification of the ORF5 and ORF7 from PRRSV

Nr.	Amplification of virus genotype – ORF	Sequence (5' to 3')	Anneal. temp.	Position LV* ¹	Position VR* ²	Amplicon size	Ref.
307	EU/NA-ORF7 S	5'-GCCCCTGCCCAI CAC-3'	55 °C	14414	14705	636 bp EU 659 bp NA	1
308	EU/NA-ORF7 AS	5'-TCGCCCTAATTGAATAGGTGA-3'		15050	15364		
310	EU-ORF5 S	5'-CAATGAGGTGGGCIACAACC-3'	60 °C	13442	-	718 bp	1
311	EU-ORF5 AS	5'-TATGTIATGCTAAAGGCTAGCAC-3'		14160	-		
313	NA-ORF5 S	5'-GCTCCATTTTCATGACACCTG-3'	60 °C	-	13699	818 bp	1
314	NA-ORF5 AS	5'-AAAGGTGCAGAAGCCCTAGC-3'		-	14517		
322	EU/NA-ORF5 S	5'-CGGTTGCTICATTTTCITGAC-3'	50 °C	13414	13694	777 bp EU 788 bp NA	2
323	EU/NA-ORF5 AS	5'-CACCTTIAGGGCITATATCA-3'		14191	14482		
301	EU-ORF5 S	5'-TGAGGTGGGCTACAACCATT-3'	55 °C	13400	-	702 bp	2
302	EU-ORF5 AS	5'-AGGCTAGCACGAGCTTTTGT-3'		14102	-		

I = Inosin, S = sense primer, AS = antisense primer, *¹LV = Lelystad virus, *²VR = VR-2332

References: 1 = Oleksiewicz et al., 1998; 2 = this work

Table 3: Summary of the genotypes of the PRRSV isolates from the 18 herds at both time points analysed

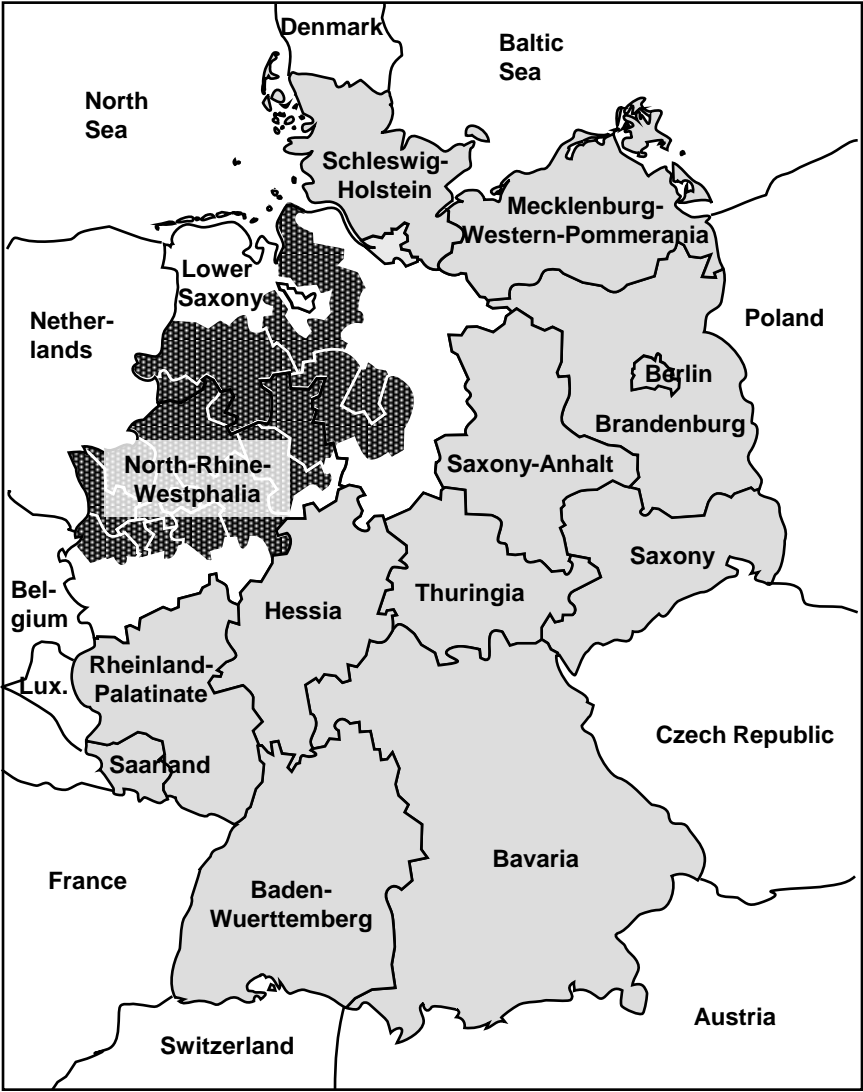
Genotypes	Isolates from herds No.	
	First sampling (2004/2005)	Second sampling (2006/2007)
EU field isolates	H-01-1defgijk; H-02-1bdefgk; H-03-abdefghjklmnop; H-05-1abcd; H-06-1abcdefg; H-10-1e; H-13-1a; H-16-1abd; H-20-1a; H-21-1a; H-23-1a; H-25-1a; H-26-1a; H-29-1bcde; H-30-1b; H-45-1a; H-46-1a; H-47-1a	H-01-2ab; H-05-2ab; H-16-2a; H-20-2a; H-26-2b; H-30-2abd; H-45-2a; H-47-2a
EU (LV cluster)	H-10-1cd	H-26-2acd
NA (VR2332 cluster)	H-01-1a; H-02-1a; H-10-1ab; H-30-1a	H-02-2a; H-03-2abde; H-16-2bce

Table 4: Nucleotide identities (in %) of the ORF5 sequences of the clusters within genotype EU1

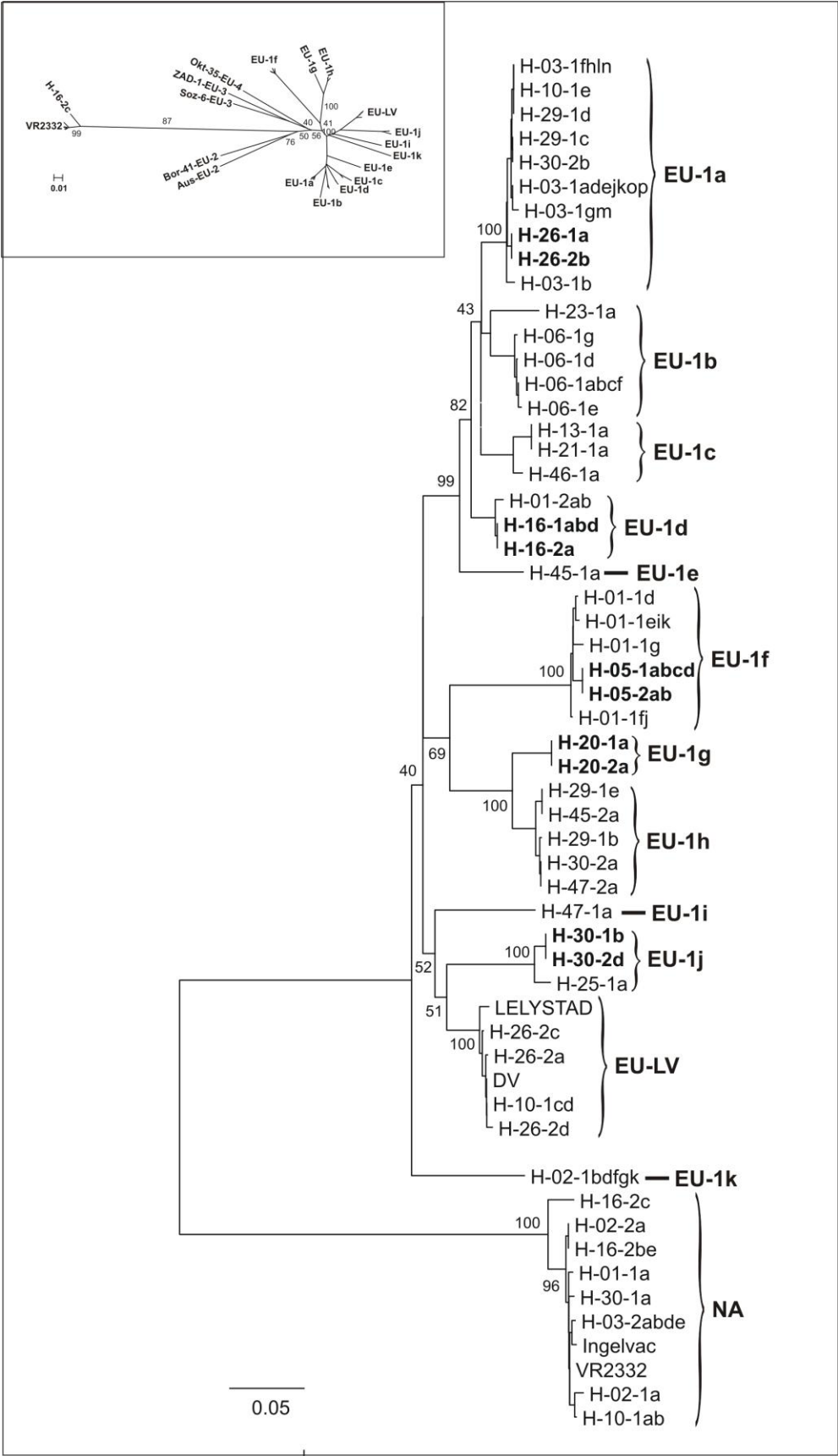
Cluster	LV	EU-1a	EU-1b	EU-1c	EU-1d	EU-1e	EU-1f	EU-1g	EU-1h	EU-1i	EU-1j	EU-1k
LV	98-100											
EU-1a	90.2-90.5	98.8-100										
EU-1b	88.7-89.7	94.3-95.8	94.8-99.8									
EU-1c	89.0-90.2	94.5-95.5	93.7-95.7	98.1-100								
EU-1d	89.6-93.2	95-95.5	93.2-95.7	93.7-95.8	99.3							
EU-1e	90.2-90.4	92.7-93	91-92	91.4-92.5	92.2-92.5	100*						
EU-1f	86.3-86.7	83.8-84.1	83.3-84.4	83.9-84.4	84.8-85.4	84.1	98.6-100					
EU-1g	87.2-87.4	86.9-87.2	84.9-87.1	86.9-87.1	87.1-87.4	86.1	85.8-86.1	100*				
EU-1h	87.2-87.7	88.1	86.1-88.1	87.2-87.4	87.1-87.7	86.7	86.4-86.7	95.2-95.7	99.1-100			
EU-1i	89.6-89.7	88.1-88.4	85.8-87.2	88.1-88.2	87.4-88.1	87.2	84.3-84.9	84.1	84.3-84.6	100*		
EU-1j	90.9-91.4	86.4-86.9	84.6-86.7	86.6-86.7	86.7-87.9	87.1	83.3-83.9	84.3-84.8	84.3-85.3	88.4-88.6	98.1-100	
EU-1k	87.6-88.1	86.7-87.1	85.9-87.1	87.2-87.4	86.4-87.1	85.6	81.5-81.8	84.4	85.1-85.8	86.1	85.4-85.8	99.9

* Single isolates

Figure



Figure



Figure

