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Molecular Survey of Latent Pseudorabies Virus Infection in Nervous Tissues of Slaughtered Pigs by Nested and Real-time PCR

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In this study, the prevalence and quantity of a latent pseudorabies virus (PrV) infection in the nervous tissues of randomly selected pigs was determined via nested and real-time PCR. The nervous tissues, including the trigeminal ganglion (TG), olfactory bulb (OB), and brain stem (BS), were collected from the heads of 40 randomly selected pigs. The majority of the nervous tissues from the selected pigs evidenced a positively amplified band on nested PCR. In particular, nested PCR targeted to the PrV glycoprotein B (gB) gene yielded positive results in all of the BS samples. Nested PCR for either the gE or gG gene produced positive bands in a less number of nervous tissues (57.5% and 42.5%, respectively). Real-time PCR revealed that the examined tissues harbored large copy numbers of latent PrV DNA, ranging between $10^{0.1}$ and $10^{7.2}$ ($1\text{-}1.58\times10^{7}$) copies per 1 μg of genomic DNA. Real-time PCR targeted to the PrV gE gene exhibited an accumulated fluorescence of reporter dye at levels above threshold, thereby indicating a higher prevalence than was observed on the nested PCR (100% for BS, 92% for OB, and 85% for TG). These results indicate that a large number of farm-grown pigs are latently infected with a field PrV strain with a variety of copy numbers. This result is similar to what was found in association with the human herpes virus.

Key words: latent infection, nested PCR, pseudorabies virus, real-time PCR

The pseudorabies virus (PrV) is an alphaherpes virus, which causes a fatal disease in swine known as Aujeszky's disease, one of the most infectious diseases affecting the swine industry (Kluge *et al.*, 1999). Economically, the greatest cost of this disease is associated with the acute phase of the infection, in which the abortion rate and neonatal mortality are quite high (Kluge *et al.*, 1999). Similarly to human alphaherpesvirus, PrV establishes a lifelong infection in a variety of the nervous tissues of the natural host, and this infection can be reactivated under experimental or natural stresses (Rock, 1993; Azmi *et al.*, 2002). Such reactivations of latent PrV cause the release of viral particles into the environment, and transmission to neighboring hosts (Brockmeier *et al.*, 1993; Enquist *et al.*, 2002; Tomishima and Enquist, 2002).

Currently, genetically live mutant PrV vaccines with a specific glycoprotein deletion have been developed, and are marked to facilitate a differentiation between vaccinated and field virus-infected swine (van Oirshot *et al.*, 1990; Vilnis *et al.*, 1998; Ferrari *et al.*, 2000). A differential anti-glycoprotein antibody ELISA technique has also been developed, in order to enable control of the spread of PrV, as well as its eradication, by the testing and removal of field virus-infected swine (Kit *et al.*, 1990; Lehmann *et al.*, 2002; Pensaert *et al.*, 2004). Therefore, it is essential that swine latently infected with field virus produce and maintain detectable levels of serum antibodies against such differential envelope glycoproteins. However, swine with latent infections, which are presumed to be free of PrV, can easily be overlooked in cases in which differential ELISA fails to detect the antibody response to the field virus. Furthermore, it has also been suggested that some swine may be latently infected with a seronegative variant of PrV (Thawley *et al.*, 1984; Jacobs *et al.*, 1996; McCaw *et al.*, 1997). Therefore, the phenomenon of the latent infection and reactivation of PrV is considered to constitute a major hindrance in its control and eradication (Maes *et al.*, 1997).

A variety of techniques have been developed to detect and/or quantify latent PrV infections (Beran *et al.*, 1980; Maes and Thacker, 1988; Lokensgard *et al.*, 1990; Schang and Osorio, 1994; Denis *et al.*, 1997). Some of these tools, based on the explantation and cocultivation of infected tissues, are both time-consuming and insufficiently sensitive,

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whereas the use of molecular techniques has been associated with great advances in both sensitivity and accuracy (McFarlane *et al.*, 1986; Lokensgard *et al.*, 1991). In particular, the advent of techniques based on the polymerase chain reaction (PCR) has heralded a significant increase in the ability to determine the existence and quantity of viruses that persist in tissues. The use of these techniques, as a result, continues to increase (Krumbholz *et al.*, 2003; Mumford *et al.*, 2004; Ozoemena *et al.*, 2004). In this study, we have used nested and real-time PCR techniques to determine both the prevalence and quantity of a latent PrV infection in the nervous tissues of randomly selected pigs. Therefore, the data collected in this report is expected to provide insight into latent PrV infections in pigs grown on Korean farms, in a similar manner to the studies conducted with the human herpes virus.

Materials and Methods

Cell and virus

The virulent PrV Yangsan (YS) strain, which was generously provided to us by the National Veterinary Research and Quarantine Service of Korea, was utilized as a control for the determination of the specificity of both the primers and probes. The PrV YS strain was propagated in the PK-15 porcine kidney cell line. The cultures were incubated in a humidified CO_2 incubator at 37°C. The viral stock was concentrated, titrated, and stored in appropriate aliquots at -80°C until needed.

Purification of PrV DNA genome

The acquired virus was suspended in a TBS buffer (10 mM Tris-HCl, 0.15 M NaCl, pH 7.4), and then disrupted with 0.6% SDS and proteinase K (400 μg/ml) for 2 h at 37°C. The released viral DNA was then extracted using a conventional phenol:chloroform:isoamyl alcohol (25:24:1) solution. The contaminating RNA was removed with RNase A (200 μg/ml), and the final viral DNA pellets were resuspended in DNase/RNase free water and stored at -20°C until needed.

Collection of nervous tissues from pigs

The heads of 40 crossbred pigs that had been transferred to the slaughter site of the Mokwoochon Corporation of Korea were randomly selected between April and September of 2004. Nervous tissues, namely the brain stem (BS), olfactory bulb (OB), and trigeminal ganglion (TG), were extracted from the cranial caves of the heads. Each of these nervous tissues was then frozen at -70°C until needed.

Purification of genomic DNA from nervous tissues

The genomic DNA from the recovered nervous tissues (approximately 100 mg per nervous tissue) was extracted with DNAzol (MRC, USA), in accordance with the manufacturer's instructions. The contaminating RNA was removed via RNase (200 μg/ml) treatment. This was followed by the phenol/chloroform extraction and ethanol precipitation of genomic DNA, and resuspension of the DNA in DNase/RNase free water, and its storage at -20°C until needed. The DNA concentrations were evaluated using a GeneQuant RNA/DNA calculator (Biochrom, UK), and adjusted to 50 ng/μl.

Nested PCR for identification of latent PrV DNA

The prevalence of latent PrV infection in the nervous tissues was then determined via nested PCR targeted to the viral glycoprotein gB (gII), gE (gI), and gG (gX) genes (Kluge *et al.*, 1999). Table 1 shows the primer sequences used, as described in previous studies (McCaw *et al.*, 1997; Balasch *et al.*, 1998a; Vilnis *et al.*, 1998). A total of

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

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

[a]The expected product size after PCR amplification

10 μl (500 ng) of genomic DNA was used to run nested PCR in a 25-μl reaction volume, containing 2.5 μM of each primer, 1.5 mM $MgCl_2$, 0.05 U *Taq* Polymerase (Promega, USA), 0.2 mM dNTP, and 1× reaction buffer. The DNA was initially amplified with a Perkin-Elmer GenAmp PCR system 9600 (Perkin-Elmer, USA) for 1 cycle of 95°C for 10 min, and 30 cycles of denaturation (94°C, 45 sec), annealing (62°C, 1 min) and extension (72°C, 1 min). A final extension time of 10 min at 72°C was included at the end of the final cycle. The second nested PCR amplification was conducted using 5 μl of the products of the first PCR, under identical conditions. The PCR products were then electrophoresed in 1.85% agarose gel (Invitrogen, USA), and visualized via gel documentation (Vilber Lourmat, France), followed by ethidium bromide staining.

Restriction fragment length polymorphism (RFLP) of nested PCR products

The specificity of the nested PCR products was then verified via restriction fragment length polymorphism (RFLP) analysis (Mishra *et al.*, 2002; Park *et al.*, 2003; Yoon *et al.*, 2003). The nested PCR products for the gB, gE, and gG genes of PrV were eluted from agarose gel, using a SV gel/PCR clean-up system (Promega), and these products were in turn digested with the *Hae*III restriction enzyme. The digested PCR products were then analyzed using 1.85% agarose gel. The nucleotide sequence data obtained from the GenBank EMBL data bank (accession number M17321 for gB gene, AF207700 for gE gene, and M10986 for gG gene) were used to search for the restriction enzyme recognition sites specific to PrV. The sequences of some of the amplified products were also determined via cloning into pGEM T-easy vector (Promega, USA).

Real- time PCR for quantification of latent PrV DNA

The quantity of latent PrV DNA within the nervous tissues was determined via real-time PCR specific for the gB and gE genes. Real-time PCR was conducted using the *Chrome 4*™ Detector for Real-Time Fluorescence Detection (MJ Research, USA). The reaction mixture (25 μl) used for the *TaqMan* assay contained 5 μl (500 ng) of genomic DNA, 12.5 μl of 2× *TaqMan* PCR Master Mix (Applied Biosystems, USA), 2.5 μl of a 900 nM solution of each of the primers, and 2.5 μl of 250 nM fluorescence-labeled probes. The thermocycling program was conducted as follows: 50 cycles of 94°C for 1 min, 60°C for 30 sec, and 72°C for 1 min. The forward primer for the PrV gB gene was 5'-ACGGCACGGGCGTGATC-3', and the reverse primer was 5'-ACTCGCGGTCCTCCAG CA-3' (Balasch *et al.*, 1998a). The TaqMan probe, 5'-CTCG-CGCGACCTCATCGAGCCCTGCAC-3', was used to detect any amplifications of the PrV gB gene. By way of contrast, the forward primer, 5'-TCGTGATGACGTGCG-TCGTCG-3', and the reverse primer, 5'-CGCGGAACCA-GTCGTCGAAGC-3', were used in the detection of the PrV gE gene (McCaw *et al.*, 1997). 5'-CTACGAGGGGCCG-TACGCGAGCCTGGA-3' was used for the TaqMan analysis. All TaqMan probes were labeled at the 5' end with the reporter dye, 6-carboxyfluorescein (FAM), and with the quencher dye, 6-carboxytetramethylrodamine (TAMRA), at the 3'-end. At each cycle, the accumulated PCR products were detected by monitoring increases in the fluorescence of the reporter dye from the *TaqMan* probes. All data was analyzed using operations manual Ver. 2.03 Supports *Optical Monitor*™ software.

Results

Specificity of the primers used for nested PCR

The specificity of the primers used for the nested PCR was evaluated via the digestion of the PCR products with restriction enzymes. The DNA extracts obtained from the

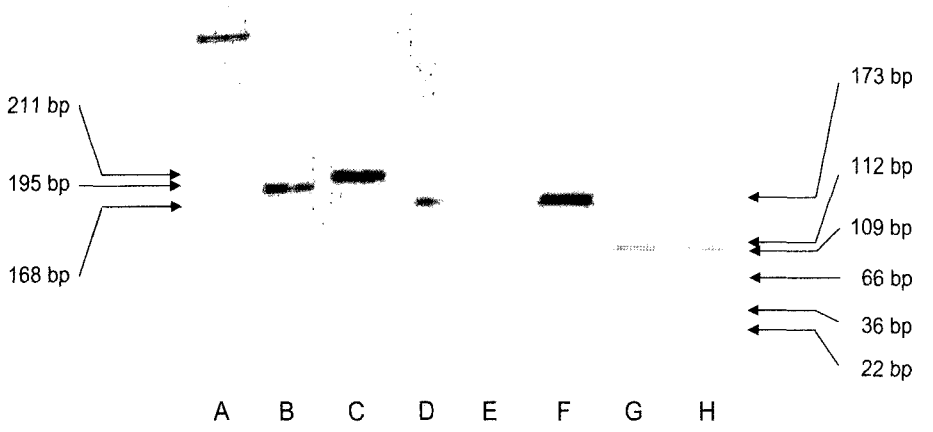

Fig. 1. Amplified products of the nested PCR targeted to three PrV genes (gB, gE, and gG) and the digested pattern of the PCR products for testing the specificity of the primers. A, size markers (100 bp DNA ladders); B, 195 bp product of gB gene; C, 211 bp product of gE gene; D, 168 bp product of gG gene; E, negative control; F, *Hae* III restriction of gB-targeted PCR product (173 and 22 bp); G, *Hae* III restriction of gE-targeted PCR product (109, 66 and 36 bp); H, *Hae* III restriction of gG-targeted PCR product (112 and 22 bp). The relative positions in the gel of predicted size are indicated by arrowheads on both sides.

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

Target gene	Nervous tissues	No. of positive tissues/[a] No. of tested tissues	Percentage of positive results in tested tissues (%)	No. of positive pigs/[b] No. of tested pigs	Percentage of positive result in tested pigs (%)
gB	BS	40/40	100	40/40	100
	OB	39/40	97.5		
	TG	33/40	82.5		
gE	BS	16/40	40	23/40	57.5
	OB	13/40	32.5		
	TG	25/40	62.5		
gG	BS	9/40	22.5	17/40	42.5
	OB	6/40	15		
	TG	10/40	25		

BS, brain stem; OB, olfactory bulb; TG, trigeminal ganglion
[a]Number of nervous tissues showing positively amplified band in total tested tissues
[b]Number of pigs harboring PrV latently infected nervous tissues in total tested pigs

PrV virion were then subjected to nested PCR amplification, using primers designed on the basis of the sequence data of the PrV target gene from the GenBank EMBL data bank. The final products were digested with the *Hae* III restriction enzyme. As is shown in Fig. 1, the digestion of the nested PCR product (195 bp) targeted to the PrV gB gene yielded two fragments (173 and 22 bp), a result consistent with the sequence data in the GenBank database. The PCR product (211 bp) of the gE gene yielded three fragments (109, 66, and 36 bp) after digestion, whereas the 168 bp product of the gG gene was divided into two fragments (112 and 22 bp). Identical digestion patterns were elicited from the genomic DNA extracted from the nervous tissues of the slaughtered pigs (data not shown). All digested patterns of the PCR products were consistent with the expected results, based on the sequence data contained in GenBank. The specific amplification of target genes via nested PCR was also detected after the cloning of the PCR products, followed by sequence analysis.

Determination of nervous tissues harboring latent PrV DNA by nested PCR

The prevalence of the nervous tissues latently infected with PrV was evaluated via the extraction of the genomic DNAs from the tissues, which were then used as templates for the nested PCR targeted to the PrV gB, gE, and gG genes. The nervous tissues were recovered from the heads of 40 crossbred pigs, which had not been previously serologically tested. Table 2 shows that almost all of the nervous tissues from the selected pigs were positive for amplified bands of the target genes. Specifically, nested PCR targeted to the PrV gB gene yielded positive results in all selected BS tissues. Nested PCR for the PrV gB gene yielded positive results in 39 (97.5%) and 33 (82.5%) of the OB and TG tissue samples, respectively. On the other hand, nested PCR targeted to either the gE or gG genes yielded positive bands in less number of samples (total 57.5% and 42.5%, respectively). 16 BS (40%), 13 OB (32.5%), and 25 TG (62.5%) samples out of a total of 40 samples each evidenced positively amplified bands of the PrV gE gene, whereas nested PCR for the PrV gG gene yielded positive results in 9 BS (22.5%), 6 OB (15%), and 10 TG (25%) samples. This demonstrates that nested PCR, using the primer sets for the amplification of the PrV gB gene detected the three target genes at the highest prevalence. Moreover, it should be noted that the majority of the nervous tissues from the randomly selected pigs were latently infected with PrV.

Quantification of latent PrV DNA by real-time PCR

The quantity and prevalence of latent PrV DNA in the selected nervous tissues were also evaluated via real-time PCR techniques. When real-time PCR using the primer sets and probes for the PrV gB and gE genes was conducted, the tissues were found to harbor substantial copy numbers of latent PrV DNA, in a range between $10^{0.1}$ and $10^{7.2}$ ($1\text{-}1.58\times10^{7}$) copies per 1 μg of genomic DNA (Fig. 2). In particular, real-time PCR targeted to the PrV gE gene evidenced an accumulated reporter dye fluorescence above threshold levels, indicating a higher prevalence for the same gene than was shown by nested PCR targeted toward the same gene (100% for BS, 92% for OB, and 85% for TG) (Fig. 2D. E, F). However, less number of OB (67%) and TG (74%) samples tested positive on real-time PCR using the primer sets and probes for the PrV gB gene than were observed on the nested PCR (Fig. 2. B, C). This suggests that the majority of pigs growing on Korean farms are latently infected with the field PrV strain, at a variety of copy numbers.

Discussion

The objective of this study was to obtain information

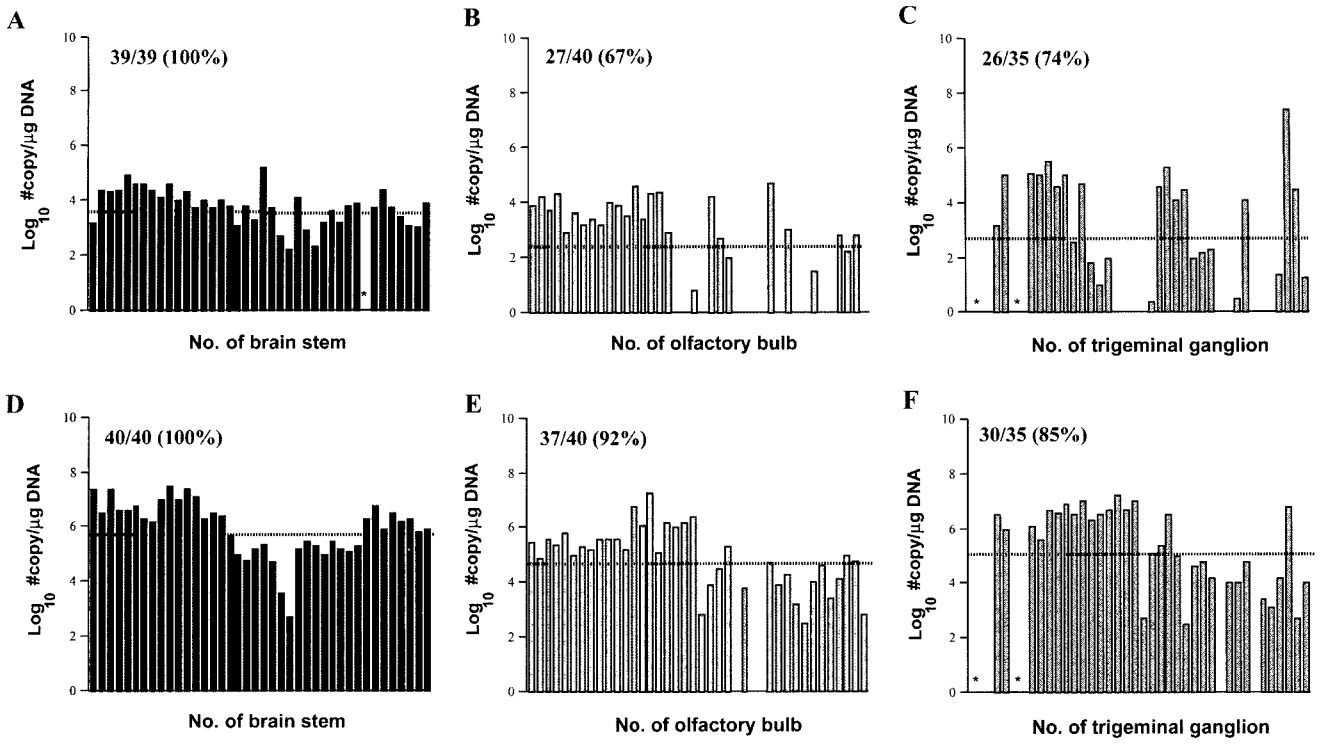

Fig. 2. The quantity of latent PrV DNA in the nervous tissues of pigs via real-time PCR. Real-time PCR was conducted using the primers and probes specific to the PrV gB (above panel A, B and C) and gE (bottom panel D, E and F) genes in BS (A and D), OB (B and E), and TG (C and F) tissues, as described in Materials and Methods. The individual bar represents the copy numbers of the target gene per one microgram of genomic DNA in each of the tested nervous tissues. The horizontal line in each of the graphs denotes the average copy number in the corresponding tissues (A, $10^{3.8}$; B, $10^{2.3}$; C, $10^{2.6}$; D, $10^{5.9}$; E, $10^{4.6}$; F, $10^{5.0}$). The values in the bar charts represent the numbers of samples exhibiting an accumulated reporter dye fluorescence above threshold levels /No. of total tested samples (percentage). *Not determined.

regarding the prevalence and quantity of latent PrV infections in the nervous tissues of randomly selected pigs, via both nested and real-time PCR. Almost all of the selected pigs were shown to be latently infected with the field PrV strain. Moreover, real-time PCR demonstrated that the levels of these latent PrV infections varied considerably from one animal to the next. Therefore, the field PrV strain appears to be widely established, in latent state, in the nervous tissues of growing pigs, in a manner similar to that of the alpha herpesvirus in humans.

Aujeszky's disease, which is caused by a PrV infection, is a significant cause of economic loss within the swine industry. Infectious viral particles are released into the environment upon the reactivation of the latent virus, which results in intermittent outbreaks. Therefore, it has been theorized that the ability of this virus to persist in a latent state should be considered a crucial factor in any eradication or control campaign (Maes *et al.*, 1997). The mechanisms controlling the latency and reactivation of the alphaherpes virus have received a considerable amount of attention, but remain poorly understood (Rock, 1993). Vaccination with an attenuated strain is one technique which has been previously used to confer partial protection of young piglets. However, such vaccinations do not, apparently, prevent the establishment of latency by infectious viruses. Furthermore, many vaccine strains have been reported to themselves establish latency (Mengeling, 1991; Volz *et al.*, 1992). Therefore, a variety of methods have been used to detect latent herpesvirus (Beran *et al.*, 1980; Maes *et al.*, 1988; Lokensgard *et al.*, 1990; Schang and Osorio, 1994; Dennis *et al.*, 1997). Some techniques detect the presence of the virus itself, including *in vivo* or *in vitro* reactivation techniques, which rely on the explantation and cocultivation of infected tissues. Recently, the molecular techniques have proven to be more powerful techniques. Nested and real-time PCR can be used to determine the prevalence and quantity of latent PrV DNA. Such PCR-based tools, which tend to be highly specific and sensitive, allow for the detection of five copies of the gene encoding for the PrV envelope glycoprotein, among 10^6 host cells. This level of sensitivity is almost identical to the radioactivity-based method, which can discern three copies of PrV DNA in 1 μg of total DNA (Schang and Osorio, 1994). Real-time PCR, however, provides more sensitive and valuable information regarding prevalence, from a quantitative standpoint. Furthermore, latent PrV infections have been found by this technique in a variety of nervous tissues, even though the TG has classically been regarded to be the primary site at which the virus is located (Beran *et al.*, 1980; Brown *et al.*, 1990; Wheeler

and Osorio, 1991; Romero *et al.*, 2003). Similarly, Balasch *et al.* reported that tested OB samples from experimentally infected pigs exhibited positive PCR amplification, but that the viral genome could not be amplified from the TG (Balasch *et al.*, 1998b). This shows that, in order to avoid false negative results, the testing of other nervous tissues, including the OB and the BS, may be as important in diagnosing PrV latency as the testing of the TG.

It has been previously suggested that some seronegative pigs may be latently infected with PrV (Thawley *et al.*, 1984; Jacobs *et al.*, 1996; McCaw *et al.*, 1997). Furthermore, passively acquired antibodies may be capable of blocking the induction of a differential antibody response after exposure to low doses of a virulent virus, without preventing latent infection (McCaw *et al.*, 1997). This would clearly hinder campaigns targeted toward the eradication and control of PrV. Several attempts to develop an appropriate procedure for the quick and easy diagnosis of latent PrV infections have focused primarily on the cerebrospinal fluid and the peripheral blood mononuclear cells (Balasch *et al.*, 1998a; Balasch *et al.*, 1998b). However, such approaches have not proven to provide valuable information regarding latent infections. It is apparent that the removal from herds of seronegative pigs with latent PrV infections would be a prerequisite with regard to the eradication of PrV. Therefore, efforts to develop simple diagnostic techniques, along with protocols for the timely eradication of latently infected animals, should be accompanied by the development of a vaccine appropriate for the prevention of latent infections. Our current molecular survey provides insight into the incidence of latent PrV infections, even though the study involved only the nervous tissues of pigs randomly selected from a single site.

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