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Molecular basis for porcine parvovirus detection in dead fetuses

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ABSTRACT. Reproductive failures are still common grounds for complaint by commercial swine producers. Porcine parvovirus (PPV) is associated with different clinical reproductive signs. The aim of the present study was to investigate PPV fetal infection at swine farms having ongoing reproductive performance problems. The presence of virus in fetal tissues was determined by nested-polymerase chain reaction assay directed to the conserved NS1 gene of PPV in aborted fetuses, mummies and stillborns. Fetuses show a high frequency of PPV infection (96.4%; N = 28). In 60.7% of the fetuses, PPV were detected in all tissue samples (lung, heart, thymus, kidney, and spleen). Viral infection differed among fetal tissues, with a higher frequency in the lung and heart ($P < 0.05$).

Fetuses with up to 99 days of gestational age and from younger sows showed a higher frequency of PPV ($P < 0.05$). No significant difference in the presence of PPV was detected among the three clinical presentations. The results suggest that PPV remains an important pathogenic agent associated with porcine fetal death.

Key words: Swine; Porcine parvovirus; Nested-polymerase chain reaction; Abortion; Stillbirth; Mummification

INTRODUCTION

Swine reproductive failures are often inconclusive about their origin. In early- and late-term fetal death, the involvement of infection should be investigated. Different infectious agents cause similar clinical signs and impair diagnosis (Kirkbride and McAdaragh, 1978; Clark, 1996). Clinical signs such as abortion, fetal mummification and stillbirth are common records by commercial swine producers and are associated with porcine parvovirus (PPV) infection throughout the world. Infection of sows with PPV by the oronasal route or via experimental infection of fetuses, does not result in significant clinical signs in the dam (Hogg et al., 1977; Mengeling et al., 2000). After viremia, the virus usually crosses the placenta and infects the developing conceptus due to predilection for actively dividing cells. In sows with specific circulating PPV antibodies, transplacental infection may be inhibited. However, spreading of the virus *in utero* could not be prevented by maternal antibodies (Lager et al., 1992).

PPV, a member of the subfamily Parvovirinae and genus *Parvovirus*, represents an autonomous replicative virus with a single-stranded DNA genome of about 5200 bp. The nonenveloped icosahedral virus of 18-25 nm is formed by three structural proteins (VP1-VP3) and one nonstructural protein (NS1). Two large open reading frames (ORFs) were identified, where the right ORF encodes the three capsid proteins and the left ORF encodes the NS1 protein. NS1 is highly conserved, with striking homology among PPV species (Ránz et al., 1989). Differences in pathogenesis among PPV strains were observed in experimental infections, mainly in late-term gestation when fetuses could survive to PPV infection due to immune response (Chaniago et al., 1978; Lager et al., 1992). Clinical diagnosis of PPV infection has become more difficult after the proof of the late-term virulence capability of PPV.

Genetic variability of PPV isolates has been demonstrated in VP2 protein, and recent experimental studies suggest the presence of a new antigenic variant or type of PPV from field isolates (Martins et al., 2003; Zeeuw et al., 2007).

The classical methods to confirm fetal infection by PPV are virus isolation, tissue extract hemagglutination and the immunofluorescence test which detects viral antigen in lungs (Joo et al., 1977; Mengeling et al., 1991). The use of the hemagglutination-inhibiting assay (HIA) to detect anti-PPV-specific antibodies has been reported in 56-70-day-old fetuses (Hogg et al., 1977). Meanwhile, the detection of viral nucleic acid in fetuses, especially after natural transplacental infection, showed more reliable results (Molitor et al., 1991; Soares et al., 1999).

The detection of PPV in dead immunocompetent fetuses using the nested-polymerase

chain reaction (PCR) directed to the conserved NS1 gene, could provide information about PPV infection and viral tissue distribution in swine reproductive diseases.

MATERIAL AND METHODS

Animals

Twenty-eight dead fetuses were collected from ten commercial swine farms located in the three Brazilian States, São Paulo (N = 5), Minas Gerais (N = 3) and Paraná (N = 2), where these farms had an ongoing history of reproductive problems. The frequent grounds for complaint among the swine producers were return to estrus (>10%), stillbirth (>5%), abortion (>3%), mummification (>2%), and fewer liveborn piglets (<10%). At all the farms, inactivated vaccines were routinely used to prevent PPV infection in the pregnant pig.

The fetuses were classified according to clinical presentation as abortion, mummies and prepartum stillbirth, originating from young (1-3 parturitions) and older sows (>4 parturitions). The fetuses were also classified according to gestational age (estimated by crown-rump measure) into group A (70-99 days) and B (≥100 days) (Table 1). Fetuses showed normal gross appearance, dark outer coloration with edema or typical mummification.

Table 1. Number of animals in each clinical presentation, types and number of tissue samples, fetus classifications according to gestational age, and number of sow parturitions.

	Clinical presentation			Sows		Fetal gestational age		Fetal tissue samples				
	Ab	Mu	Sb	Y	O	A	B	L	H	T	K	S
Fetuses (N = 28)	6	6	16	13	13	21	7	28	28	14	16	22

Ab = abortion; Mu = mummified; Sb = stillbirth; Y = young, 1-3 parturitions; O = older, >4 parturitions; group A = 70-99 days; group B = ≥100 days; L = lung; H = heart; T = thymus; K = kidney, and S = spleen.

Sample, DNA extraction and amplification

A total of 108 tissue fragments of lung, heart, thymus, kidney, and spleen were individually collected and frozen at -70°C until tested by nested-PCR assay (Table 1). To avoid cross-contamination between samples, a new set of sterile instruments was used for each sample. Twenty-three samples of fetal thoracic fluids were collected to be assayed by HIA.

The tissue samples and PPV prototype (strain NADL-2 EMBRAPA, Concórdia, Brazil), cultured in the swine kidney cell line SK6, were homogenized in 20% (v/w) phosphate-buffered saline (0.01 M, pH 7.4). The homogenates were frozen three times and clarified at 2000 *g* for 20 min at 4°C. The extractions were carried out as follows: 40 µL TNE buffer (100 mM Tris-HCl, pH 7.4, 100 mM NaCl, 20 mM EDTA, pH 8.0), 20 µL 10% SDS and 10 µL proteinase K (Sigma; 10 mg/mL) were added to 130 µL tissue homogenate supernatant. After overnight incubation at 37°C, DNA extractions were carried out with phenol/chloroform (1:1), refrigerated and centrifuged at 11,600 *g*. Fresh chloroform was added and centrifugation repeated. DNA precipitation was performed with 95% ethanol for 18 h at -20°C and pellets were diluted in 25 µL sterile distilled water and stored at -20°C until the nested-PCR.

The amplification of the non-structural protein gene (NS1) by nested-PCR was carried out based on the procedure described by Soares et al. (1999) with the following modification: initial heating at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 60 s, and final extension at 72°C for 10 min. Nested-PCR products of 137 bp were electrophoresed on 2% agarose gels in standard TBE (0.045 M Tris-borate, 1 mM EDTA) and stained with 0.5 mg/mL ethidium bromide.

Cloning and sequence analysis

The nested-PCR amplified products of 137 bp from lungs of stillborns and mummies (N = 4) were excised from the gels, purified and cloned into the plasmid vector pGEM-T Easy (Promega, Madison, WI, USA). Sequencing was performed with the same primers used for DNA amplification (Soares et al., 1999) using the Big Dye TM Terminator-cycle sequencing ready reaction kit (Applied Biosystem, Foster City, CA, USA). The sequences were determined with the automated sequencer (ABI PRISM 310) according to manufacturer instructions. The four NS1 PPV sequences obtained from this study were aligned with three sequences from GenBank, using BLASTN 2.2.3 (Altschul et al., 1997).

Hemagglutination inhibition assay

Clarified and heat-inactivated fetal thoracic fluids were added to a 10% guinea pig erythrocyte suspension at 4°C for 4 h. Four hemagglutinating units of viral antigen prototype were added to serially diluted fluids. After a 2-h incubation at room temperature, 0.8% guinea pig erythrocyte suspension was added (Gouveia et al., 1984; Rodriguez et al., 2003). Total inhibition of hemagglutination reaction in positive controls determined the end point for HIA titer. Titers ≥1:16 were considered positive for the presence of anti-PPV-specific antibodies. Sow sera were used as positive controls (HIA titer 1:2048) and sera of colostrum-deprived piglets as negative controls.

Statistical analyses

The model was adjusted utilizing the theory of generalized linear models proposed by Nelder and Wedderburn (1972), using the GLIMMIX procedure of the SAS software. Mathematical expectation of E[Yt] equal to μt, becomes the linear expression applied by natural logarithm, obtaining a linear predictor, linked function on μt by logit. Adjustment of the model was performed considering a binomial distribution of the results of nested-PCR, with mean μ and variance σ^2 having the logarithm a link function, Ln(t), with t being the explanatory variable. The model considered the fixed effects of the clinical presentation (fetuses), fetal tissue samples, fetal age group, sow classes, and HIA titer results, as well as the effect of repeated measures in the fetus (N = 26) with a structure of autoregressive co-variance of first order, beyond the random residual effect.

Both interaction effects were not significant ($P > 0.10$) and could not be estimated due to inappropriate distribution of the data; they were excluded from the final analysis model. The least square means were compared with the Student *t*-test, shown in the original scale, for easier interpretation.

RESULTS

PPV detection in fetal tissues

The nested-PCR product yielded a 137-bp fragment, identifying the NS1 gene of PPV genome in 89 of 108 tissue samples of dead fetuses (Figure 1).

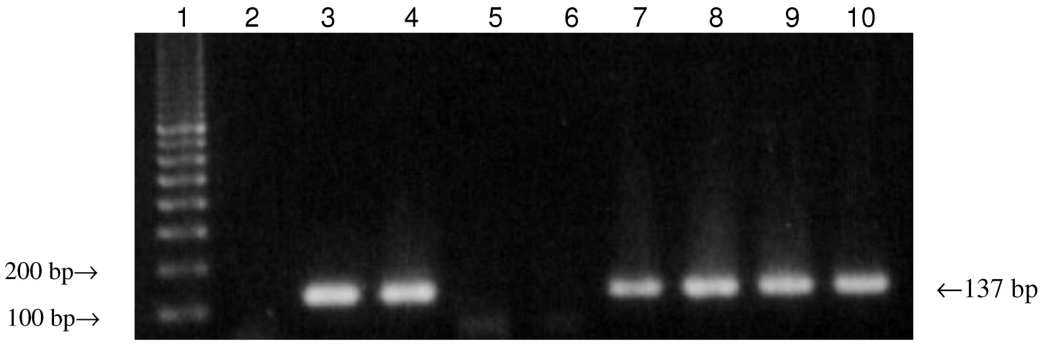

Figure 1. Nested-polymerase chain reaction products of eight fetal tissue samples on agarose gel eletrophoresis stained with ethidium bromide. *Lane 1*, Molecular weight ladder, 100 bp; *lane 2*, negative control; *lane 3*, lung of stillbirth, 100 days; *lane 4*, lung of stillbirth, 105 days; *lane 5*, heart of stillbirth, 105 days; *lane 6*, spleen of stillbirth, 105 days; *lane 7*, kidney of stillbirth, 105 days; *lane 8*, lung of mummie, 81 days; *lane 9*, heart of mummie, 81 days; *lane 10*, positive control, PPV strain NADL-2.

From the 28 dead fetuses evaluated, 27 were positive for PPV (96.4%). Fetuses were considered to be positive for PPV when at least one tissue sample was positive by nested-PCR. PPV detection in tissue samples did not differ significantly for the three clinical presentations ($P > 0.05$). Nevertheless, PPV tended to be more frequent in the prepartum stillborns than in mummies and aborted fetuses (Table 2).

Table 2. Mean (in percent) and standard error (in parentheses) of positive results by nested-polymerase chain reaction (nested-PCR) for porcine parvovirus detection in tissue samples of aborted (Ab), mummified (Mu) and stillborn fetuses (Sb).

Fetuses	Positive nested-PCR
Ab	79.98 (15.94)[a]
Mu	79.48 (16.91)[a]
Sb	87.43 (6.59)[a]
P value	0.90

Means with the same letter were not statistically different by the Student *t*-test ($P > 0.05$).

In 60.7% of the fetuses, PPV genome was detected in all tissue samples (lung, heart, spleen, thymus, and kidney). PPV was detected more frequently in lung (96.99%) and heart (93.33%), regardless of the fetus class. The spleen was less positive (54.91%; $P < 0.05$) (Table 3).

Table 3. Mean (in percent) and standard error (in parentheses) of positive results by nested-polymerase chain reaction (nested-PCR) for porcine parvovirus detection, in different tissue samples from 28 dead fetuses.

Fetal tissue	Positive nested-PCR
Lung	96.99 (3.48)[a]
Heart	93.33 (5.93)[a,b]
Kidney	69.56 (17.10)[b,c]
Thymus	66.00 (18.28)[b,c]
Spleen	54.91 (16.76)[c]
P value	0.05

Means with the same letter were not statistically different by the Student *t*-test (P > 0.05).

In dead fetuses with 70-99 days of gestation, the frequency of positive results for viral genome was higher (94.76%) than in older fetuses (55.57%; P < 0.05) (Table 4). Tissues from fetuses of younger sows, when compared to tissues from fetuses of older sows, showed 92.28 and 65.43% of positive results, respectively (P < 0.05) (Table 5).

Table 4. Mean (in percent) and standard error (in parentheses) of positive results by nested-polymerase chain reaction (nested-PCR) for porcine parvovirus detection in fetal tissues, related to fetal gestational age.

Fetal gestational age	Positive nested-PCR
A	94.76 (2.87)[a]
B	55.57 (23.77)[b]
P value	0.03

Means with different letters were statistically different by the Student *t*-test (P < 0.05).

Table 5. Mean (in percent) and standard error (in parentheses) of positive results by nested-polymerase chain reaction (nested-PCR) for porcine parvovirus detection in fetal tissues, related to sow class (number of parturitions).

Sow class	Positive nested-PCR
Young	92.28 (5.67)[a]
Old	65.43 (11.34)[b]
P value	0.03

Means with different letters were statistically different by the Student *t*-test (P < 0.05).

From 23 thoracic fluids tested by HIA, 21 samples (91.30%) were positive for anti-PPV-specific antibodies. Stillborns showed the greatest HIA titers. No statistical difference was detected in nested-PCR results among fetuses with lower and higher HIA titers (Table 6).

Table 6. Mean (in percent) and standard error (in parentheses) of positive results by nested-polymerase chain reaction (nested-PCR) for porcine parvovirus detection in fetal tissues, related to hemagglutination-inhibiting assay (HIA) results in fetal thoracic fluids.

HIA	Positive nested-PCR
Titer 0-1:64	83.94 (9.80)[a]
Titer 1:128-1:1024	81.23 (8.73)[a]
P value	0.80

Means with the same letter were not statistically different by the Student *t*-test (P > 0.05).

Sequencing of amplified DNA

The sequence analyses of products with 137 bp from amplified NS1 gene of PPV by nested-PCR showed high homology (90%) with NS1 sequence of PPV strains (accession numbers AY502114, AY686601, U44978). The four sequences were performed at least twice to avoid artifacts.

DISCUSSION AND CONCLUSIONS

Clinical expression and reproductive performance data are first used in the diagnosis of reproductive disease in swine herds. Similar clinical expressions give rise to doubts about origin. For pathogen identification and their epidemiology in reproductive problems, the application of laboratory diagnostic methods is necessary. Among the viral reproductive pathogens in swine, parvovirus is a recognized important infectious agent (Gouveia et al., 1984; Soares et al., 1999; Schneider et al., 2001; Rodriguez et al., 2003). In naturally infected swine herds, molecular methods to confirm PPV infection are indicated due to specificity and samples containing low amount of virus. The use of nested-PCR, directed to the conserved NS1 gene, was able to detect PPV genome in the dead fetuses of several swine farms demonstrating an ongoing history of reproductive failures.

PPV has a requirement to replicate in cells in a permissive S-phase of DNA mitosis, and infection is cytolytic. Small litter size and mummification are the most typical clinical findings when fetuses are infected up to about 70 days of gestation, especially during an acute outbreak (Choi et al., 1987; Clark, 1996; Mengeling et al., 2000). The infection is detected in fetal tissues, mainly in the lungs. Fetal immune competence after 60 to 70 gestational days could lead to immune response and surveillance (Liebermann et al., 1988; Clark, 1996). The HIA detected specific anti-PPV antibodies in almost all fetuses (90.4%). Evidence of immunocompetence due to fetal infection was apparently unable to prevent viral replication and fetal death. In aborted, mummified and stillborn fetuses, PPV was found in similar high frequency, independent of fetal gross appearance. PPV genome was present in all tissue samples of 60.7% of the fetuses. Similar to previous reports (Soares et al., 1999), the present study demonstrated that the lung showed a high frequency of viral genome in fetuses collected in commercial swine herds.

Cellular permissivity differs among PPV strains. Differences in pathogenicity between PPV NADL-8 and Kresse strains in late-term gestation have been described, indicating the Kresse strain as more virulent (Choi et al., 1987; Oraveerakul et al., 1993). Comparing relative virulence of the same two prototype strains in experimentally infected porcine fetuses at immune competence age showed that the NADL-8 strain causes more fetal mortality (Lager et al., 1992). Tissue tropism of PPV, replication in selective sites and quantity of virus produced may explain the distinction in pathogenesis. Viral antigen and viral DNA replication in the brain were demonstrated only in late-term fetal infection with the Kresse strain (Choi et al., 1987; Oraveerakul et al., 1993). In the present study, the replicative form of PPV genome was not demonstrated in tissue samples and PPV was not investigated in nervous tissue, but the viral genome was detected in many tissues, showing evidence of PPV spreading and tissue damage in transplacentally infected fetuses from vaccinated sows. Although there is a great difference in pathogenicity between different PPV strains, they share an identical genomic organization and a high degree of sequence identity. Molecular studies of PPV DNA have been

carried out and a small variability in capsid protein is responsible for distinct biological properties (Bergeron et al., 1996). Recent studies indicated genetic diversity in field strains and distinct genotypes of PPV (Soares et al., 2003). Pathogenic types of PPV have to be further investigated and antigenic variants may explain low neutralization antibody activity (Zeeuw et al., 2007). As younger sows seem to be more susceptible to PPV infection, sample collections are recommended from dams with up to three parturitions. Besides that, lung and heart are indicated as tissues of choice for PPV detection and diagnosis purpose in fetuses, independent of their clinical presentation. Live attenuated vaccines are not available in Brazil. Therefore, field isolates from reproductive disturbances should be considered pathogenic.

The effect of viral agents on porcine reproductive performance is a worldwide concern, but the identification of pathogens shows regional differences and selected swine reproductive diseases. PPV is clearly associated with embryonic death and increased number of mummified fetuses, but it was not directly associated with abortion until recently (Mengeling et al., 2000). Afterwards, in a study about abortifacient viruses in Spain, PPV infection was not identified in tissues of aborted fetuses and stillborn neonates, using an antigen capture ELISA test (Maldonado et al., 2005). Also, the porcine reproductive and respiratory syndrome virus is not recognized as a reproductive pathogen in Brazil, and a complete study of the effect of porcine circovirus type 2 (PCV-2) on swine reproductive failures has not yet been conducted. The results indicate that PPV remains an important pathogenic infectious viral agent in porcine fetal death, even after immune competence. According to local management systems, fetal co-infection of PPV and PCV-2 cannot be ruled out. Epidemiological studies are desirable for these highly stable virions to reduce economic losses, as eradication programs are very difficult to conduct.

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