



Semen alterations in porcine rubulavirus-infected boars are related to viral excretion and have implications for artificial insemination

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Accepted 29 January 2007

Abstract

Porcine rubulavirus (PoRV), also known as blue eye disease (BED) of swine, causes respiratory and reproductive problems in pigs at several developmental stages. To study the effect of PoRV infection on semen production, five boars were infected with 1×10^6 TCID₅₀/ml of PoRV strain PAC-3 and evaluated for 59 days post inoculation (DPI). Infected boars developed reproductive tract pathology that included swelling of the testes and epididymides. Analysis of the semen showed that the infection had little effect on semen production in four animals, but semen from one boar showed severe alterations in sperm concentration, motility, and morphology. When motility was analyzed in BTS-diluted semen after 24, 48, or 72 h, alterations were detected in all boars. Furthermore, viral antigen was detected in semen, the seminal plasma fraction, or sperm fraction from all boars. These results showed that PoRV is excreted via semen and, therefore, artificial insemination is a potential route of dissemination.

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Keywords: Swine; Semen; Virus diseases transmission; Porcine rubulavirus; Artificial insemination

1. Introduction

Blue eye disease (BED) is a viral disease of swine first recognized in La Piedad Michoacán, Mexico, in 1980 in association with an outbreak of encephalitis and corneal opacity in piglets (Stephano et al., 1988). The causal agent of BED was subsequently named La Piedad Michoacan virus (LPMV). LPMV is an enveloped, single-stranded RNA virus, with hemagglutinating, hemolytic, and syncytia-forming properties (Moreno-Lopez et al., 1986). Gene sequencing data showed that LPMV is closely related to mumps virus (MuV), simian virus 5 (SV5), parainfluenza virus type 2 (PIV-2) and type 4 (PIV-4) (Berg et al., 1991; Sundqvist

et al., 1992; Berg et al., 1992; Berg et al., 1997; Svenda et al., 1997), supporting the classification of LPMV in the genus *Rubulavirus*, family *Paramyxoviridae*, order *Mononegavirales* (Rima et al., 1995; Van Regenmortel et al., 2000).

The clinical signs of BED are age-dependent. In newborn pigs, porcine rubulavirus (PoRV) infection produces neuropathological disorders consisting of hyperexcitability, motor incoordination, ataxia, hind limb paralysis, conjunctivitis, unilateral or bilateral corneal opacity, respiratory alterations, and death in 90% of pigs infected at <20 days of age (Stephano et al., 1988). In pregnant sows, BED is characterized by an increased number of animals returning to estrus, stillbirths, mummified fetuses, and a reduction in the number of live born pigs (Stephano, 1999). Infected boars show orchitis and epididymitis (Ramírez-Mendoza et al., 1997), including swelling of the head and body of the epididymis, sperm granulomas, vacuolar degeneration

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of ductular epithelium, mononuclear cell infiltration, interstitial fibroplasia, testicular atrophy associated with degeneration of seminiferous tubes, and giant cell formation (Ramírez-Mendoza et al., 1997). PoRV antigen has been identified by immunofluorescence in the head of the epididymis of infected boars (Ramírez-Mendoza et al., 1997). BED has been identified in farms using artificial insemination, suggesting the possibility that porcine rubulavirus may be excreted in semen and disseminated by natural and artificial mating. Since many infected pigs do not develop overt clinical signs, the objective of this investigation was to assess the impact of porcine rubulavirus infection on semen quality and to determine whether viral antigen is shed in the semen of infected boars.

2. Materials and methods

2.1. Virus and cells

The PoRV isolate PAC-3 (Jalisco/1992) was isolated by our laboratory in conjunction with a clinical outbreak of BED characterized by high infertility rates, stillbirths, and fetal mummification in pregnant females, as well as testicular and epididymal lesions in sexually mature males. The virus was propagated on Vero cells grown in 25 cm² cell culture flasks (430168, Corning, NY, USA) using Eagle's Minimal Essential Medium (EMEM; M0268, Sigma, St Louis, MO, USA) supplemented with 5% fetal bovine serum (FBS; 16000-044, GIBCO, Gland Island, NY, USA), 100 UI ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin (P4458, Sigma, St Louis, MO, USA), at 37 °C, in a humidified 5% CO₂ incubator. When cytopathic effect (CPE) was apparent, cell cultures were freeze-thawed twice and the cell lysates were centrifuged at 1 × 650g at 4 °C. The supernatant was collected, titrated, and stored at -70 °C. Virus titer in the inoculum was determined by performing serial 10-fold dilutions on Vero cells grown in 96-well plates (3596, Corning, NY, USA). Cytopathic effect (CPE) was used to determine which wells were infected. The virus titer was calculated using the Spearman-Kärber method (Hamilton et al., 1977) and expressed in tissue culture infective dose 50% (TCID₅₀) ml⁻¹.

2.2. Animals

Five 9-month-old sexually mature York-Landrace boars were obtained from a farm free of PoRV, porcine reproductive and respiratory syndrome virus, and Aujeszky's disease virus. Three weeks prior to the beginning of the experiment, each boar was placed into individual housing. Prior to inoculation, boars were trained for semen collection, and semen samples ($n = 6$ during 2 weeks before infection) were collected and analyzed for concentration, motility, color, viability in fresh semen or in BTS-diluted semen after 24, 48 and 72 h, and for the presence of viral antigen in semen and serum. These baseline values were used for comparisons of samples collected after infection.

On day zero of the study, pigs were intranasally inoculated with 5 ml of PAC-3 virus at a titer of 1×10^6 TCID₅₀/ml. After infection, temperature and clinical signs were recorded daily. Head and body of the epididymis and testicular length and width were evaluated to determine epididymitis and orchitis.

2.3. Collection and semen analysis

Semen was collected in sterilized, pre-warmed beakers with the gloved-hand technique (Shipley, 1999). Semen was filtered through sterile gauze to remove gelatinous material. The sperm-rich and sperm-poor fractions were collected separately. Semen was maintained at room temperature for approximately 1.5 h after collection while macroscopic and microscopic characteristics of the freshly collected semen were evaluated. Semen volume was determined in a sterilized and pre-warmed volumetric glass tube. Color was evaluated by visual inspection. Sperm motility was analyzed by microscopy (Olympus, Tokyo, Japan) (Dowsett and Knott, 1996). Spermatozoa concentration was determined in a Neubauer cell chamber (Neild et al., 2003). For sperm abnormalities, 40 µl of semen was stained with crystal violet and analyzed by microscopy (Olympus, Tokyo, Japan). One hundred spermatozoa were randomly selected from the stained preparations and classified as either morphologically normal or abnormal. Abnormalities concerned abnormal heads or acrosomes, coiled tails and proximal cytoplasmic droplets. Sperm integrity was evaluated using propidium iodide by flow cytometry (Marchetti et al., 2002). Semen viability was evaluated at different times after infection (2, 4, 7, 9, 11, 16, 18, 21, 23, 25, 32, 38, 42, 45, 49, 52, and 59 days). Six semen samples from each boar were obtained during 2 weeks before inoculation and used as negative controls. These values are presented as mean ± SD at time 0.

2.4. Semen dilution in BTS solution

The semen was diluted to a concentration of 3.75×10^8 cells ml⁻¹ in Beltsville Thawing Solution [BTS: 60.0 g D-glucose (G7021, Sigma), 3.7 g sodium citrate (71497, Sigma), 3.7 g EDTA (E6758, Sigma), 1.2 g sodium bicarbonate (S5761, Sigma), 100 UI ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin] following published artificial insemination procedures (Dubé et al., 2004). Diluted samples were stored in conical flasks at 17 °C, protected from light, and viability was evaluated after 24, 48, and 72 h.

2.5. Virus isolation

Ten milliliters of each semen sample (fresh semen, FS) was centrifuged at 105g for 15 min at room temperature. Thereafter, the seminal plasma fraction (supernatant; SP) and the sperm fraction (pellet; SF) were collected and stored at -194 °C. FS was stored similarly. SP, SF, and FS were thawed twice and clarified by centrifugation at 650g for 30 min at 4 °C and the supernatant was inoculated on to

confluent Vero cells grown in 96-well microculture plates (Corning, NY, USA), at three dilutions (1:10, 1:100, and 1:2001). After incubating for 1 h at 37 °C, the cells were washed with EMEM medium and then fresh EMEM medium with FBS was added. Thereafter, plates were incubated for 5 days at 37 °C in a 5% CO₂ incubator. Viral infection was indicated by CPE and confirmed by hemagglutination and immunofluorescence assays. In the absence of CPE and after 5 days, a second passage was carried out by inoculating the supernatant directly onto fresh Vero cells. For the second passage no adsorption procedure was used.

2.6. Hemagglutination

Hemagglutinating activity in infected cell supernatants was determined with a serial dilution assay. In brief, 100 µl of serial dilutions (1:2 to 1:1024) of supernatants were incubated for 45 min at room temperature with 100 µl of 0.5% chicken red blood cells in 96-well round bottom microculture plates (3799, Corning, NY, USA). The titer was expressed in hemagglutinating units (HAU) (Ramírez-Mendoza et al., 1996)

2.7. Indirect immunofluorescence (IFA)

Rabbit polyclonal antibody was prepared by inoculating rabbits ($n = 2$) with PoRV with complete Freund's adjuvant followed by two more inoculations at 2 week intervals with PoRV with incomplete Freund's adjuvant. One week after the last inoculation, serum was collected, pooled, and the optimal dilution determined. To perform the IFA assay, cells inoculated with supernatant in the second passage were fixed with 50:50 methanol (45,354-6, Sigma)/acetone (53,406-4, Sigma) for 10 min at room temperature, washed with 1X PBS (pH 7.2), incubated with a rabbit polyclonal antibody against porcine rubulavirus for 30 min at 37 °C, and then washed twice with PBS. Thereafter, cells were incubated with FITC-conjugated goat anti-rabbit IgG (1:100) (Zymed, San Francisco, CA, USA) for 30 min at 37 °C, washed twice with PBS, and analyzed in an inverted fluorescent microscope.

2.8. Statistical analysis

In order to identify differences through the experimental time, a one-way repeated measures analysis of variance or Friedman analysis was employed. When differences were observed ($p < 0.05$), a multiple comparisons versus group (Holm-Sidak test) or Wilcoxon signed ranks test was run using SigmaStat 3.1 or SPSS 10 for Windows.

3. Results

3.1. Clinical signs

Clinical signs observed in all infected pigs included fever (40–41 °C), anorexia, constipation, and conjunctivitis

between 3 and 7 days post-infection (PI). Unilateral epididymitis, particularly severe in boar No. 1, was observed between 7 and 21 days PI. No other clinical signs were observed.

3.2. Semen analysis

The analysis of the ejaculate volume before and after infection revealed no significant differences among boars ($p > 0.05$, data not show). Semen was milky-white in color after infection in four of the five infected boars. In boar No. 1, semen was milky-white in color for the first 16 days PI; milky-white in color, but stained with blood at 21 days PI, and aqueous brown in color at 25 to 38 days PI. The color gradually cleared, but the ejaculate with a watery appearance (45 days PI) until the milky-white color reappeared at 49 days PI. The concentration of spermatozoa was variable during the first 10 days PI in all boars (Fig. 1), but the concentration decreased thereafter (25, 28, and 32 days PI; $p < 0.05$). Spermatozoa loss was most severe in boar No. 1, with an about 70% reduction. Likewise, PoRV infection decreased spermatozoa viability (Fig. 1) after 16 days PI ($p < 0.05$), and abnormalities in spermatozoa morphology increased after 21 days PI ($p < 0.05$). As for spermatozoa concentration, loss of viability and motility, and increases in abnormalities were more severe in boar No. 1. To avoid the influence of boar No. 1 in the results, we analyzed the data individually comparing the results before and after infection (data not shown). This analysis showed significant ($p < 0.05$) abnormalities and death spermatozoa (PI positive) in all pigs. With respect to the concentration and motility, no significant differences were observed in boars No. 2–5, only in boar No. 1.

3.3. Viability of semen diluted on BTS solution

In order to evaluate whether semen of PoRV-infected pigs was viable for artificial insemination, samples were diluted in BTS solution and sperm motility was evaluated at the collection time (fresh semen) and after 24, 48, and 72 h of storage (Table 1). No significant differences ($p > 0.05$) in motility were observed in fresh semen, even though the range of motility among boars progressively widened over time. When semen was analyzed 24 h after dilution in BTS solution, motility significantly decreased by 16 days PI ($p < 0.05$) to approximately 20%. When analyzed after 48 h, motility significantly decreased by 11 day PI ($p < 0.05$) and by 14 days PI motility was zero. After 72 h of storage, motility significantly decreased by 9 days PI ($p < 0.05$).

3.4. Detection of viral antigen on semen, seminal plasma fraction, and sperm fraction

To determine if spermatozoa alterations were associated with PoRV in semen, the presence of viral antigen was eval-

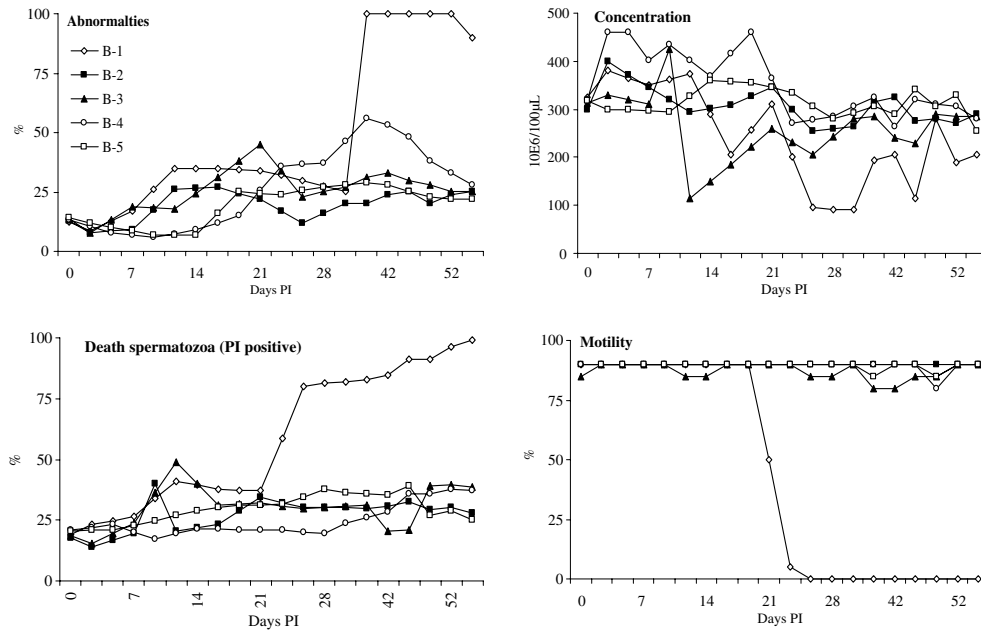


Fig. 1. Semen analysis of boars infected with porcine rubulavirus. Six semen samples from each boar were collected during two weeks prior to inoculation (day 0) and at different days PI.

Table 1
Percentage of motile spermatozoa motility in fresh semen or diluted in BTS and analyzed at 24, 48, or 72 h post collection

Boar	Days	0	2	4	7	9	11	14	16	18	21	23	25	28	32	38	42	45	49	52	59
<i>Fresh semen</i>																					
1		90	90		90		90		90		50		0		0	0	0	0	0	0	0
2		90	90		90		90		90		90		90		90	90	90	90	90	90	90
3		86	90		90		90		90		90		85		90	80	80	85	85	90	90
4		87		90		90		90		90		90		90		80	90	90	90	80	90
5		90		90		90		90		90		90		90		85	90	90	85	90	90
<i>24 h</i>																					
1		80	80		80		70		20 ^a		0		0		0	0	0	0	0	0	0
2		81	80		80		80		20		20		0		0	20	0	10	10	20	30
3		79	85		80		70		20		20		0		0	10	0	0	10	10	20
4		81		80		80		80		60		0		0		20	0	0	0	10	10
5		79		85		70		80		30		30		10		30	10	10	30	30	40
<i>48 h</i>																					
1		74	75		65		50 ^a		0		0		0		0	0	0	0	0	0	0
2		78	70		70		70		10		0		0		0	0	0	0	0	0	0
3		73	75		65		50		0		0		0		0	0	0	0	0	0	0
4		74		70		70		0		0		0		0		20	0	0	0	0	0
5		74		70		65		0		10		0		0		10	0	0	0	0	0
<i>72 h</i>																					
1		65	65		60		0		0		0		0		0	0	0	0	0	0	0
2		66	60		65		0		0		0		0		0	0	0	0	0	0	0
3		64	70		60		0		0		0		0		0	0	0	0	0	0	0
4		67		60		50 ^a		0		0		0		0		20	0	0	0	0	0
5		65		75		40		0		10		0		0		10	0	0	0	0	0

Clear squares indicate no semen collection.

^a Significant difference ($p < 0.05$) from this PI day until the end of the experiment.

uated. In addition, semen was fractionated into seminal plasma fractions and sperm fraction in order to identify whether the virus predominated in one compartment. Vero cells were inoculated with semen or either seminal plasma

or sperm fractions and PoRV virus was identified at first and second passage by CPE, HA, and IFA. Because of the toxicity of semen for Vero cells and the subsequent potential for false negatives, only the results of the second

Table 2
HA titer in semen, seminal plasma, and sperm fractions

Boar	Days																			
	0	2	4	7	9	11	14	16	18	21	23	25	28	32	38	42	45	49	52	59
<i>Fresh semen</i>																				
1	–	–	–	1:32	–	–	–	1:32	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	–	1:64	–	–	–	1:32	–	–	–	–	–	–	1:256	–	–	–	–	–
3	–	–	–	–	–	–	–	1:16	–	–	–	–	–	–	1:64	–	–	–	–	–
4	–	–	–	–	1:8	–	–	–	1:64	–	–	–	1:64	–	–	–	–	–	–	–
5	–	–	–	–	–	–	1:32	–	–	–	–	–	–	–	1:32	–	–	–	–	–
<i>Sperm fraction</i>																				
1	–	–	–	1:64	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	–	–	–	1:32	–	1:64	–	–	–	1:32	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1:64	–
4	–	–	–	–	1:32	–	–	–	–	–	–	–	–	–	–	–	–	–	1:32	–
5	–	–	–	–	–	–	1:64	–	1:32	–	–	–	–	–	–	–	–	–	1:64	–
<i>Seminal plasma fraction</i>																				
1	–	1:32	–	1:64	–	–	–	1:64	–	1:64	–	–	–	1:64	–	–	–	–	1:32	–
2	–	–	–	–	–	1:16	–	–	–	–	–	1:64	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	1:32	–	–	–	1:32	–	–	–	–	–	–	1:64	–
4	–	–	–	–	1:8	–	–	–	1:32	–	–	–	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	1:64	–	1:32	–	–	–	–	1:32	–	–	–	–	1:32	–

The titer was obtained after second passage of semen, seminal plasma, and sperm fraction in Vero cells. Clear squares indicate no semen collection.

passage of samples diluted 1:200 in the first passage are presented. In the first passage, 63 samples were positive (data not shown). Upon second passage, 43 samples were positive. Table 2 shows the HA titer in semen, seminal plasma fraction and sperm fraction. The HA titer was 1:8 to 1:64 among all pigs. PoRV was detected at 2 days PI in the seminal plasma fraction of boar No. 1 and until 49 days PI in the sperm fraction of boar No. 3, and seminal plasma fraction of boar No. 1. Semen ($n = 14$) or seminal plasma fraction ($n = 17$) samples positive by IFA were also positive by HA. However, 12 sperm fraction samples were positive by IFA and 10 of these 12 were also positive by HA. All boars shed the virus intermittently in semen for 38 (boar 2), 45 (boars 4 and 5), or 49 (boars 1 and 3) days post inoculation.

4. Discussion

After experimental infection of boars with porcine rubulavirus, in four of five boars, damage was temporary, but one boar became infertile during the last phase of this experiment (25–53 days PI) due to sperm losses, and increased spermatozoa abnormalities, death and reduced motility. This same boar showed changes in semen color due to a severe inflammatory reaction and hemorrhage. Since porcine rubulavirus does not affect accessory glands (Ramírez-Mendoza et al., 1997), the ejaculate's volume was not affected, but sperm concentration, motility, and morphology were significantly affected ($p < 0.05$). Previous research reported that PoRV affected sperm ducts and could lead to complete loss of sperm cells (Ramírez-Mendoza et al., 1997). A complete loss of sperm cells was observed in one boar in this study, although macroscopic characteristics were apparently normal after 21 days PI,

suggesting that the inflammatory response was transitory, but could lead to permanent consequences. In the other boars, the inflammatory response was apparently less severe and the only effect was an increase in the proportion of morphological sperm abnormalities. No effects were seen in the volume of the ejaculate, sperm concentration, or motility.

The different susceptibility found in the boars could be explained at least in two ways: (a) immune resistance, and (b) differential expression of virus receptors on target cells. The immune resistance has been described by others as a factor that could increase or not the susceptibility to some pathogens. In the case of PRRS virus (Royae et al., 2004), differences in the ability to produce IFN-alpha even among pigs growing in the same conditions have been reported, suggesting that genetic factors associated with the immune system could be involved. In addition to differences in IFN-alpha production, pigs with high or low antibody and cell-mediated immune response have been reported by other authors (Raymond et al., 1998; Crawley et al., 2005). Considering the importance of antibody response and CMI in the control of viral infections (Tortorella et al., 2000), as well as in porcine rubulavirus infection (Hernandez et al., 1998; Hernandez et al., 2001), it is possible to suppose that the differences in susceptibility among pigs could be due, at least in part, to the participation of the immune system genes. On the other hand, considering that virus receptors are involved in this susceptibility, PoRV-gonadotropism is supported by the affinity of viral particles to sialic acid molecules (Rajalakshmi and Prasad, 1968). We and other authors have reported the presence of sialic acid in testis, epididymis, deferent duct, and male accessory glands of pigs and other species (Reyes-Leyva et al., 1997). Sialic acids residues have been identified as

important constituents of sperm acrosomes, since they participate in both sperm maturation and capacitation, as well as in the fertilization process (Toowicharanont and Chulavatnatol, 1983). Future work addressing the molecular events involved in viral-host interactions will provide further insights into mechanisms to address this important disease.

PoRV infection in adult boars proceeded with transitory clinical signs and without evident macroscopic alterations in semen. An important result of this work showed that, when fresh semen was diluted in BTS solution as is typically done in artificial insemination, motility was reduced in only one individual; motility in the other specimens was normal. However, a marked decrease in sperm motility was observed after 24, 48, and 72 h PI. In previous reports, PoRV was detected in the reproductive tracts of boars and sows (Ramírez-Mendoza et al., 1997), suggesting that PoRV is excreted in semen, as are many other paramyxoviruses (Nathalie and Bernard, 2001) and other porcine viruses (Guérin and Pozzi, 2005). In this study, PoRV was detected in the semen of infected pigs from 2 days PI until 49 days PI. Likewise, the virus was detected free in seminal plasma or in association with sperm fractions. Previously, our group reported that PoRV is associated with red blood cells from 4 to 12 days PI, and with leukocytes from day 12 until the end of the experiment (20 days PI) (Reyes-Leyva et al., 2004), suggesting that these cells are used by the virus to spread it throughout the body, including reproductive tract or directly into semen. In the reproductive tract, PoRV affects sperm ducts and could lead to complete loss of sperm cells (Ramírez-Mendoza et al., 1997), which could explain the lower seminal quality. An important observation was the normal appearance of semen from most PoRV-infected boars, suggesting that it may be considered acceptable for artificial insemination when evaluated at the time of collection, but may not be suitable for AI when used later. When diluted for artificial insemination at a later time, the expected consequence would be decreased motility of spermatozoa and reduced fertilization. This could be due to the presence of PoRV in excreted semen, as shown here. However, a more sensitive test as Real Time PCR is needed to confirm this observation. From our results it is possible that semen collected from PoRV-infected boars could be an important route of virus transmission among and within farms.

In sum, this work shows that porcine rubulavirus induces reproductive alterations characterized by a transitory reduction in sperm quality. However, when this sperm looks apparently healthy and is used for artificial insemination a significant reduction in motility is observed. This reduced sperm quality is due, at least in part, to the porcine rubulavirus infection.

References

- Berg, M., Sundqvist, A., Moreno-López, J., Linné, T., 1991. Identification of the porcine paramyxovirus LPMV matrix protein gene: comparative sequence with other paramyxovirus. *Journal of General Virology* 72, 1045–1050.
- Berg, M., Hjertner, B., Moreno-López, J., Linné, T., 1992. The P gene of the porcine paramyxovirus encodes three possible polypeptides P, V and C: the P gene mRNA is edited. *Journal of General Virology* 73, 1195–1200.
- Berg, M., Bergvall, A.C., Sveda, M., Sundqvist, A., Moreno-López, J., Linné, T., 1997. Analysis of the fusion protein gene of the porcine rubulavirus LPMV: analysis of the paramyxovirus F protein. *Virus Genes* 14, 57–61.
- Crawley, A.M., Mallard, B., Wilkie, B.N., 2005. Genetic selection for high and low immune response in pigs: effects on immunoglobulin isotype expression. *Veterinary Immunology and Immunopathology* 108, 71–76.
- Dowsett, K.F., Knott, L.M., 1996. The influence of age and breed on stallion semen. *Theriogenology* 46, 397–412.
- Dubé, C., Beaulieu, M., Reyes-Moreno, C., Guillemette, C., Bailey, J., 2004. Boar sperm storage capacity of BTS and Androhep Plus: viability, motility, capacitation, and tyrosine phosphorylation. *Theriogenology* 62, 874–886.
- Guérin, B., Pozzi, N., 2005. Viruses in boar semen: detection and clinical as well as epidemiological consequences regarding disease transmission by artificial insemination. *Theriogenology* 63, 556–572.
- Hamilton, M.A., Russo, R.C., Thurston, R.V., 1977. Trimed Spearman-Kärber method for estimating mean lethal concentration in toxicity bioassays. *Environment Science Technology* 11, 714–719.
- Hernandez, J., Reyes-Leyva, J., Zenteno, R., Ramirez, H., Hernandez-Jauregui, P., Zenteno, E., 1998. Immunity to porcine rubulavirus infection in adult swine. *Veterinary Immunology and Immunopathology* 64, 367–381.
- Hernandez, J., Garfias, Y., Nieto, A., Mercado, C., Montano, L.F., Zenteno, E., 2001. Comparative evaluation of the CD4+CD8+ and CD4+CD8- lymphocytes in the immune response to porcine rubulavirus. *Veterinary Immunology and Immunopathology* 79, 249–259.
- Marchetti, C., Obert, G., Deffosez, A., Formstecher, P., Marchetti, P., 2002. Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. *Human Reproduction* 17, 1257–1265.
- Moreno-Lopez, J., Correa-Girón, P., Martínez, A., Ericsson, A., 1986. Characterization of a paramyxovirus isolated from the brain of a piglet in Mexico. *Archives of Virology* 91, 221–231.
- Nathalie, D., Bernard, J., 2001. Viruses in the mammalian male genital tract and their effects on the reproductive system. *Microbiology and Molecular Biology Reviews* 65, 208–231.
- Neild, D.M., Gadella, B.M., Chaves, M.G., Miragaya, M.H., Colenbrander, B., Agüero, A., 2003. Membrane changes during different stages of a freeze-thaw protocol for equine semen cryopreservation. *Theriogenology* 59, 1693–1705.
- Rajalakshmi, M., Prasad, M.R.M., 1968. Changes in the sialic acid content of the accessory glands of the male rats. *Journal of Endocrinology* 41, 471–476.
- Ramírez-Mendoza, H., Carreón, N.R., Mercado, G.C., Rodríguez, T.J., 1996. Hemoaglutinación e inhibición de la hemoaglutinación del paramixovirus porcino a través de la modificación de algunas variables que participan en la prueba. *Veterinaria Mexico* 27, 257–259.
- Ramírez-Mendoza, H., Hernández-Jauregui, P., Reyes-Leyva, J., Zenteno, E., Moreno-López, J., Kennedy, S., 1997. Lesions in the reproductive tract of boars experimentally infected with porcine rubulavirus. *Journal of Comparative Pathology* 117, 237–252.
- Raymond, C., Wilkie, B., Mallard, B., Quinton, M., 1998. Natural killer cell frequency and function in Yorkshire pigs selectively bred for high or low antibody and cell-mediated immune response. *Natural Immunity* 16, 127–136.
- Reyes-Leyva, J., Espinosa, B., Hernández, J., Zenteno, R., Vallejo, V., Hernández-Jauregui, P., Zenteno, E., 1997. NeuAc2,3Gal-Glycoconjugate expression determines cell susceptibility to the porcine rubulavirus LPMV. *Comparative Biochemistry Physiology* 118B, 237–332.
- Reyes-Leyva, J., García-Morales, O., Santo-López, G., Vallejo, V., Ramírez-Mendoza, H., Hernández, J., 2004. Detección de viremia en

- la infección experimental por Rubulavirus porcino. *Archivos de Medicina Veterinaria* 36, 39–47.
- Rima, B., Alexander, D.J., Billeter, M.A., Collins, P.L., Kingsbury, D.J., Lipkind, M.A., Nagai, Y., Orvell, C., Pringle, C.R., Ter Meulen, V., 1995. Family paramyxoviridae. In: Murphy, F.A., Fauquet, C.M., Bishop, D.H.L. (Eds.), *Virus Taxonomy. Classification and Nomenclature of Viruses*. Springer, Wien, p. 268.
- Royae, A.R., Husmann, R.J., Dawson, H.D., Calzada-Nova, G., Schnitzlein, W.M., Zuckermann, F.A., Lunney, J.K., 2004. Deciphering the involvement of innate immune factors in the development of the host response to PRRSV vaccination. *Veterinary Immunology and Immunopathology* 102, 199–216.
- ShIPLEY, C., 1999. Breeding soundness examination in the boar. *Journal of Swine Health and Reproduction* 7, 117–120.
- Stephano, H.A., 1999. Blue eye disease. In: Leman, A.D., Glock, R.D., Mengeling, W.L. (Eds.), *Disease of swine*, fifth ed. Iowa State University, Ames Iowa, pp. 103–112.
- Stephano, H.A., Gay, G.M., Ramírez, T.C., 1988. Encephalomyelitis, reproductive failure and corneal opacity (Blue eye) in pigs, associate with a paramyxovirus infection. *Veterinary Record* 112, 6–10.
- Sundqvist, A., Berg, M., Moreno-López, J., Linné, T., 1992. The hemagglutinating-neuraminidase glycoprotein of porcine paramyxovirus LPM: comparison with others paramyxovirus revealed the closest relationship to Simian virus 5 and Mumps virus. *Archives of Virology* 122, 331–340.
- Svenda, M., Berg, M., Moreno-López, J., Linné, T., 1997. Analysis of the large (L) protein gene of the porcine rubulavirus LPMV: identification of possible functional domains. *Virus Research* 48, 57–70.
- Toowicharanont, P., Chulavatnatol, M., 1983. Direct assay of sialic acids on rat spermatozoa from the caput and cauda epididymidis. *Journal of Reproduction and Fertility* 67, 275–280.
- Tortorella, D., Gewurz, B.E., Furman, M.H., Schust, D.J., Ploegh, H.L., 2000. Viral subversion of the immune system. *Annual Review of Immunology* 18, 861–926.
- Van Regenmortel, H.M.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E., Estes, M.K., Lemon, S., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R., Wickner, R., 2000. *Virus Taxonomy: Seventh Report of the International Committee on Taxonomy of Viruses*. Academic Press, San Diego, 1167 pp.