Detection of Porcine Circovirus from Lesions of a Pig with Wasting Disease in Japan

Atsushi ONUKI, Keiichi ABE, Katsuhiro TOGASHI[1)], Kenji KAWASHIMA[2)], Atsushi TANEICHI and Hiroshi TSUNEMITSU[2)]

Yamagata Central Livestock Hygiene Service Center, 736 Urushiyama, Yamagata 990–2161, [1)]Shonai Livestock Hygiene Service Center, Amarume, Yamagata 999–7781 and [2)]Shichinohe Research Unit, National Institute of Animal Health, Shichinohe, Aomori 039–2586, Japan

(Received 26 April 1999/Accepted 22 June 1999)

ABSTRACT. A wasting disease characterized by progressive weight loss and dyspnea has been observed in weaning pigs on a farm in Yamagata Prefecture in 1998. Histopathologic findings in an affected pig were bronchointerstitial pneumonia and intracytoplasmic clusters of basophilic inclusions in macrophages of lymph nodes, which were similar to those in pigs with postweaning multisystemic wasting syndrome (PMWS) recently reported in North America and Europe. Porcine circovirus (PCV)-like particles were observed in bronchial lymph node of the pig by electron microscopy, and PCV antigens were detected in the lesions by immunohistochemical staining. PCV DNA was also detected in the lung and tonsil by PCR, and restriction fragment length polymorphism analysis of the PCR products with *Hin*fI showed the same type of the PCV associated with PMWS (pmws PCV). Homology of nucleotide sequences between the PCR product and corresponding regions of published pmws PCV genomes was very high. These results indicated that virus detected in this study was pmws PCV. To our knowledge, this is the first report on the presence of pmws PCV in Japan.—KEY WORDS: immunohistochemistry, PCR, PMWS, porcine circovirus.

J. Vet. Med. Sci. 61(10): 1119–1123, 1999

Porcine circovirus (PCV) was originally detected as a noncytopathic contaminant of porcine kidney (PK15) cell line [17]. PCV has been classified into a new virus family called the *Circoviridae* [10], on the basis of its circular single-stranded DNA genome [15]. PCV as PK15 cells contaminant was nonpathogenic for experimentally infected pigs [16].

Postweaning multisystemic wasting syndrome (PMWS) in pigs was first reported in western Canada in 1991 [2]. It was characterized by progressive weight loss, respiratory signs, and jaundice. Gross and histologic lesions were granulomatous interstitial pneumonia, lymphadenopathy, and lymphocytic, granulomatous hepatitis and nephritis. Similar syndromes have been described in pigs from the United States [3] and Europe [1]. PCV antigen and nucleic acid have been demonstrated in tissues of pigs with PMWS [1, 4]. PCV from PMWS (pmws PCV) was antigenically and genetically distinguished from nonpathogenic PCV (np PCV) [1, 6]. These findings have led to speculation that a new or modified virulent PCV may have participated in those lesions in pigs with PMWS.

This report describes the detection of PCV from tissues of a pig with a wasting disease in Yamagata Prefecture, Japan, and compares the partial length of this PCV genome to the published corresponding sequences from pmws PCV and np PCV.

MATERIALS AND METHODS

Clinical samples: A wasting disease has been observed in weaning pigs on a farm in Yamagata Prefecture since May of 1998. About 20 of 400 weaning pigs showed progressive weight loss and dyspnea, followed by death. Samples of lung, liver, kidney, heart, intestine, brain, tonsil, spleen, bronchial and mesenteric lymph nodes, and tracheal swab were collected from an affected pig aged 50 days in this farm. These samples were examined for bacteria by routine methods. Virus isolation from the samples was carried out using porcine alveolar macrophage cultures as described previously [19]. The samples were also examined for porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus and hog cholera virus by PCR [8, 12, 18].

Pathological Examination: At necropsy, the tissues were fixed in 10% buffered formalin and embedded in paraffin wax by standard techniques. Four to five μm thick sections were stained with haematoxylin and eosin (HE), and also used in immunohistochemistry.

Immunohistochemistry: Hyperimmune rabbit antiserum to np PCV (CCL-33) as PK15 cells contaminant was kindly provided by Dr. Imada, National Institute of Animal Health, Japan. Porcine immune serum (kindly provided by Dr. Haritani, National Institute of Animal Health) was from a healthy pig from a herd in which PMWS had been diagnosed by histopathologic examination (unpublished data). This serum was biotinylated with a commercial kit according to the manufacturer's instructions (Pharmacia Biotech, Ltd.). Immunohistochemical identification of PCV was performed with a streptavidin-biotin-complex immunoperoxidase kit (Nichirei Co.) as previously described [4]. Briefly, after removal of paraffin and rehydration, sections were incubated with either rabbit anti-PCV serum (diluted 1:500) or biotinylated porcine immune serum (diluted 1:16). Sections stained with rabbit anti-PCV serum were then incubated with biotinylated goat anti-rabbit IgG. Following reaction with biotinylated sera, these sections were reacted with a

streptavidin-biotin-complex immunoperoxidase reagent and with 3,3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Ltd.). The sections were then counterstained with methyl green. Tissue sections from non-infected gnotobiotic pigs and sera from a non-immunized rabbit and a non-infected gnotobiotic pig were used for control purpose.

Electron microscopy: Transmission electron microscopic examination of bronchial lymph node of the pig was performed using negative staining and ultrathin section. For negative staining, the tissue was frozen and thawed at −70°C (3 cycles), homogenized in 50 mM Tris buffer containing 100 mM NaCl and 1 mM $CaCl_2$ (TBS; pH 7.5), and sonicated in an ice bath. Cell debris was removed by centrifuging the lysate at 1,812 × g for 10 min and then at 7,730 × g for 20 min. The supernatant was transferred to an ultracentrifuge tube and centrifuged at 57,600 × g for 2 hr. The pellet was then resuspended in TBS. This suspension was applied to a carbon-coated collodion grid for 10 min, and negatively stained with 2% phosphotungstic acid. For ultrathin section, formalin-fixed tissue of the bronchial lymph node was refixed with 1% osmium tetroxide for 2 hr, dehydrated in alcohol, and enbedded in Epon mixture. The ultrathin section was stained with 2% uranyl acetate and lead citrate.

DNA extraction, PCR amplification, and sequencing: Viral genomic DNA was extracted from lung and tonsil of the pig and from PK15 cells with a commercial DNA isolation kit (SepaGene, Sanko Junyaku Co., Ltd.). PCR for detection of PCV was conducted with the primers reported by Hamel *et al.* [6]. The primer pair N1f and N4r resulted in 815 bp and 802 bp PCR products of pmws PCV and np PCV genes, respectively, which were located in the open-reading-frame 1 coding for the Rep proteins [6]. The PCR was performed as follows. A mixture of 2 μl of sample DNA, 1 μl each of primer (100 pmol), 77 μl of water and 19 μl of the reagent mixture of Takara Ex Taq (Takara Shuzo Co.) prepared according to the instructions of the supplier was subjected to 35 amplification cycles, each cycle consisting of 30 sec denaturation, 30 sec annealing and 30 sec elongation steps at temperatures of 94, 58 and 72°C, respectively. In the first cycle, the denaturation step continued for 5 min at 94°C. The PCR was concluded with a final elongation step of 7 min at 72°C. The amplified products were separated by electrophoresis on 2% agarose gels and visualized with UV light after staining with ethidium bromide. The PCR products were also digested with restriction enzyme *Hin*fI for 1 hr at 37°C, and analyzed by electrophoresis.

The PCR product from the lung tissue was sequenced directly with these PCR primers by cycle sequencing with an auto sequencer (ABI PRISM 377; Perkin-Elmer Applied Biosystems). The DDBJ accession number for the PCR product is AB025276.

RESULTS

Pathological and microbiological examination: The lung was noncollasped, rubbery and edematous. Histologically, the characteristic lesion in the lung was bronchointerstitial pneumonia. The lesion included filling of alveolar septa and bronchioles with macrophages, edema fluid, inflammatory cells and debris. In some lobules, purulent exudate was predominant in alveolar lumen. Bronchial and mesenteric lymph nodes were enlarged. Small necrotic areas were seen in follicles of the lymph nodes. In these areas, intracytoplasmic clusters of basophilic inclusions were seen in macrophages (Fig. 1). *Streptococcus suis* type 1 was isolated from lung and tracheal swab. PRRSV was isolated from lung in alveolar macrophage cultures, and also detected from lung, brain and tonsil by PCR. Other viral and bacterial agents were not detected.

Immunohistochemistry: Immunohistochemical identification using porcine immune serum was attempted. PCV antigens agreed with basophilic cytoplasmic inclusions in bronchial lymph node, mesenteric lymph node and spleen (Fig. 3). PCV antigen was also observed in the lung. Immunohistochemical identification using hyperimmune rabbit antiserum in the tissue showed faint positive antigens agreeing with basophilic cytoplasmic inclusions (Fig. 2).

Electron microscopy: Circovirus-like particles (17 nm in diameter) were observed in the extract of bronchial lymph node by negative staining (Fig. 4). In ultrathin sections of the bronchial lymph node, the inclusions seen on light microscopy were composed of electron-dense granules approximate 20 nm in diameter that formed crystalline arrays (Fig. 5).

PCR amplification and genetic characterization: PCR products of the expected sizes were detected in both lung and tonsil of the affected pig and in PK15 cells (Fig. 6).

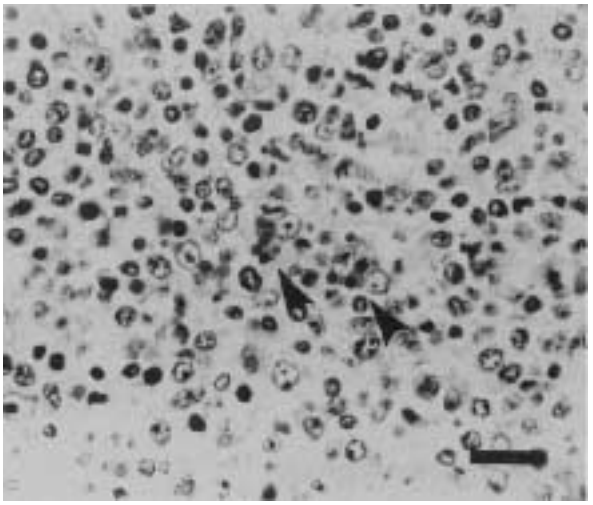

Fig. 1. Bronchial lymph node of a pig with wasting disease. Macrophages in lymphoid follicle are distended with globular intracytoplasmic inclusion bodies (arrowhead). Haematoxylin and eosin. Bar=25 μm.

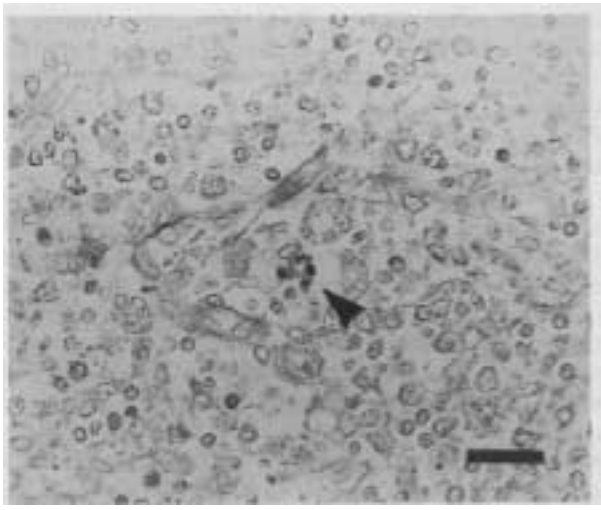

Fig. 2. Circoviral antigen in accordance with intracytoplasmic inclusion bodies in macrophages in bronchial lymph node of a pig with wasting disease (arrowhead). Immunohistochemical staining using anti-PCV rabbit serum. Bar=25 μm.

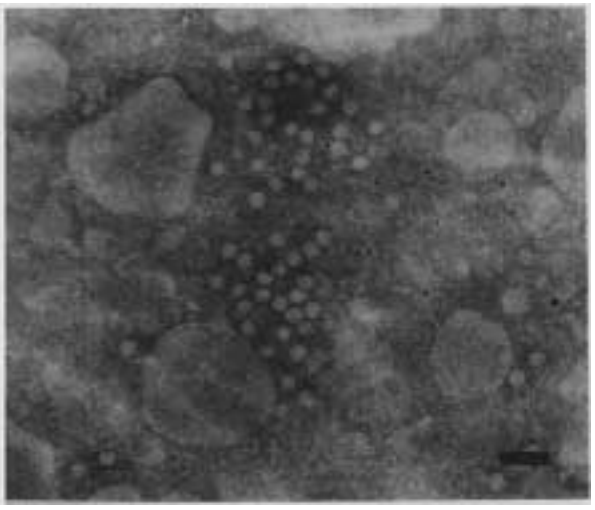

Fig. 4. Electron micrograph of small circovirus-like particles in homogenate of bronchial lymph node of a pig with wasting disease. Negative staining. Bar=50 nm.

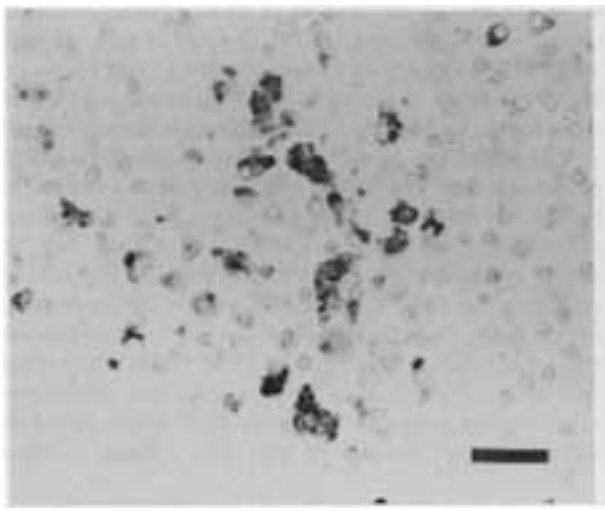

Fig. 3. Positive cytoplasmic staining for circovirus in macrophages in bronchial lymph node of a pig with wasting disease. Immunohistochemical staining using porcine immune serum. Bar=25 μm.

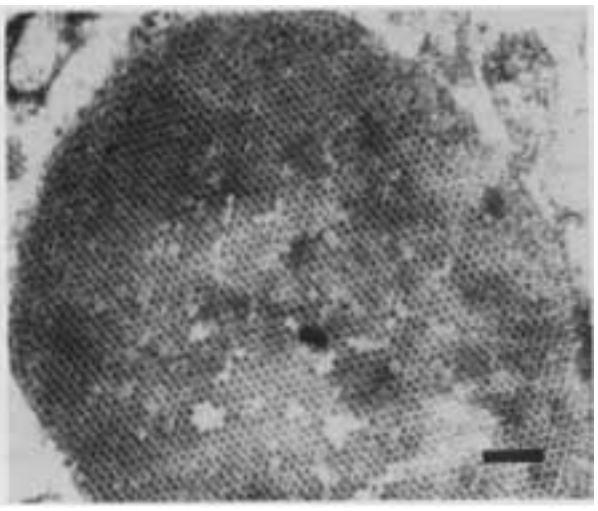

Fig. 5. Electron micrograph showing crystalline arrays of circovirus-like particles in cytoplasmic inclusions in bronchial lymph node of a pig with wasting disease. Uranyl acetate and lead citrate staining. Bar=100 nm.

The PCR products from both tissues were digested with restriction enzyme *Hin*fI into 2 fragments, 437 bp and 378 bp (Fig. 6). All of 13 pmws PCV genomes published in the GenBank database had the same cleavage site with *Hin*fI as determined by computer program. By contrast, the PCR product from PK15 cells was not digested with this enzyme (Fig. 6). In this region, *Hin*fI had no recognition site in all 3 published np PCV genomes.

The 766 bp of the PCR product from the lung sample was sequenced and compared with the corresponding regions of 13 pmws PCV and 3 np PCV genomes. Nucleotides identity of the detected PCV, designated the Yamagata strain, was 95.7–97.7% to pmws PCV strains, and 80.5–80.9% to np PCV. Deduced amino acid (aa) identity of the Yamagata PCV (246 aa) was 95.5–99.6% and 88.9–89.3% to pmws PCV (246 aa) and np PCV (243 aa), respectively. Figure 7 showed aa alignments of the Yamagata and one of each pmws PCV and np PCV. There was a potential glycosylation site located at aa 23 to 25 in the Yamagata and pmws PCV, and at 20 to 22 aa in np PCV.

DISCUSSION

PMWS appeared to be an emerging disease problem in

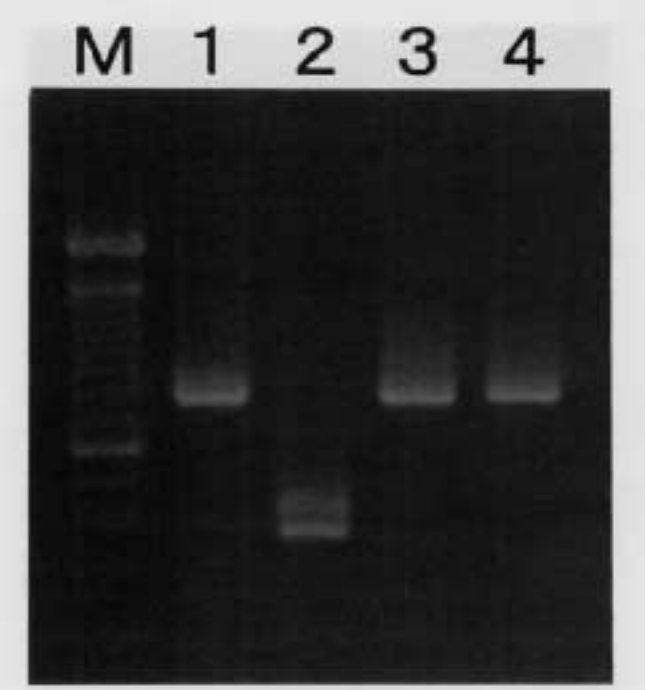

Fig. 6. PCR detection of PCV DNA in lung of a pig with wasting disease and in PK15 cells. PCR products were treated with restriction enzyme *Hin*fI (lanes 2 and 4) and visualized on 2% agarose gel stained with ethidium bromide. Lanes: M, molecular weight marker; 1, lung of the affected pig; 2, lung of the affected pig (digested with *Hin*fI into 2 fragments, 437 bp and 378 bp); 3, PK15 cells; 4, PK15 cells (treated with *Hin*fI).

North American and European swine herds [1–3]. Several papers reported that PCV was detected from PMWS-affected pigs. PCV from PMWS was distinguished from np PCV both antigenically and genetically [1, 4, 6]. Serological surveys using indirect immunofluorescence assays indicated that antibodies to PCV were very common in North American and European swine herds [9, 16]. In Japan, although antibodies to PCV have been found in approximately 30% of pigs (T. Imada, personal communication), it is difficult to determine whether these antibodies in pigs were produced by np PCV or by pmws PCV infection due to the fact that np PCV and pmws PCV share common antigens [1, 4].

In the present case, a wasting disease was observed in a swine herd. PCV antigens were detected by immunohistochemistry, and PCV-like particles were detected by electron microscopy. PCV gene was detected in the lung and tonsil by PCR, and restriction fragment length polymorphism analysis with the PCR products showed the same type of pmws PCV. Homology of nucleotide sequences between the PCR product and the corresponding regions of the published pmws PCV genomes were very high. These results indicated that the PCV detected in this study was pmws PCV. To our knowledge, this is the first report on the presence of pmws PCV in Japan.

On the basis of detection of viral antigens and particles in the bronchial and mesenteric lymph nodes, follicular necrosis with intracellular inclusion bodies was presumed to be induced by replication of PCV. These lymphoid lesions in this pig were similar to those of pigs affected with PMWS reported in Canada and the United States [1, 4]. However, emaciation and dyspnea, which are characterized by PMWS, are also prominent clinical features of PRRS-affected pigs [14]. Pathologically, interstitial pneumonia and lymphadenopathy are also common to PMWS and PRRS. Thus, at least some clinical signs and pathological findings in PMWS and PRRS may be indistinguishable. Further, PRRSV was also detected in some previous PMWS-affected [7, 13] and the present pig. On the basis, the etiology of the present wasting disease may be concurrent infections of PCV, PRRSV and other infectious agents including *Streptococcus suis*. Moreover, experimental infection of pmws PCV alone failed to reproduce the disease in pigs [5]. Thus, a clear

```
1   MPSKKNGRSGPQPHKRWVFTLNNPSEDERKKIRELPISLFDYFIVGEEGNEEGRTPHLQGFANFVKKQTFNKVKWYFGAR  Yamagata PCV
1   ..............................................................L...  pmws PCV
1   .....---.................E.KN.............VC....L.............A.....  np PCV

81  CHIEKAKGTDQQNKEYCSKEGNLLIECGAPRSQGQRSDLSTAVSTLLESGSLVTVAEQHPVTFVRNFRGLAELLKVSGKM  Yamagata PCV
81  .Y.............................................................................  pmws PCV
78  .....................HI........N..K.............T.........F...Y.....................  np PCV

161 QKRDWKTNVHVIVGPPGCGKSKWAANFADPETTYWKPPRNKWWDGYHGEEVVVIDDFYGWLPWDDLLRLCDRYPLTVETK  Yamagata PCV
161 .............................................................................  pmws PCV
158 .Q.....A.............Q..R...E.RD.....S................L.........................  np PCV

241 GGTVPF                                                                        Yamagata PCV
241 ......                                                                        pmws PCV
238 ......                                                                        np PCV
```

Fig. 7. Alignments of amino acid (aa) sequences (243 or 246 aa) of a part of ORF 1 in the Yamagata PCV (DDBJ accession no. AB025276), pmws PCV (GenBank accession no. AF027217 [6]), and np PCV (GenBank accession no. U49186 [11]).

understanding of the pathogenesis of the detected PCV requires further investigation, such as epidemiological studies.

REFERENCES

1. Allan, G. M., McNeilly, F., Kennedy, S., Draft, B., Clarke, E. G., Ellis, J. A., Haines, D. M., Meehan, B. M. and Adair, B. M. 1998. Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. *J. Vet. Diagn. Invest.* 10: 3–10.
2. Clark, E. G. 1997. Post-weaning multisystemic wasting syndrome. *Proc. Am. Assoc. Swine. Pract.* 28: 499–501.
3. Daft, B., Nordhausen, R. W., Latimer, K. S. and Niagro, F. D. 1996. Interstitial pneumonia and lymphadenopathy associated with circoviral infection in a six week-old piglet. *Proc. Am. Assoc. Vet. Lab. Diagn.* 39: 32.
4. Ellis, J., Hassard, L., Harding, J., Allan, G., Willson, P., Strokappe, J., Martin, K., McNeilly, F., Meehan, B., Todd, D. and Haines, D. 1998. Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. *Can. Vet. J.* 39: 44–51.
5. Ellis, J., Krakowka, S., Lairmore, M., Haines, D., Bratanich, A., Clark, E., Allan, G., Konoby, C., Hassard, L., Meehan, B., Martin, K., Harding, J., Kennedy, S. and McNeilly, F. 1999. Reproduction of lesions of postweaning multisystemic wasting syndrome in gnotobiotic piglets. *J. Vet. Diagn. Invest.* 11: 3–14.
6. Hamel, A. L., Lin, L. L. and Nayar, G. P. S. 1998. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. *J. Virol.* 72: 5262–5267.
7. Harding, J. C. 1997. Post-weaning multisystemic wasting syndrome (PMWS): preliminary epidemiology and clinical presentation. *Proc. Am. Assoc. Swine. Pract.* 28: 503.
8. Hennings, J. C., Nelson, E. A., Nelson, J. K., Hines, R. J., Swenson, S. L., Hill, H. T., Zimmerman, J. J., Katz, J. B., Yaeger, M. J., Chase, C. C. L. and Benfield, D. A. 1995. Detection of porcine reproductive and respiratory syndrome virus in boar semen by PCR. *J. Clin. Microbiol.* 33: 1730–1734.
9. Hines, R. K. and Lukert. P. D. 1995. Porcine circovirus: a serological survey of swine in the United states. *Swine. Health. Prod.* 3: 71–73.
10. Lukert, P., de Boer, G. F., Dale, J. L. *et al*. 1995. The circoviridae. pp. 166–168. *In*: Virus Taxonomy, 6th Report of the International Committee on Taxonomy of Viruses (Murphy, F. A., Fauquet, C. M. and Bishop, D. H. L., eds.), Springer-Verlag, New York.
11. Meehan, B. M., Creelan, J. L., McNulty, M. S. and Todd, D. 1997. Sequence of porcine circovirus DNA : affinities with plant circovirus. *J. Gen. Virol.* 78: 221-227.
12. Molitor, T. W., Oraveerakul, K., Zhang, Q. Q., Choi, C. S. and Ludemann, L. R. 1991. Polymerase chain reaction (PCR) amplification for the detection of porcine parvovirus. *J. Virol. Methods* 32: 201–211.
13. Morozov, I., Sirinarumitr, T., Sorden, S. D., Halbur, P. G., Morgan, M. K., Yoon, K-J. and Paul, P. S. 1998. Detection of a novel strain of porcine circovirus in pigs with postweaning multisystemic wasting syndrome. *J. Clin. Microbiol.* 36: 2535–2541.
14. Rossow, K. D. 1998. Porcine reproductive and respiratory syndrome. *Vet. Pathol.* 35: 1–20.
15. Tischer, I., Gelderblom, H., Vetterman, W. and Koch M. A. 1982. A very small porcine virus with circular single-stranded DNA. *Nature (Lond.)* 295: 64–66.
16. Tischer, I., Mields, W., Wolff, D., Vagt, M. and Griem, W. 1986. Studies on epidemiology and pathogenicity of porcine circovirus. *Arch. Virol.* 91: 271–276.
17. Tischer, I., Rasch, R. and Tochtermann, G. 1974. Characterization of papovavirus and picornavirus-like particles in permanent piglet kidney cell lines. *Zentralbl. Bakteriol. Orig. A.* 226: 153–167.
18. Vilček, Š., Herring, A. J., Herring, J. A., Nettleton, P. F., Lowings, J. P. and Paton, D. J. 1994. Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. *Arch. Virol.* 136: 309–323.
19. Wensvoort, G., Terpstra, C., Pol, J. M. A., Ter Laak, E. A., Bloemraad, M., De Kluyver, E. P., Kragten, C., Van Buiten, L., Den Besten, A., Wagenaar, F., Broekhuijsen, J. M., Moonen, P. L. J. M., Zetstra, T., De Boer, E. A., Tibben, H. J., De Jong, M. F., Van't Veld, P., Groenland, G. J. R., Van Gennep, J. A., Voets, M. Th., Verheijden, J. H. M. and Braamskamp, J. 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. *Vet. Quart.* 13: 121–130.