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Authors: Carlos H. Romero, Paul N. Meade, Bruce L. Homer, Joseph E. Shultz, and Gene Lollis

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## POTENTIAL SITES OF VIRUS LATENCY ASSOCIATED WITH INDIGENOUS PSEUDORABIES VIRUSES IN FERAL SWINE

Carlos H. Romero,<sup>1,3</sup> Paul N. Meade,<sup>1</sup> Bruce L. Homer,<sup>1</sup> Joseph E. Shultz,<sup>1</sup> and Gene Lollis<sup>2</sup>

<sup>1</sup> Department of Pathobiology, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32610, USA

<sup>2</sup> MacArthur Agro-Ecology Research Center, Archbold Biological Station, Lake Placid, Florida 33852, USA

<sup>3</sup> Corresponding author (email: romeroc@mail.vetmed.ufl.edu)

**ABSTRACT:** Free-ranging feral swine (*Sus scrofa*) are known to be present in at least 32 states of the USA and are continuously expanding their range. Infection with pseudorabies virus (PRV) occurs in feral swine and the primary route of transmission in free-living conditions seems to be venereal. Between 1995 and 1999, naturally infected feral swine and experimentally infected hybrid progeny of feral and domestic swine, were kept in isolation and evaluated for occurrence of latent PRV indigenous to feral swine in sacral and trigeminal ganglia and tonsil. Sacral ganglia were shown, by polymerase chain reaction (PCR) amplification of the thymidine kinase (TK) gene of PRV, to be the most frequent sites of latency of PRV. Nine (56%) of 16 sacral ganglia, seven (44%) of 16 trigeminal ganglia, and five (39%) of 13 tonsils from naturally infected feral swine were positive for PCR amplification of TK sequences of PRV. These tissues were negative for PRV when viral isolation was attempted in Vero cells. DNA sequencing of cloned TK fragments from the sacral ganglia of two feral swine, showed only one nucleotide difference between the two fragments and extensive sequence homology to fragment sequences from various domestic swine PRV strains from China, Northern Ireland, and the USA. The hybrid feral domestic swine, experimentally inoculated with an indigenous feral swine PRV isolate by either the genital or respiratory route, acquired the infection but showed no clinical signs of pseudorabies. Virus inoculated into either the genital or respiratory tract could, at times, be isolated from both these sites. The most common latency sites were the sacral ganglia, regardless of the route and dose of infection in these experimentally infected hybrids. Nine of 10 sacral ganglia, six of 10 trigeminal ganglia, and three of 10 tonsils were positive for PCR amplification of TK sequences. No virus was isolated from these tissues in Vero cells. The demonstration of the sacral ganglia as the most common sites of latency of pseudorabies viruses indigenous to feral swine, supports the hypothesis that these viruses are primarily transmitted venereally, and not by the respiratory route as is common in domestic swine, in which the trigeminal ganglia are the predominant sites of virus latency.

**Key words:** Feral swine, latency, pseudorabies virus, transmission.

### INTRODUCTION

Pseudorabies virus (PRV) is an *Alpha-herpesvirus* of significant economic importance for the swine industry worldwide. Pseudorabies virus is the cause of pseudorabies or Aujeszky's disease, and has predilection for respiratory and nervous tissue; hence, most clinical signs in domestic swine are associated with a dysfunction of these organs (Kluge et al., 1999). The disease is characterized by high mortality in neonatal piglets, weight loss and poor feed conversion in growers, and abortions in sows (Kluge et al., 1999). Chronically infected adult domestic swine are considered to be the main PRV reservoir for perpetuating the infection in nature (Shope, 1935; McFerran and Dow, 1964).

Free-living feral swine are known to exist in at least 32 states of the US and have been shown to be infected with PRV (Nettles and Erikson, 1984; Van Der Leek et al., 1993). Their continuous migration and range expansion across the central US (Gipson et al., 1998) pose a serious threat to completion of the National Pseudorabies Eradication Program from the national domestic swine herd. Immediately after infection, alphaherpesviruses travel centripetally along the peripheral sensory nerves towards neurons in ganglia (Hill, 1985) in which they persist latent as an episome in a non-integrated form (Aurelian, 1989). Sporadically, and under the influence of environmental stress, latent alphaherpesviruses are reactivated, and travel centrifugally along the axons towards

the original site of entry, with or without causing overt clinical signs (Hill, 1985). In domestic swine, the trigeminal ganglia have been identified as the primary sites of PRV latency (Gutekunst et al., 1980; Brockmeier et al., 1993; Tham et al., 1994) with the tonsils and brain also being affected (Sabo and Rajcani, 1976; Beran et al., 1980; Galeota-Wheeler and Osorio, 1991; Tham et al., 1994). Thus, latency and reactivation are the hallmarks of infection with herpesviruses.

Transmission of PRV in domestic swine usually occurs by the oropharyngeal route after direct contact of infected and susceptible animals and the ingestion or aspiration of infected aerosols, secretions, and excretions (McFerran and Dow, 1964; Christensen et al., 1993). Recently, we reported that transmission of PRVs indigenous to feral swine occurs mainly by the genital route during the mating of PRV-infected feral swine to susceptible feral or domestic swine (Romero et al., 1997, 2001). Since the mode of transmission of PRV in feral swine seems to differ from that in domestic swine, we speculated that latency might occur at sites different from those observed in domestic swine. Latency sites in domestic swine have been reevaluated using the polymerase chain reaction (PCR) on total DNA extracted from selected tissues (Belak et al., 1989; Maes et al., 1990; Galeota-Wheeler and Osorio, 1991; Brockmeier et al., 1993; Tham et al., 1994; Thiery et al., 1996). In the present study, we have used PCR amplification of DNA extracted from sacral and trigeminal ganglia and tonsil to determine latency sites after natural and experimental infection with PRV indigenous to feral swine.

## MATERIALS AND METHODS

### Animals and housing

Feral swine were trapped and maintained at the Buck Island Ranch, MacArthur Agro-Ecology Research Center (Lake Placid, Florida, USA; 27°10'N, 81°21'W), between 1995 and 1999. Animals were ear-tagged and a small blood sample was tested immediately for antibodies to PRV (PRV Agglutination Test Kit, Vi-

ral Antigens, Inc., Memphis, Tennessee, USA), and *Brucella* sp. (*Brucella* Card Test, Becton-Dickinson Co., Inc., Baltimore, Maryland, USA). Animals were treated for gastrointestinal nematodes with Ivermectin (Meriel, Rahway, New Jersey, USA) and separated by both sex and PRV antibody status. Only animals testing negative for antibodies to *Brucella* were used for experimentation. Animals were kept outdoors in two wooden slat pens, measuring 5×10 m, with dirt floors, food troughs, and a continuous source of water. There was no roof over the pens, but nearby trees provided shade. Although located in an open field, the pens were secure to avoid contact of the experimental animals with free-roaming feral swine that may have been in the area. Animals were fed a diet consisting mainly of corn and feed pellets once daily.

### Collection of swabs and tissues

Nasal and genital secretions for virus isolation were collected with sterile Dacron swabs (Curtin Matheson Scientific, Houston, Texas, USA) in cold transport medium and processed as described previously for virus isolation in African green monkey kidney (Vero) cells (Romero et al., 2001). All swine were euthanized with a captive bolt pistol applied over the brain's frontal lobe and immediately exsanguinated by severing the axillary venous plexus. Sacral and trigeminal ganglia and tonsil were aseptically removed, frozen immediately in dry ice, and transported to the laboratory. For virus isolation, approximately 10% suspensions of the above tissues were prepared in Dulbecco's modified Eagle medium (DMEM) containing 2% fetal bovine serum (FBS) and antibiotics (penicillin 200 U/ml, streptomycin 200 µg/ml, amphotericin B 25 U/ml) and inoculated directly on drained monolayers of Vero cells. Inoculated cultures were observed for 10 days for the development of cytopathogenic changes characteristic of herpesvirus growth.

### Natural and experimental infection with PRV

Seventeen naturally infected, PRV antibody positive, adult feral swine that had been trapped and kept in captivity between 2–3 yr were used as tissue donors for direct virus isolation in Vero cells and for the detection of latency by PCR. Hybrids of feral and domestic swine were obtained after mating PRV antibody positive feral sows with PRV free domestic boars and were used in the experimental infection trial. The hybrid pigs were monitored monthly, during 8 mo, for PRV antibodies (Romero et al., 1997) and for PRV excretion in nasal and genital swabs (Romero et al., 2001).

Pseudorabies virus was not isolated at any time from these animals and except for low-titer maternal antibodies detected during the first month of life, the hybrid progeny remained PRV antibody negative. Seven female and three male hybrid swine were separated by sex and housed in two wooden slat pens. An additional male hybrid pig was kept in isolation as un inoculated control. Ten hybrid pigs were experimentally infected at 8 mo of age by instillation in the vaginal or preputial cavity, or in the nostrils with  $10^6$  median tissue culture infectious doses (TCID<sub>50</sub>) (high dose) or  $10^3$  TCID<sub>50</sub> (low dose) of PRV FS268 strain initially isolated from the vaginal tract of a naturally infected free-living feral sow (Romero et al., 1997). Genital and nasal swabs for virus isolation were obtained at 0, 1, 2, 3, 4, 5, 6, 7, and 10 days and at 2, 3, 4, 6, 8, 10, and 12 wk after experimental infection. A blood sample (~5 ml) was also obtained from each animal by venipuncture of the jugular vein at each of these times and the serum tested for the presence of neutralizing antibodies as previously described (Romero et al., 1997). All animals were euthanized utilizing a captive bolt pistol 3 mo after the experimental infection.

#### Swab infectivity and PCR assays

To determine the amount of infectious virus contained per collected swab, swab fluids positive for PRV were titrated by decimal dilutions in 96-well microtiter plates using Vero cells as indicators of infection. The virus titer of each swab was expressed as the number of TCID<sub>50</sub> contained in 2 ml of swab fluid. Total DNA from the sacral and trigeminal ganglia and tonsil tissues was extracted from approximately 200 mg of tissue using Trizol LS (Life Technologies, Baltimore, Maryland, USA) following the manufacturer's protocol. The thymidine kinase (TK) gene of PRV was targeted for PCR amplification using forward primer FP 5'-TCT GTT CGA CAC GGA CAC-3' and reverse primer RP 5'-GGG ATG ACA TAC ACA CAT TGG C-3', as previously described (Dangler et al., 1992). Polymerase chain reactions were performed in 100 µl volumes and contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 400 nM of each primer, 200 nM of each deoxynucleoside triphosphate (dNTP), 5% dimethylsulfoxide (DMSO), 500 ng of DNA template, and 2U of Taq DNA polymerase (Life Technologies). A total of 40 cycles was performed in a PTC-100 thermal cycler (MJ Research, Inc., Waltham, Massachusetts, USA). Each cycle consisted of denaturation at 94 C for 1 min, annealing at 51 C for 1 min, and extension at 72 C for 1 min. Elongation during

the last cycle was performed during 8 min. These conditions direct the amplification of a DNA fragment of 953 bp (Dangler et al., 1992). Half (50 µl) of the amplification reaction was resolved by electrophoresis in agarose (0.8%) gels containing ethidium bromide (0.5 µg/ml) and the DNA bands visualized by ultraviolet transillumination.

#### Sequencing of amplified DNA fragments

Two DNA fragments of the predicted size (953 bp) amplified from total DNA extracted from the sacral ganglia of two naturally infected feral swine (FS166 and FS170) were cloned into the plasmid vector pGEM-T (Promega, Madison, Wisconsin, USA) and sequenced in duplicate from the vector T7 and SP6 promoters utilizing the Big Dye Terminator sequencing kit (PE Applied Biosystems Division, Foster City, California, USA). Sequences were confirmed using the Chromas 1.55 program (Technelysium Pty Ltd., Queensland, Australia) and the nucleotide and deduced amino acid sequences aligned using, respectively, the Gap, PileUp and Pretty software of the Wisconsin Package Version 10.0 (Genetics Computer Group [GCG], Madison, Wisconsin, USA). The derived sequences were deposited in GenBank (undated; Accession numbers AF362082 and AF362083) and were compared to TK gene sequences from virulent PRV NIA-3 strain from Northern Ireland (Accession number X55001), virulent PRV Ea strain from the Republic of China (Accession number AF080571), and unclassified PRV strain from the USA (Accession number I02601) stored in the GenBank database.

## RESULTS

Polymerase chain reaction amplification of total DNA extracted from tissues of feral swine naturally infected with PRV showed that the sacral ganglia are the most common sites of latency of PRV indigenous to feral swine. Nine (56%) of 16 sacral ganglia, seven (44%) of 16 trigeminal ganglia, and five (39%) of 13 tonsils were positive by PCR amplification (Table 1). None of the approximately 10% suspensions prepared from these tissues yielded virus after inoculation in Vero cell cultures indicating that the detected DNA corresponded to latent and not replicating virus. DNA fragments of the predicted 953 bp were amplified and resolved by agarose gel electrophoresis (Fig. 1). Two of these frag-

TABLE 1. Potential sites of pseudorabies virus (PRV) latency in tissues of naturally infected adult feral swine, detected by polymerase chain reaction (PCR) amplification of the viral thymidine kinase (TK) gene sequences.

FS <sup>a</sup>	Sex	Sacral ganglia	Trigeminal ganglia	Tonsils	Antibody titer
36	M	+ <sup>b</sup>	- <sup>b</sup>	-	64 <sup>c</sup>
39	M	+	-	-	32
44	F	-	-	-	32
122	F	-	-	nd <sup>d</sup>	16
123	F	+	nd	+	64
124	F	+	+	+	64
166	M	+	-	-	64
168	F	nd	-	-	64
170	F	+	+	nd	64
196	M	-	+	-	24
198	M	-	-	+	32
199	M	-	+	-	48
267	M	-	-	-	64
388	M	+	-	+	48
389	M	+	+	+	32
417	F	-	+	nd	16
424	F	+	+	-	16

<sup>a</sup> Naturally infected adult feral swine (FS) had been kept in captivity between 2–3 yr before they were euthanized and tissues evaluated for the presence of TK gene sequences by PCR. Nasal and genital swabs and tissue suspensions were negative for PRV on the day of euthanasia.

<sup>b</sup> Total DNA extracted was PCR positive (+) or negative (-) for the viral TK gene sequences.

<sup>c</sup> Animals tested for serum neutralizing antibodies on the day of euthanasia. Antibody titers are expressed as the reciprocal of the highest serum dilution that neutralized 100 median tissue culture infectious doses of the Shope strain of PRV.

<sup>d</sup> nd = not done.

ments were cloned into a bacterial plasmid and shown by sequence analysis to be very similar to available TK gene sequences derived from the virulent PRV NIA-3 strain from Northern Ireland, the virulent PRV Ea strain from the Republic of China, and an unclassified PRV strain from the USA (Fig. 2). The nucleotide sequences of the PRV TK gene fragments from feral swine FS166 and FS170 are shown, minus the primer sequences used for PCR amplification (Fig. 2). These sequences differed only at position 430 in which a consensus nucleotide T in strain FS166 was replaced by a C nucleotide in strain FS170, changing the deduced amino acid from valine to alanine, both nonpolar hydrophobic amino

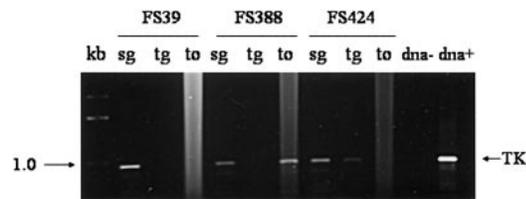


FIGURE 1. Agarose gel electrophoresis of amplified DNA fragments corresponding to the thymidine kinase gene of pseudorabies virus (PRV) from tissues of feral swine naturally infected with indigenous PRV. Sg=sacral ganglia, Tg=trigeminal ganglia, To=tonsils, kb=1 kb molecular weight ladder, dna<sup>-</sup>=no dna in the PCR reaction tube, dna<sup>+</sup>=purified DNA from the PRV FS268 isolate in the PCR reaction tube.

acids. Several nucleotide differences were observed when the PRV TK sequences derived from the feral swine isolates were compared to homologous sequences from domestic swine strains of PRV (Fig. 2). However, the percentage nucleotide homologies between the sequences derived from the PRV TK fragments from feral swine and those derived from domestic swine strains obtained from the GenBank database varied between 98.5 and 99.5%. Although all naturally infected feral swine had PRV neutralizing antibodies at the time of euthanasia (titers ranged from 1:16 to 1:64), latency could not be demonstrated in the tissues of four of the 17 feral swine (Table 1).

Experimental infection of hybrid feral domestic swine with the PRV-FS268 strain indigenous to feral swine, initially isolated from the vagina of a naturally infected feral sow, resulted in the establishment of infection in all inoculated animals. This was evidenced by the development of PRV serum neutralizing antibodies (titers ranged from 1:4 to 1:64, data not shown). However, virus was only sporadically isolated from nasal or vaginal swabs during the first 10 days following infection (Table 2). Pseudorabies virus deposited in the genital tract was isolated from the genital tract and the nasal passages. Similarly, virus deposited in the nasal cavity was also isolated from the genital tract (Table 2). Virus titers recovered in



TABLE 2. Isolation of pseudorabies virus from swabs after experimental infection of hybrid feral domestic swine with the PRV-FS268 strain indigenous to feral swine.

FS <sup>a</sup> num- ber	Swab	Infection		Virus isolation after infection at days								
		Route <sup>a</sup>	Dose <sup>a</sup>	1	2	3	4	5	6	7	10	
143	Nasal	IN	LD	- <sup>b</sup>	-	-	-	-	-	-	-	-
	Vaginal			-	-	-	-	-	-	-	-	-
144	Nasal	IN	LD	-	-	-	-	-	-	-	-	-
	Vaginal			-	-	-	-	-	-	-	-	-
145	Nasal	IV	LD	-	-	-	-	-	3.7 <sup>c</sup>	-	-	-
	Vaginal			-	-	-	-	+ <sup>b</sup>	-	-	-	-
146	Nasal	IV	LD	-	-	-	-	-	-	-	-	-
	Vaginal			-	2.2	4.9	5.7	5.7	3.2	5.6	3.8	
147	Nasal	IN	LD	-	-	-	-	-	4.6	1.8	5.8	-
	Preputial			-	-	-	-	-	-	-	-	-
148	Nasal	IP	LD	-	-	-	-	-	-	-	-	-
	Preputial			-	-	-	-	-	-	-	-	-
149	Nasal	IP	HD	-	-	-	-	-	-	-	-	-
	Preputial			-	-	-	-	-	-	-	-	-
150	Nasal	IV	HD	-	-	5.2	5.0	7.4	nd <sup>d</sup>	6.4	nd	-
	Vaginal			4.6	5.2	nd	4.7	5.4	nd	5.0	nd	
174	Nasal	IN	HD	4.0	5.5	6.3	6.5	6.3	5.2	1.3	-	-
	Vaginal			-	+	-	-	-	-	-	-	-
175	Nasal	IN	HD	-	-	-	-	-	-	-	-	-
	Vaginal			-	-	-	-	-	-	-	-	-
902	Nasal	Uninfected		-	-	-	-	-	-	-	-	-
	Preputial	-	-	-	-	-	-	-	-	-	-	-

<sup>a</sup> Feral swine (FS) hybrids were infected intranasally (IN), intravaginally (IV), or intrapreputially (IP) with a low dose (LD),  $10^3$  median tissue culture infectious doses (TCID<sub>50</sub>), or a high dose (HD),  $10^6$  TCID<sub>50</sub>, of the feral swine isolate PRV-FS268.

<sup>b</sup> Virus negative (-) swabs and virus positive (+) untitrated swabs. Swabs collected at day 0 and weeks 2, 3, 4, 6, 8, 10, and 12 after experimental infection were virus negative (not shown).

<sup>c</sup> Log<sub>10</sub> of TCID<sub>50</sub> per 2 ml of swab fluid.

<sup>d</sup> nd = not done.

vaginal swabs varied from log<sub>10</sub> 2.2 to 5.7 TCID<sub>50</sub> per 2 ml of swab fluid. Virus titers recovered from nasal swabs varied between log<sub>10</sub> 1.3 and 7.4 TCID<sub>50</sub> per 2 ml of swab fluid. The most common latency sites after the experimental infection with the feral swine isolate PRV-FS268, regardless of the route and dose of the infection, were the sacral ganglia. Nine of 10 (90%) sacral ganglia, six of 10 (60%) trigeminal ganglia, and three of 10 (30%) tonsils were positive by PCR amplification using the PRV TK gene as target template (Table 3). None of the approximately 10% tissue suspensions inoculated onto Vero cell cultures yielded virus.

#### DISCUSSION

Demonstration of higher frequency of latency in the sacral ganglia instead of the

trigeminal ganglia of naturally and experimentally infected feral or hybrid swine is consistent with previous results that indicate that the main route of transmission of PRVs indigenous to feral swine is venereal (Romero et al., 1997, 2001) and not respiratory as is the case of PRV strains in domestic swine (McFerran and Dow, 1964; Christensen et al., 1993). An example of a herpesvirus primarily transmitted venereally is herpes simplex virus 2 (HSV-2), the cause of genital herpes in humans in which latency has been shown to occur mainly in the sacral dorsal root (S2-S4) ganglia (Aurelian, 1989). In facial herpetic infections in humans caused by HSV-1, latent virus is mainly localized in the trigeminal ganglia (Aurelian, 1989). Although venereal transmission of PRV among feral swine is

TABLE 3. Detection of potential latency sites of pseudorabies virus indigenous to feral swine in tissues of experimentally infected hybrid pigs by polymerase chain reaction.

Pig number	Route of infection <sup>a</sup>	Infective dose <sup>b</sup>	Sacral ganglia	Trigeminal ganglia	Tonsils
143	IN	LD	+ <sup>c</sup>	- <sup>c</sup>	-
144	IN	LD	+	-	-
145	IV	LD	+	-	-
146	IV	LD	+	-	-
147	IN	LD	+	+	-
148	IP	LD	+	+	+
149	IP	HD	+	+	+
150	IV	HD	+	+	+
174	IN	HD	-	+	-
175	IN	HD	+	+	-
902	Uninfected	na <sup>d</sup>	-	-	-

<sup>a</sup> Pigs infected intranasally (IN), intravaginally (IV), or intrapreputially (IP).

<sup>b</sup> Feral swine strain PRV-FS268 administered at a low dose (LD) =  $10^3$  median tissue culture infectious doses (TCID<sub>50</sub>) or a high dose (HD) =  $10^6$  TCID<sub>50</sub>. na = not applicable.

<sup>c</sup> Positive (+) and negative (-) by polymerase chain reaction amplification of viral thymidine kinase nucleotide sequences in pig tissues.

<sup>d</sup> na = not applicable.

typical for PRV indigenous to this species, demonstration of latency in the trigeminal ganglia and tonsils indicates that these viruses have the potential for transmission by the respiratory route and may be transmitted by this route, but at a lower frequency.

Published information on the pathogenicity of PRVs indigenous to feral swine is limited. In the experimental infection study in which 8 mo old feral domestic hybrid swine were infected with  $10^3$  or  $10^6$  TCID<sub>50</sub> PRV FS268 by the genital or respiratory route, clinical pseudorabies was not observed. In domestic swine, efficiency of colonization (latency) of the trigeminal ganglia is dependent on the dose and strain of PRV, with virulent strains being more efficient than attenuated strains (Schang et al., 1994). If PRVs indigenous to feral swine are naturally attenuated as our study and those of others (Hahn et al., 1997) seem to indicate, then this would partially explain the relatively low levels of latency encountered in the sacral ganglia (56%), trigeminal ganglia (44%), and tonsils (39%) in the naturally infected antibody positive feral swine. These levels, however, were shown to be higher in the

sacral ganglia (90%), trigeminal ganglia (60%), and tonsils (30%) of experimentally infected hybrids. However, the unknown time since natural infection and dose of virus responsible for this infection may also account for these differences. Also, it is not known whether hybrids may be more susceptible to PRV than free-living feral swine.

In one experimental animal (FS150), infectious PRV deposited in the vagina at a high dose, spread to the respiratory tract, from where it could be isolated at high titers on consecutive days during the first week after infection. Virus spread from the genital to the respiratory tract could not be clearly demonstrated when the dose of infectious virus deposited in the vagina was low. In two animals, virus spread from the genital to the respiratory tract (FS145) and from the nasal to the genital tract (FS174) was demonstrated once. Virus was not isolated in five of 10 experimentally infected hybrid swine and this most likely indicates the limitation and poor sensitivity of the virus isolation technique when the amount of virus in nasal or genital passages is small.

Detection of latency in tissues of do-

mestic swine experimentally infected with virulent PRV using PCR is a practical and sensitive approach that may identify 100% of trigeminal ganglia latently infected with virulent PRV (Brockmeier et al., 1993). In the present studies, PRV sequences corresponding to the TK gene of, most likely, naturally attenuated strains of PRV indigenous to feral swine were identified in the sacral and trigeminal ganglia and in tonsil tissue of naturally infected feral swine. Similar DNA fragments could be amplified from the same tissues obtained from experimentally infected hybrid swine. The demonstration of latency at higher frequencies in the sacral than in the trigeminal ganglia reinforces the concept that PRVs indigenous to feral swine are primarily transmitted by the venereal route.

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