

## Investigation of Pseudorabies Virus Latency in Nervous Tissues of Seropositive Pigs Exposed to Field Strain

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(Received 12 April 2005/Accepted 25 October 2005)

**ABSTRACT.** The prevalence and quantity of latent pseudorabies virus (PrV) in nervous tissues of pigs exposed to field strain in Korea was investigated by nested and real-time PCR. Nervous tissues including trigeminal ganglion (TG), olfactory bulb (OB), and brain stem (BS) were collected from 94 seropositive pigs. PrV latent infection in nervous tissues was initially investigated by nested PCR targeting three glycoprotein genes (gB, gE, and gG). Based on the obtained result, latent infection was detected in 95.7% of screened animals. Furthermore, it was revealed that the examined tissues harbored different copy numbers of latent PrV genome ranging from  $<10^{2.0}$  to  $10^{7.1}$  copies per microgram of genomic DNA in real-time PCR analysis. These results show that under normal conditions, levels of latent PrV in the nervous tissues of pigs can vary across a wide range. Therefore, the data presented here provides information regarding control of the endemic state of PrV in Korea.

**KEY WORDS:** latent infection, nested PCR, pseudorabies virus, real-time PCR.

*J. Vet. Med. Sci.* 68(2): 143-148, 2006

Pseudorabies virus (PrV) is an *alphaherpesvirus* that causes a fatal disease called Aujeszky's disease in swine [14]. Depending on the age and immunological status of the animals, the clinical feature range from being inapparent to fatal. Similar to other herpesvirus infections, PrV is capable of causing lifelong latent infection in various nervous tissues of the natural host, which can reactivate under experimental or natural stress conditions [25]. Reactivation of latent PrV leads to release of viral particles into the environment and subsequent transmission to new uninfected hosts [5, 10]. Furthermore, the currently used attenuated vaccines do not completely prevent the establishment of latent infection [22, 27]. Thus, latency and reactivation of PrV is considered to be a major obstacle in the campaign to control and eradicate this disease [18].

Several methods have been developed to detect and/or quantify the latency of PrV [4, 9, 16, 19, 28]. Some of the techniques based on explantation and cocultivation of infected tissues are time-consuming, and their sensitivity is also relatively low [4, 19, 26]. In contrast, the use of molecular techniques has enhanced the sensitivity and accuracy in detection of PrV latency [16, 21]. Many studies have also demonstrated the efficiency of polymerase chain reaction (PCR) in detecting latent PrV infection in various tissues, such as the trigeminal ganglion (TG), olfactory bulb (OB), brainstem (BS), and even the tonsils [1, 17]. The required sensitivity and quantification of latent genome number is difficult to achieve by following conventional PCR methods. Several modified PCR methods have been described to quantify the amount of latent PrV [9, 27, 28, 31]. Such

quantitative PCR methods involve co-amplification of an internal control template or make use of a chemiluminescent probe [28]. The advent of real-time PCR has enabled us to detect the exact amount of persistently infected viruses in tissues and use of this technique is increasing [15, 23, 24]. Real-time PCR using fluorescence reporter dye accomplishes constant measurement of PCR products, allowing easy, rapid, and quantitative detection of a target gene in tissues. In the current study, we investigated the prevalence and quantity of latent PrV in nervous tissues of pigs exposed to field virus by employing nested and real-time PCR.

### MATERIALS AND METHODS

*Cell and virus:* The virulent PrV Yangsan (YS) strain, kindly provided from the National Veterinary Research and Quarantine Service of Korea, was used as a control to determine the specificity of primers and probes. The PrV YS strain was propagated in a porcine kidney cell line, PK-15. The cultures were incubated in a humidified CO<sub>2</sub> incubator at 37°C. The virus stock was concentrated, titrated, and stored in aliquots at -80°C until use.

*Purification of PrV DNA genome:* The acquired virus was suspended in TBS buffer (10 mM Tris-HCl, 0.15 M NaCl, pH 7.4) and disrupted with 0.6% SDS and proteinase K (400 µg/ml) at 37°C for 2 hr. The released viral DNA was extracted with the conventional phenol:chloroform:isoamyl alcohol (25:24:1) solution. The contaminating RNAs were removed using RNase A (200 µg/ml), and the final viral DNA pellets were resuspended in 8 mM NaOH and kept at -20°C until use.

*Collection of nervous tissues from pigs:* During the time period between September 2002 and August 2003, 94 cross-

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bred pigs that had been exposed to field strain, as identified by gpI antibody test (IDEXX HerdCheck anti-PrV gpI, Westbrook, Maine, U.S.A.), were collected in Wang-Gung, Chonbuk province, Korea. All the tested pigs were randomly selected 36–48-month old females devoid of vaccination with marker vaccine (gE-negative inactivated vaccine). Nervous tissues designated as BS, OB, and TG were incised from the cranial cavity of the selected pigs. Each of the nervous tissues was then frozen at  $-70^{\circ}\text{C}$  until use.

**Purification of genomic DNA from nervous tissues:** The genomic DNAs from the nervous tissue samples (approximately 100 mg per nervous tissue) were extracted with DNAzol (MRC, Cincinnati, OH), as per the prescribed information by manufacturer. Contaminating RNA was removed by RNase (200  $\mu\text{g}/\text{mL}$ ) treatment followed by phenol/chloroform extraction. Ethanol-precipitated genomic DNAs were then resuspended in 8 mM NaOH and kept at  $-20^{\circ}\text{C}$  until use. The DNA concentration was determined by GeneQuant RNA/DNA calculator (Biochrom, Cambridge, UK) and was adjusted to a final concentration of 50  $\text{ng}/\mu\text{L}$ .

**Nested PCR for identification of latent PrV DNA:** The prevalence of latent PrV infection in the nervous tissues was determined by nested PCR targeted for viral glycoprotein genes, gB (gII), gE (gI), and gG (gX) [14]. The sequences of the primers, which have been previously described, are represented in Table 1 [3, 20, 32]. A total of 10  $\mu\text{L}$  (500  $\text{ng}$ ) of genomic DNA was used for nested PCR in 25- $\mu\text{L}$  of reaction mixture containing 2.5  $\mu\text{M}$  of each of the primers, 1.5 mM  $\text{MgCl}_2$ , 0.05 U *Taq* Polymerase (Promega, Madison, WI, U.S.A.), 0.2 mM dNTP, and 1  $\times$  reaction buffer. Primarily, DNA was amplified with the Perkin-Elmer GeneAmp PCR system 9600 (Perkin-Elmer, Wellesley, MA, U.S.A.) using the following application procedure: 1 cycle of  $95^{\circ}\text{C}$  for 10 min, and 30 cycles of denaturation ( $94^{\circ}\text{C}$ , 45 s), annealing ( $62^{\circ}\text{C}$ , 1 min), and extension ( $72^{\circ}\text{C}$ , 1 min). A final extension time of 10 min at  $72^{\circ}\text{C}$  was included at the end of the last cycle. The second amplification of nested PCR was carried out using 5  $\mu\text{L}$  of the first PCR product under similar conditions. The PCR products were electrophoresed in 1.85% agarose gel (Invitrogen, Carlsbad, CA, U.S.A.) and visualized with a gel documentation system (Vilber Lourmat, Torcy, France) after staining with ethid-

Table 1. Sequences of the primers used in the nested PCR assays

Target gene	Amplification	Primers	Sequence 5'-3'	Length (bp) <sup>a)</sup>
gB	1 st	gB first 05 gB first 03	ATG GCC ATC TCG CGG TGC ACT CGC GGT CCT CCA GCA	334
	2nd	gB second 05 gB second 03	ACG GCA CGG GCG TGA TC GGT TCA GGG TCA CCC GC	195
gE	1 st	gE first 05 gE first 03	TCG TGA TGA CGT GCG TCG TCG GGG TCC ATT CGT CAC TTC CGG	377
	2nd	gE second 05 gE second 03	CCC ACG CAC GAG GAC TAC TAC CGC GGA ACC AGT CGT CGA AGC	211
gG	1 st	gG first 05 gG first 03	ATG TTG TCG TTT GAT CCC GTC AGC CGC GAG AGT AGT CCG TCC	327
	2nd	gG second 05 gG second 03	GAA TGT GGA CCG TAT AAA ACG GC TGG CCG TAG CAG AGC TCC	168

a) The expected product size after PCR amplification.

Table 2. Prevalence of nervous tissues latently infected with PrV determined by nested PCR targeted at the gB, gE, and gG genes

Target gene	Nervous tissues	No. of positive tissues/No. of <sup>a)</sup> tested tissues (%)	No. of positive pigs/No. of <sup>b)</sup> tested pigs (%)
gB	BS	66/94 (70.2)	79/94 (84.0)
	OB	67/93 (72.0)	
	TG	55/94 (58.5)	
gE	BS	22/94 (23.4)	42/94 (44.7)
	OB	25/93 (26.9)	
	TG	34/94 (36.1)	
gG	BS	69/94 (73.4)	84/94 (89.4)
	OB	66/93 (70.9)	
	TG	52/94 (55.3)	

BS: brain stem, OB: olfactory bulb, TG: trigeminal ganglion.

a) Number of nervous tissues showing a positively amplified band of the total number of tested tissue samples.

b) Number of pigs harboring PrV latently infected nervous tissues of the total number of tested pigs.

ium bromide. The specificity of the primers was confirmed by digestion of the amplified products with *Hae* III restriction enzyme. Based on the GenBank EMBL data bank (accession number M17321 for gB gene, AF207700 for gE gene, and M10986 for gG gene), the obtained restriction patterns were compared with the expected patterns. Only a few amplified products were randomly selected and cloned into pGEM-Teasy vector (Promega, Madison, WI, U.S.A.). The authenticity of amplification was also confirmed by determining the sequence of the nucleotides.

**Real-time PCR for quantification of latent PrV DNA:** The quantity of latent PrV DNA in the nervous tissues was determined by real-time PCR specific for gB and gE gene. Real-time PCR was performed using a TaqMan real-time PCR SDS 7700 instrument (ABI prism, Perkin-Elmer). The reaction mixture (50  $\mu$ l) for TaqMan assay contained 550 ng of genomic DNA, 1  $\times$  TaqMan PCR Master Mix (PE Applied Biosystems), 900 nM of each primer, and 250 nM of fluorescence-labeled probe. The thermal cycling conditions employed were 40 cycles of 95°C for 15 s and 60°C for 1 min, with an initial cycle of 50°C for 2 min and 95°C for 10 min. For the PrV gB gene, the forward primer was 5'-ACGGCACGGGCGTGATC-3' and the reverse primer was 5'-ACTCGCGGTCTCCAG CA-3' [3]. For the TaqMan probe, 5'-CTCGCGGACCTCATCGAGCCCTGCAC-3' was used to detect amplification of the PrV gB gene. For detection of the PrV gE gene, 5'-TCGTGATGACGTGCGTCGTCG-3' was chosen as the forward primer, 5'-CGCGGAACCAGTCGTCGAAGC-3' was chosen as the reverse primer [20], and 5'-CTACGAGGGGCCGTACGC-GAGCCTGGA-3' was used for TaqMan probe. All TaqMan probes were labeled with the reporter dye, 6-carboxyfluorescein (FAM), at the 5'-end and with the quencher dye, 6-carboxytetramethylrhodamine (TAMRA) at the 3'-end. Accumulation of PCR products was detected for each cycle by monitoring the increase in fluorescence of the reporter dye from the TaqMan probes. All data was analyzed using the GeneAmp 5700 SDS Ver. 1.7 software.

## RESULTS

**Prevalence of latent PrV infection in nervous tissues by nested PCR:** Nested PCR targeted for PrV gB, gE, and gG genes was employed to assess the prevalence of latent PrV infection in the nervous tissues of pigs. Genomic DNA extracted from sample tissues were used as templates for nested PCR targeting of PrV gB, gE, and gG genes. Nervous tissue samples were recovered from 94 pigs that were exposed to field strain and identified by serological testing. As summarized in Table 2, 66 of the 94 BS tissue samples (70.2%) elicited positively amplified bands of 195 bp PCR products when nested PCR targeting of gB was performed. In the case of OB tissues, 67 of the 93 tissue samples (72%) showed positive results, whereas 55 TG tissue samples (58.5%) showed positive bands in 94 tissue samples. Nested PCR of the gB gene showed positively amplified bands in 79 of the 94 pigs (84.0%) in the case of re-evalua-

tion of all the selected tissues. On the other hand, nested PCR targeting of the gE gene elicited positively amplified bands (211 bp) in 22 of the 94 BS nervous tissues (23.4%), as shown in Table 2. Similarly, 25 of 93 OB (26.9%) and 34 of 94 TG (36.1%) tissue samples showed positive results by gE-targeted nested PCR. These results revealed positively amplified gE PCR products in 42 of the 94 pigs (44.7%). With regard to nested PCR performed for the gG gene, 69 of 94 BS (73.4%), 66 of 93 OB (70.9%), and 52 of 94 TG (55.3%) tissue samples showed amplified bands (168 bp) on agarose gel. Thus, 85 of the 94 seropositive pigs (91.4%) showed positive results in the case of nested PCR targeted at the gG gene. Finally, it was revealed that 90 of the 94 screened pigs (95.7%) were latently infected with field PrV, taking all of the positive data obtained from nested PCR targeted at the three genes into consideration. On the other hand, only 8 of the 94 pigs (8.5%) showed all positive data from nested PCR targeted at the three genes in three selected tissues, whereas 4 pigs (4.3%) exhibited all negative data from nested PCR targeted at the three genes.

**Quantification of latent PrV DNA by real-time PCR:** Viral genome load in the nervous tissue samples, which showed positive results in nested PCR, was further quantified by real-time PCR focusing on glycoprotein genes, gB and gE. Basically, real-time PCR was performed using 550 ng of total DNA. As shown in Fig. 1, real-time PCR was initially standardized by using gB- or gE-encoded plasmid DNA as positive templates. Detection and quantification of the two genes were linear over a range of  $10^2$  to  $10^8$  copies. Real-time PCR targeted for the gB gene showed accumulation of fluorescence over the threshold line at a lower cycle number than for the gE gene. The PrV genome load in latently infected tissues was found to vary from  $<10^{2.0}$  to  $10^{7.1}$  copies per 1  $\mu$ g of genomic DNA (Fig. 2). In real-time PCR using primers and a probe for the gB gene, 36 of 66 BS (54.5%), 34 of 67 OB (50.7%), and 21 of 50 TG (42%) tissue samples showed accumulation of fluorescence for reporter dye above the threshold line (Figs. 2 A, B and C). The copies of the gB gene were evaluated to range from  $<10^{2.0}$  to  $10^{5.5}$  per 1  $\mu$ g of DNA. On the other hand, a smaller number of tested tissue samples reached the fluorescence threshold in the results of real-time PCR targeted for the gE gene (Figs. 2D, E, and F). From the gE-focused real-time PCR, the latent PrV genome was detected in the range of  $10^{2.8}$  to  $10^{7.1}$  copies.

## DISCUSSION

The present study was designed to gain more information on the prevalence and quantity of latent PrV in the nervous tissues of pigs previously exposed to field strain. Nested PCR revealed that the prevalence rate of nervous tissues latently infected with field virus varies depending on the target gene and tissue. It was also revealed that most of the examined pigs (95.7%) were latently infected, taking all of the positive data from nested PCR targeted at the three genes into consideration. There were 8 out of 94 pigs that showed

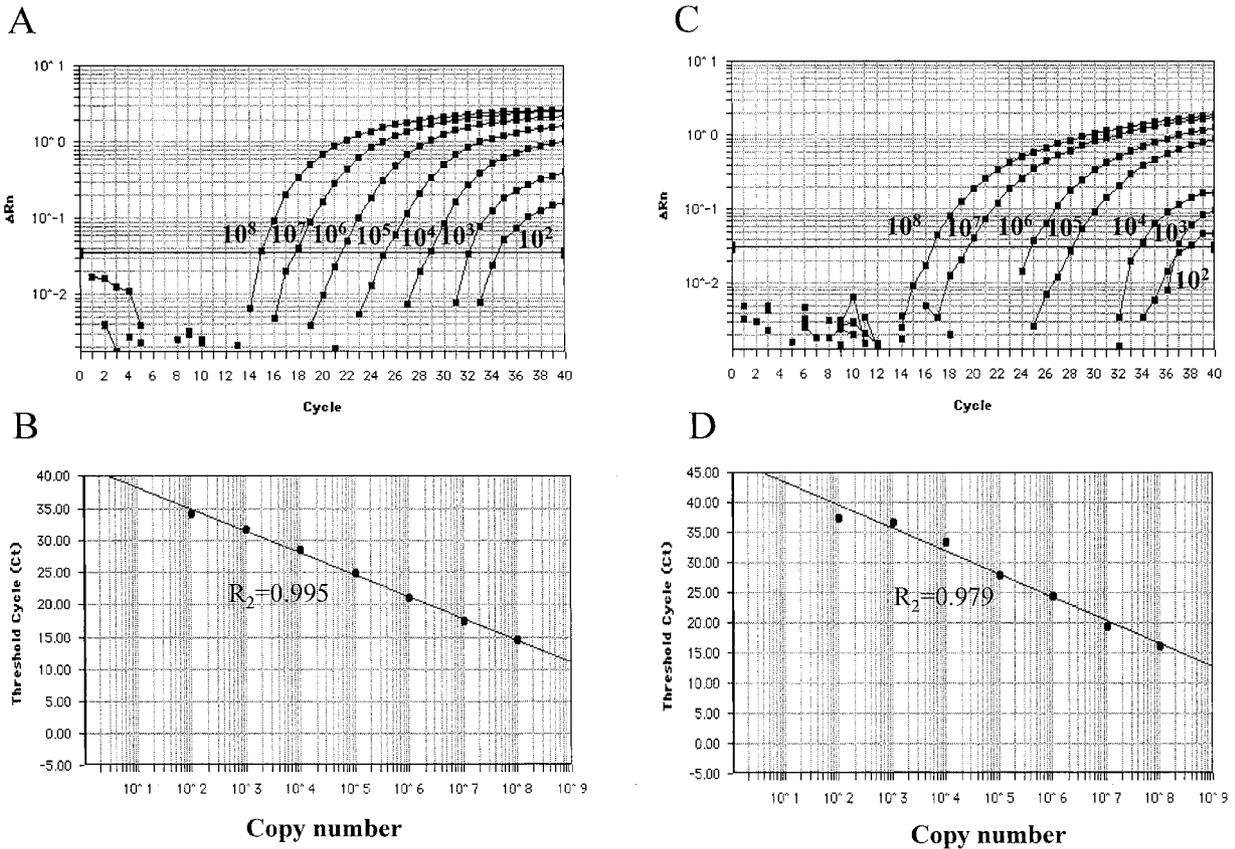


Fig. 1. Amplification and standard curve of the real-time PCR specific for the gB gene (A and B) and gE gene (C and D) of PrV. The upper panel demonstrates the amplification curve (A and C), and the lower panel (B and D) reveals the standard curve. The horizontal bars in the upper panel indicate the threshold line. The horizontal x-axis of the upper panel and vertical y-axis of the lower panel represents the cycle number, and the vertical x-axis of the upper panel indicates the monitored fluorescence. The horizontal x-axis of the lower panel indicates the estimated copy number when fluorescence reached the threshold line. The amplification was linear in the range of  $10^2$ – $10^8$  (correlation coefficient=0.995 for the gB gene, and 0.979 for the gE gene).

positive results for the three target genes in all of the three nervous tissues, whereas 4 pigs showed no amplified product in all the selected tissues. Moreover, the data obtained from real-time PCR indicated that the quantitative level of latent PrV varied considerably from one animal to another. These results imply that field PrV could establish a latency state in the nervous tissues of pigs at various levels over a long period of time.

Several studies on latent infection of PrV have been performed using experimentally infected animals. We chose previously exposed pigs living on farms, and not experimentally infected pigs, to get a real picture of the conditions in the field. Latent infection of PrV has been detected in various nervous tissues, but TG has been regarded as the primary site [4, 6, 33]. A previous report has claimed that OB showed positive amplification by PCR in experimentally infected pigs, for which the viral genome could not be amplified from TG [2]. Considering these facts, we believed that testing of other nervous tissues, including OB and BS, is as important as testing TG for the diagnosis of

PrV latency to avoid false negative results.

We quantified, for the first time, the viral genome of latent PrV by real-time PCR. Real-time PCR was capable of detecting low quantity levels of latent infection, less than 5 copies. It is postulated that the diversity in the quantity of latent PrV could be caused by several factors, such as field viral strain, the dose of the exposed virus, the route of exposure, and immunological status and age of the animals. According to other reports, the quantity of latent infection showed differences from one animal to another, even when they were inoculated with the same dose of virus [31]. Furthermore, the detection limit and quantity of latent infection determined by real-time PCR seemed to depend on the primers and probes used for real-time PCR, as revealed by the discrepancy in results arising due to the primers and probes for the gB and gE genes. Thus, the PCR method for detection of latent PrV DNA should be carefully adjusted depending on the target gene, selected tissue samples, and vaccinated/unvaccinated animals.

Various methods have also been tried for detection and/or

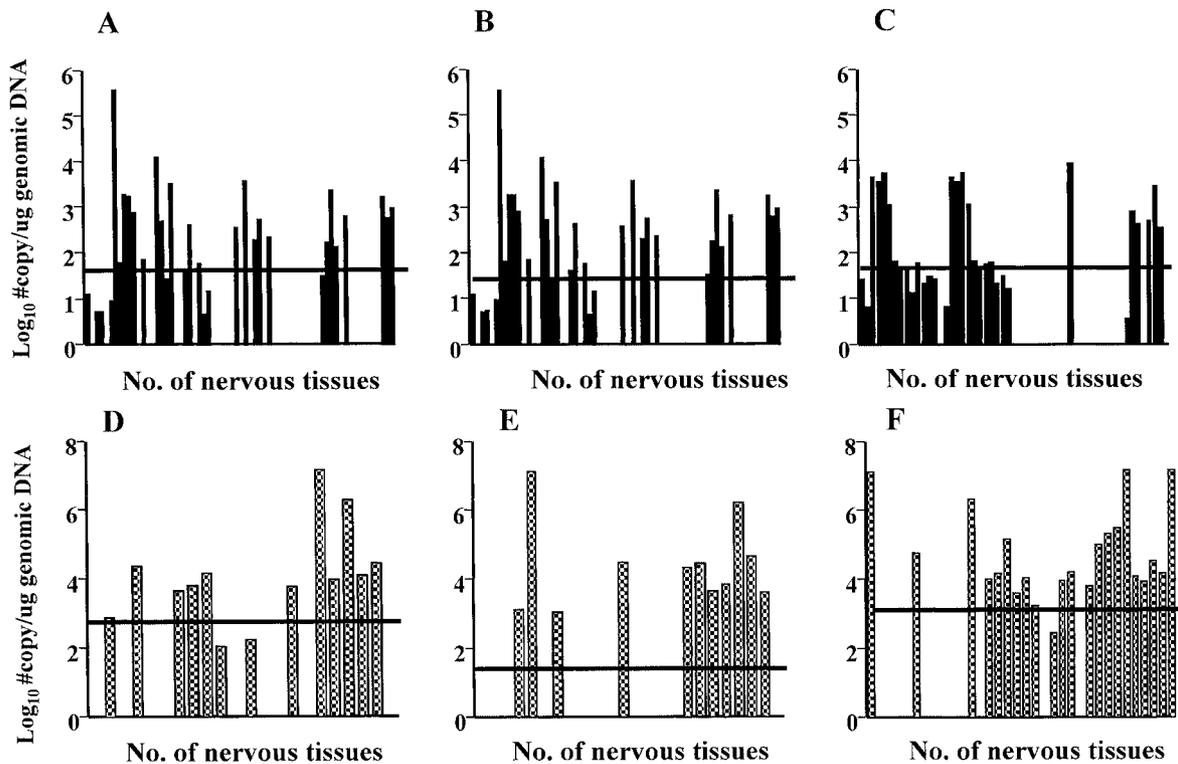


Fig. 2. The quantity of latent PrV DNA in the nervous tissues of pigs determined by real-time PCR. Real-time PCR was performed using primers and a probe specific for PrV gB (upper panel A, B, and C) and gE (lower panel D, E, and F) in BS (A and D), OB (B and E), and TG (C and F) tissue samples. The individual bar represents the copy number of the target gene per microgram of genomic DNA in each nervous tissue. The horizontal bar in each graph reveals the average copy number in each of the corresponding tissues (A:  $10^{1.50}$ , B:  $10^{1.17}$ , C:  $10^{1.33}$ , D:  $10^{2.51}$ , E:  $10^{1.91}$ , F:  $10^{3.13}$ ).

quantification the latency of PrV. Amongst such methods, PCR assay is regarded as a powerful tool for detection of PrV latent infection because of its convenience, reproducibility, and sensitivity [1, 5, 7, 17]. There are reports showing that animals experimentally inoculated with virulent or avirulent strains have been latently infected, as demonstrated by PCR amplification, independent of the dose of infection and previous existence of immunity against PrV [8, 11, 31, 32]. Latently infected pigs could be left in herds, and presumed to be free of PrV if an antibody response to viral infection is not detected by differential ELISA for gE or other glycoproteins. The possible existence of seronegative pigs latently infected with field PrV has been previously postulated [20, 30]. Furthermore, passively acquired antibodies may be able to block the induction of a detectable differential antibody response after exposure to a low dose of a virulent virus without preventing any latent infection [20]. Such a case is troublesome during the campaign to eradicate and control PrV. Several studies have also tried to evaluate adequate procedures for quick and easy diagnosis of PrV latent infection by placing emphasis on cerebrospinal fluid and peripheral blood mononuclear cells [2, 3]. However, such approaches did not provide information about true latent infection. It is apparent that removal of seronegative PrV latently infected pigs from herds may be critical

for an eradication program. Thus, we are currently investigating the prevalence and quantity of PrV latent infection in the nervous tissues of seronegative pigs.

Efforts to detect and eradicate PrV latently infected animals should be accompanied by the development of an appropriate vaccine to prevent latent infection. Several vaccine types and routes of administration have been investigated for establishment of PrV latent infection [11–13, 22, 27, 29, 32]. Consequently, this report demonstrates that PrV could be latently present in apparently normal animals without them showing any clinical signs. A continuous survey for latently infected pigs and defined vaccination strategies to prevent latency establishment could contribute to an eradication program for PrV.

**ACKNOWLEDGEMENTS.** We would like to thank Mrs. Soon-Ok No for technical assistance and generous help. We are also thankful to the members of Jeonbuk Livestock Development and Research Institute for collection of samples. This study was supported by the Technology Development Program for Agriculture and Forestry of the Ministry of Agriculture and Forestry, a research grant from the Bio-Safety Research Institute, Chonbuk National University, and the Brain Korea 21 Project in 2003 of the Republic of Korea. HA Yoon was supported in part by a Post-Doc. Pro-

gram grant from Chonbuk National University (2004).

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