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Temporal and Morphologic Characterization of the Distribution of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) by In Situ Hybridization in Pigs Infected with Isolates of PRRSV that Differ in Virulence

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Abstract

Three groups of 5-week-old cesarian-derived, colostrum-deprived pigs were inoculated intranasally with either a high-virulence isolate (VR2385) or a low-virulence isolate (VR2431) of porcine reproductive and respiratory syndrome virus (PRRSV) or with uninfected cell culture and media. Formalin-fixed, paraffin-embedded tissues from pigs euthanatized at 10, 21, and 28 days post-inoculation were examined by in situ hybridization for PRRSV nucleic acid using a digoxigenin-labeled antisense RNA probe approximately 1,000 nucleotides in length. Alveolar macrophages were positive in the lungs of 9/9, 2/2, and 0/2 VR2385-inoculated pigs and 7/9, 1/2, and 2/3 VR2431-inoculated pigs at 10, 21, and 28 days post-inoculation, respectively. More positive cells were detected in lungs from VR2385-inoculated pigs compared to VR2431-inoculated pigs at 10 and 21 days post-inoculation. Positive cells within lymph nodes were tingible body macrophages in germinal centers and macrophages or interdigitating dendritic cells within the paracortical areas. VR2385 was detected in the tracheobronchial lymph node (TBLN) and mediastinal lymph node (MLN) of 7/9 and 9/9 pigs at 10 days post-inoculation, but was only detected in the TBLN of 1/2 and 0/2 pigs and in the MLN of 0/2 and 1/2 pigs at 21 and 28 days post-inoculation, respectively. In contrast, VR2431 was detected in the TBLN and MLN of 5/9 and 2/9 pigs at 10 days post-inoculation and in the TBLN of 0/2 and 1/3 pigs and in the MLN of 0/2 and 0/3 pigs at 21 and 28 days post-inoculation, respectively. There were more positive cells in TBLN and MLN in pigs inoculated with VR2385 at 10 days post-inoculation. Macrophages located at the epitheliallymphoid interface of tonsilar crypts and within the paracortical areas were positive in tonsils of 9/9, 2/2, and 1/2 VR2385-inoculated pigs and 7/9, 1/2, and 1/3 VR2431-inoculated pigs at 10, 21, and 28 days post-inoculation, respectively. Positive cells in the thymic medulla were multinucleate and were only detected at 10 days post-inoculation in 2/9 VR2385-inoculated pigs and 4/9 VR2431-inoculated pigs. Positive cells within the spleen were few, spindle-shaped, located within smooth muscle trabecula, and only present at 10 days post-inoculation in 3/9 VR2385-inoculated pigs. We conclude that the tissue tropism and distribution of positive cells within tissues is similar for VR2385 and VR2431. However, tissues from more pigs and more cells within tissues were positive in pigs inoculated with VR2385 than VR2431 at 10 and 21 days post-inoculation. These findings indicate that the more virulent isolate VR2385 may replicate better in vivo than the less virulent isolate VR2431. This supports the hypothesis that an increased ability to replicate in vivo contributes to increased virulence of PRRSV.

Disciplines

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Temporal and Morphologic Characterization of the Distribution of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) by In Situ Hybridization in Pigs Infected with Isolates of PRRSV that Differ in Virulence

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Abstract. Three groups of 5-week-old cesarian-derived, colostrum-deprived pigs were inoculated intra-nasally with either a high-virulence isolate (VR2385) or a low-virulence isolate (VR2431) of porcine repro-ductive and respiratory syndrome virus (PRRSV) or with uninfected cell culture and media. Formalin-fixed, paraffin-embedded tissues from pigs euthanatized at 10, 21, and 28 days post-inoculation were examined by in situ hybridization for PRRSV nucleic acid using a digoxigenin-labeled antisense RNA probe approximately 1,000 nucleotides in length. Alveolar macrophages were positive in the lungs of 9/9, 2/2, and 0/2 VR2385-inoculated pigs and 7/9, 1/2, and 2/3 VR2431-inoculated pigs at 10, 21, and 28 days post-inoculation, respec-tively. More positive cells were detected in lungs from VR2385-inoculated pigs compared to VR2431-inoculated pigs at 10 and 21 days post-inoculation. Positive cells within lymph nodes were tingible body macrophages in germinal centers and macrophages or interdigitating dendritic cells within the paracortical areas. VR2385 was detected in the tracheobronchial lymph node (TBLN) and mediastinal lymph node (MLN) of 7/9 and 9/9 pigs at 10 days post-inoculation, but was only detected in the TBLN of 1/2 and 0/2 pigs and in the MLN of 0/2 and 1/2 pigs at 21 and 28 days post-inoculation, respectively. In contrast, VR2431 was detected in the TBLN and MLN of 5/9 and 2/9 pigs at 10 days post-inoculation and in the TBLN of 0/2 and 1/3 pigs and in the MLN of 0/2 and 0/3 pigs at 21 and 28 days post-inoculation, respectively. There were more positive cells in TBLN and MLN in pigs inoculated with VR2385 at 10 days post-inoculation. Macrophages located at the epithelial–lymphoid interface of tonsilar crypts and within the paracortical areas were positive in tonsils of 9/9, 2/2, and 1/2 VR2385-inoculated pigs and 7/9, 1/2, and 1/3 VR2431-inoculated pigs at 10, 21, and 28 days post-inocu-lation, respectively. Positive cells in the thymic medulla were multinucleate and were only detected at 10 days post-inoculation in 2/9 VR2385-inoculated pigs and 4/9 VR2431-inoculated pigs. Positive cells within the spleen were few, spindle-shaped, located within smooth muscle trabecula, and only present at 10 days post-inoculation in 3/9 VR2385-inoculated pigs. We conclude that the tissue tropism and distribution of positive cells within tissues is similar for VR2385 and VR2431. However, tissues from more pigs and more cells within tissues were positive in pigs inoculated with VR2385 than VR2431 at 10 and 21 days post-inoculation. These findings indicate that the more virulent isolate VR2385 may replicate better in vivo than the less virulent isolate VR2431. This supports the hypothesis that an increased ability to replicate in vivo contributes to increased virulence of PRRSV.

Key words: In situ hybridization; interdigitating dendritic cells; lymphoid tissue; macrophages; porcine reproductive and respiratory syndrome virus; swine.

Porcine reproductive and respiratory syndrome was first recognized in the United States in 1987 and sub-sequently in other countries. The causative agent, a virus, was first identified in 1991 in Lelystad, The Netherlands, and named the Lelystad virus.[11] In 1992, a similar virus was isolated from swine in the United States.[1,2] This virus is named the porcine reproductive and respiratory syndrome virus (PRRSV) and is a pos-itive-stranded RNA virus tentatively classified in the Arterivirus group of the family Togaviridae, which also includes lactate dehydrogenase-elevating virus, simian hemorrhagic fever virus, and equine arteritis virus.[3,9] Infection with PRRSV can be detected sero-logically, by virus isolation, and immunohistochemi-cally utilizing a monoclonal antibody against nucleo-capsid protein.[1,4,12] Immunohistochemistry has proven

to be extremely useful in detecting viral antigen within alveolar macrophages, dendriticlike cells and macrophages in tonsil and lymph nodes, and endothelium of coronary vessels.[5,7]

PRRSV will replicate within porcine alveolar macrophages in vitro and presumably in vivo as well.[11] Because PRRSV nucleocapsid antigen has been detected in dendriticlike cells and macrophages by immunohistochemistry, we hypothesize that PRRSV also replicates in these cell populations.[7] This could be confirmed by in situ hybridization (ISH), a technique that will detect as little as 10–20 copies of target nucleic acid within cells.[10]

PRRSV isolates differ in virulence; some isolates induce severe clinical pneumonia and pulmonary lesions, whereas other isolates induce very mild transient clinical disease and lesions.[6,8] Isolate VR2385 was found to be highly virulent, and PRRSV antigen was detected in larger amounts in more tissues and for a longer time period than was PRRSV antigen in the low-virulence isolate VR2431.[6,7] The mechanisms that result in different degrees of virulence for different PRRSV isolates are not yet understood. We hypothesize that some isolates of PRRSV replicate better in vivo and that this characteristic makes them more virulent.

Our objectives for this study were to develop a digoxigenin-labeled RNA probe that will detect PRRSV by in situ hybridization and to use it to temporally characterize the distribution of PRRSV in the respiratory and lymphoid systems of pigs experimentally infected with a high- (VR2385) or a low- (VR2431) virulence US isolate of PRRSV.

Archived formalin-fixed, paraffin-embedded tissues were used from an experiment where three groups of 25 5-week-old cesarian-derived, colostrum-deprived (CDCD) pigs were infected with a high-virulence isolate (VR2385) or a low-virulence isolate (VR2431) of PRRSV or with uninfected cell culture and media. Histopathologic descriptions of lesions and results of immunohistochemical (IHC) examination for the presence of PRRSV antigens in these tissues has been recently reported.[6,7] The pigs were inoculated intranasally with $10^{4.8}$ median tissue culture infective doses $(TCID_{50})/5$ ml. Tissues from nine pigs from each group necropsied at 10 days post-inoculation, two pigs necropsied at 21 days post-inoculation, and two pigs (VR2385) or three pigs (VR2431, controls) necropsied at 28 days post-inoculation were used for ISH examination. Tissues were fixed in 10% neutral buffered formalin for 1 to 3 days and routinely processed to paraffin blocks in an automated tissue processor.

A cDNA copy of the genome segment of PRRSV strain VR2385 containing the entire sequences of open reading frame (ORF) 7, ORF 6, the 3' noncoding region and 50 nucleotides of the 3' end of ORF 5 was cloned into a pBluescript II SK+ plasmid vector (Promega, Madison, WI); the total length of the cloned segment was 1,103 base pairs. Following amplification, the plasmid was isolated, linearized with Eco R1 (Stratagene, La Jolla, CA), and then run on a 1% agarose gel (Gibco BRL, Gaithersburg, MD) in tris-acetate-EDTA buffer to determine that linearization was complete. The band of linearized cDNA was cut from the agarose and purified with the glass milk procedure (Geneclean®, Bio 101 Inc., La Jolla, CA). After the concentration of the cDNA template was determined, transcription was carried out using T7 RNA polymerase and a mixture of nucleotides including digoxigenin-labeled UTP (Genius 4® kit, Boehringer Mannheim, Indianapolis, IN). Following transcription, the cDNA template was destroyed by DNase I (Sigma, St. Louis, MO) and the RNA probe was purified with a Quick Spin® G-25 sephadex column (Boehringer Mannheim). The resulting probe was an antisense, digoxigenin-labeled strand of RNA approximately 1,000 base pairs in length. The concentration of the labeled probe was determined by dot blot for detection of digoxigenin following the protocol supplied with the Genius 4 kit. Briefly, the probe was compared to serial dilutions of a digoxigenin-labeled RNA standard that were baked on to a nylon membrane and treated sequentially with sheep-antidigoxigenin-alkaline phosphatase complex (Boehringer Mannheim) and color substrate solution (nitro blue tetrazolium with 5-bromo-4-chloro-indoyl phosphate toluidinium) (Boehringer Mannheim). Specificity of the PRRSV RNA probe was confirmed with slot blot hybridization on a nitrocellulose membrane utilizing RNA from PRRSV isolates VR2385 and VR2431; the probe did not hybridize with RNA extracted from the uninfected ATCC CRL 11171 cells in which the PRRSV was propagated.

For in situ hybridization, sections of the accessory lobe of the lung, tracheobronchial and mediastinal lymph nodes, thymus, spleen, and tonsil were harvested at 10, 21, and 28 days post-inoculation. For each tissue, a single similarly sized section was cut at 3 μm, mounted on a silanized slide (Cel-Tek, Inc., Glenview, IL), deparaffinized, treated with Proteinase K (1 μg/ml) for 15 minutes at 37 C, then rinsed in DEPC water (Sigma). Sections were acetylated for 10 minutes at room temperature, rinsed, dehydrated with ethanol, and air dried. The hybridization mixture for each slide was composed of 100 pg of the PRRSV RNA probe added to 51 μl of hybridization buffer; this mixture was heated at 80 C for 2 minutes, immediately chilled on ice, placed on each section, cover slipped, and incubated overnight at 52 C. Following hybridization, treatment with RNase A (Sigma) and a

series of stringency washes, the sections were incubated in 3% blocking reagent (Boehringer Mannheim) for 30 minutes at room temperature. Sections were then incubated for 2 hours at room temperature with sheep-antidigoxigenin–alkaline phosphatase complex, diluted 1 : 300 in buffer with 3% normal sheep serum. The antidigoxigenin–alkaline phosphatase complex solution was removed and the sections were rinsed three times (10 minutes each) in buffer. The color substrate solution (nitro blue tetrazolium with 5-bromo-4-chloro-indoyl phosphate toluidinium) was then applied to the sections and incubated in the dark for 30 minutes at room temperature. After a final rinse in ultrapure water, the sections were counterstained with nuclear fast red and cover slipped. Negative controls consisted of lung and lymph node from age-matched non-PRRSV-infected pigs, additional sections from PRRSV-infected pigs in which a commercial digoxigenin-labeled non-PRRSV RNA probe (Genius 4 kit) was substituted for the PRRSV RNA probe and sections pretreated with RNase. The total number of cells with a positive PRRSV nucleic acid hybridization signal were counted in each section of tissue. As a positive control, additional sections were examined with immunohistochemistry using monoclonal antibody SDOW-17 as previously described.[4]

Lung, lymph node, and tonsil were the tissues in which cells with a positive hybridization signal were most readily identified for either VR2385 or VR2431. Generally, positive cells were scattered and individual; however, occasionally there was a focus in which several positive cells were aggregated. Positive cells in all tissues had a strong signal characterized by abundant black, granular reaction product covering only the cytoplasm. VR2385 was detected in the lungs of all challenged pigs at 10 and 21 days post-inoculation, but not in lungs at 28 days post-inoculation (Table 1). In contrast, VR2431 was detected in most lungs at 10, 21, and 28 days post-inoculation. There were more positive cells detected in lungs challenged with VR2385 compared to VR2431 at 10 and 21 days post-inoculation. Positive cells morphologically were characteristic of alveolar macrophages and were scattered throughout the lungs within alveolar lumens and occasionally alveolar walls (Fig. 1). VR2385 was detected more frequently and with a larger number of positive cells in tracheobronchial and mediastinal lymph nodes than VR2431 at 10 days post-inoculation. In addition, VR2385 was detected in one of these nodes at 21 and 28 days post-inoculation, whereas VR2431 was only detected in one node at 28 days post-inoculation. Positive cells within germinal centers were interpreted as tingible body macrophages because they had abundant cytoplasm, irregular cytoplasmic borders, and contained 2–5-μm-diameter globules of

Table 1. Number of pigs in which porcine reproductive and respiratory syndrome virus (PRRSV) nucleic acid was detected and the estimated amount of PRRSV nucleic acid in tissues from pigs infected with one of three isolates (VR2385, VR2431, Lelystad) of PRRSV.

Tissue*	VR2385	VR2431	Control
10 Days post-inoculation ($n = 9$)			
Lung	9/9 (2.8)†	7/9 (1.1)	0/9
TBLN	7/9 (1.4)	5/9 (0.6)	0/9
MLN	9/9 (1.1)	2/9 (0.3)	0/9
Thymus	2/9 (0.2)	4/9 (0.4)	0/9
Spleen	3/9 (0.4)	0/9	0/9
Tonsil	9/9 (1.4)	7/9 (1.4)	0/9
21 Days post-inoculation ($n = 2$)			
Lung	2/2 (1.5)	1/2 (0.5)	0/2
TBLN	1/2 (0.5)	0/2	0/2
MLN	0/2	0/2	0/2
Thymus	0/2	0/2	0/2
Spleen	0/2	0/2	0/2
Tonsil	2/2 (1.5)	1/2 (0.5)	0/2
28 days post-inoculation ($n = 2$ or 3)			
Lung	0/2	2/3 (0.7)	0/3
TBLN	0/2	1/3 (0.3)	0/3
MLN	1/2 (0.5)	0/3	0/3
Thymus	0/2	0/3	0/3
Spleen	0/2	0/3	0/3
Tonsil	1/2 (0.5)	1/3 (0.3)	0/3

* TBLN = tracheobronchial lymph node; MLN = mediastinal lymph node.

† ( ) = mean score of the estimated amount of PRRSV nucleic acid detected by in situ hybridization and scored as (0) = no positive cells, (1) = 1–10 positive cells per section, (2) = 11–30 positive cells per section, (3) = 31–100 positive cells per section, (4) = more than 100 positive cells per section of tissue examined.

pyknotic debris. In addition, positive cells within the paracortical areas were interpreted as either macrophages or interdigitating dendritic cells because they had abundant cytoplasm and long cytoplasmic processes in contact with lymphocytes (Fig. 2). Most pigs inoculated with VR2385 or VR2431 had positive cells detected within the tonsil at 10, 21, and 28 days post-inoculation. Positive cells interpreted as macrophages were located at the epithelial–lymphoid interface of tonsilar crypts (Fig. 3). In addition, positive cells interpreted as macrophages or interdigitating dendritic cells were present within the paracortical areas. Positive cells were detected in the thymus only at 10 days post-inoculation from pigs inoculated with VR2385 or VR2431. Positive cells were limited to the medulla and had abundant cytoplasm with one to four round nuclei and prominent nucleoli. Only pigs inoculated with VR2385 had positive cells within the spleen, and these were only present at 10 days post-inoculation. Positive cells were few, spindle-shaped, and located within smooth muscle trabecula. Negative control sec-
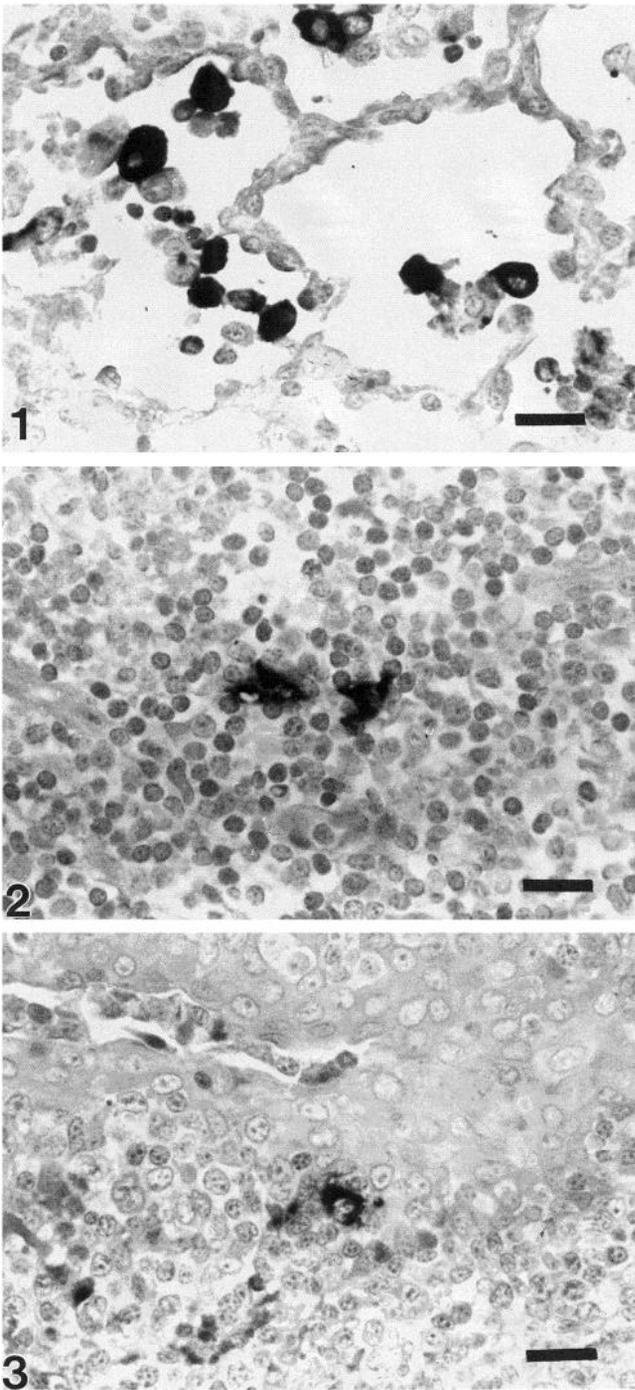
tions (noninfected tissue, infected tissue pretreated with RNase, and infected tissue in which a digoxigenin-labeled non-PRRSV RNA probe was used in place of the PRRSV RNA probe) did not contain any positive cells. In addition, positive control sections of lung, tracheobronchial lymph node, and thymus from pigs infected with VR2385 or VR2431 contained macrophage that were positive by immunohistochemistry.

This study documents the development and use of a digoxigenin-labeled RNA probe that uses ISH to detect the genome of two PRRSV isolates that vary in virulence. Because a cell must contain 10–20 copies of target nucleic acid to produce a positive hybridization signal, we conclude that positive cells contain actively replicating PRRSV.[10] The tissue tropism and distribution of positive cells within tissues was similar for VR2385 and VR2431. However, tissues from more pigs and more cells within tissues were positive in swine infected with VR2385 compared to VR2431 at 10 and 21 days post-inoculation. At 28 days post-inoculation, both isolates were detected in lymph node, but only VR2431 was detected within lung. These findings indicate that viremia with either isolate peaks by 10 days post-inoculation and that the more virulent isolate VR2385 may replicate better in vivo than the less virulent isolate VR2431. This is consistent with a previous report in which PRRSV was isolated from more tissues and PRRSV antigen was detected in more tissues of VR2385-infected pigs than in VR2431 or Lelystad virus-infected pigs and supports the hypothesis that VR2385 is more virulent than other isolates, at least in part, because it replicates better in vivo.[7]

The tissues that were positive for PRRSV nucleic acid by ISH and the distribution of the positive cells within these tissues was similar to that reported for PRRSV antigen by IHC, with the exception of lymph node. In an earlier study using the same archived blocks of tissues, we were able to detect PRRSV antigen in the tracheobronchial lymph nodes of 3/9 VR2385-infected pigs and 1/9 VR2431-infected pigs

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cleic acid detected in cells interpreted to be interdigitating dendritic cells in the paracortical area. In situ hybridization, with digoxigenin-labeled riboprobe detected by anti-digoxigenin antibody–alkaline phosphatase complex, counterstained with nuclear fast red. Bar = 20 μm.

Fig. 3. Tonsil; pig inoculated intranasally 10 days previously with porcine reproductive and respiratory syndrome virus (PRRSV) VR2431. PRRSV nucleic acid detected in a macrophage at the epithelial–lymphoid interface adjacent to a crypt. In situ hybridization, with digoxigenin-labeled riboprobe detected by anti-digoxigenin antibody–alkaline phosphatase complex, counterstained with nuclear fast red. Bar = 20 μm.

Fig. 1. Lung; pig inoculated intranasally 10 days previously with porcine reproductive and respiratory syndrome virus (PRRSV) VR2385. PRRSV nucleic acid detected in macrophages within alveolar lumens. Hybridization product is abundant and limited to the cytoplasm. In situ hybridization, with digoxigenin-labeled riboprobe detected by anti-digoxigenin antibody–alkaline phosphatase complex, counterstained with nuclear fast red. Bar = 20 μm.

Fig. 2. Tracheobronchial lymph node; pig inoculated intranasally 10 days previously with porcine reproductive and respiratory syndrome virus (PRRSV) VR2385. PRRSV nu-

at 10 days post-inoculation, whereas by ISH in this study we detected PRRSV in 7/9 and 5/9 of the VR2385-infected pigs and VR2431-infected pigs, respectively.[7] These findings indicated that in situ hybridization may be more sensitive than IHC for detecting PRRSV-infected cells within lymph nodes.

Replication of PRRSV in macrophages of the lung and lymphoid system in vivo is expected because PRRSV will replicate in porcine alveolar macrophages in vitro.[11] The identity of the cells that morphologically appear to be interdigitating dendritic cells needs to be confirmed. Macrophages and interdigitating dendritic cells are antigen-presenting cells, which have an important role in producing an effective immune response to infectious agents. Destruction or damage to these cells by PRRSV may decrease the ability of pigs to mount an effective immune response, resulting in prolonged viremia, persistent infection, and increased susceptibility to other viral and bacterial infections.

Acknowledgements

References

1 Benfield DA, Nelson E, Collins JE, Harris L, Goyal SM, Robison D, Christianson WT, Morrison RB, Gorcyca D, Chladek D: Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). J Vet Diagn Invest 4:127–133, 1992

2 Collins JE, Benfield DA, Christianson WT, Harris L, Hennings JC, Shaw DP, Goyal SM, McCullough S, Morrison RB, Joo HS, Gorcyca D, Chladek D: Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. J Vet Diagn Invest 4:117–126, 1992

3 Conzelmann K-K, Visser N, Van Woensel P, Thiel HJ: Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the Arterivirus group. Virology 193:329–339, 1993

4 Halbur PG, Andrews JJ, Huffman EL, Paul PS, Meng XJ, Niyo Y: Development of a streptavidin–biotin immunoperoxidase procedure for the detection of porcine reproductive and respiratory syndrome virus antigen in porcine lung. J Vet Diagn Invest 6:254–257, 1994

5 Halbur PG, Miller LD, Paul PS, Meng XJ, Huffman EL, Andrews JJ: Immunohistochemical identification of porcine reproductive and respiratory syndrome virus (PRRSV) antigen in the heart and lymphoid system of three-week-old colostrum-deprived pigs. Vet Pathol 32:200–204, 1995

6 Halbur PG, Paul PS, Frey ML, Landgraf J, Eernisse K, Meng XJ, Lum MA, Andrews JJ, Rathje JA: Comparison of the pathogenicity of two U.S. porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet Pathol 32:648–660, 1995

7 Halbur PG, Paul PS, Frey ML, Landgraf J, Eernisse K, Meng XJ, Andrews JJ, Lum MA, Rathje JA: Comparison of the antigen distribution of two U.S. porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet Pathol 33:159–170, 1996

8 Halbur PG, Paul PS, Meng X-J, Lum MA, Andrews JJ, Rathje JA: Comparative pathogenicity of nine U.S. porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a 5-week-old cesarean-derived-colostrum-deprived pig model. J Vet Diagn Invest 8:11–20, 1996

9 Meulenberg JJM, Hulst MM, De Meijer EJ, Moonen PLJM, den Besten A, de Kluyver EP, Wensvoort G, Moormann RJM: Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. Virology 192:62–72, 1993

10 Nuovo GL: PCR In Situ Hybridization. Protocols and Applications, 2nd ed. Raven Press, New York, NY, 1994

11 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, Zestra T, de Boer EA, Tibben HJ, de Jong MF, van't Veld P, Groenland GJR, van Gennep JA, Voets MT, Verheijden JHM, Braamskamp J: Mystery swine disease in The Netherlands: isolation of Lelystad virus. Vet Q 3:121–130, 1991

12 Yoon K-J, Zimmerman JJ, Swenson SL, McGinley MJ, Eernisse KA, Brevik A, Rhinehart LL, Frey ML, Hill HT, Platt KB: Characterization of the humoral immune response to porcine reproductive and respiratory syndrome (PRRS) virus infection. J Vet Diagn Invest 7:305–312, 1995