Acta virologica **57**: 363 – 368, 2013 doi:10.4149/av_2013_03_363

Genetic typing of porcine reproductive and respiratory syndrome virus isolates from central European countries

A. JACKOVÁ, M. VLASÁKOVÁ, R. MANDELÍK, Š. VILČEK*

Department of Epizootiology and Parasitology, University of Veterinary Medicine and Pharmacy, Komenského 73, 041 81 Košice, Slovak Republic

Received January 31, 2013; accepted July 12, 2013

Summary. – All thirty-three but one porcine reproductive and respiratory syndrome virus (PRRSV) isolate originating from pigs in Austria, Czech Republic and Slovakia were typed on the basis of partial ORF5 sequence as PRRSV-1, subtype EU-1. The single isolate of PRRSV-2 originated from Slovakia.

Keywords: porcine reproductive and respiratory syndrome virus; genotyping; ORF5

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) (the family *Arteriviridae*, the genus *Arterivirus*) is the etiological agent of porcine reproductive and respiratory syndrome (PRRS). This syndrome is characterized by reproductive failure in sows and respiratory problems in piglets (Snijder and Meulenberg, 1998). PRRS has been considered one of the most economically devastating diseases for the swine industry worldwide (Neumann *et al.,* 2005). The syndrome was first reported in North America in 1987 (Keffaber, 1989) and a few years later in Europe (Wensvoort *et al.,* 1991). At the same time, outbreaks of PRRS occurred in Asia (Hirose *et al.,* 1995).

Two genotypes have been defined for PRRSV to date, PRRSV type 1 (PRRSV-1, EU genotype) and PRRSV type 2 (PRRSV-2, NA genotype) and they are represented by the reference strain Lelystad (LV) and the strain VR-2332 (Snijder *et al.,* 2005). Later, the analysis of nucleotide sequences of viral isolates from Poland, Lithuania, Belarus and Russia expanded the knowledge about the PRRS virus in this part of Europe (Stadejek *et al.,* 2006, 2008). Consequently, the division of PRRSV-1 into three subtypes has been proposed: the pan-European subtype 1 (subtype EU-1) and the East European subtypes 2 and 3 (subtypes EU-2 and EU-3) (Stadejek *et al.,* 2008).

PRRSV is a small enveloped virus with a positive-sense single-stranded RNA genome approximately 15 kb in length (Meulenberg *et al.,* 1993). The viral genome contains nine overlapping open reading frames (ORFs): ORF1a, 1b, 2a, 2b, and 3–7. ORF5 encodes the major envelope glycoprotein (GP5) (Mardassi *et al.,* 1995), which is essential for virus infectivity and correlates with virus neutralisation (Ansari *et al.,* 2006). GP5 represents the most variable protein of PRRSV (Murtaugh *et al.,* 1995).

So far, only limited genetic information is available on PRRSV isolates from Austria and Czech Republic (Indik *et al.,* 2000; 2005). We have recently analyzed ORF7 of PRRSV isolates originating from those countries and Slovakia and found two unique isolates from Slovakia with a new length polymorphism of the nucleocapsid (N) protein (Jackova *et al.,* 2012). Since most PRRSV isolates collected worldwide have been analyzed for ORF5, we decided to analyze our isolates from Austria, Czech Republic and Slovakia in the same genomic region. By comparing the obtained sequences with those from the database we could assign almost all these isolates to PRRSV-1, subtype EU-1. Just one isolate, originating from Slovakia, was identified as PRRSV-2. This work

*Corresponding author. E-mail: vilcek@uvm.sk; phone: +421-915-984654.

Abbreviations: GP5 = glycoprotein 5; PRRSV-1 = porcine reproductive and respiratory syndrome virus type 1; PRRSV-2 = porcine reproductive and respiratory syndrome virus type 2

represents the first ORF5-based genotyping of PRRSV circulating in Slovakia.

Materials and Methods

Clinical samples. The samples (serum, lymph nodes, lung tissue; n = 33) used in this study originated from pig farms in Austria, Czech Republic and Slovakia. They were obtained in the period between 2007 and 2012 from pigs aged from 6 to 24 weeks showing clinical signs compatible with PRRS. Austrian samples (n = 7) were collected from five farms in Lower Austria, Czech samples (n = 15) originated from 9 farms in seven country districts, Slovak samples (n = 11) were obtained from four farms in south-western Slovakia. Lung and lymph node samples were homogenized in phosphate-buffered saline (Sigma-Aldrich, USA) to make 20% (w/v) suspensions. Virus was not cultivated from original clinical samples. In the interpretation of results it is supposed that the detection of PRRSV sequence in clinical sample by RT-PCR or sequencing indicates the presence of a PRRSV isolate.

RNA extraction. Total RNA was extracted using Trizol Reagent (Invitrogen, USA) from 200 µl samples according to the manufacturer´s recommendation. The extracted RNA was resuspended in 20 µl of nuclease-free water (Serva, Germany).

RT-PCR. RNA was transcribed to cDNA using 2 µmol/l outer antisense PCR primer and 200 U SuperScript III Reverse Transcriptase (Invitrogen, USA) according to manufacturer's instructions. The ORF5 region was amplified using a nested RT-PCR. PRRSV-1 was detected using ORF5 primers published by Oleksiewicz *et al.* (1998). The nested primers for the second round of amplification of PRRSV were as described by Suarez *et al.* (1996). The PRRSV-2-specific ORF5 primers (Oleksiewicz *et al.,* 1998) were used for detection of PRRSV-2.

Sequencing, sequence analysis, and phylogeny. Purified PCR products were sequenced by a commercial company using the Sanger method employing fluorescent labeled ddNTPs. Both strands of the PCR products were sequenced with the same primers as used for the nested PCR amplification. The nucleotide sequences were deposited in GenBank under the following accession numbers: Austria – KC522616 to KC522622, Czech Republic – KC522623 to KC522637, and Slovakia – KC522638 to KC522648. The nucleotide sequence and the deduced amino acid sequence identities were calculated using the MegAlign program from the DNASTAR package (DNASTAR, Lasergene 8.1, Madison, USA). Phylogenetic tree was constructed using the neighbor-joining method from the computer program MEGA version 4.

Results

Sequence and phylogenetic analysis of ORF5

The nucleotide and deduced amino acid sequence identity among Austrian, Czech and Slovak PRRSV isolates in ORF5 was between 82.9–100% and 84.0–100%, respectively.

Similar results were obtained when nucleotide sequences of the Central European viral isolates were compared to sequences of the reference strain Lelystad of PRRSV-1, but lower values were observed when they were compared with sequences for the strains Sid and

Table 1. ORF5 sequence identities in PRRSV isolates

Type Subtype Strain	Identity %		
	Austrian isolates	Czech isolates	Slovak isolates[1]
PRRSV-1	89.4–98.6 (nt)	85.0–98.6 (nt)	85.4–89.1 (nt)
EU-1			
Lelystad	84.7–97.9 (aa)	87.5–97.9 (aa)	86.1–90.3 (aa)
PRRSV-1	75.5–78.7 (nt)	75.9–80.3 (nt)	77.5–79.6 (nt)
EU-2			
Sid (LT)	81.2–84.7 (aa)	79.9–86.1 (aa)	84.7–85.4 (aa)
PRRSV-1	80.8–83.3 (nt)	80.3–83.6 (nt)	78.9–84.0 (nt)
EU-3			
Yuz (BY)	82.6–86.8 (aa)	83.3–86.1 (aa)	81.9–87.5 (aa)
PRRSV-2	63.0–66.7 (nt)	63.4–66.2 (nt)	62.7–65.3 (nt)
IAF-EXP 91	60.4–63.2 (aa)	59.0–61.8 (aa)	59.7–61.8 (aa)
PRRSV-2	65.3–68.3 (nt)	64.4–68.3 (nt)	64.8–66.7 (nt)
VR-2332	61.1–66.0 (aa)	60.4–64.6 (aa)	61.1–62.5 (aa)

[1]Except the isolate 36M (PRRSV-2).

Yuz (Table 1) from Lithuania and Belarus belonging to PRRSV-1 subtypes EU-2 and EU-3. The lowest identity was observed between Central European viral isolates and North American isolates, e.g. 62.7–68.3% (nt) and 59.0–66.0% (aa) (Table 1).

The viral isolate 36M from Slovakia was the one showing the lowest sequence identity to the Lelystad strain, 63.4% (nt) and 60.4 % (aa). On the other hand, this isolate was more similar to VR-2332, a reference strain of the PRRSV-2, 86.6% (nt) and 88.2% (aa), and had a very close relationship with the isolate IAF-EXP 91, 91% (nt) and 92.4% (aa).

Phylogenetic analysis (Fig. 1) revealed that 32 of 33 Austrian, Czech and Slovak isolates belonged to PRRSV-1, subtype EU-1. The Slovak PRRSV isolates were clustered into three phylogenetic branches according to their farm of origin. The Czech and Austrian isolates originating from different farms were mostly located as single nodes. The most significant cluster of isolates from neighboring countries was observed between Czech and Slovak isolates (see grouping of isolates 28M and 29M with Czech isolates) but this observation was not a general phenomenon. None of the isolates collected from pig farms in the south-western part of Slovakia close to Hungary were grouped with the subtype EU-1 of Hungarian isolates. Interestingly, although several Danish companies manage the pig farms in Slovakia, no isolate from Slovakia has been grouped with the phylogenetic clade of Danish isolates.

When present isolates were compared to other Austrian and Czech isolates analyzed several years ago, common grouping was observed mostly with older and recent Austrian isolates but only partially with isolates from Czech Republic.

The isolate 36M originating from one of the Slovak pig farms fell into PRRSV-2. This isolate was grouped with the Canadian isolate IAF-EXP 91, with the closest position to two isolates from Hungary (Fig. 1). Three isolates of PRRSV-2 from Austria described in older study fell into a separate cluster together with strain VR-2332 and the Ingelvac PRRS vaccine strain.

Glycosylation and conservation of glycoprotein GP5

The alignment of deduced GP5 amino acid sequences of Central European PRRSV isolates confirmed the occurrence of three N-glycosylation sites corresponding to amino acids 37, 46, and 53. However, only two N-glycosylation sites were found in two Slovak isolates 10M and 11M (lacking N37) and in the Czech isolate P4 (lacking N46). The stretch of amino acids surrounding the conserved N-glycosylation sites at amino acids 38–55 was highly conserved. The most variable region of GP5 appeared to be located between amino acids 89–109 (data not shown).

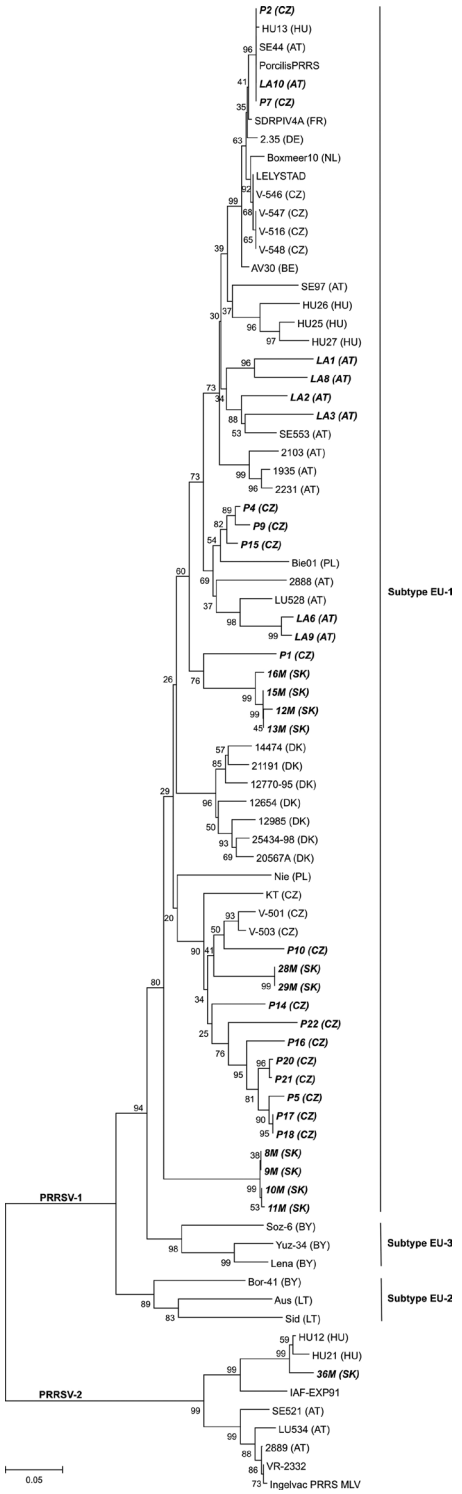

Fig. 1

A phylogenetic tree based on partial ORF5 sequences

A 432 nucleotide fragment corresponding to positions 97–528 of ORF5 was analyzed. The sequences analysed in this work are labeled in bold italics. The country of origin of isolates is in parenthesis. Branch numbers indicate bootstrap values (in %; 1000 replicates). Bar: number of substitutions per site.

Discussion

In this study, the genetic analysis of PRRSV isolates originating from three Central European countries has been carried out. All but one PRRSV isolate studied were typed as PRRSV-1, subtype EU-1 and none of the isolates clustered into subtypes EU-2 or EU-3. Recently, the partial results on genetic typing of PRRSV isolates originating from Hungary, Croatia, Serbia, Poland and Romania have been presented (Balka *et al.,* 2012). The isolates were typed as PRRSV-1, subtype EU-1, similar as the isolates in this work. These data indicate that in Central and Eastern European countries circulate isolates of subtype EU-1, similar as in Western Europe. The highly diverse isolates, which belong to subtypes EU-2 and EU-3, circulating in Belarus, Lithuania, Latvia and Russia have not penetrated deeper into the European continent.

When compared to the phylogenetic tree constructed from ORF5 sequences (this work) and ORF7 sequences (Jackova *et al.,* 2012) the genetic typing of isolates was the same. However, a more detailed inspection of the phylogenetic trees revealed that although clustering of isolates into discrete branches was similar, it has not been exactly the same in both trees studied.

A comprehensive study of the genetic diversity of PRRSV isolates in ORF5 originating from many European countries has indicated partial geographic clustering into three phylogenetic groups of subtype EU-1, namely the Lelystad-like clade (the isolates from the Netherlands, Belgium, France, Germany, Spain, and UK), Italian-like and the cluster of Danish isolates (Forsberg *et al.,* 2002). Later Balka *et al.* (2008) described 5 subgroups, other teams even 12 clusters (Shi *et al.,* 2010). In this study of 32 PRRSV-1 isolates, only two Czech isolates (P2 and P7) and one Austrian isolate (LA10) fell into the Lelystad-like cluster and none of the Slovak isolates were found in this cluster. Other isolates from Central Europe fell into two or more previously described phylogenetic clusters, there being no relationship between their clustering and their geographical origin.

The PRRSV-2 has been detected sporadically in Europe. In our study, only a single Slovak isolate 36M was typed as PRRSV-2 with higher nucleotide sequence identity to the isolate IAF-EXP 91 (also labeled as Quebec strain) than to the strain VR-2332. This isolate, detected near the Slovak-Hungarian border, was highly similar (96–97%) to two isolates from Hungary (HU12 and HU21) reported earlier by Balka *et al.* (2008). One could speculate that the isolate 36M was introduced to the farm by the PRRS vaccine. The authorized vaccine in Slovakia is Ingelvac PRRS–MLV based on strain VR-2332 (Boehringer Ingelheim, Germany), which is genetically distinct from the isolate 36M. The introduction of PRRSV-2 isolates has been observed not only in Hungary and Slovakia, but also in Austria (Indik *et al.,* 2005). The origin of the PRRSV-2 isolates in Central Europe is unknown so far. The use of the American-type vaccine is not allowed in Austria and Hungary. However, a non-authorised use of the American-type vaccine or the import of vaccinated animals and subsequent introduction of a vaccine strain into pig herds cannot be excluded as it has been observed in Denmark (Madsen *et al.,* 1998).

The ORF5 encodes the main envelope protein GP5, where the neutralizing viral epitopes are located. The glycosylation of GP5 is important for antibody neutralization, because neutralizing antibodies against PRRSV were bound less effectively to the glycosylated GP5 (Ansari *et al.,* 2006). Most of the isolates analyzed in this study were characterized by three N-glycosylation sites at amino acid positions 37, 46, and 53 as has been observed in many Western European isolates (Pesch *et al.,* 2005). Two glycosylation sites corresponding to amino acids at the positions 46 and 53 determined in two Slovak isolates (10M and 11M) have also been observed in the Lelystad strain, in most Czech isolates and isolates from Poland (Meulenberg *et al.,* 1995; Indik *et al.,* 2000; Stadejek *et al.,* 2002). One Czech isolate (P4) missed the N-46 glycosylation site, which was declared as highly conserved (Ansari *et al.,* 2006). Most PRRSV-2 isolates contain three glycosylation sites (Andreyev *et al.,* 1997), but only two glycosylation sites have been found in the Slovak isolate 36M.

In summary, PRRSV-1 isolates of subtype EU-1 circulate in pig farms in Austria, Czech Republic and Slovakia. No isolates of subtypes EU-2 and EU-3 were found in this geographic region. Occasionally PRRSV-2 isolates with no clear origin were detected in Central European countries.

Acknowledgements. This work was supported by APVV-0379-10, VEGA 1/0704/11 and INFEKTZOON, ITMS: 26220120002 in the frame of COST Action project FA0902. The authors thank Dr. Wigbert Rossmanith from The State Veterinary Institute in St. Polten (Austria), Dr. Alexander Nagy from The State Veterinary Institute in Prague (Czech Republic) and Dr. Vladimir Bony from Merial Slovakia for providing clinical samples of PRRSV.

References

Andreyev VG, Wesley RD, Mengeling WL, Vorwald AC, Lager KM (1997): Genetic variation and phylogenetic relationships of 22 porcine reproductive and respiratory syndrome virus (PRRSV) field strains based on sequence analysis of open reading frame 5. Arch. Virol. 142, 993–1001. http://dx.doi.org/10.1007/s007050050134

Ansari IH, Kwon B, Osorio FA, Pattnaik AK (2006): Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibod-

ies. J. Virol. 80, 3994–4004. http://dx.doi.org/10.1128/JVI.80.8.3994-4004.2006

Balka G, Hornyak A, Balint A, Kiss I, Kecskemeti S, Bakonyi T, Rusvai M (2008): Genetic diversity of porcine reproductive and respiratory syndrome virus strains circulating in Hungarian swine herds. Vet. Microbiol. 127, 128–135. http://dx.doi.org/10.1016/j.vetmic.2007.08.001

Balka G, Cadar D, Novosel D, Petrovic T, Biksi I, Rusvai M, Szymanek K, Stadejek T (2012): Phylogentic analysis of PRRSV strains from selected Central and Eastern European countries. Proc. EuroPRRS2012 Symposium, Budapest, pp. 67–70.

Forsberg R, Storgaard T, Nielsen HS, Oleksiewicz MB, Cordioli P, Sala G, Hein J, Botner A (2002): The genetic diversity of European type PRRSV is similar to that of the North American type but is geographically skewed within Europe. Virology 20, 38–47. http://dx.doi.org/10.1006/viro.2002.1450

Hirose O, Kudo H, Yoshizawa S, Hiroike T, Nakane T (1995): Prevalence of porcine reproductive and respiratory syndrome virus in Chiba prefecture. J. Jpn. Vet. Med. Assoc. 48, 650–653.

Indik S, Valicek L, Klein D, Klanova J (2000): Variations in the major envelope glycoprotein GP5 of Czech strains of porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 81, 2497–2502.

Indik S, Schmoll F, Sipos W, Klein D (2005): Genetic variability of PRRS virus in Austria: consequences for molecular diagnostics and viral quantification. Vet. Microbiol. 107, 171–178. http://dx.doi.org/10.1016/j.vetmic.2005.01.024

Jackova A, Vlasakova M, Leskova V, Vilcek S (2012): Identification of a new unusual length polymorphism of the nucleocapsid protein in porcine reproductive and respiratory syndrome virus. Virus Genes 45, 590–592. http://dx.doi.org/10.1007/s11262-012-0797-7

Keffaber KK (1989): Reproductive failure of unknown etiology. Am. Assoc. Swine Pract. Newsl. 1, 1–19.

Madsen KG, Hansen CM, Madsen ES, Strandbygaard B, Botner A, Sorensen KJ (1998): Sequence analysis of porcine reproductive and respiratory syndrome virus of the American type collected from Danish swine herds. Arch. Virol. 143, 1683-1700. http://dx.doi.org/10.1007/s007050050409

Mardassi H, Mounir S, Dea S (1995): Molecular analysis of the ORF3-7 of porcine reproductive and respiratory syndrome virus, Quebec reference strain. Arch. Virol. 140, 405–1 418.

Meulenberg JJM, Hulst MM, de Meijer EJ, Moonen PL, Petersen den Besten A, de Kluyver EP, Wensvoort G, Moormann RJ (1993): Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. Virology 192, 62–72. http://dx.doi.org/10.1006/viro.1993.1008

Meulenberg JJM, Petersen den Besten A, de Kluyver EP, Moormann RJM, Schaaper WMM, Wensvoort G (1995): Characterization of proteins encoded by ORFs 2 to 7 of Lelystad virus. Virology 206, 155–163. http://dx.doi.org/10.1016/S0042-6822(95)80030-1

Murtaugh MP, Elam MR, Kakach LT (1995): Comparison of the structural protein coding sequences of the VR-2332 and Lelystad virus strains of the PRRS virus. Arch. Virol. 140, 1451–1460. http://dx.doi.org/10.1007/BF01322671

Neumann EJ, Kliebenstein JB, Johnson CD, Mabry JW, Bush EJ, Seitzinger AH, Green AI, Zimermann JJ (2005): Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. J. Am. Vet. Med. Assoc. 227, 385–392. http://dx.doi.org/10.2460/javma.2005.227.385

Oleksiewicz MB, Botner A, Madsen KG, Storgaard T (1998): Sensitive detection and typing of porcine reproductive and respiratory syndrome virus by RT-PCR amplification of whole viral genes. Vet. Microbiol. 64, 7–22. http://dx.doi.org/10.1016/S0378-1135(98)00254-5

Pesch S, Meyer C, Ohlinger VF (2005): New insights into the genetic diversity of European porcine reproductive and respiratory syndrome virus (PRRSV). Vet. Microbiol. 107, 31–48. http://dx.doi.org/10.1016/j.vetmic.2005.01.028

Shi M, Lam TT, Hon CC, Hui RK, Faaberg KS, Wennblom T, Murtaugh MP, Stadejek T, Leung FC (2010): Molecular epidemiology of PRRSV: a phylogenetic perspective. Virus Res. 154, 7–17. http://dx.doi.org/10.1016/j.virusres.2010.08.014

Snijder EJ, Meulenberg JJM (1998): The molecular biology of arteriviruses. J. Gen. Virol. 79, 961–979.

Snijder EJ, Brinton MA, Faaberg K, Godeny EK, Gorbalenya AE, MacLachlan NJ, Mengeling WL, Plagemann GW (2005): Family Arteriviridae. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (Eds) (2005): Virus taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses, Elsevier/Academic Press, Amsterdam.

Stadejek T, Oleksiewicz MB, Potapchuk D, Podgorska K (2006): Porcine reproductive and respiratory syndrome virus strains of exceptional diversity in eastern Europe support the definition of new genetic subtypes. J. Gen. Virol. 87, 1835–1841. http://dx.doi.org/10.1099/vir.0.81782-0

Stadejek T, Oleksiewicz MB, Scherbakov AV, Timina AM, Krabbe JS, Chabros K, Potapchuk D (2008): Definition of subtypes in the European genotype of porcine reproductive and respiratory syndrome virus: nucleocapsid characteristics and geographical distribution in Europe. Arch. Virol. 153, 1479–1488. http://dx.doi.org/10.1007/s00705-008-0146-2

Suarez P, Zardoya R, Martin MJ, Prieto C, Dopazo J, Solana A, Castro JM (1996): Phylogenetic relationships of European strains of porcine reproductive and respiratory syndrome virus (PRRSV) inferred from DNA sequences of putative ORF-5 and ORF-7 genes. Virus Res. 42, 159–165. http://dx.doi.org/10.1016/0168-1702(95)01305-9

Wensvoort G, Terpstra C, Pol JMA, Ter Laak EA, Bloemraad M, De Kluyver EP, Kragten C, Van Buiten L, Den Besten A, Wagenaar F, Broekhuijsen JM, Moonen PLJM, Zetstra

T, De Boer EA, Tibben HJ, De Jong MF, Van ,t Veld P, Groenland GJR, Van Gennep JA, Voets MT, Verheijden JHM, Braamskamp J (1991): Mystery swine disease in the Netherlands: the isolation of Lelystad virus. Vet. Q 13, 121–130. http://dx.doi.org/10.1080/01652176.1991.9694296