JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1997, p. 264–267 Vol. 35, No. 1
0095-1137/97/$04.00+0

Typing of Porcine Reproductive and Respiratory Syndrome Viruses by a Multiplex PCR Assay

S. A. GILBERT,[1] R. LAROCHELLE,[2] R. MAGAR,[2] H. J. CHO,[1] AND D. DEREGT[1]*

Animal Diseases Research Institute, Agriculture and Agri-Food Canada, Lethbridge, Alberta,[1] and Laboratoire d'hygiène vétérinaire et alimentaire, Agriculture et Agroalimentaire Canada, St-Hyacinthe, Québec,[2] Canada

Received 15 July 1996/Accepted 16 October 1996

A rapid multiplex PCR assay was developed to distinguish between North American and European genotypes of porcine reproductive and respiratory syndrome (PRRS) virus after a portion of the polymerase gene (open reading frame 1b) was sequenced for two North American PRRS virus strains. DNA products with unique sizes characteristic of each genotype were obtained.

Porcine reproductive and respiratory syndrome (PRRS) is a new viral disease of swine first observed in the United States in 1987 and in Europe in 1990 (3, 9, 28). PRRS is characterized by reproductive failure through abortion, stillbirth, and mummified fetuses; the birth of weak piglets; and severe respiratory disease in newborn and nursing pigs (7).

PRRS virus is a member of the arteriviruses, a group of small, enveloped, positive-strand RNA viruses (4, 19, 24). The virus has a genome of approximately 15 kb containing 8 open reading frames (ORFs) (19). ORFs 1a and 1b encode the polymerase, and ORFs 5 to 7 encode the major structural proteins of the virion (20). Antigenic and subsequent genetic analyses of PRRS viruses isolated from North America and Europe have revealed clear differences between viruses originating on the two continents (5, 8, 11, 12, 14, 15, 17, 18, 21–23, 27). Although the complete genomic sequence has only been reported for the Lelystad virus (LV) (19), a European strain, the existence of two genotypes is evident from the comparison of sequences of ORFs 2 to 7 of European and North American PRRS virus strains. These ORFs or their predicted proteins show sequence homologies of only about 50 to 70%, the exception being the ORF 6 product, which demonstrates close to 80% homology (8, 11, 14, 15, 17, 18, 21, 22).

Serological evidence suggests that infections with a LV-like PRRS virus (with the presumed European genotype) have occurred in at least 10% of the surveyed PRRS-infected swine farms in the United States (1). Isolation in North America of PRRS viruses with the European genotype, however, has not been reported.

In several studies, American and Canadian isolates have been differentiated from European strains with the use of monoclonal antibodies in immunoperoxidase and immunofluorescent assays (5, 12, 23). The ability to differentiate between the North American and European genotypes of PRRS virus based on the detection of specific nucleic acid sequences is desirable, both for identification of the causative agent in PRRS virus outbreaks and to determine and confirm the ability of monoclonal antibodies to type PRRS viruses. In this report, a multiplex PCR assay for differentiation of European and North American genotypes of PRRS virus is described.

PCR primers were designed on the basis of ORF 1b, as sequences from this gene were expected, because of their conservation among arteriviruses (19), to be more conserved within and between the two PRRS virus genotypes than those of other genes. Initial primers were designed from LV sequences because of the lack of sequence information for North American strains of PRRS virus for this region of the genome. Type-specific and type-common primers for multiplex or nested multiplex PCR (Table 1) were designed after sequencing a portion of ORF 1b from two North American strains of PRRS virus: Minnesota MN-1b (11, 29) and Quebec LHVA-93-3 (13) isolates. Primers were designed by using Primer Designer for Windows, Version 2.0 (Scientific and Educational Software, State Line, Pa.), and synthesized with an Applied Biosystems 391 DNA synthesizer.

Viral RNA was extracted directly from 100 μl of the supernatants from virus-infected, MARC-145 cell (10) or porcine alveolar macrophage cultures by using TRIzol (Canadian Life Technologies Inc., Burlington, Ontario, Canada) and following the manufacturer's protocol with modifications, essentially as described previously (6). RNA was precipitated in isopropanol with 20 μg of glycogen, and the RNA pellet was resuspended in 25 μl of water. Serial 10-fold dilutions of virus in Eagle's minimal essential medium (beginning with 10^5 50% tissue culture infective doses [$TCID_{50}$]/100 μl) were used for RNA extraction to determine the lower limit of detection by PCR. Ten serum samples (100-μl volumes) from two pigs which had been inoculated intranasally at 3 weeks of age with 5×10^6 $TCID_{50}$ of LV or U.S. isolate 89-46448 were also used for RNA extraction and PCR.

Five microliters of the RNA was mixed with 375 ng of random hexamers (Promega, Madison, Wis.) in a total volume of 21.5 μl and incubated at 70°C for 10 min. A reverse transcription mixture was added to the RNA-hexamer solution to give a final volume of 50 μl and final concentrations of 0.4 U of RNAguard RNase inhibitor (Pharmacia, Baie D'Urfe, Quebec, Canada) per μl, 0.5 mM each deoxynucleoside triphosphate (dNTP) (Pharmacia), 50 mM Tris HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, and 10 U of Superscript II reverse transcriptase (Canadian Life Technologies) per μl. The combined mixture was incubated at room temperature for 10 min to promote hexamer annealing, and reverse transcription was carried out at 37°C for 1 h, followed by reaction inactivation at 94°C for 5 min.

A 600-bp portion of ORF 1b of the MN-1b and LHVA-93-3 strains of PRRS virus was amplified for PCR cycle sequencing. Upstream primer SU (5′ CATCCTGGGCACCAACA 3′) and complementary downstream primer SD (5′ GACGGTTTTCT TTGGGTC 3′), corresponding to nucleotides 8412 to 8428 and

* Corresponding author. Mailing address: Animal Diseases Research Institute, Agriculture and Agri-Food Canada, P.O. Box 640, Lethbridge, Alberta, Canada T1J 3Z4. Phone: 403 382 5500. Fax: 403 381 1202.

TABLE 1. Oligonucleotide primers for PCR amplification and typing of PRRS viruses

Primer type and sequence (5′ to 3′)	Position in genome[a]	Size of PCR product (bp)	Type detected
Multiplex (or nested multiplex)			
U1 GTATGAACTTGCAGGATG	8634–8651	186	European
D1 GCCGACAATACCATGTGCTG	8800–8819		
U2 GGCGCAGTGACTAAGAGA	8713–8730	107	North American
D2 GTAACTGAACACCATATGCTG	8799–8819		
External for nested PCR			
EU CCTCCTGTATGAACTTGC	8628–8645	255	Common
ED AGGTCCTCGAACTTGAGCTG	8863–8882		

[a] Nucleotide positions are numbered according to the sequence of LV (19).

9004 to 9021, respectively, of the LV sequence (19), were used for amplification. For PCR, 2.5 μl of cDNA was added to a mixture containing 2 mM $MgCl_2$, PCR buffer (20 mM Tris HCl [pH 8.4], 50 mM KCl), 0.2 mM dNTPs, primers SU and SD (10 μg of each per ml), and 5 U of *Taq* DNA polymerase (Canadian Life Technologies) in a total volume of 100 μl. After denaturation at 94°C for 3 min, the reactions were cycled 4 times at 94°C for 20 s, 42°C for 1 min, and 72°C for 1 min and then 40 times at 94°C for 20 s, 47°C for 1 min, and 72°C for 1 min, with a final extension step of 72°C for 15 min. The PCR product was purified from a 1.5% agarose gel with Geneclean (BIO 101). For PCR cycle sequencing, primers SU and SD, upstream primer U2 (Table 1), and complementary degenerate downstream primer SD2 (5′ TTCTG(G/T)GCATA(A/G)ATTACCAG 3′) or SD3 (5′ GTGCTGTGCATA(A/G)AT(T/C)ACCA 3′), corresponding to nucleotide positions 8785 to 8804 and 8786 to 8805 of LV, respectively, were used. PCR products were fully sequenced in both directions by using the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Mississauga, Ontario, Canada) with 0.25 pmol of template and 1 μM primer. Sephadex G-50 spin columns were used to desalt the sequencing mixture, and the reaction mixtures were vacuum dried and stored at −20°C. Automated fluorescence sequencing was performed with an Applied Biosystems 373A sequencer. The obtained sequence was analyzed with SeqEd 1.0.3 (Applied Biosystems) and MacDNASIS (Hitachi Software Engineering, San Bruno, Calif.) software.

The nucleotide sequences obtained from ORF 1b for North American strains MN-1b and LHVA-93-3 are shown in Fig. 1. The MN-1b and LHVA-93-3 sequences revealed 89% homology (i.e., identity) with each other and 68 and 70% homology with the ORF 1b sequences of LV, respectively (for deduced amino acid sequences, the homologies were 98, 79, and 80%,

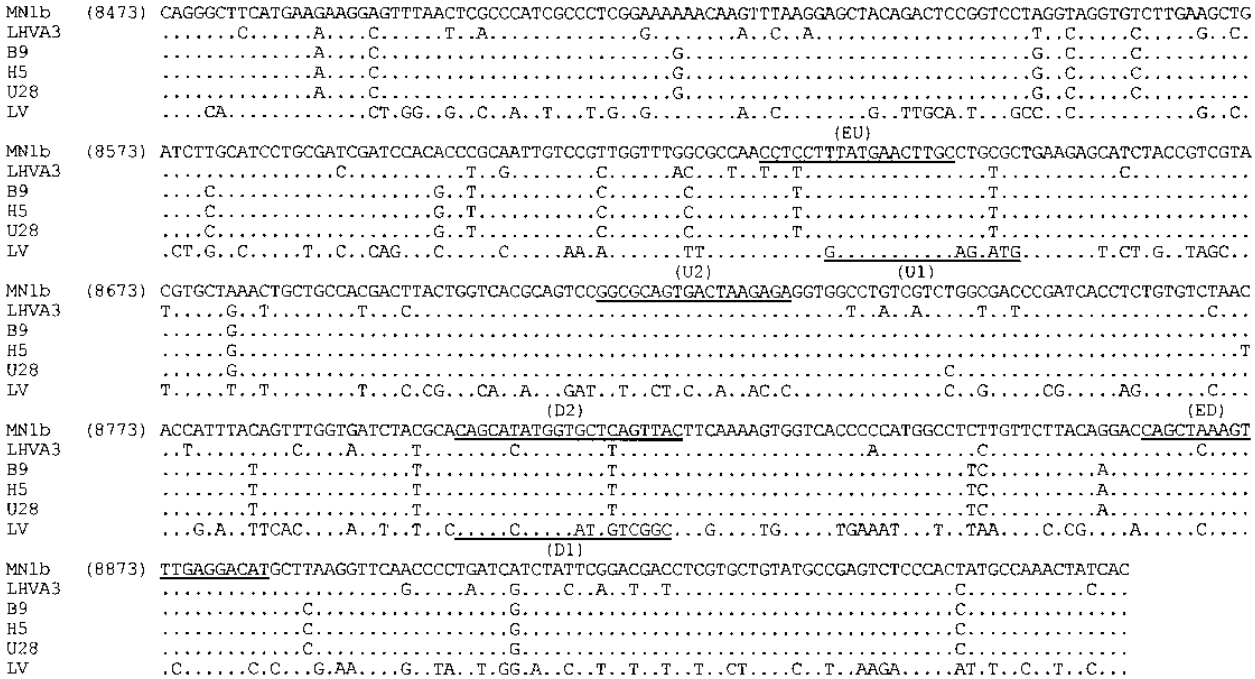

FIG. 1. Comparison of the nucleotide sequences of a portion of the polymerase gene (ORF 1b) of Minnesota strain MN-1b; Quebec strain LHVA-93-3 (LHVA3), Albertan PRRS virus isolates B-9, H-5, and U-28; and the European reference strain, LV (19). Only differences are indicated. The positions of PCR primers listed in Table 1 are underlined.

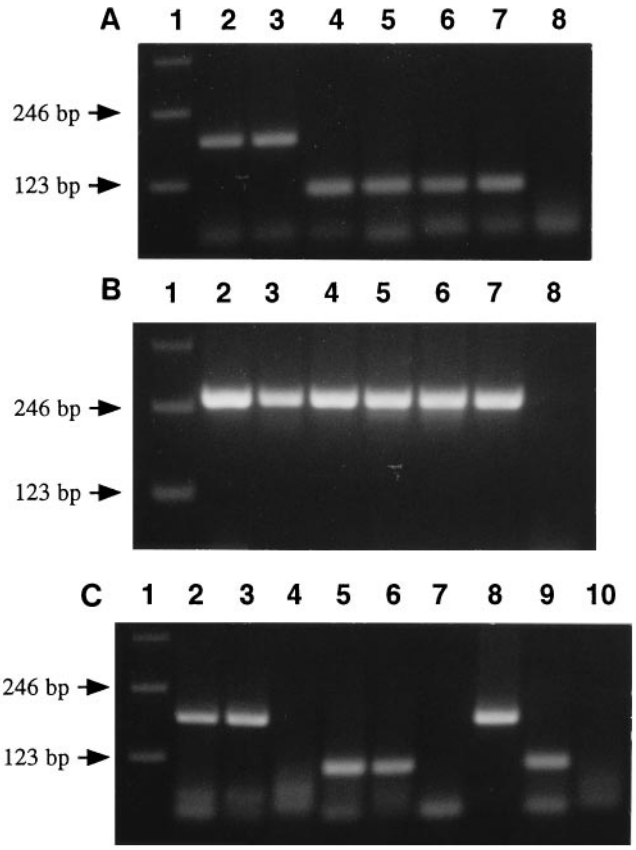

FIG. 2. PCR for PRRS virus typing. (A) Multiplex PCR. Lanes: 2, LV; 3, H1; 4, MN-1b; 5, 89-46448; 6, LHVA-93-3; 7, H-5; 8, negative control (medium from uninfected MARC-145 cell culture). (B) PCR using type-common primers and the same viruses and control as in panel A. (C) Nested multiplex PCR of virus in tissue culture medium. Lanes: 2, 100 $TCID_{50}$ of LV; 3, 10 $TCID_{50}$ of LV; 4, 1 $TCID_{50}$ of LV; 5, 100 $TCID_{50}$ of MN-1b; 6, 10 $TCID_{50}$ of MN-1b; 7, 1 $TCID_{50}$ of MN-1b; 8, serum (collected 25 dpi) containing 400 $TCID_{50}$, from a pig infected with LV; 9, serum (collected 25 dpi) containing 100 $TCID_{50}$, from a pig infected with isolate 89-46448; 10, negative control serum. In all panels, lane 1 contained a 123-bp DNA ladder.

respectively). The nucleotide homology between genotypes was similar to that of the most conserved structural gene, ORF 6 (18). The sequences of the same region for three Albertan PRRS virus isolates (B-9, H-5, and U-28) that were PCR typed are also shown in Fig. 1 (see below).

For multiplex PCR, 2.5 μl of the cDNA was added to a mixture containing 1.25 mM $MgCl_2$, PCR buffer, 0.2 mM dNTPs, the primers (listed in Table 1), U1 and U2 (5 μg of each per ml) and D1 and D2 (2.5 μg of each per ml), and 5 U of *Taq* DNA polymerase in a total volume of 100 μl. After denaturation at 94°C for 3 min, the reactions were cycled 35 times at 94°C for 20 s, 47°C for 30 s, and 72°C for 30 s, with a final extension step of 72°C for 15 min. The final product (10 to 25 μl) was electrophoresed on a 2.5% agarose gel and stained with ethidium bromide. When nested multiplex PCR was performed, type-common primers EU and ED (Table 1; 10 μg of each per ml) were first utilized in a primary PCR with the same conditions and cycle parameters as for the multiplex PCR, except that 1.0 mM $MgCl_2$ was utilized. Two microliters of the PCR product (undiluted or diluted 1/100 in water) was then utilized as the template in the nested multiplex PCR assay.

The magnesium concentration, cycle parameters, and primer concentrations were optimized for the multiplex PCR assay. The primers predicted the amplification of a 186-bp product for LV (European genotype) and a 107-bp product for North American strains MN-1b and LHVA-93-3 (Table 1). By using RNA extracted from the medium of infected cell cultures for reverse transcription and PCR, products consistent with the predicted sizes were obtained for the above reference strains (Fig. 2A). These products were PCR cycle sequenced and confirmed to be PRRS virus specific (data not shown). A total of 21 PRRS virus strains and isolates propagated in cell culture were tested in the multiplex assay. The European viruses had the European genotype, whereas all of the North American viruses had the North American genotype (Fig. 2A and Table 2). No PCR products were obtained from tissue culture samples containing equine arteritis virus, a related arterivirus, or the porcine viruses transmissible gastroenteritis virus, porcine influenza virus, porcine rotavirus type A, and porcine parvovirus (data not shown).

The multiplex PCR assay produced prominent DNA products for different PRRS viruses with titers that ranged from 1×10^4 to 3×10^5 $TCID_{50}$/100 μl. The limit of detection for the assay using RNA extracted from 10-fold dilutions of the MN-1b strain was 1×10^3 $TCID_{50}$ (data not shown).

The ORF 1b region of three Albertan PRRS isolates, B-9, H-5, and U-28, was sequenced to further confirm the ability of the assay to type PRRS viruses. The nucleotide sequences of these isolates, shown in Fig. 1, reveal 95% homology with the sequences of the MN-1b strain (North American genotype), and thus an accurate assignment was made on the basis of the multiplex PCR results (Table 2).

A nested multiplex assay was investigated by utilizing type-common primers EU and ED (Table 1) in a first round of PCR amplification. The resulting PCR product was then used as the template for the nested multiplex PCR assay. The products of the first round of PCR are shown in Fig. 2B. Viruses with both genotypes produced a PCR product of ~255 bp, consistent with the predicted size of the product. The products of the nested assay using virus in tissue culture medium or sera from experimentally infected pigs are shown in Fig. 2C. As shown, the nested multiplex PCR assay could detect 10 $TCID_{50}$, an improvement from the 10^3 $TCID_{50}$ that could be detected by the multiplex PCR assay. From 10 serum samples of two pigs infected with PRRS virus with either genotype, the nested PCR assay produced products of the predicted sizes (Fig. 2C) and showed full agreement with virus isolation. PCR products were obtained from all seven serum samples, collected 7 to 25

TABLE 2. Multiplex PCR of PRRS viruses

Virus origin/name	PCR product size/type[a]
Europe/LV[b], H1[c]	186/E
United States/MN-1b,[d] 89-46448,[e] 93-24025,[e] 93-44927[e]	107/NAm
Quebec and Ontario, Canada/LHVA-92-1, -92-2, -93-3, -93-4, -93-5, -93-6, -94-7	107/NAm
Alberta, Canada/B-9, T-20, T-39, H-5, U-24, U-27, U-28, U-29	107/NAm

[a] Estimated product sizes are in base pairs. E, European; NAm, North American.
[b] Obtained from G. Wensvoort, Central Veterinary Institute, Lelystad, The Netherlands.
[c] Obtained from T. Drew, Central Veterinary Laboratory, New Haw, United Kingdom.
[d] Obtained from H. S. Joo, University of Minnesota.
[e] Obtained from M. L. Frey, National Veterinary Services Laboratories, Ames, Iowa.

days postinfection (dpi), which were positive by virus isolation (100 to 560 $TCID_{50}$), whereas, three serum samples (collected 0 and 7 dpi) were negative for both virus isolation and PCR.

Several PCR assays have been developed for the detection of PRRS virus (2, 16, 25, 26). Most of these methods used primers designed to amplify nucleocapsid protein (ORF 7) sequences. One PCR assay utilized an absence of 37 nucleotides in the 3′ end of the genome of European strains to differentiate between Canadian and European strains (16).

The main advantage of the multiplex PCR assay described herein is the ability to type PRRS virus to one of two genotypes directly from the supernatants of virus-infected cell cultures. All of the viruses propagated in cell culture achieved titers above the detection limit of the multiplex PCR assay. Thus, the assay was sufficiently sensitive that PCR products could be visualized by ethidium bromide staining without the need for additional detection methodology. Combined with a rapid RNA extraction procedure, the multiplex PCR assay was user friendly. The additional steps involved in the nested PCR assay described herein may be required in only a few instances for isolates which propagate to very low titers. The higher sensitivity of the nested version of the multiplex PCR assay was an advantage in that it permitted direct typing of PRRS virus from the porcine serum samples used in this study. The general utility of the nested assay with clinical samples, however, remains to be more thoroughly investigated.

The multiplex PCR assay described herein was rationally designed after the sequencing of a portion of ORF 1b of two North American PRRS virus strains. There was sufficient sequence divergence between the two genotypes for the design of type-specific primers and sufficient homology to design type-common primers for a nested assay. The multiplex PCR assay shows promise for the typing of a wide array of PRRS virus strains.

We acknowledge the excellent technical assistance of W. Orr, I. van Kempen, S. Prins, and S. Entz. We also thank T. Graham for helpful advice and E. Blake for assistance with the preparation of the manuscript.

REFERENCES

1. **Bautista, E. M., S. M. Goyal, and J. E. Collins.** 1993. Serological survey for Lelystad and VR-2332 strains of porcine respiratory and reproductive syndrome (PRRS) virus in US swine herds. J. Vet. Diagn. Invest. **5:**612–614.
2. **Christopher-Hennings, J., E. A. Nelson, J. K. Nelson, R. J. Hines, S. L. Swenson, H. T. Hill, J. J. Zimmerman, J. B. Katz, M. J. Yaeger, C. C. L. Chase, and D. A. Benfield.** 1995. Detection of porcine reproductive and respiratory syndrome virus in boar semen by PCR. J. Clin. Microbiol. **33:** 1730–1734.
3. **Collins, J. E., D. A. Benfield, W. T. Christianson, L. Harris, J. C. Hennings, D. P. Shaw, S. M. Goyal, S. McCollough, R. B. Morrison, H. S. Joo, D. E. Gorcyca, and D. W. Chladek.** 1992. 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.
4. **Conzelmann, K. K., N. Vissner, P. van Woensel, and H.-J. Thiel.** 1993. Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the Arterivirus group. Virology **193:**329–339.
5. **Drew, T. W., J. J. M. Meulenberg, J. J. Sands, and D. J. Paton.** 1995. Production, characterization and reactivity of monoclonal antibodies to porcine reproductive and respiratory syndrome virus. J. Gen. Virol. **76:**1361–1369.
6. **Gilbert, S. A., P. J. Timoney, W. H. McCollum, and D. Deregt.** 1995. Single-step extraction of viral RNA from semen for RT-PCR. Focus **17:**18–19.
7. **Goyal, S. M.** 1993. Porcine reproductive and respiratory syndrome. J. Vet. Diagn. Invest. **5:**656–664.
8. **Katz, J. B., A. L. Shafer, K. A. Eernise, J. G. Landgraf, and E. A. Nelson.** 1995. Antigenic differences between European and American isolates of porcine reproductive and respiratory syndrome virus (PRRSV) are encoded by the carboxy terminal portion of viral open reading frame 3. Vet. Microbiol. **44:**65–76.
9. **Keffaber, K. K.** 1989. Reproductive failure of unknown etiology. Am. Assoc. Swine Practitioners Newsl. **2:**1–10.
10. **Kim, H. S., J. Kwang, I. J. Yoon, H. S. Joo, and M. L. Frey.** 1993. Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogeneous subpopulation of MA-104 cell line. Arch. Virol. **133:**477–483.
11. **Kwang, J., H. S. Kim, and H. S. Joo.** 1994. Cloning, expression, and sequence analysis of the ORF 4 gene of porcine reproductive and respiratory syndrome virus MN-1b. J. Vet. Diagn. Invest. **6:**293–296.
12. **Magar, R., R. Larochelle, S. Dea, C. A. Gagnon, E. A. Nelson, J. Christopher-Hennings, and D. A. Benfield.** 1995. Antigenic comparison of Canadian and US isolates of porcine reproductive and respiratory syndrome virus using monoclonal antibodies to the nucleocapsid protein. Can. J. Vet. Res. **59:** 232–234.
13. **Magar, R., Y. Robinson, C. Dubuc, and R. Larochelle.** 1995. Isolation and experimental oral transmission in pigs of a porcine reproductive and respiratory syndrome virus isolate, p. 139–144. *In* P. J. Talbot and G. A. Levy (ed.), Corona- and related viruses: current concepts in molecular biology and pathogenesis. Plenum Press, New York, N.Y.
14. **Mardassi, H., S. Mounir, and S. Dea.** 1994. Identification of major differences in the nucleocapsid protein genes of a Quebec strain and European strains of porcine reproductive and respiratory syndrome virus. J. Gen. Virol. **75:**681–685.
15. **Mardassi, H., S. Mounir, and S. Dea.** 1995. Molecular analysis of ORFs 3 to 7 of porcine reproductive and respiratory syndrome virus, Quebec reference strain. Arch. Virol. **140:**1405–1418.
16. **Mardassi, H., L. Wilson, S. Mounir, and S. Dea.** 1994. Detection of porcine reproductive and respiratory syndrome virus and efficient differentiation between Canadian and European strains by reverse transcription and PCR amplification. J. Clin. Microbiol. **32:**2197–2203.
17. **Meng, X.-J., P. S. Paul, and P. G. Halbur.** 1994. Molecular cloning and nucleotide sequencing of the 3′-terminal genomic RNA of the porcine reproductive and respiratory syndrome virus. J. Gen. Virol. **75:**1795–1801.
18. **Meng, X.-J., P. S. Paul, P. G. Halbur, and M. A. Lum.** 1995. Phylogenetic analyses of the putative M (ORF 6) and N (ORF 7) genes of porcine reproductive and respiratory syndrome virus (PRRSV): implication for the existence of two genotypes of PRRSV in the U.S.A. and Europe. Arch. Virol. **140:**745–755.
19. **Meulenberg, J. J. M., M. M. Hulst, E. J. de Meijer, P. L. J. M. Moonen, A. den Besten, E. P. de Kluyer, G. Wensvoort, and R. J. M. Moormann.** 1993. Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS) is related to LDV and EAV. Virology **192:**62–74.
20. **Meulenberg, J. J. M., A. Petersen-den Besten, E. P. de Kluyver, R. J. M. Moormann, W. M. M. Schaaper, and G. Wensvoort.** 1995. Characterization of proteins encoded by ORFs 2 to 7 of Lelystad virus. Virology **206:**155–163.
21. **Morozov, I., X.-J. Meng, and P. S. Paul.** 1995. Sequence analysis of open reading frames (ORFs) 2 to 4 of a U.S. isolate of porcine reproductive and respiratory syndrome virus. Arch. Virol. **140:**1313–1319.
22. **Murtaugh, M. P., M. R. Elam, and L. T. Kakach.** 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.
23. **Nelson, E. A., J. Christopher-Hennings, T. Drew, G. Wensvoort, J. E. Collins, and D. A. Benfield.** 1993. Differentiation of U.S. and European isolates of porcine reproductive and respiratory syndrome virus by monoclonal antibodies. J. Clin. Microbiol. **31:**3184–3189.
24. **Plagemann, P. G. W., and V. Moennig.** 1991. Lactate dehydrogenase-elevating virus, equine arteritis virus, and simian hemorrhagic fever virus: a new group of positive-strand RNA viruses. Adv. Virus Res. **41:**99–192.
25. **Suarez, P., R. Zardoya, C. Prieto, A. Solana, E. Tabares, J. M. Bautista, and J. M. Castro.** 1994. Direct detection of the porcine reproductive and respiratory syndrome (PRRS) virus by reverse polymerase chain reaction (RT-PCR). Arch. Virol. **135:**89–99.
26. **Van Woensel, P., J. van der Wouw, and N. Visser.** 1994. Detection of porcine reproductive respiratory syndrome virus by the polymerase chain reaction. J. Virol. Methods **47:**273–278.
27. **Wensvoort, G., E. P. de Kluyer, E. A. Luijtze, A. den Besten, L. Harris, J. E. Collins, W. T. Christianson, and D. Chladek.** 1992. Antigenic comparison of Lelystad virus and swine infertility and respiratory syndrome (SIRS) virus. J. Vet. Diagn. Invest. **4:**134–138.
28. **Wensvoort, G., C. Terpstra, J. M. A. Pol, E. A. ter Laak, M. Bloemraad, E. P. de Kluyer, C. Kragten, L. van Buiten, A. den Besten, F. Wagenaar, J. M. Broekhuijsen, P. L. J. M. Moonen, T. Zetstra, E. A. de Boer, H. J. Tibben, M. F. de Jong, P. van't Veld, G. J. R. Groenland, J. A. van Gennep, M. T. H. Verheijden, and J. Braamskamp.** 1991. Mystery swine disease in the Netherlands: the isolation of Lelystad virus. Vet. Q. **13:**121–130.
29. **Yoon, I. J., H. S. Joo, W. T. Christianson, H. S. Kim, J. E. Collins, J. H. Carson, and S. A. Dee.** 1992. Isolation of a cytopathic virus from weak pigs on farms with a history of swine reproductive and respiratory syndrome. J. Vet. Diagn. Invest. **4:**139–143.