Artificial insemination of gilts with porcine reproductive and respiratory syndrome (PRRS) virus-contaminated semen

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Summary: *Six gilts were artificially inseminated (AI) with extended semen from a boar free of porcine reproductive and respiratory syndrome (PRRS) virus infection. One week later, the same boar was inoculated intranasally with PRRS virus. Seven days after inoculation, the boar was used to AI an additional five gilts. All 11 gilts were bred 3 days in a row using freshly collected and extended semen on each of the 3 days. Gilts were bled on a weekly basis until they were euthanized. Serum samples were tested for the presence of PRRS virus antibodies by the indirect-fluorescent antibody (IFA) test and for the presence of PRRS virus using virus isolation on porcine alveolar macrophages. Due to the cytotoxic nature of semen for continuous cell lines, a swine bioassay was used to confirm the presence of PRRS virus in the semen. The boar was euthanized on day 21 post-challenge. The control gilts were euthanized on day 40 and the gilts exposed to PRRS virus-contaminated semen were euthanized on day 34 following the first insemination. Reproductive tract tissues were collected for virus isolation and histopathologic examination.*

No clinical signs of PRRS were noted in the 11 gilts. The boar was depressed and anorexic for several days following challenge, but was physically normal by the time of collection 7 days post-challenge. Sperm motility and morphology were within normal acceptable limits for AI. Virus was detected in undiluted aliquots of semen collected on days 7 and 8 post-challenge, but not in the four samples collected prior to challenge or in the semen collected on days 9, 14, or 21 post-challenge. At the time of euthanasia, four of six control gilts were pregnant and one of five gilts inseminated with PRRS virus-contaminated semen was pregnant. None of the gilts seroconverted on the IFA test and virus was not isolated from the serum or reproductive tracts. Virus was not isolated from the reproductive tract of the boar. No histopathologic lesions were noted in the reproductive tracts of the gilts or boar. There was no significant difference in the pregnancy rates between the control and virus-exposed gilts. Transmission of PRRS virus through virus-contaminated semen was not detected based on development of PRRS virus antibodies, virus isolation from serum, or virus isolation from reproductive tracts.

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Porcine reproductive and respiratory syndrome (PRRS) was first reported in the United States in 1987.[1,2] Infection in sows and gilts has been reported to cause reproductive failure characterized by delayed returns to estrus, reduced conception rates, abortions, early farrowings, and an increased number of pigs born dead. Little is known about the role of the boar in female reproductive failure or the transmission of PRRS virus (PRRSV) via semen. There is conflicting evidence regarding the effect of PRRSV infection on measures of semen quality. Decreases in semen quality have been described in infected boars at artificial insemination centers[3] and in experimentally infected boars;[4] however, there are other reports in which alterations in semen quality following experimental infection of boars were not observed.[5,6] An epidemiologic study conducted in Britain concluded that there was circumstantial evidence that PRRSV was spread to non-infected herds via purchased semen.[7] Experimental infection of boars has led to seminal shedding of virus for up to 43 days following infection,[5] and insemination of gilts with undiluted semen from experimentally infected boars resulted in two of two gilts seroconverting and zero of two gilts pregnant.[6] Currently, there is enough concern in the swine industry regarding PRRSV-contaminated semen that Australia and South Africa have stopped importing semen from countries in which PRRS has been reported.[8] The purpose of this research was to:

- document the course of clinical signs of gilts inseminated with PRRSV-contaminated semen;
- determine whether there was a difference in pregnancy rates of gilts receiving normal and PRRS virus-contaminated semen;

- determine whether gilts would seroconvert following exposure to PRRSV via the semen; and
- evaluate the lesions resulting from PRRSV infection in reproductive tissues.

Materials and methods

Animals and housing

Eleven gilts and one boar were obtained from PRRSV-free herds and subsequently confirmed to be serologically negative for PRRSV antibodies by the indirect-fluorescent antibody (IFA) test. Gilts were housed in individual isolation facilities. Four- to 8-week-old pigs used in the PRRSV swine bioassay (SB) and as a source of porcine alveolar macrophages (PAMs) were also obtained from a PRRSV-free herd and verified to be PRRSV-antibody negative using the IFA test. Pigs were moved to individual isolation facilities prior to semen inoculation.

Boar inoculation

The PRRSV (ATCC VR-2402) is a plaque-purified isolate originally derived from a pool of tissues from clinically affected young pigs obtained from a herd undergoing a PRRS outbreak. The boar was inoculated intranasally with 2 mL per naris of $10^{6.5}$ $TCID_{50}$ per mL PRRS virus 7 days after the first insemination of the control gilts. The dose of virus used was previously shown to induce seminal shedding in breeding-age boars.[5]

Estrus synchronization and pregnancy evaluation

The gilts were randomly divided into a control group (six gilts) and a group exposed to virus via semen (five gilts). The estrus cycles were synchronized so that the virus-exposed group came into heat 14 days after the control group and 7 days after the boar was infected with PRRSV. The dose, route, and times of hormonal treatment were the same for both groups of gilts. Altrenogest (Regu-Mate®, Hoechst-Roussel, Somerville, New Jersey) was given orally in the feed at a dose of 11 mg per gilt every 24 hours for 13 days. Dinoprost tromethamine (Lutalyse®, Upjohn Company, Kalamazoo, Michigan) at a dose of 10 mg per gilt was injected intramuscularly (IM) the morning of day 14 and repeated 8–10 hours later. A single dose of a product (PG 600®, Intervet Inc., Millsboro, Delaware) containing 300 IU per gilt human chorionic gonadotropin (HCG) and 600 IU per gilt pregnant mare serum gonadotropin (PMSG) was injected IM on day 15.

Pregnancy status was determined at the time of euthanasia. Statistical significance of pregnancy status between groups of gilts was determined using Fisher's Exact test.

Semen collection

Fresh semen was collected and extended on each day the gilts were artificially inseminated (AI). Semen was collected into prewarmed thermos bottles lined with a semen collection bag as two gel-free fractions, sperm-rich and sperm-poor, using the gloved hand technique.[9] Polyvinyl chloride gloves (S/P® Brand diSPo® gloves, Baxter Healthcare, McGaw Park, Illinois) were used during semen collection. To remove the gel fraction, ejaculate was directed onto a sterile gauze covering the mouth of the thermos. Following semen collection, the gauze containing the gel fraction was discarded, semen was evaluated, and a small volume of each fraction of semen was stored at -80°C in 4–5 mL aliquots. Semen was collected 8, 7, and 6 days prior to challenge of the boar, at the time of challenge, and 7, 8, 9, 14, and 21 days following challenge.

Semen evaluation

Sperm motility was assessed on prewarmed slides within 30 minutes of collection. Sperm concentration was determined by diluting an aliquot of the sperm-rich fraction in a 2.9% sodium citrate solution and comparing the optical density to standard spectrophotometric reference values. Sperm morphology slides were made at the time of collection by mixing one drop of semen with one drop of eosin-nigrosin stain.[10] Slides were stored at room temperature until they were evaluated at the termination of the experiment. To avoid bias, all slides were assigned randomly ordered numbers and evaluated sequentially using differential interference contrast microscopy at ×1250 (oil immersion).

Semen extension and artificial insemination

Semen extender (Modena Boar Semen Extender, Swine Genetics International, LTD, Cambridge, Iowa) was prepared according to manufacturer instructions using filtered and heat-sterilized water. Fresh extender was prepared when the boar was collected for AI in control gilts. Fresh semen was collected 3 days in a row for AI. Extender remaining after the first semen extension was refrigerated until use the next day, at which time the required volume of extender was warmed prior to mixing with semen. The same procedure was followed on the third day. Extender remaining after the third day of collection and AI of control gilts was discarded. Fresh extender was prepared at the time of first collection of the boar for AI in exposed gilts. Semen was extended such that each gilt received a total volume of 80 mL (15 mL semen and 65 mL extender) at each insemination and sufficient motile spermatozoa for pregnancy to occur.

Gilts were artificially inseminated using commercially available disposable spiral catheters, semen bottles, and lubricant. Back pressure was applied to induce the immobility response and the spirette was locked into the cervix following lubrication of the spirette. Extended semen was slowly deposited into the uterus. Gilts were artificially inseminated 72, 96, and 120 hours after the HCG/PMSG injection. Extended semen was inseminated into gilts within 2 hours of collection.

Blood collection

Blood was collected from each gilt monthly until the time of first insemination, and then weekly until the time of euthanasia. The boar was bled 3 weeks prior to challenge, the day of challenge, and following challenge on days 7, 14, and 21, after which the boar was euthanized. Serum for virus isolation and

IFA was stored at -80°C. Prior to serological testing, serum samples were randomized and pig or date identifiers were removed. Serum samples were evaluated for the presence of PRRSV antibodies using the IFA test.[5]

Virus isolation

Virus isolation was done on porcine alveolar macrophages (PAMs). Macrophages were collected from young PRRS-free pigs and stored at -80°C until needed. Macrophages were then thawed, diluted in growth medium composed of RPMI 1640 (Sigma, St. Louis, Missouri) supplemented with glucose (Sigma, St. Louis, Missouri), fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah), gentamicin sulfate (Schering, Omaha, Nebraska), penicillin (Sigma, St. Louis, Missouri), streptomycin sulfate (Sigma, St. Louis, Missouri), amphotericin B (Squibb and Sons, Rolling Meadow, Illinois), and HEPES (Sigma, St. Louis, Missouri), and seeded onto 24 well plates (Costar Corp., Cambridge, Massachusetts).

The boar was euthanized 21 days after challenge. Tissues collected for virus isolation included: lung, spleen, kidney, bone marrow from the femur, vas deferens, epididymis, testicle, prostate, seminal vesicles, bulbourethral gland, prepuce, and penis. Samples collected from the gilts for virus isolation at the time of necropsy included: ovary, uterus, cervix, placenta, fetuses, and amniotic fluid. Tissue homogenates were centrifuged at 2000 × g for 15 minutes and inoculated onto 18- to 24-hour PAMs in 24 well plates after the growth medium was removed. Serum diluted 1 : 5 in growth medium was inoculated onto 18- to 24-hour PAMs in 24 well plates after the growth medium was removed. Inoculated cultures were incubated for 1 hour at 37°C, after which 0.8 mL of growth medium was added to each well. Cultures were then incubated at 37°C and observed periodically for 1 week for cytopathic effects. All samples were subinoculated onto MA104 cells in eight chamber slides. Seventy-two hours after inoculation, slides were fixed and stained with PRRS conjugate (D. Benfield, Department of Veterinary Science, South Dakota Center for Livestock Disease Control, South Dakota State University, Brookings, South Dakota) and read.

Histopathology

Tissues collected from the boar at the time of euthanasia included: lung, spleen, vas deferens, epididymis, testicle, prostate, seminal vesicles, bulbourethral gland, prepuce, and penis. Tissues collected from the gilts at the time of necropsy included: ovary, uterus, cervix, placenta, and fetuses. Tissues were fixed in 10% neutral buffered formalin. Following routine processing, tissues were embedded in paraffin and 5-µm sections were stained with hematoxylin-eosin stain.

Bioassay of semen samples

The presence of PRRSV in semen was determined by a SB. Uninfected pigs were inoculated with an unextended semen sample, then serologically monitored for evidence of PRRSV infection. Bioassay pigs were housed individually in isolation facilities to preclude exposure to PRRSV from other sources. Each pig was inoculated intraperitoneally with a 13- to 15-mL sample of semen (equal volumes of sperm-rich and sperm-poor fractions) from a single boar collection using a 20-mL syringe and 20-gauge needle. Serum samples were collected from SB pigs at the time of intraperitoneal inoculation and at weekly intervals thereafter. Two or more consecutive IFA-positive results from weekly samples were considered indicative of the presence of infectious PRRSV in the semen inoculum. Otherwise, SB pigs were followed for a total of 5 weeks after inoculation.

Results

Boar

The boar was depressed and anorexic for 3 days post-challenge (PC). By the time of semen collection on day 7 PC, his behavior and appetite were back to normal and he was willing to mount the dummy. The boar did not have any other clinical signs through day 21 PC, when he was euthanized. Spermatozoa motility and morphology remained within normal limits throughout the study. The boar was seronegative for PRRSV antibodies by IFA at the time of challenge. The IFA titer was 1 : 2560 on day 7 PC and rose to 1 : 5120 by day 21 PC. Virus was not isolated from the tissues and lesions were not detected by gross or histopathological examination.

Bioassay of semen samples

All of the bioassay pigs remained clinically healthy following inoculation with unextended semen. Pigs inoculated with semen collected from the boar prior to challenge, on the day of challenge, and on days 9, 14, and 21 PC remained seronegative, indicating that virus was not present in the semen. Pigs inoculated with semen samples collected on days 7 and 8 PC seroconverted, indicating the presence of virus in the semen.

Gilts

All of the gilts remained clinically healthy throughout the study and had a strong standing response on at least 1 of the 3 days on which they were bred. Four of the six control gilts were pregnant at the time of euthanasia 40 days after first insemination. The four pregnant control gilts had 5, 9, 11, and 11 fetuses within the uterus, respectively. One of the control gilts appeared to have cycled but was not pregnant and the other control gilt had a small, anestrus reproductive tract. One of the five exposed gilts was pregnant at the time of euthanasia 34 days after the first insemination. This gilt had 12 fetuses in the left horn and 9 fetuses in the right horn. The fetuses were grossly normal except for two that had signs of hemorrhage in the tissues. Three of the gilts that were not pregnant had old corpora lutea and developing follicles indicating they were coming back into estrus. The fourth exposed gilt had a small, anestrus reproductive tract similar in appearance to the control gilt's tract. The difference in pregnancy rates was not significantly different ($P = .24$). All 11 gilts remained IFA negative throughout the study and virus was not isolated from the reproductive tracts or serum.

Discussion

The role of semen in the transmission of PRRSV is not clearly understood. Epidemiologic information suggests that semen from infected boars may have been responsible for the transmission of PRRSV into uninfected herds.[7] Unextended semen from experimentally infected boars has been shown to transmit PRRSV to naive gilts.[6] In this study, transmission by AI was not detected even though the same amount of unextended semen used to artificially inseminate gilts was shown to be infectious when inoculated intraperitoneally into 4- to 8-week-old pigs.

The factors most likely to be responsible for the absence of transmission by AI in this study are route of exposure and dose of virus. The effect of route on transmission of viruses has been demonstrated. For lactate dehydrogenase-elevating virus (LDV), a virus closely related to PRRSV, the minimum infectious dose for mice has been shown to vary considerably depending on the route of exposure.[11] Exposure to LDV through intraperitoneal or tail cartilage injections revealed a minimum infectious dose of 1, while mucosal exposure via the ocular, vaginal, or oral routes required a minimum infectious dose of $1 \times 10^{5.3}$. Cows artificially inseminated with a combination of semen and ephemeral fever virus did not seroconvert, although these same cows were found to be susceptible to infection when inoculated intravenously with 1/10 the dose of virus used in AI.[12] Although pigs are susceptible to PRRSV infection by a variety of routes, the minimum infectious dose has not been established for each route. Our work suggests that a higher minimum infectious dose may be required for intrauterine transmission than for intraperitoneal transmission.

The boar in this study shed PRRSV in the semen for a shorter time than other PRRSV-infected boars we have studied. Previous studies at Iowa State University have shown that experimentally infected boars typically shed PRRSV in the semen by day 3–5 postchallenge and for 3 or more weeks.[5] Days 7–9 postchallenge were chosen for semen collection in this study based on the previously mentioned study in which one boar was no longer shedding after day 13 postchallenge. In this study, virus was present in semen on days 7 and 8 postchallenge, but not on days 9, 14, or 21, suggesting that this particular boar may have been shedding unusually low quantities of PRRSV in semen. Thus, the combination of intrauterine route and low dose of virus may not have met the requirements for transmission.

Other possible causes for the absence of transmission of PRRSV include:

- inactivation of the virus by a virucidal component of the extender;
- inactivation by a virucidal component of the semen; or
- virus inactivation by an interaction of the two.

However, these appear to be unlikely possibilities. The time from semen collection until insemination was relatively short (approximately 2 hours). Further, a preliminary study to evaluate extender and temperature effects indicated that infectious PRRSV was still present in extended semen held at 25°C for 1 hour, then frozen and stored at -80°C until intraperitoneal inoculation (Swenson, unpublished observations).

In total, four of six (66%) of control gilts and one of five (20%) of exposed gilts were pregnant. Because of the relatively small sample size, this distribution was not significantly different; that is, the lower pregnancy rate in the virus-exposed gilts could reflect normal variation.

Attempts were made to reduce variation by using one boar to inseminate the 11 gilts and, within each group of gilts, using estrus synchronization to compress the breeding period. Thus, given the identical treatment of the groups and the field observations of early infertility problems, the proportional difference in pregnancy rates (66% versus 20%) is suggestive of an effect of PRRSV on conception or early gestation and justifies further studies. If PRRSV affects conception or pregnancy, the mechanism may involve one or more stages of reproduction:

- spermatogenesis and the subsequent ability of spermatozoa to fertilize eggs; or
- fertility of the eggs; or
- development of the conceptus — either by direct virus effects or indirectly through changes in the uterine environment.

Other infectious agents are known to interfere with fertilization, development, or implantation of the fertilized egg. Cows infected with infectious bovine rhinotracheitis virus[13] and gilts exposed to pseudorabies virus[14] have been shown to develop endometritis. Preimplantation murine embryos exposed to cytomegalovirus were found to develop normally; however, embryos from mice inoculated intraperitoneal with cytomegalovirus were found to be developmentally retarded.[15] When these embryos were transferred to uninfected mice they developed normally, indicating that alterations in the maternal environment rather than the virus were responsible for the observed effects. Intrauterine exposure of cows to bovine viral diarrhea virus interferes with fertilization and development of embryos.[16,17] It is also reported that fertilized porcine eggs exposed to porcine parvovirus are developmentally retarded compared to control eggs.[18]

In summary, transmission of PRRSV via extended virus-contaminated semen was not detected in this study. This does not mean that transmission through the use of extended semen from infected boars will never occur. Seminal shedding of PRRSV for an extended period of time, as previously reported, indicates that the risk of PRRSV transmission via semen exists. Until additional trials of this type are performed, we cannot rule out the possibility of PRRSV transmission via extended semen. Furthermore, although a small number of gilts were used in this study, PRRSV may be associated with early infertility, as has been reported in field infections. Further studies are needed to confirm these results and, if corroborated, determine the stage(s) of reproduction affected.

Implications

- Transmission of PRRSV by exposure of females to PRRSV-contaminated semen has been reported, but was not demonstrated in this study.
- Lower pregnancy rates in gilts artificially inseminated with PRRSV-contaminated semen suggested a possible association with infertility. Additional research is needed to prove whether PRRSV-contaminated semen actually causes infertility.
- Swine practitioners should be aware of the possibility that boars may shed PRRSV in semen, that there is the potential for transmission of PRRSV in semen to susceptible females, and that PRRSV-contaminated semen may play a role in early infertility. Further research is needed to clarify these issues.

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