Science in China Series C: Life Sciences

© 2008 SCIENCE IN CHINA PRESS

Springer

www.scichina.com
life.scichina.com
www.springerlink.com

Complete nucleotide sequence of a novel porcine circovirus-like agent and its infectivity *in vitro*

WEN LiBin[1], HE KongWang[1†], YANG HanChun[2†], NI YanXiu[1], ZHANG XueHan[1], GUO RongLi[1] & PAN QunXin[1]

[1] Key Laboratory of Animal and Poultry Diseases Diagnostic of the Ministry of Agriculture, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China;

[2] Key Laboratory of Preventive Veterinary Medicine of Ministry of Agriculture, College of Veterinary Medicine, China Agricultural University, Beijing 100094, China

A novel agent (hence termed as P2) was isolated from pig sera in China, which contained covalently bound circular genomic DNAs of 993 nucleotides. Sequence analyses indicated that the agent was closely related to the porcine circovirus (PCV). The molecular clone of P2 was constructed subsequently and used for the following studies. Intracytoplasmic inclusions and intranuclear inclusions were only found in PK-15 cells transfected with the tandem dimer of P2 molecular DNA clone. Intracytoplasmic inclusions were round or irregular in shape and 0.1—0.4 μm in diameter, and intranuclear inclusions were electronically denser than intracytoplasmic inclusions and had two general shapes: round/small (0.1 μm in diameter) and hexagonal/large (0.5—1.4 μm in diameter). The inclusions were not membranously bound. The cells transfected with the tandem dimer of P2 molecular DNA clone were tested positive for P2 DNA at passages 5. The P2 antigen could be detected in both transfected and passaged PK-15 cells. This is the first report regarding the complete nucleotide sequence of a small DNA genome in a circovirus-like infectious agent *in vitro*.

P2, porcine circovirus, molecular clone, PK-15 cells

Porcine circovirus (PCV) contains circular, single-stranded DNA genome[1−3] and belongs to a newly constituted family of *Circoviridae*, the smallest known autonomously replicating viruses, pathogens of plants, birds and swine. Two genotypes of PCV (PCV1 and PCV2) have been identified. PCV1 was originally isolated as a contaminant of a porcine kidney cell line[4]. Serologic surveys have indicated that PCV1 is common in swine, but causes no clinical diseases, and is thus considered nonpathogenic to pigs[5,6]. Meanwhile, PCV2 is considered the causative agent of postweaning multisystemic wasting syndrome (PMWS) which has been reported throughout the world[9]. It primarily affects pigs between 5 and 18 weeks of age, characterized by progressive weight loss or dyspnea, unthriftiness, enlarged lymph nodes, pallor, jaundice, and diarrhea[10,11]. Thus, PMWS casts serious impacts on the swine industry worldwide.

PCV2 may require other factors or agents to induce a full spectrum of clinical signs and lesions of PMWS. In this article, we reported the complete DNA sequence and additional sequence analyses of a novel porcine circovirus-like agent isolated from pig sera by polymerase chain reaction (PCR). Meanwhile, to rescue the agent from the limited serum and avoid interference of the other swine agents in serum, we constructed a molecular DNA cloning of the agent in order to acquire a biologi-

Received August 26, 2007; accepted December 19, 2007
doi: 10.1007/s11427-008-0053-5
†Corresponding author (email: hekongwang@yahoo.com; yanghanchun1@cau.edu.cn)
Supported by the State Key Basic Research Project (973 project) of China (Grant No. 2007CB116308), Planned Projects for Postdoctoral Research Funds of Jiangsu Province, China (Grant No. 5910602) and Postdoctoral Funds of Jiangsu Academy of Agricultural Sciences, China (Grant No. 6510501)

cally pure agent for further studies. We also reported here that the agent DNA clone containing two tandem copies genomes was infectious when transfected with PK-15 cells.

1 Materials and methods

1.1 The isolation of porcine circovirus-like agent (P2)

In our diagnosis, we found that the positive rate for PCV2 detection in PCR was enhanced by the amplification of PCV2 ORF2 rather than the amplification of PCV2 ORF1 or complete PCV2 genome (data not shown). We therefore suspected that the agents having similar sequence to that of PCV2'S ORF2 were present in porcine sera. Based on the published PCV2 ORF2 sequence, a reverse PCR was performed using a pair of primers that could specifically amplify the ORF2 region of the genome in samples. PCR products were cloned into the pMD18-T vector (TaKaRa Biotechnology Co. Ltd. China) and sequenced at a commercial facility (Invitrogen Biotechnology Co., Ltd. China). The sequences of fragments generated in PCR were then assembled into a consecutive sequence using DNAMAN software (Version 5.2.2, Lynnon Biosoft, 1994). Multiple alignments of nucleotides and amino acid sequences were also done using DNAMAN software. The following representatives of PCV2[12] and PCV1 with their accession numbers were used in this study: PCV2 (CHN-2A, AF381175; CHN-2B, AF381176; CHN-2C, AF381177; CHN-2D, AY181948; CHN-2F, AY181947; CHN-2G, AF538325); PCV1, Y09921.

A total of 278 serum samples were collected from unthrifty pigs (ranging from 1 to 3 months old) with multi-systemic lesions and clinical signs of PMWS in Hebei, Beijing, Shandong and Jiangsu from 2002 to 2006. The serum samples were used for DNA extraction and PCR.

1.2 Molecular cloning of the P2 genome

A pair of PCR primers (5′- TGA GGA TCC ACT AGT AAC GGC CGC -3′ and 5′- AGT GGA TCC TCA TTT AGG GTT TAA GTG -3′) were designed to amplify the complete genome of P2, including a overlapping region containing *Bam*HI restriction enzyme site. The steps of the amplification reaction were briefly described: pre-denaturation at 94℃ for 5 min, followed by 35 cycles of denaturation at 94℃ for 45 s, annealing at 56℃ for 45 s, extension at 72℃ for 1 min, and a final extension at 72℃ for 10 min. The PCR products with the expected size were separated by gel electrophoresis and purified with AxyPrep™ DNA Gel Extraction Kit (AXYGEN Bio, USA).

The PCR product containing complete P2 genomes was first introduced into the pMD18-T vector, and then transformed into *Escherichia coli* DH5α competent cells. The recombinant plasmids underwent PCR, restriction enzyme digestion and DNA sequencing. The full-length P2 genomic DNA strand was excised from the pMD18-T vector by digestion of *Bam*H I restriction enzyme and then cloned into the pBluescript SK (pSK) vector (Stratagene). The molecular clone contained a single copy of the P2 genome and was ligated with T4 DNA ligase (Promega) at 22℃ for 3 h, which favored sticky-end ligations to form a tandem dimer. The tandem dimer was subsequently cloned into the pSK vector. Recombinant plasmids containing a single genomic copy and tandem dimers of P2 were confirmed by PCR, restriction enzyme digestion and DNA sequencing. The DNA concentration of the recombinant plasmids was determined spectrophotometrically.

1.3 Cell

PK-15 cell lines were grown at 37℃ in RPMI 1640 (GIBCO™, USA) supplemented with 10% heat-inactivated fetal calf serum and 5% CO_2. PK-15 cells that were free of PCV1, PCV2, mycoplasma and retroviruses were confirmed by PCR or RT-PCR.

1.4 Transfection

PK-15 cells seeded into 6-well culture plates were grown to approximately 85% confluency. The transfection of recombinant plasmid DNA (4.0 μg) was carried out with a commercially available lipofectamine™ 2000 reagent (Invitrogen) in the presence of Opti-MEM® I medium. The DNA/lipofectamine mixture (0.5 mL) was dispensed into each culture that was freshly rinsed with Opti-MEM® I medium. After incubation for 5 h at 37℃, the DNA/lipofectamine mixture was replaced with RPMI 1640 supplemented with 10% fetal calf serum. PK-15 cells transfected with empty pSK vector and the untransfected cells were tested as control.

1.5 Transmission electron microscopy (TEM) examination

PK-15 cells seeded in 6-well culture plates were harvested at 72 h post-transfection. All cells and parts of the

culture medium were scraped into a 1.5-mL eppendorf tube. The eppendorf tubes were centrifuged at 2000 r/min for 10 min and the supernatant was decanted. Ten different volumes of phosphate-buffered glutaraldehyde (2.5%) were added gently to the tube to prefix the samples. After dehydration, the cell pellet was postfixed in osmium tetroxide (1%) for 60 min at 4℃. Postfixed pellet were embedded in Epon812, and four-micrometer-thick sections were stained with a mixture of lead citrate and uranyl acetate following routine methods. Sections were observed and photographed using a JEM-1200EX TEM.

1.6 Preparation of P2 inoculum

The transfected cells were harvested as "virus passages 1" at 3 days posttransfection, while the untransfected cells were also harvested. In order to isolate P2 DNAs from the infected cells, the cells were frozen and thawed three times and then centrifuged. The supernatant of every generation was passaged in PK-15 cells. The cells harvested at various passages were biologically characterized and used for PCR analyses and immunochemical staining.

1.7 PCR analysis

To ensure that P2 DNA was not synthesized by the transfected plasmids, complete genomic sequences of the virus at passages 5 were determined by PCR. The forward primer was 5′- TTC ACA CCC AAA CCT GTT CTT GAC TCC ACT -3′ (nucleotide positions 768-797), and the reverse primer was 5′- TTT GGG TGT GAA GTA ACG GGA GTG GTA GGA -3′ (nucleotide positions 779-750). The steps of the reaction were as follows: 35 cycles of denaturation at 94℃ for 45 s; annealing at 58℃ for 45 s; and extension at 72℃ for 45 s. The PCR was finished with a final extension step at 72℃ for 10 min. The PCR products were sequenced to confirm the identity.

1.8 Immunochemical staining

PK-15 cells seeded in 24-well culture plates were rinsed with PBS twice, and then submerged in a PBS solution containing 40% acetone and 0.2% BSA (–20℃) for 15 min, blocked out with 30% hydrogen peroxide and methanol (1:50) and dried for 30 min at room temperature. The cells were then incubated with anti-PCV2 serum diluted with binding buffer (0.01% Tween 20 and 0.5 mol/L NaCl in PBS) for 1 h at room temperature. The cells were washed twice with PBS containing 0.05% Tween 20 (PBSW), and then incubated with Protein G conjugated with horseradish peroxidase (1:100) (Wuhan boster biological technology Ltd. China) for 30 min, and finally rinsed with PBSW twice. Color development was carried out with 3-amino-9-ethylcarbazole (AEC). Viral antigens were stained brown in this assay.

To determine the infectivity titers of P2, the PK-15 cells were infected with a 10-fold serial dilution of P2. After 3 days of incubation, the infected cells were determined by immunochemical staining. Viral infectivity titers were calculated using the Kärber method.

2 Results

A novel agent (designated as P2) containing high homologous sequences of ORF2 of PCV2 was determined from 4 samples originated from two pig farms in Hebei and Jiangsu provinces. The complete genome of P2 is suggested to be a circular stranded DNA by using inverse PCR and 993 nucleotides (nt) in size (The nucleotide sequence date reported in this manuscript has been deposited in GenBank database under the accession number of EF514717) (Figure 1).

P2 shared approximately 32.9%—35.4% sequence similarity in nucleotide sequence with other PCV2 strains, and 31.2% with PCV1. The five ORFs that could potentially encode proteins on either strand of P2 were listed here: ORF1, 7.1 kDa (nt 94—289); ORF2, 3.0 kDa (nt 412—499); ORF3, 27.7 kDa (nt 288—987); ORF4, 3.9 kDa (nt 497—392); ORF5, 6.6 kDa (nt 186—3). The largest P2 ORF (ORF3) shared 64.7%—97.4% similarity in amino acid sequence with ORF2 of other PCV isolates.

Only PK-15 cells transfected with the tandem dimer of P2 molecular DNA clone had intracytoplasmic inclusions (ICI) and intranuclear inclusions (INI), while inclusions were not present in non-tranfected PK-15 cells, empty pSK vector or PK-15 cells transfected with a single copy genome of P2 agent. ICI were not membranously bound and distributed in the cytoplasm with various electronically density. Their shape varied from round to irregular, and ranged from 0.1—0.4 μm in diameter. They were granular with no visible virions (Figure 2). INI had 2 general types: the small inclusions that were round and about 0.1 μm in diameter and the larger inclusions which were hexagonal and 0.5—1.4 μm in

```
  1 ggatccacta gtaacggccg ccagtgtgct ggaattaatt cgctgtctgc gagggccagc
 61 tgttggggtg agtactccct ctcaaaagcg ggcatgactt ctgcgctaag attgtcagtt
121 tccaaaaacg aggaggattt gatattcacc tggcccgcgg tgatgccttt gagggtggcc
181 tcgtccatct ggtcagaaaa gacaatcttt ttgttgtcaa gcttgttaat acgactcact
241 atagggagac ccaagcttgg taccgagctc ggatcgatat ctcagctatg acgtatccaa
301 ggaggcgtta ccgcagaaga agacaccgcc cccgcagcca tcttggccag atcctccgcc
361 gccgcccctg gctcgtccac ccccgccacc gctaccgttg gagaaggaaa aatggcatct
421 tcaacacccg cctctcccgc accttcggat atactgtcaa ggctaccaca gtcagaacgc
481 cctcctgggc ggtggacatg atgagattta atattgacga ctttgttccc ccgggagggg
541 ggaccaacaa aatctctata ccctttgaat actacagaat aagaaaggtt aaggttgaat
601 tctggccctg ctcccccatc acccagggtg ataggggagt gggctccact gctgttattc
661 tagatgataa ctttgtacca aaggccacag cccaaaccta tgacccatat gtaaactact
721 ccacccgcca tacaatcccc caacccttct cctaccactc ccgttacttc acacccaaac
781 ctgttcttga ctccactatt gattacttcc aaccaaataa caaaaggaat cagctttggc
841 tgaggataca aacctctgga aatgtagacc acgtaggcct cggcactgcg ttcgaaaaca
901 gtatatacga ccaggactac aatatccgtg taaccatgta tgtacaattc agagaattta
961 atcttaaaga ccccccactt aaaccctaaa tga
```

Figure 1 Genomic nucleotide sequence of P2 agent.

diameter. Both of them were more electronically dense than ICI. Both types of INI were closely associated with reticulated nucleoli (Figures 3 and 4).

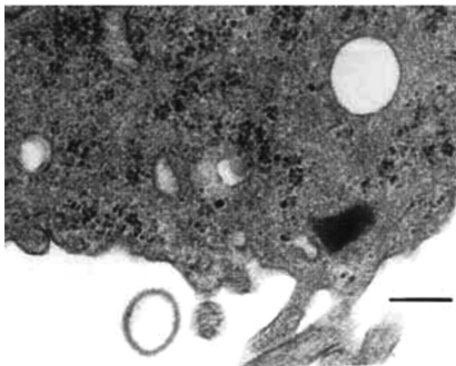

Figure 2 PK-15 cell transfected with P2 molecular clone. An electronically dense irregular inclusion body is near the cell membrane. Methods: uranyl acetate and lead citrate. Bar = 200 nm.

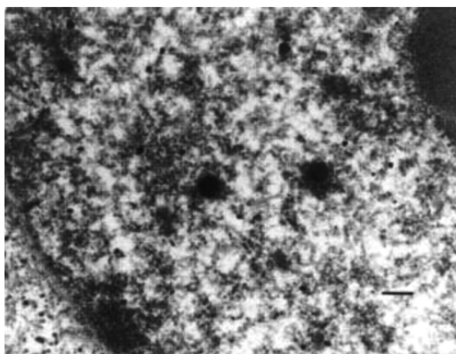

Figure 3 PK-15 cell transfected with P2 molecular clone. The small, round, more electronically dense intranuclear inclusions. Methods: uranyl acetate and lead citrate. Bar = 200 nm.

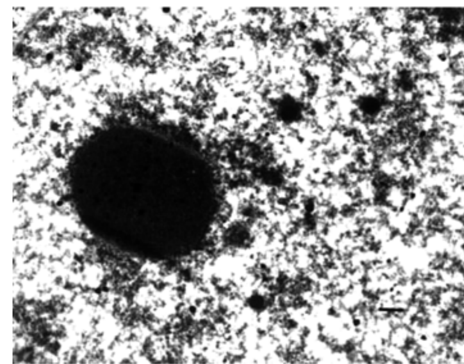

Figure 4 PK-15 cell transfected with P2 molecular clone. A large, hexagonal, more electronically dense intranuclear inclusion is within a nucleolus. Methods: uranyl acetate and lead citrate. Bar =200 nm.

No visible macroscopic changes or cytopathogenic effects were observed in the serial passaged PK-15 cells. P2 DNA was detected in the fifth generation of cell cultures, which were transfected with the tandem dimer of P2 molecular DNA clone (Figure 5).

An experiment was set up to investigate whether P2 proteins were expressed in transfected and serial passaged PK-15 cells. The cells were immunochemically stained with PCV2-specific antiserum. The results showed that only PK-15 cells transfected with the molecular DNA clone contained a tandem dimer of P2 agent and such serial passaged cultures were stained (Figure 6). The infectivity titer of P2 agent at passages 5 was $10^{3.5}$ 50% tissue culture infective doses.

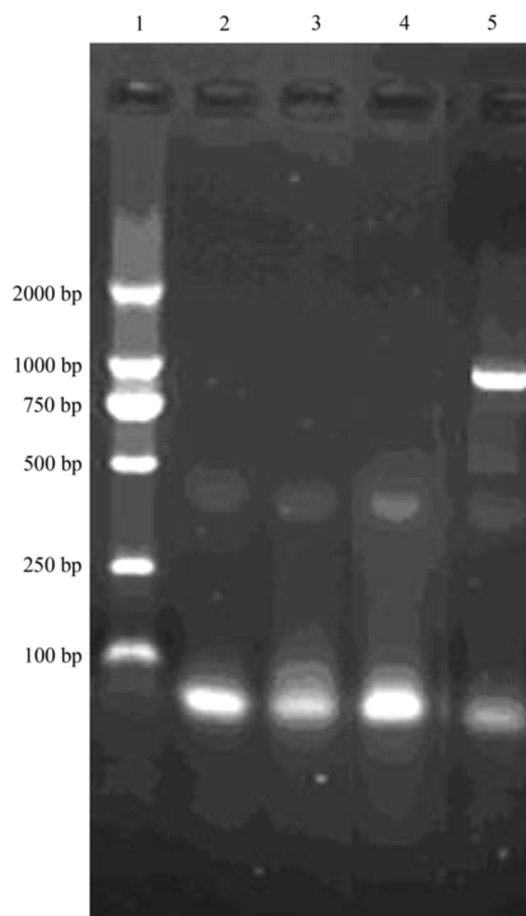

Figure 5 Detection of P2 DNA from PK-15 cell samples at passages 5 by PCR. PCR amplification of about 1000-bp fragment from cell samples transfected with the molecular DNA clone containing a tandem dimer of P2 agent (lanes 5) compared with samples negative, which transfected with a molecular DNA clone containing a single copy genome of P2 agent, empty pSK vector and untransfected (lanes 2, 3, 4). Lane 1, DL-2000 DNA ladder marker.

3 Discussion and conclusion

P2 with small circular DNA genome is structurally similar to circoviruses, and we suspected that P2 might be a member of *Circoviridae*, although all circoviruses have circular DNA genomes. However, there were few similarities at the levels of nucleotide sequence or protein between P2 and other members of *Circoviridae*, except for PCV. Therefore, P2 was suggested to associate more closely with PCV2 based on their similar nucleotide sequences, and P2, PCV1 and PCV2 might have evolved from a common ancestor. Based on ORF1 sequence analyses, PCV was believed to be an evolutionary link between the geminiviruses and the plant circoviruses[13], and also regarded as a product of recombination between a plant nanovirus and an animal picorna-like RNA virus[14]. P2 agent could further facilitate our understanding on the evolutionary history of PCV.

Like PCV2 molecular clone[15], only P2 tandem dimer molecular clones could replicate in PK-15 cells, which might be explained by the rolling-circle mechanism. In order to test the infectivity of molecular DNA clone *in vitro*, passaged and transfected PK-15 cells were applied to P2 morphology, DNA and antigen studies. This was the first report on the ultrastructure of PK-15 cells that were transfected with P2 molecular clone. PK-15 cells transfected with P2 had contained ICI and INI which were similar to PK-15 cells infected by PCV[16]. Unlike the large ICI described in PCV-infected PK-15 cells, ICI in PK-15 cells transfected with P2 molecular clone were round or irregular in shape and non-membranously bound, which resembled the small ICI as described above. ICI in PK-15 cells transfected with the P2 molecular clone composed of granular material and which might be the site for virion assembly or maturation. Unlike the ICI in PCV-infected PK-15 cells, the icosahedral virions aggregated loosely or in paracrystalline arrays, which was not observed in our study.

The INI observed ultrastructurally in PK-15 cells transfected with the P2 molecular clone were more electronically dense and hexagonally shaped, which could be distinguished from those described in PCV-infected PK-15 cells where INI were indistinct particles, finely granular and ring-shaped.

Newly synthesized P2 DNA was studied by PCR. The results indicated that PK-15 cell cultures transfected with the molecular DNA clone containing a tandem dimer of P2 were infectious and could infect normal PK15 cells. After infection, the virus replications were detectable in the fifth generation of the cell cultures.

Likewise, P2 antigens were detected by immunochemical staining in PK-15 cells transfected with the molecular DNA clone containing the tandem dimer of P2 and serial passages. Since P2 was a novel agent, no anti-P2 serum was available at present. Considering P2 ORF3 of P2 shared high similarity in the -amino acid sequences with ORF2 of other PCV2 isolates, we have used anti-PCV2 serum for a trial and the results showed it was feasible.

To our knowledge, P2 possessed small genomic DNA described to date. Much is not yet clear about P2, but the successfully constructed infectious DNA clone of P2 agent will provide fundamental materials for further studies. PMWS is one of the serious diseases in swine industry. We hope P2 described here will be useful for

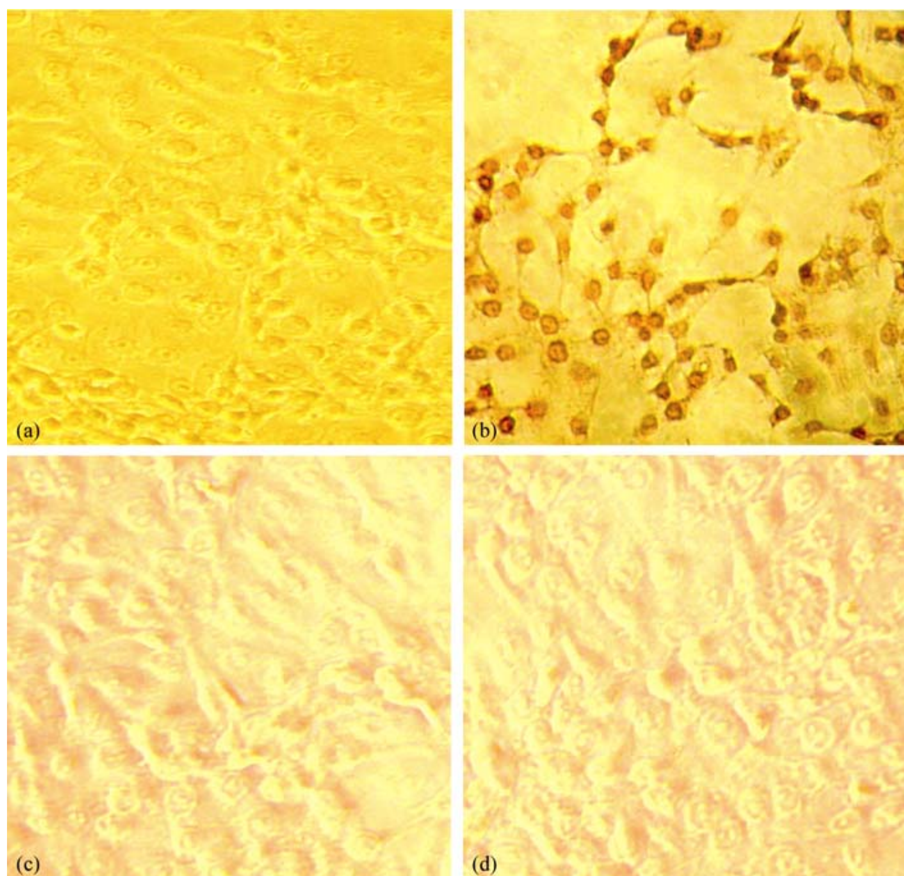

Figure 6 Detection of P2 protein expression by immunochemical staining of transfected and/or serial passaged cultures with PCV2 serum. (a) The molecular DNA clone containing a single copy genome of P2 agent; (b) the molecular DNA clone containing a tandem dimer of P2 agent; (c) empty pSK vector; (d) PK-15 cells.

improving our understanding of the PMWS and its diagnostic procedures.

We gratefully acknowledge the help of Dr. JIANG shanJun and ZHENG XiaoGang in TEM preparation and photography and Prof. HAN JiuQuan and YE RongLing in checking manuscripts.

1 Gelderblom H, Kling S, Lurz R, et al. Morphological characterization of chicken anaemia agent (CAA). Arch Virol, 1989, 109: 115－120 [DOI]
2 Ritchie B, Niagro F, Lukert P, et al. Characterization of a new virus from cockatoos with psittacine beak and feather disease. Virology, 1989, 171: 83－88 [DOI]
3 Tischer I, Gelderblom H, Vettermann W, et al. A very small pocine virus with circular single-stranded DNA. Nature, 1982, 295: 64－66 [DOI]
4 Tischer I, Rasch R, Tochtermann G. Characterizationof papovavirus and picornavirus-like particles in permanent pig kidney cell lines. Zentralbl Bakteriol Hyg A, 1974, 226: 153－167
5 Allan G M, McNeilly F, Cassidy J, et al. Pathogenesis of porcine circovirus: Experimental infections of colostrum deprived piglets and examination of pig fetal material. Vet Microbiol, 1995, 44: 49－64 [DOI]
6 Tischer I, Mields W, Wolff D, et al. Studies on epidemiology and pathogenicity of porcine circovirus. Arch Virol, 1986, 91: 271－276 [DOI]
7 Clark E G. Pathology of the post-weaning multisystemic wasting syndrome of pigs. Proc West Can Assoc Swine Pract, 1996: 22－25
8 Harding J C. Post-weaning multisystemic wasting syndrome (PMWS): Preliminary epidemiology and clinical presentation. Proc West Can Assoc Swine Pract, 1996: 21
9 Allan G M, Ellis J A. Porcine circoviruses: A review. J Vet Diagn Invest, 2000, 12: 3－14
10 Ellis J, Hassard L, Clark E, et al. Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. Can Vet J, 1998, 39: 44－51
11 Harding J C, Clark E, Strokappe J, et al. Postweaning multisystemic wasting syndrome: Epidemiology and clinical presentation. Swine Health Prod, 1998, 6: 249－254
12 Wen L B, Guo X, Yang H C. Genotyping of porcine circivirus type 2 from a variety of clinical conditions in China. Vet Microbiol, 2005, 110: 141－146 [DOI]
13 Niagro F D, Forsthoefel A N, Lawther R P, et al. Beak and feather disease virus and porcine circovirus genomes: Intermediates between the geminiviruses and plant circovirus. Arch Virol, 1998, 143: 1723－1744 [DOI]
14 Gibbs M J, Weiller G F. Evidence that a plant virus switched hosts to infect a vertebrate and then recombined with a vertebrate-infecting virus. Proc Natl Acad Sci USA, 1999, 96: 8022－8027 [DOI]
15 Fenaux M, Halbur P G, Haqshenas G, et al. Cloned genomic DNA of type 2 porcine circovirus is infectious when injected directly into the liver and lymph nodes of pigs: Characterization of clinical disease, virus distribution, and pathologic lesions. J Virol, 2002, 76: 541－551 [DOI]
16 Stevenson G W, Kiupel M, Mittal SK, et al. Ultrastructure of porcine circovirus in persistently infected PK-15 cells. Vet Pathol, 1999, 36: 368－378 [DOI]