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BRIEF REPORT

Efficient production of type 2 porcine circovirus-like particles by a recombinant baculovirus

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Abstract The capsid protein of PCV2 was expressed by using a recombinant baculovirus with insect Tn5 cells. A large amount of 28-kDa protein was released into the culture medium and self-assembled into PCV2-like particles (PCV2-LPs) with a buoyant density of 1.365 g/cm^3 and a diameter of 20 nm. PCV2-LPs were efficiently expressed, yielding 1 mg of purified particles per 10^7 Tn5 cells. The PCV2-LPs have antigenicity similar to that of authentic PCV2 particles, allowing us to develop a method for sensitively detecting PCV2-specific IgG antibodies. In addition, the PCV2-LPs appeared to be the most promising PCV2 vaccine candidate, by virtue of their potent immunogenicity.

Postweaning multisystemic wasting syndrome (PMWS) is a disease with low morbidity but high mortality in swine. PMWS has a serious economic impact on the global swine industry. It was first reported in western Canada in 1991 and later identified in the United States, Mexico, Europe, and Asia [2]. Porcine circovirus type 2 (PCV2) is the primary causative agent of PMWS, though the etiology of this disease has not been fully elucidated. The isolation of viruses from tissues of affected swine led to the identification of PCV2 [5–8, 20]. PCV2, a member of the family *Circoviridae* [25], is a small spherical nonenveloped virus with a single-stranded closed circular genomic DNA of 1.7 kb in length [1]. Two major open reading frames (ORFs) oriented in opposite directions have been identified. ORF1 encodes two proteins: Rep and its truncated form, Rep′, both of which are essential for viral DNA replication. ORF2 encodes a major structural protein that has type-specific epitopes [17, 22] and is very immunogenic and strongly associated with the induction of neutralizing antibodies [24], suggesting its potential use in diagnostic assays as well as vaccine development. In the present study, we expressed the PCV2 capsid protein by using a recombinant baculovirus in Tn5 insect cells. The capsid protein self-assembled into PCV2-like particles (PCV2-LPs) and was released into the culture medium. The PCV2-LPs possess similar antigenicity to that of the native PCV2 particles and appear to be a good antigen for the sensitive detection of PCV2-specific antibodies. Our study also demonstrated that PCV2-LPs are the most promising PCV2 vaccine candidate.

Viral DNA was extracted from the PCV2 Yamagata strain [23], and the full-length ORF2 of PCV2 (PCV2-ORF2) was amplified by PCR with forward primer PCV2-D1 (5′-AA**GGATCC**ATGACGTATCCAAGGAGGCGTT-3′) and reverse primer PCV2-U1 (5′-GC**TCTAGA**TTAGGGT TTAAGTGGGGGGTCT-3′). The forward primer contained the *Bam*HI site before the start codon, and the reverse primer

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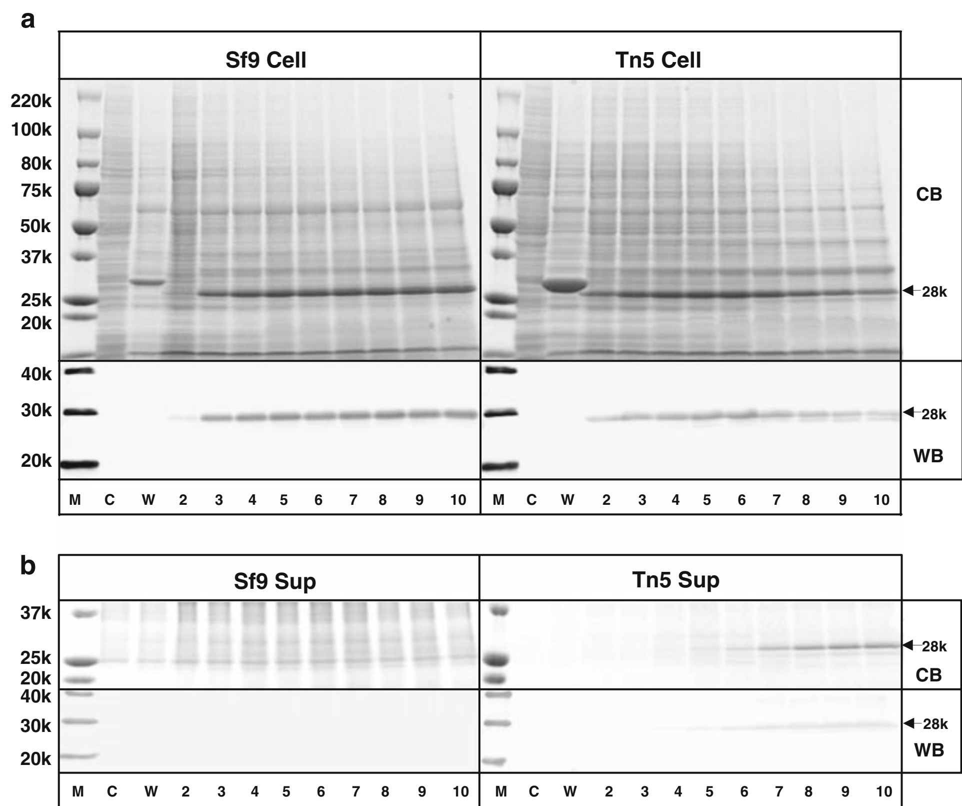

Fig. 1 Time course of the expression of PCV2 capsid protein in insect cells. Insect Sf9 and Tn5 cells were infected with the recombinant baculovirus AcPCV2-ORF2, incubated at 26.5°C, and harvested on the indicated days (2–10 days). Five microliters of the culture medium and the lysate from 10^5 cells were analyzed by SDS-PAGE. Protein bands were visualized by Coomassie blue staining (*CB*) or by Western blot assay with anti-PCV2 rabbit serum (*WB*). The cell lysate (**a**) and culture medium (**b**) were analyzed separately. *M* molecular weight marker, *C* uninfected cell, *W* wild-type baculovirus-infected cells, *lanes* 2–10, 2–10 days p.i

contained the *Xba*I site after the stop codon. The amplified ORF2 fragment was purified by using a gel extraction kit (Qiagen, Valencia, CA) and was first digested with *Bam*HI and then partially digested with *Xba*I. The purified 700-bp fragment was ligated into transfer vector pVL1393 (Pharmingen, San Diego, CA) by a ligation kit (Takara, Shiga, Japan), and a transfer plasmid pVL1393/PCV2-ORF2 was constructed.

A recombinant baculovirus was constructed and capsid proteins were expressed as previously described [14, 16]. Insect Sf9 and Tn5 cells were infected with a recombinant baculovirus, AcPCV2-ORF2, containing the entire PCV2 capsid protein. The infected cells were harvested daily until 10 days p.i. The proteins expressed in infected cells were analyzed by SDS-PAGE followed by Coomassie blue staining and by Western blot assay using a rabbit anti-PCV2 antibody (Fig. 1). A major band with a molecular mass of 28 kDa was observed in cell lysate of both Sf9 and Tn5 cells (Fig. 1a). The 28-kDa protein was first detected on day 2 in both Sf9 and Tn5 cells, and peaked on day 5 p.i. This 28-kDa protein was detected on day 4 p.i. in the supernatant of Tn5 cells and increased until day 10 p.i. (Fig. 1b), whereas it was not detected in the supernatant of Sf9 cells.

The culture medium of the AcPCV2-ORF2-infected Tn5 cells was harvested at 7 days p.i., and the PCV2 capsid protein was purified by CsCl gradient centrifugation. The 28-kDa protein appeared mainly in fractions 3, 4, and 5, which had an average density of 1.365 g/ml^3 (Fig. 2a). Examination of these fractions by electron microscopy revealed spherical particles with diameters of $\sim$20 nm. The morphology of these particles was similar to that of the authentic PCV2 particles (Fig. 2b), indicating that the 28-kDa protein formed virus-like particles (VLPs) (PCV2-LPs). The yield of the purified VLPs was 1 mg per 10^7 Tn5 cells in culture medium. We tried to purify the PCV2 capsid protein from the infected Sf9 cells, but only a few PCV2-LPs were obtained from the cell lysate, and no VLPs were obtained from the supernatant (data not shown).

An ELISA to detect antibodies was developed by using PCV2-LPs as an antigen. This antibody ELISA showed a low background, probably because the PCV2-LPs were highly purified. The cutoff value of IgG was determined by using 30 serum samples from wild boar that were negative for anti-PCV2 IgG by Western blot assay (data not shown). The OD values of these sera were between 0.036 and 0.249, and the mean value was 0.062 with a standard deviation (SD) of 0.046. Therefore, the cutoff value, the mean value+3SD, was calculated as 0.200. The PCV2-specific IgG elicited in swine experimentally infected with the PCV2 Yamagata strain was detected by the antibody ELISA. As depicted in Fig. 3a, significant IgG antibody titers were observed, indicating that the purified PCV2-LPs had similar antigenicity to native PCV2. A panel of 105

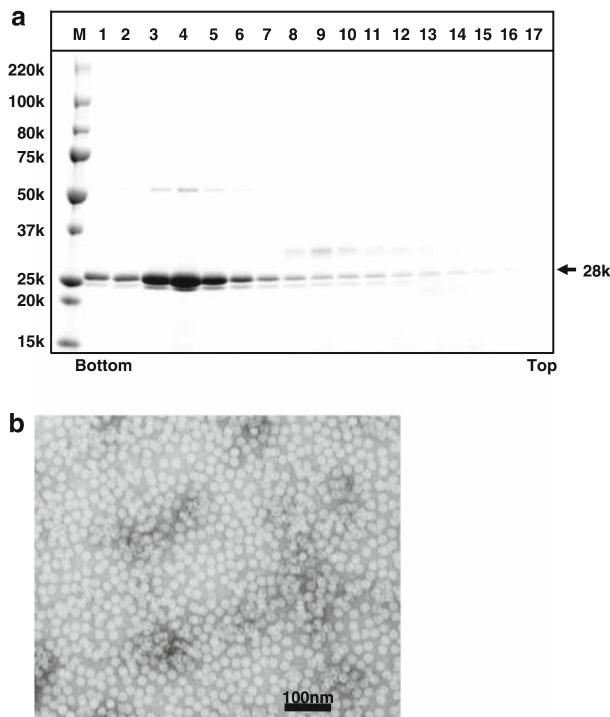

Fig. 2 **a** Purification PCV2-LPs by CsCl gradient centrifugation. The supernatant of the recombinant baculovirus-infected Tn5 cells was centrifuged for 3 h at 31,000 rpm in a Beckman SW32Ti rotor. The pellet was resuspended in 4.5 ml ExCell 405 and mixed with 2.1 g CsCl, then centrifuged for 24 h at 35,000 rpm in an SW55Ti rotor. Aliquots from the gradient were analyzed by electrophoresis on a 5–20% polyacrylamide gel and stained with Coomassie blue. The bottom and top of the gradient and the positions of molecular weight standards are indicated. **b** Electron microscopy of PCV2-LPs. Purified PCV2-LPs were stained with 2% uranyl acetate and observed by EM. *Bar* 100 nm

swine serum samples was collected from a slaughterhouse in Japan in 2006. All of the serum samples were diluted 1:200 for the ELISA test. The result showed that all sera from healthy slaughtered domestic pigs were positive for IgG against PCV2, and the OD values were above 0.65 without exception (data not shown).

Antibodies to the PCV2-LPs were prepared in rabbits and guinea pigs by subcutaneous injection of the purified PCV2-LPs. After being injected two times, the animals produced high levels of IgG antibodies, and the titers reached levels as high as 1:1,638,400 in the antibody ELISA. Immunogenicity of the VLPs was examined using an ELISA with rabbit and guinea pig hyperimmune sera as the capture and detector antibodies, respectively. As shown in Fig. 3b, the sensitivity reached 0.16 ng/ml of PCV2-LPs when a cutoff OD value of 0.2 was used, and native PCV2 particles were detected in the culture medium of PCV2-infected PK-15 cells, yielding an antigen titer of 1:32. Native PCV2 particles concentrated by centrifugation ($100{,}000 \times g$, 2 h) showed an increased titer of 1:256 (data not shown). These results demonstrated that the PCV2-LPs were immunogenic and able to elicit antibodies capable of binding to native PCV2 particles.

PCV2 is a pivotal causative agent of PMWS and is recognized as a major economic problem in the porcine industry worldwide [2, 10, 12, 26]. In addition, PCV2 infection is subclinical in some swine, and these swine become carriers and cause longer virus circulation in herds. However, the development of a vaccine and diagnosis are hampered by a low yield of PCV2 in cell culture. PCV2 capsid protein is the major structural protein and is highly

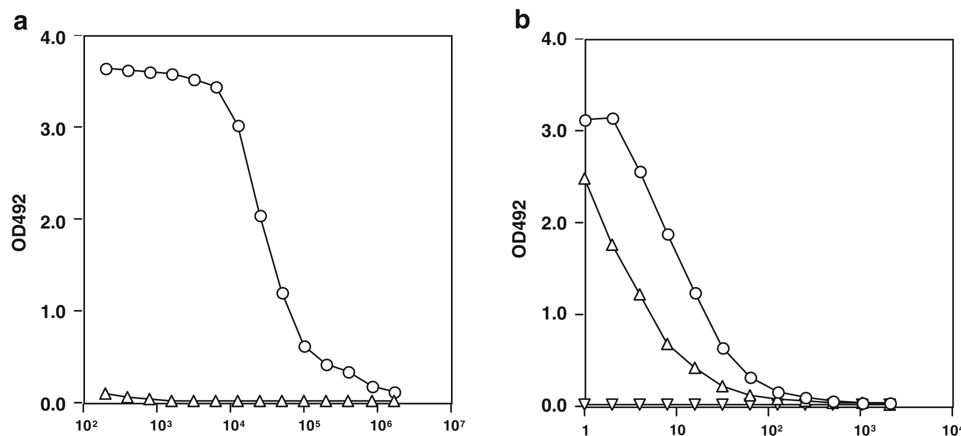

Fig. 3 **a** Antigenicity of PCV2-LPs. A swine was experimentally infected. The pre-serum was obtained before PCV2 infection, and the post-serum was obtained 4 weeks after infection. Both pre- (*open triangle*) and post-sera (*open circle*) were examined for IgG antibody by twofold dilutions starting from 1:200. **b** Immunogenicity of PCV2-LPs. Anti-PCV2-LP serum from a rabbit was used to coat a 96-well microplate. The binding of recombinant PCV2-LPs (10 ng/ml) (*open circle*) and native PCV2 particles produced in the supernatant of Yamagata-strain-infected PK-15 cells (*open triangle*) was examined by antigen ELISA. The OD value was considered positive when the absorbance was $\geq$0.150. The culture medium from uninfected PK-15 cells (*open inverted triangle*) was used as a negative control

immunogenic. As both neutralizing monoclonal antibodies and neutralizing swine sera have been shown to react with the capsid protein [13, 19, 24], the capsid protein is an attractive immunogen for vaccine development and diagnosis.

The recombinant baculovirus expression system has long been used to express proteins as well as to generate VLPs in various DNA and RNA viruses [3, 11, 14, 15, 18]. This system has many advantages over other systems: efficient expression, proper post-translational modification, correct conformation, and self-assembly of the capsid protein into VLPs, which usually retain the immunogenicity as well as physicochemical properties of their native virions. Sf9 and Tn5 cells are commonly used cell lines for the baculovirus expression system. Previous studies have shown that PCV2 capsid protein expressed in Sf9 cells self-assembled into VLPs [22]. However, although VLPs were generated in the Sf9 cells, they were not released into the culture medium. When we expressed the PCV2 capsid in Sf9 cells, the same result was observed. In contrast, when the recombinant PCV2 capsid protein was expressed in Tn5 cells, the capsid protein efficiently self-assembled into VLPs and, interestingly, was released into the culture medium, making purification of the VLPs easy. The yield of the purified VLPs reached 1 mg per 10^7 Tn5 cells, providing a virtually unlimited supply of highly purified PCV2-LPs.

The feasibility of the capsid protein-based PCV2 vaccine was demonstrated in several previous studies, where the successful induction of specific serum antibodies was observed [4, 9, 27]. It was also found that the absence of PCV2-neutralizing antibodies is well correlated with the virus replication and development of PMWS [21], indicating that the humoral immune response plays an important role in the prophylaxis of PCV2 infection. There are several advantages to using PCV2-LPs as a vaccine: First, the PCV2-LPs have an excellent safety profile, since there is no viral genome in the particles. Second, the PCV2-LPs have antigenicity and immunogenicity similar to those of the native PCV2. Third, the PCV2-LPs are morphologically the same as the native PCV2, and the conformational antigenic epitopes may be properly retained. Therefore, the PCV2-LPs share the same immunogenicity with the native PCV2 virion. In fact, antibodies elicited with PCV2-LPs strongly reacted with the native virion. These results clearly demonstrate that PCV2-LPs are a promising PCV2 vaccine candidate.

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References

1. Allan GM, Ellis JA (2000) Porcine circoviruses: a review. J Vet Diagn Invest 12:3–14
2. Allan GM, Mc Neilly F, Meehan BM, Kennedy S, Mackie DP, Ellis JA, Clark EG, Espuna E, Saubi N, Riera P, Botner A, Charreyre CE (1999) Isolation and characterisation of circoviruses from pigs with wasting syndromes in Spain, Denmark and Northern Ireland. Vet Microbiol 66:115–123
3. Baumert TF, Vergalla J, Satoi J, Thomson M, Lechmann M, Herion D, Greenberg HB, Ito S, Liang TJ (1999) Hepatitis C virus-like particles synthesized in insect cells as a potential vaccine candidate. Gastroenterology 117:1397–1407
4. Blanchard P, Mahe D, Cariolet R, Keranflec'h A, Baudouard MA, Cordioli P, Albina E, Jestin A (2003) Protection of swine against post-weaning multisystemic wasting syndrome (PMWS) by porcine circovirus type 2 (PCV2) proteins. Vaccine 21:4565–4575
5. Ellis J, Hassard L, Clark E, Harding J, Allan G, Willson P, Strokappe J, Martin K, McNeilly F, Meehan B, Todd D, Haines D (1998) Isolation of circovirus from lesions of pigs with post-weaning multisystemic wasting syndrome. Can Vet J 39:44–51
6. 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, McNeilly F (1999) Reproduction of lesions of postweaning multisystemic wasting syndrome in gnotobiotic piglets. J Vet Diagn Invest 11:3–14
7. Ellis JA, Bratanich A, Clark EG, Allan G, Meehan B, Haines DM, Harding J, West KH, Krakowka S, Konoby C, Hassard L, Martin K, McNeilly F (2000) Coinfection by porcine circoviruses and porcine parvovirus in pigs with naturally acquired post-weaning multisystemic wasting syndrome. J Vet Diagn Invest 12:21–27
8. Fenaux M, Halbur PG, Gill M, Toth TE, Meng XJ (2000) 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 38:2494–2503
9. Fenaux M, Opriessnig T, Halbur PG, Elvinger F, Meng XJ (2004) A chimeric porcine circovirus (PCV) with the immunogenic capsid gene of the pathogenic PCV type 2 (PCV2) cloned into the genomic backbone of the nonpathogenic PCV1 induces protective immunity against PCV2 infection in pigs. J Virol 78:6297–6303
10. Hasslung F, Wallgren P, Ladekjaer-Hansen AS, Botner A, Nielsen J, Wattrang E, Allan GM, McNeilly F, Ellis J, Timmusk S, Belak K, Segall T, Melin L, Berg M, Fossum C (2005) Experimental reproduction of postweaning multisystemic wasting syndrome (PMWS) in pigs in Sweden and Denmark with a Swedish isolate of porcine circovirus type 2. Vet Microbiol 106:49–60
11. Ishii Y, Ozaki S, Tanaka K, Kanda T (2005) Human papillomavirus 16 minor capsid protein L2 helps capsomeres assemble independently of intercapsomeric disulfide bonding. Virus Genes 31:321–328
12. Kim J, Chung HK, Jung T, Cho WS, Choi C, Chae C (2002) Postweaning multisystemic wasting syndrome of pigs in Korea: prevalence, microscopic lesions and coexisting microorganisms. J Vet Med Sci 64:57–62
13. Lekcharoensuk P, Morozov I, Paul PS, Thangthumniyom N, Wajjawalku W, Meng XJ (2004) Epitope mapping of the major

capsid protein of type 2 porcine circovirus (PCV2) by using chimeric PCV1 and PCV2. J Virol 78:8135–8145
14. Li TC, Yamakawa Y, Suzuki K, Tatsumi M, Razak MA, Uchida T, Takeda N, Miyamura T (1997) Expression and self-assembly of empty virus-like particles of hepatitis E virus. J Virol 71:7207–7213
15. Li TC, Takeda N, Kato K, Nilsson J, Xing L, Haag L, Cheng RH, Miyamura T (2003) Characterization of self-assembled virus-like particles of human polyomavirus BK generated by recombinant baculoviruses. Virology 311:115–124
16. Li TC, Takeda N, Miyamura T, Matsuura Y, Wang JC, Engvall H, Hammar L, Xing L, Cheng RH (2005) Essential elements of the capsid protein for self-assembly into empty virus-like particles of hepatitis E virus. J Virol 79:12999–13006
17. Mahe D, Blanchard P, Truong C, Arnauld C, Le Cann P, Cariolet R, Madec F, Albina E, Jestin A (2000) Differential recognition of ORF2 protein from type 1 and type 2 porcine circoviruses and identification of immunorelevant epitopes. J Gen Virol 81:1815–1824
18. Matsuo E, Tani H, Lim C, Komoda Y, Okamoto T, Miyamoto H, Moriishi K, Yagi S, Patel AH, Miyamura T, Matsuura Y (2006) Characterization of HCV-like particles produced in a human hepatoma cell line by a recombinant baculovirus. Biochem Biophys Res Commun 340:200–208
19. McNeilly F, McNair I, Mackie DP, Meehan BM, Kennedy S, Moffett D, Ellis J, Krakowka S, Allan GM (2001) Production, characterisation and applications of monoclonal antibodies to porcine circovirus 2. Arch Virol 146:909–922
20. Meehan BM, McNeilly F, Todd D, Kennedy S, Jewhurst VA, Ellis JA, Hassard LE, Clark EG, Haines DM, Allan GM (1998) Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. J Gen Virol 79(Pt 9):2171–2179
21. Meerts P, Misinzo G, Lefebvre D, Nielsen J, Botner A, Kristensen CS, Nauwynck HJ (2006) Correlation between the presence of neutralizing antibodies against porcine circovirus 2 (PCV2) and protection against replication of the virus and development of PCV2-associated disease. BMC Vet Res 2:6
22. Nawagitgul P, Morozov I, Bolin SR, Harms PA, Sorden SD, Paul PS (2000) Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. J Gen Virol 81:2281–2287
23. Onuki A, Abe K, Togashi K, Kawashima K, Taneichi A, Tsunemitsu H (1999) Detection of porcine circovirus from lesions of a pig with wasting disease in Japan. J Vet Med Sci 61:1119–1123
24. Pogranichnyy RM, Yoon KJ, Harms PA, Swenson SL, Zimmerman JJ, Sorden SD (2000) Characterization of immune response of young pigs to porcine circovirus type 2 infection. Viral Immunol 13:143–153
25. Pringle CR (1999) Virus taxonomy-1999. The universal system of virus taxonomy, updated to include the new proposals ratified by the International Committee on Taxonomy of Viruses during 1998. Arch Virol 144:421–429
26. Rodriguez-Arrioja GM, Segales J, Calsamiglia M, Resendes AR, Balasch M, Plana-Duran J, Casal J, Domingo M (2002) Dynamics of porcine circovirus type 2 infection in a herd of pigs with postweaning multisystemic wasting syndrome. Am J Vet Res 63:354–357
27. Wang X, Jiang P, Li Y, Jiang W, Dong X (2007) Protection of pigs against post-weaning multisystemic wasting syndrome by a recombinant adenovirus expressing the capsid protein of porcine circovirus type 2. Vet Microbiol 121:215–224