J Microbiol Immunol Infect 2010;43(2):85–92

Contents lists available at ScienceDirect

Journal of Microbiology, Immunology and Infection

Journal homepage: http://www.e-jmii.com

Original Article

Fast Diagnosis and Quantification for Porcine Circovirus Type 2 (PCV-2) Using Real-Time Polymerase Chain Reaction

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BACKGROUND/PURPOSE: The postweaning multisystemic wasting syndrome, caused by the porcine circovirus type 2 (PCV-2), is a major disease that poses a significant threat to the global swine industry. The purpose of this study was to establish a real-time polymerase chain reaction (PCR) method for the quantification of PCV-2 and to enable the rapid differentiation of porcine circoviruses type 1 and 2 (PCV-1 and PCV-2). Such a method would significantly speed up the process of clinical diagnosis, and could also be used to study the pathogenic mechanisms of diseases associated with PCV-2.

METHODS: Multiplex real-time PCR, together with LightCycler PCR data analysis software, was used for the quantification of PCV-2, and for the rapid differentiation of PCV-1 and PCV-2. A 263-bp DNA fragment was amplified from the 3' end of the open reading frame-2 of PCV-2 by nested PCR, and its DNA sequence was verified as having 100% identity with a PCV-2 standard (NCBI accession number: AF055394). The 263-bp DNA fragment was cloned into the pGEM-T easy vector, and the recombinant plasmid was serially diluted and quantified using real-time PCR. A standard curve was then constructed for quantification of the PCV-2 levels in field samples. The differentiation of PCV-1 and PCV-2 was carried out by analyzing the melting temperatures of the genotype-specific PCR products.

RESULTS: To quantify the PCV-2 levels in field samples, a standard curve (1×10^2–1×10^9 copies/μL) was constructed. PCV-2 concentrations as low as 1×10^2 copies/μL could be detected in specimens taken from the lymph nodes or infected tissues in samples of PCV-2-infected pigs. The diagnosis of PCV-1 and PCV-2 infections and the quantification of the viral load in the field samples could be completed within 45 minutes after extracting the viral DNA using a commercial extraction kit.

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Article History:
Received: March 10, 2009
Revised: May 1, 2009
Accepted: Jun 11, 2009

CONCLUSION: This study demonstrate that real-time PCR is a clinically feasible method for the accurate quantification of PCV-2, and for the rapid differentiation of PCV-1 and PCV-2.

KEYWORDS: circovirus typing, multiplex real-time PCR, porcine circovirus type 2

Introduction

Porcine circovirus (PCV) was first identified by Tischer et al in 1974 as a non-cytopathic contaminant of the pig kidney cell line, PK-15 (ATCC CCL33), and was described as a Picornaviridae-like virus.[1] In 1982, PCV was further characterized by Tischer's group as a single stranded DNA virus with a circular genome having a diameter of about 17 nm. Since it was originally detected in porcine cells, and its antibodies have been detected in pig sera, it was named porcine circovirus[2] and was classified in 1993 by the International Committee on Taxonomy of Virus as a member of the virus family *Circoviridae*.

In 1986, Tischer et al utilized indirect immunofluorescence assays to determine the presence of porcine circovirus type 1 (PCV-1) antibody in serum collected from pigs between 1979 and 1982 at different slaughterhouses in Germany, and reported a PCV-1 antibody-positivity rate of 77–95%.[3]

In 1991, the postweaning multisystemic wasting syndrome (PMWS) was first reported in Canada.[4] The clinical signs of the disease include progressive weight loss and dyspnea. Gross pathologic findings associated with porcine circovirus type 2 (PCV-2) include granulomatous interstitial pneumonia, lymphadenopathy and, less frequently, hepatitis and nephritis.[5] Since 1991, PMWS in pigs has been reported in North America,[6] Europe,[7] and Asia.[8] In the late 1990s, PCV was linked to PMWS.[6,9]

In 1995, Tischer et al used enzyme-linked immunosorbent assays to recheck the presence of PCV-1 antibody in the serum of pigs from the different slaughterhouses in Germany and found that, while the PCV-1 antibody positive rate dropped to 35% overall, it was 95% in postpartum sows, suggesting the accumulation of antibodies with increasing age.[10]

In 1999, Allan et al isolated PCV from tissues taken from PMWS-infected pigs from Spain, Denmark, and Northern Ireland, and determined their antigenicity by indirect immunofluorescence assays using polyclonal and monoclonal antibodies prepared against previously isolated PCVs.[11] These PCV isolates were found to be both antigenically and genomically similar to previously reported isolates of PCV from PMWS-infected pigs, but were distinct from the PCV isolates from the PK-15 cell lines. Sequence analysis of the PMWS-associated PCV revealed that it was antigenically and genomically different from the earlier described PCV derived from persistently infected PK-15 cell lines.[12,13] The nonpathogenic type was designated as PCV-1 and the pathogenic PMWS-associated PCV was designated as PCV-2. PCV-2 has been identified as the cause of PMWS.[13,14]

In 2000, Magar et al tested the serum of pigs collected in Canada in 1985, 1989, and 1997, and found an antibody-positive rate of 28% for PCV-1 and 49% for PCV-2; confirming that both PCV-1 and PCV-2 existed as early as 1985.[15] In the same year, Allan et al extended the immunofluorescence assays test to detect PCV-2 antibodies in the serum of cows, goats, and humans; however, the result was negative.[16]

Also in 2000, Larochelle et al detected PCV-2 DNA in boar semen using nested PCR after intranasal inoculation of PCV-2. This suggests that PCV-2 can be transmitted through semen.[17] In the same year, Allan et al isolated the virus from aborted swine fetuses and stillborn piglets, thus demonstrating vertical transmission of PCV-2 from dam to piglet. Post-mortem examination revealed pathologic abnormalities such as congestive heart failure and diffuse myocarditis.[16]

In 2001, Sanchez et al inoculated PCV-2 into the uteri of sows, and demonstrated the ability of the virus to replicate in fetuses at different stages of gestation, and to cause pathologic abnormalities in fetuses including stillbirths.[18]

In 2002, Rovira et al tried to determine whether PMWS could be mimicked by inoculating healthy pigs with porcine reproductive and respiratory syndrome virus (PRRSV), PCV-2, or with both PRRSV and PCV-2.[19] Inoculated pigs were subjected to histopathology, serology analysis, and

necropsy up to 32 days post-inoculation to detect the presence of antibodies and clinical signs of PMWS in the lesions. The techniques used for the detection of PCV-2 and PRRSV included *in situ* hybridization, PCR, quantitative PCR and reverse-transcription (RT)-PCR. They found that infection with PCV-2 subsequent to PRRSV infection might have caused immunosuppression, and potentiated the replication and distribution of the PCV-2 virus because more PCV-2 than expected was detected in the lung, liver, kidneys, and lymph nodes of the infected pigs. As a consequence, the virus nucleotide content was found to be much higher in the lesions of the PRRSV/PCV-2 infected pigs than that found in the lesions of PCV-2-infected pigs. Likewise, the symptoms of wasting and respiratory disease were more severe in the cases of PRRSV/PCV-2 infection.[19]

A variety of diagnostic tests for PCV-2 were recently reviewed by Opriessnig et al.[20] Conventional methods used for detecting PCV-2 in cases of co-infection include *in situ* hybridization,[14,21,22] which is complex and time-consuming, and antibody-based immunohistochemistry methods,[23] which are also time consuming. Neither is as sensitive as molecular diagnostic methods, such as PCR for post-mortem specimens.

Most of the reported real-time PCR methods were used in cases where PCV-2 was the only virus present,[24,25] or where PCV-2 was co-infected with either porcine parvovirus,[26,27] or PRRSV.[28]

Fenaux et al reported the differentiation of PCV-1 and PCV-2 using a universal PCR-restriction fragment length polymorphism (PCR-RFLP) diagnostic assay.[6] This method requires restriction enzyme digestion analysis following the conventional PCR amplification of the PCV-1 or PCV-2-specific DNA fragments.

Larochelle et al used multiplex PCR to differentiate PCV-1 and PCV-2.[29] This method operates on the same principle as ours, except that a 349-bp PCV-1-specific DNA fragment (as opposed to our 74-bp fragment) was amplified, and that gel electrophoresis was used to differentiate the PCV-specific PCR products.

The possibility of mixed infection with PCV-1 and PCV-2 has already been reported[30] (and is common on pig farms in Taiwan), as has the concurrent presence of both PCV-1 and PCV-2 in the same herd.[31] Therefore, it is necessary to develop a fast and direct method for the simultaneous detection and differentiation of PCV-1 and PCV-2 to avoid erroneous diagnosis during epidemiological outbreaks. Therefore, the aim of this study was to develop an improved multiplex real-time PCR method for the rapid diagnosis and differentiation of PCV-1 and PCV-2 in cases of co-infection.

Methods

Extraction of viral DNA

Viral DNA was extracted from homogenates of clinical tissue samples using the "High Pure" Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

PCR primers

Primers were designed using the Universal Probe Library software (Roche Diagnostics). For PCV-1, primers UPL-F: 5′-GTG GCG GGA GGA GTA GTT AAT-3′ (1,277–1,297 bp) and UPL-R: 5′-TCT TGG ATG CCA ACT TTG TAA C-3′ (1,350–1,329 bp) were used and these primers were designed to amplify a 74-bp PCV-1-specific DNA fragment from standard PCV-1 (PK15 cell line; ATCC CCL33). A locked nucleic acid probe #22: 5′-TGG TGG AG-3′ (1,318–1,325 bp), which responded positively only to PCV-1, was also included to test the PCV-1-specificity of the selected primers. For PCV-2, primers 263F: 5′-TAG GTT AGG GCT GTG GCC TT-3′ (1,323–1,342 bp) and 263R: 5′-CCG CAC CTT CGG ATA TAT ACT G-3′ (1,586–1,567 bp) were used. These primers were designed to amplify a 263-bp PCV-2-specific DNA fragment, which was selected from the PCV-2 open reading frame-2 region.[19] A locked nucleic acid probe #79: 5′-CCA GGA GG-3′ (537–544 bp), which responded positively only to PCV-2, was also included to test the PCV-2-specificity of the selected primers. PCV-2 from strain NPUST-2K1[32] was used to establish a standard curve.

The PCR product was subjected to DNA sequencing and compared with the DNA sequence of PCV-1 and PCV-2 deposited in NCBI (accession number: AY193712 and AF055394, respectively) to confirm the specificity of PCR.

Establishment of a standard curve for PCV-2 quantification

The 263-bp PCV-2-specific DNA fragment was amplified and inserted into the pGEM-T easy vector to obtain

the recombinant plasmid. To ensure that the 263-bp DNA fragment was correctly inserted, the recombinant plasmid was digested with *Eco*RI and then electrophoresed on an agarose gel to confirm the presence of DNA insert. The cloned plasmid was then sequenced and compared with that of NCBI (accession number: AF055394).

A standard curve, relating the PCR cycle number as a function of the viral DNA concentration, was constructed by serial dilution of the plasmid containing the 263-bp DNA insert, and each dilution was quantified using real-time quantitative PCR and used as the copy reference to construct the standard curve (1×10^2–1×10^9 copies/μL) for the quantification of PCV-2 levels in field samples. The DNA concentration was determined by optical density at 260 nm and converted into copies per mole as described.[33]

Real time quantitative PCR

Quantification of PCV-1 and PCV-2

Real-time PCR was performed using the LightCycler FastStart DNA Master SYBR® Green I kit (Roche Diagnostics).The amplification was carried out in a 20 μL reaction containing 1 μM of each forward and reverse primer, 3 mM Mg^{2+}, 2 μL of 1 × Master SYBR Green I Mix (Roche Diagnostics) and 5 μL DNA template (NPUST 2K1). The PCR conditions were as follows: 95°C for 10 minutes, followed by 30 cycles of amplification at 95°C for 0 seconds, 62°C for 5 seconds and 72°C for 11 seconds. Melt curve analysis was performed at 95°C for 0 seconds, 65°C for 15 seconds and 95°C for 0 seconds; and cooling at 40°C for 30 seconds.

For the quantification of PCV-2 concentrations in field specimens, viral DNA was extracted from the lymph nodes of PCV-2-infected pigs, and the PCV-2-specific DNA fragments were amplified by real-time PCR using the PCV-2-specific primer pair. The PCV-2 concentration (virus copies/μL) was quantified by correlation with the standard curve.

The same PCR conditions were used for the quantification and identification of unknown PCV-1 in sample tissues, except PCV-1-specific UPL-F and UPL-R primers and a DNA template extracted from the standard PCV-1 (PK15, ATCC CCL33) were used.

Differentiation of unknown PCV in tissue samples

To differentiate PCV-1 from PCV-2, we used a multiplex PCR protocol, which enables the simultaneous amplification of more than one target sequence in a single PCR step using two pairs of primers. The LightCycler software (Roche Diagnostics) for PCR data analysis was used to determine the characteristic melting temperature (T_m) of the PCR products obtained from standard PCV-1 (PK15, ATCC CCL33), PCV-2 (NPUST-2K1 strain) and PCVs of unknown type.

Results

The melting curve analysis results from the genotyping of 10 field samples showed that the melting temperatures of PCV-1 and PCV-2 PCR products were 82.54±0.15°C and 86.15±0.17°C, respectively (Table). The melting curve is shown in Figure 1. A difference of about 3.6°C between the two melting temperatures amplified by multiplex real-time PCR procedure is evident. This suggests that real-time PCR is a reliable method for the discrimination of PCV-1 from PCV-2.

Figure 2 shows the change in fluorescence with increasing PCR cycle number when PCV-2-specific primers were used. Since only PCV-2-positive samples show an increase in fluorescence during the PCR reaction, the efficiency and specificity of PCV-2-specific primers is clear.

Figure 3 shows the typical change in fluorescence with increasing PCR cycle number when PCV-1 specific primers were used. Likewise, as only the PCV-1-positive samples show an increase in fluorescence during the PCR, the efficiency and specificity of PCV-1-specific primers is clear.

Table. Result of genotyping by melting temperature analysis for porcine circovirus type 1 and type 2 sample amplified by multiplex real-time polymerase chain reaction (n=10)*

Primers	T_m (°C)		T_m difference (°C)
	PCV-1	PCV-2	
UPL-F & UPL-R	82.54±0.15	–	3.61±0.18
263F & 263R	–	86.15±0.17	

*Data presented as mean±standard deviation. T_m=Melting temperature; PCV-1=porcine circovirus type 1; PCV-2=porcine circovirus type 2.

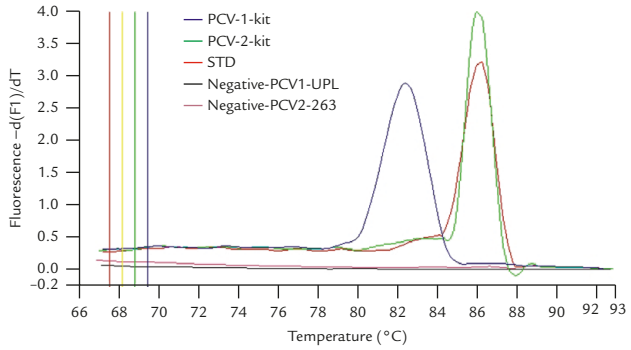

Figure 1. Comparison of the melting curves between multiplex real-time polymerase chain reaction-amplified porcine circovirus type 1- and type 2-specific fragments show a 3.6°C difference in their melting temperatures. Blue traces, positive control for PCV-1 (T_m=82.54°C); green traces, positive control for PCV-2 (T_m=86.15°C); red traces, a field sample whose melting curve appeared in the same melting range as the positive control for PCV-2; black and pink traces, the negative controls for PCV-1 and PCV-2, respectively.

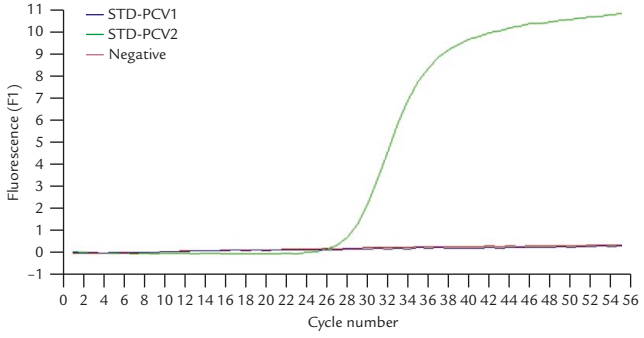

Figure 2. Fluorescent signal for both the field and standard samples of porcine circovirus type-1 and type-2 after real-time polymerase chain reaction using PCV-2-specific primers 263F and 263R, together with locked nucleic acid probe #79, which responded positively only to PCV-2. The result indicates that only PCV-2 responded positively. Blue trace, standard PCV-1 reference (PK15, ATCC CCL33); green trace, standard PCV-2 reference (NPUST-2K1); red trace, negative control (H_2O).

Figure 4 shows the individual fluorescent signals of each dilution of plasmid DNA (263-bp fragment) during real-time PCR and the standard curve constructed from these individual runs. The DNA sequence of the 263-bp fragment was found to have 100% identity when compared with that of the PCV-2 standard (accession number: AF055394). As expected, a good linear relationship between the threshold PCR cycle number and the logarithmic DNA concentration was obtained, since the concentration of viral DNA was exponentially multiplied during PCR amplification. It is worth mentioning that a

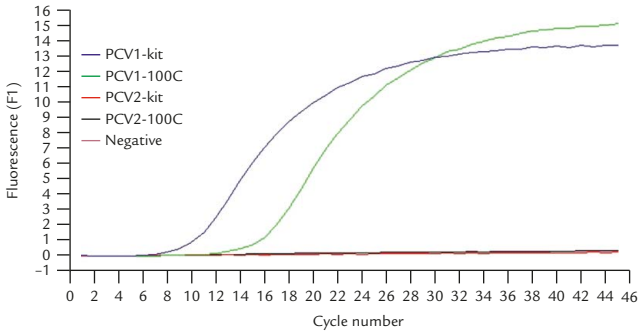

Figure 3. Fluorescent signal for field and standard samples of porcine circovirus type 1 and type-2 after real-time polymerase chain reaction using the PCV-1-specific primers UPL-F and UPL-R, together with locked nucleic acid probe #22, which responded positively only to PCV-1. The result indicates that only PCV-1 responded positively. Blue trace, standard PCV-1 reference (PK15 ATCC CCL33); green trace, PCV-1 field sample; red trace, standard PCV-2 reference (NPUST-2K1); black trace, PCV-2 field sample; pink trace, negative control (H_2O).

viral concentration of PCV-2 as low as 10^2 copies/µL was detected, indicating the sensitivity of this method for PCV-2 quantification.

When the standard PCV-2 plasmid was used to evaluate the reproducibility of our real-time PCR for PCV-2, the intra- and inter-assay coefficients of variation for the PCR threshold cycle (C_T) values were found to range between 1.00–1.64%, and 1.16–1.83%, respectively.

Figure 5 shows the PCR amplification plot of a field sample of PCV-2, together with a positive control. The viral load of the field specimen was found to be 3.1×10^6 copies/µL according to the standard curve established in Figure 4.

Discussion

The purpose of this study was to establish a real-time PCR method for the quantification of PCV-2, and for the rapid differentiation of PCV-1 and PCV-2 in order to speed up the process of clinical diagnosis. A conventional diagnostic procedure for PCV-2 takes about 7–8 hours and involves PCR, nested PCR, and agarose gel electrophoresis. In comparison, our real-time PCR method takes about 45 minutes and has great potential as a practical tool for clinical diagnosis.

The PCR-RFLP assay developed by Fenaux et al to differentiate PCV-1 and PCV-2 identified two fragments

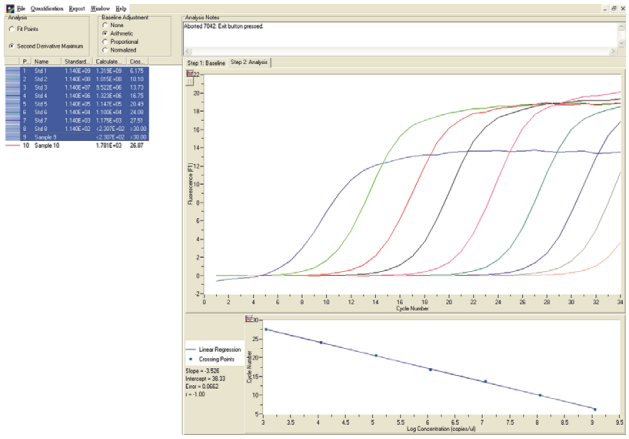

Figure 4. (A) Fluorescent signals during real-time polymerase chain reaction for each serial dilution of plasmid DNA (263 bp fragment; 1×10^2–1×10^9 copies/μL), and (B) the standard curve established for these individual runs.

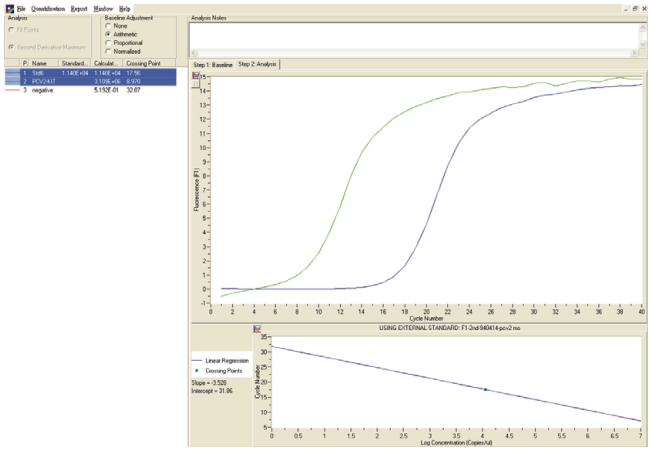

Figure 5. (A) Amplification plot of the real-time polymerase chain reaction-amplified positive control (left curve) and field sample (right curve) of viral DNA taken from the lymph nodes of porcine circovirus (PCV) type-2-infected pigs. (B) The cross point at the standard curve quantified the viral load of field sample as 3.1×10^6 copies/μL.

(168 bp and 75 bp) from the amplified PCV-2 isolates, and one fragment of 243 bp from PCV-1 isolates.[6] In clinical samples from pigs with dual infection by PCV-1 and PCV-2, all three fragments (243, 168, and 75 bp) were detected, indicating the reliability of the method for use in the dual infection cases. In comparison, our method requires only one multiplex real-time PCR procedure to accurately differentiate PCV-1 and PCV-2, and is relatively time-saving. The Larochelle also used multiplex PCR method,[29] but used gel electrophoresis to differentiate the PCV-specific PCR products by their molecular size. In comparison, our method takes advantage of the built-in melting curve analysis function of the PCR instrument to differentiate PCV-1 PCR product from the PCV-2 PCR product, and considerably shortens the time required for the PCV typing step.

For the quantification of PCV-2 levels in field samples, a 263-bp DNA fragment was amplified from the 3′ end of the open reading frame-2 of the PCV-2 standard by nested PCR, and its DNA sequence was verified by comparison with that of NCBI. The recombinant plasmid containing the 263-bp insert from PCV-2 was serially diluted, and quantified using real-time PCR to construct a standard curve. For field sample quantification, PCV-2 concentrations as low as 1×10^2 copies/μL were detectable in specimens taken from the lymph nodes or infected tissues of PCV2-infected pigs.

Since SYBR Green I stains any double-stranded DNA, the PCR incorporated both positive and negative controls, and the T_m of the PCR products was also analyzed to rule out contamination.

This rapid PCV-2 quantification method can also be used for the determination of the PCV-2 levels in serum as an indicator of viremia; the early detection of PCV-2-associated diseases such as PMWS (since PCR is sensitive enough to detect small amounts of viral DNA in tissues and organs); for the post vaccination tracking of the viral load in different tissues and organs of pigs in pathogenesis studies; and for evaluating the potency of developmental vaccines.

Several research groups have reported detection of PCV-2 and PRRSV in the same tissues of co-infected pigs by PCR, and that PCV-2 DNA was most prevalent in monocyte/macrophages within the lymphoid tissues.[8,11,34] Thus, in the future application of our PCV-2 test for detecting PCV-2 related diseases in pigs, we recommend sampling the monocyte/macrophage cells in the blood or lymphoid tissues as the first priority.

Overall, this study shows that real-time PCR is a clinically feasible method for the accurate quantification of PCV-2, and for the rapid differentiation of PCV-1 and PCV-2.

Acknowledgments

We are grateful to the Council of Agriculture, Executive Yuan, Taiwan, for their financial support through the award of grant (96-As-14.6-BQ-B7).

References

1. Tischer I, Rasch R, Tochtermann G. Characterization of papovavirus- and picornavirus-like particles in permanent pig kidney cell lines. Zen*tralbl Bakteriol Mikrobiol Hyg Ser. A* 1974;226:153–67.
2. Tischer I, Gelderblom H, Vettermann W, Koch M A. A very small porcine virus with circular single-stranded DNA. *Nature* 1982; 295:64–6.
3. Tischer I, Mields W, Wolff D, Vagt M, Griem W. Studies on epidemiology and pathogenicity of porcine circovirus. *Arch Virol* 1986;91:271–6.
4. Harding JC, Clark EG. Recognizing and diagnosing postweaning multisystemic wasting syndrome (PMWS). *Swine Health and Production* 1997;5:201–3.
5. Ellis J, Hassard L, Clark E, Harding J, Allan G, Willson P, et al. Isolation of circovirus from lesions of pigs with postweaning multisystemic wasting syndrome. *Can Vet J* 1998;39:44–51.
6. Fenaux M, Halbur PG, Gill M, Toth TE, Meng XJ. Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. *J Clin Microbiol* 2000;38:2494–503.
7. Mankertz A, Hillenbrand B. Replication of porcine circovirus type proteins encoded by the viral rep gene. *Vir*ology 2001; 279:429–38.
8. Choi C, Chae C. Colocalization of porcine reproductive and respiratory syndrome virus and porcine circovirus 2 in porcine in dermatitis and nephrology syndrome by double-labeling technique. *Vet Pathol* 2001;38:436–41.
9. Allan GM, Meehan B, Todd D, Kennedy S, McNeilly F, Ellis J, et al. Novel porcine circoviruses from pigs with wasting disease syndromes. *Vet Rec* 1998;142:467–8.
10. Tischer I, Bode L, Peters D, Germann B. Distribution of antibodies to porcine circovirus in swine populations of different breeding farms. *Arch Virol* 1995;140:737–43.
11. Allan GM, Kennedy S, McNeilly F, Foster JC, Ellis JA, Krakowka SJ, et al. Experimental reproduction of severe masting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. *J Comp Path* 1999;121:1–11.
12. Hamel AL, Lin LL, Nayar GP. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. *J Virol* 1998;72:5262–7.
13. Mahe D, Blanchard P, Truong C, Amauld C, Le Cann P, Cariolet R, et al. Differential recognition of ORF2 protein from type 1 and type 2 porcine circoviruses and identification of immunorelevant epitopes. *J Gen Virol* 2000;81:1815–24.
14. Nawagitgul P, Morozov I, Sirinarumitr T, Sorden SD, Paul PS. Development of probes to differentiate porcine circovirus types 1 and 2 in vitro by *in situ* hybridization. *Vet Microbiol* 2000; 75:83–9.
15. Magar R, Müller P, Larochelle R. Retrospective serological survey of antibodies to porcine circovirus type 1 and type 2. *Can J Vet Res* 2000;64:184–6.
16. Allan GM, McNeilly F, Ellis J, Krakowka S, Meehan B, McNair I, et al. Experimental infection of colostrums deprived piglets with porcine circovirus 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRS) potentiates PCV2 replication. *Arch Virol* 2000;145:2421–9.
17. Larochelle R, Bielanski A, Müller P, Magar R. PCR Detection and Evidence of Shedding of Porcine Circovirus Type 2 in Boar Semen. *J Clin Microbiol* 2000;38:4629–32.
18. Sanchez Jr. RE, Nauwynck HJ, McNeilly F, Allan GM, Pensaert MB. Porcine Circovirus 2 infection in wine fetuses inoculated at different stages of gestation. *Vet Microbiol* 2001;83:69–176.
19. Rovira A, Balasch M, Segalés J, Garcia L. Plana-Durán J, Rosell C, et al. Experimental inoculation of conventional pigs with Porcine Reproductive and Respiratory Syndrome Virus and Porcine Circovirus 2. *J Virol* 2002;76:3232–9.
20. Opriessnig T, Meng XJ, Halbur PG. Porcine circovirus type 2–associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. *J Vet Diagn Invest* 2007;19:591–615.
21. Kim J, Chae C. Differentiation of porcine circovirus 1 and 2 in formalin-fixed, paraffin-wax-embedded tissues from pigs with postweaning multisystemic wasting syndrome by in-situ hybridisation. *Res Vet Sci* 2001;70:265–9.
22. Calsamiglia M, Segalés J, Quintana J, Rosell C, Domingo M. Detection of porcine circovirus types 1 and 2 in serum and tissue samples of pigs with and without postweaning multisystemic wasting syndrome. *J Clin Microbiol* 2002;40:1848–50.
23. Haa Y, Junga K, Choia C, Hwang KK, Chaea C. Synthetic peptide-derived antibody-based immunohistochemistry for the detection of porcine circovirus 2 in pigs with postweaning multisystemic wasting syndrome. *J Comp Pathol* 2005;133:201–4.
24. Opriessnig T, Yu S, Gallup JM, Evans RB, Fenaux M, Pallares F, et al. Effect of vaccination with selective bacterins on conventional pigs infected with type 2 porcine circovirus. *Vet Pathol* 2003;40:521–9.
25. Sibila M, Olvera A, Calsamiglia M, Segalés J, Domingo M. Quantification of PCV2 Load by Real Time PCR in PMWS and PDNS Naturally Affected Pigs. In: Martelli P, Cavirani S, Lavazza A, eds. *PRRS - PMWS - Swine Influenza*. 4th International Symposium on Emerging and Re-emerging Pig Diseases, June 29–July 2. 2003, Palazzo dei Congressi, Rome.
26. Cao S, Chen H, Zhao J, Lu J, Xiao S, Jin M, et al. Detection of porcine circovirus type 2, porcine parvovirus and porcine pseudorabies virus from pigs with postweaning multisystemic wasting syndrome by multiplex PCR. *Vet Res Commun* 2005;29:263–9.
27. Kim J, Choi C, Han DU, Chae C. Simultaneous detection of porcine circovirus type 2 and porcine parvovirus in pigs with PMWS by multiplex PCR. *Vet Rec* 2001;149:304–5.
28. Chung WB, Chan WH, Chaung HC, Lien Y, Wu CC, Huang YL. Real-time PCR for quantitation of porcine reproductive and respiratory

syndrome virus and porcine circovirus type 2 in naturally-infected and challenged pigs. *J Virol Methods* 2005;124:11–9.

29. Larochelle R, Antaya M, Morin M, Magar R. Typing of porcine circovirus in clinical specimens by multiplex PCR. *J Virol Methods* 1999;80:69–75.
30. Allan GM, McNeilly F, Kennedy S, Daft B, Clark EG, Ellis JA, et al. Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. *J Vet Diagn Invest* 1998;10:3–10.
31. LeCann P, Blanchard P, Arbauld C, Albina E, Hutet E, Madec F, et al. Id*entification of porcine circovirus associated with piglet wasting disease*. In: S, Thomson J, Varley M, eds. Proceedings of International Pig Veterinary Society Congress 1998. Nottingham: Nottingham University Press, 1998.
32. Chang GN, Su BS, Chiou JH, Liu HJ, Tsai SS. Identification of porcine circovirus infection in Taiwan by using polymerase chain reaction, analysis of DNA sequence and *in situ* hybridization. *Taiwan Vet J* 2002;28:186–94.
33. Wang CC. *Immunomodulatory and anti-inflammatory effects of polysaccharides from mycelia and fermented culture broth of antrodia camphorata*. Master Thesis: National Yang-Ming University, Institute of Clinical Medicine, Taiwan. 2009;19.
34. Duan X, Nauwynck HJ, Pensaert MB. Virus quantification and identification of cellular targets in the lungs and lymphoid tissues of pigs at different time intervals after inoculation with porcine reproductive and respiratory syndrome virus (PRRSV). *Vet Microbiol* 1997;56:9–19.