Porcine reproductive and respiratory syndrome virus: a persistent infection

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Abstract

Persistent infection with porcine reproductive and respiratory syndrome virus (PRRSV) was shown in experimentally infected pigs by isolation of virus from oropharyngeal samples for up to 157 days after challenge. Four 4 week old, conventional, PRRSV antibody-negative pigs were intranasally inoculated with PRRSV (ATCC VR-2402). Serum samples were collected every 2 to 3 days until day 42 post inoculation (PI), then approximately every 14 days until day 213 PI. Fecal samples were collected at the time of serum collection through day 35 PI. Oropharyngeal samples were collected at the time of serum collection from 56 to 213 days PI by scraping the oropharyngeal area with a sterile spoon, especially targeting the palatine tonsil. Turbinate, tonsil, lung, parotid salivary gland, spleen, lymph nodes and serum were collected postmortem on day 220 PI. Virus isolation (VI) on porcine alveolar macrophage cultures was attempted on all serum, fecal and oropharyngeal samples, as well as tissues collected postmortem. Postmortem tonsil tissues and selected fecal samples were also assayed for the presence of PRRSV RNA by the polymerase chain reaction (PCR). Serum antibody titers were determined by IFA, ELISA and SVN. Virus was isolated from all serum samples collected on days 2 to 11 PI and intermittently

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for up to 23 days in two pigs. No PRRSV was isolated from fecal samples, but 3 of 24 samples were PCR positive, suggesting the presence of inactivated virus. Oropharyngeal samples from each pig were VI positive 1 or more times between 56 and 157 days PI. Oropharyngeal samples from 3 of 4 pigs were VI positive on days 56, 70 and 84 PI. Virus was isolated from one pig on day 157 PI, 134 days after the last isolation of virus from serum from this animal. Virus was isolated from oropharyngeal samples for several weeks after the maximum serum antibody response, as measured by IFA, ELISA and SVN tests. All tissues collected postmortem were VI negative and postmortem tonsil samples were also negative by PCR. An important element in the transmission of PRRSV is the duration of virus shedding. The results of this study provided direct evidence of persistent PRRSV infection and explain field observations of long-term herd infection and transmission via purchase of clinically normal, but PRRSV infected, animals. Effective prevention and control strategies will need to be developed in the context of these results. © 1997 Elsevier Science B.V.

Keywords: Porcine reproductive and respiratory syndrome; PRRS virus; Epidemiology; Persistent infection; Transmission

1. Introduction

As an emerging virus, porcine reproductive and respiratory syndrome virus (PRRSV), has been the focus of an intense research effort since the first report of its isolation in 1991 (Wensvoort et al., 1991). One of the outstanding features of PRRSV has been its high degree of transmissibility. First recognized in 1987, PRRS spread rapidly through domestic swine populations in Europe, North America and Asia (Owen et al., 1992; Wensvoort et al., 1992; Bautista et al., 1993; Chang et al., 1993).

Although fundamental to the development of effective prevention and control strategies, the transmission of PRRSV among swine is not yet clearly understood. Swine infected with PRRSV are known to shed infectious virus by several routes. Virus has been found in semen from experimentally inoculated boars (Swenson et al., 1994). Also, transmission occurred when gilts were inseminated with fresh semen from infected boars (Yaeger et al., 1993). Virus has also been reported in feces, nasal secretions and urine (Yoon et al., 1993; Rossow et al., 1994), suggesting other routes of transmission, as well. The purpose of this study was to expand our knowledge of PRRSV shedding patterns. We report the prolonged isolation of infectious PRRSV from oropharyngeal samples and new evidence that PRRSV produces a persistent infection in swine.

2. Materials and methods

2.1. PRRS virus

The PRRSV isolate (ATCC VR-2402) used in the experiment was originally derived from a pool of tissues from clinically affected young pigs from a herd undergoing
clinical PRRS. Inoculation of tissue homogenates into a gnotobiotic pig was followed by
virus isolation (VI) in porcine alveolar macrophages (PAMs). The isolate was purified
by 3 rounds of limiting dilution in PAMs, then plaque purified twice in an African
monkey kidney continuous cell line (MA 104).

The titer of virus inoculum used in the study was determined by making serial 10 fold
dilutions of virus in 96 well microtitration plates (Corning Glass Works, Corning, NY),
using a high-glucose minimum essential medium (JRH Biosciences, Lenexa, KS)
supplemented with 30 μg of neomycin sulfate/ml (Sigma Chemical Company, St.
Louis, MO) and 1.2 mg of sodium bicarbonate/ml. Virus dilutions were inoculated onto
confluent MA-104 cells in replicates of 8. Wells were observed for cytopathic effects at
4 to 5 days after inoculation. The wells were fixed with 80% acetone/water and allowed
to air dry. The cell monolayer was flooded with PRRSV fluorescent monoclonal
antibody conjugate SDOW17 (David Benfield, South Dakota State University, Brook-
ings, SD) and placed in a humid 37°C incubator for 30 min. Plates were rinsed in a
phosphate-buffered saline solution bath for 5 min and a distilled water bath for 1 min.
After air drying, plates were observed under a fluorescent microscope. Tissue culture
infective dose titers (TCID$_{50}$/ml) were calculated using the Kärber method (Schmidt
and Emmons, 1989).

2.2. Experimental animals

Four 4 week old pigs were obtained from a herd periodically tested for PRRSV and
known to be free of the virus. Pigs were determined to be seronegative for PRRSV
antibodies by indirect fluorescent antibody (IFA), serum virus neutralization (SVN) and
enzyme linked immunosorbent assay (ELISA). Animals were housed in isolation
facilities throughout the experiment. After a four day acclimatization period, pigs were
intranasally inoculated with 1 ml of PRRSV at a concentration of $10^{3.2}$ TCID$_{50}$/ml by
instilling 0.5 ml of the inoculum into each nostril during inspiration.

2.3. Biological samples

Serum samples were collected for virus isolation (VI) every 2 to 3 days up to day 42
post-inoculation (PI) and then approximately every 14 days until day 213 PI. Serum
samples were also collected for VI at necropsy on day 220 PI. Blood samples were
drawn and the serum separated after 30 min at room temperature by centrifugation at
1000 × g for 10 min. Serum samples were refrigerated until VI procedures were started
later in the same day. Samples for serological tests were stored at −80°C until the tests
were performed.

Fecal samples for VI were collected at the time of serum collection through day 35
PI. Approximately 0.5 g of feces were collected with a fecal loop and suspended in 10
ml of Hanks' balanced salt solution (HBSS; Sigma Chemical Company, St. Louis, MO)
containing 0.5% bovine serum albumin (BSA; Sigma Chemical Company, St. Louis,
MO) and antibiotic–antimycotics (500 IU/ml penicillin, 500 μg/ml streptomycin, 250
μg/ml gentamicin, 125 μg/ml amphotericin B). The suspension was clarified by centrifugation at 2500 x g for 30 min and supernatants were sequentially filtered through 0.45 μm and 0.22 μm nitrocellulose membrane filters (Costar Corporation, Cambridge, MA). Virus isolation and polymerase chain reaction (PCR) procedures were performed on the final filtrates.

Oropharyngeal samples for VI were collected at the time of serum collection on days 56 through 213 PI. Animals were restrained with a nose snare and the mouth held open with an oral speculum. A stainless steel spoon with an elongated handle was used to scrape the oropharyngeal area, specifically targeting the palatine tonsil. With the aid of a Dacron sterile swab (Baxter Healthcare Corporation, McGaw Park, IL), the material collected on the spoon was placed into a capped tube containing 1 ml of sterile HBSS supplemented with 0.5% BSA and antibiotic-antimycotics. The swab was twirled in the medium, broken off, and left in the tube. Samples were vortexed thoroughly and the swabs removed aseptically with forceps. Afterwards, the suspension was clarified by centrifugation at 1500 x g for 10 min and filtered through a 0.22 μm nitrocellulose membrane filter. Virus isolation was carried out on the filtrates.

The pigs were euthanatized by electrocution and exsangination under the supervision of United States Department of Agriculture, Food Safety and Inspection Service inspectors. Turbinate, tonsil, lung, parotid salivary gland and spleen samples were collected, as well as tracheobronchial, mediastinal, iliac, mesenteric and parotid lymph nodes. Minced tissue specimens were suspended in 20 ml of cold HBSS supplemented with 50 μg/ml gentamicin and antibiotic-antimycotics (100 IU/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml fungizone). The tissue suspension was homogenized in a Stomacher 400 (Tekmar, Cincinnati, OH) for 20 s, then centrifuged at 2000 x g for 15 min. The supernatants were aliquoted and frozen at -80°C until submitted for VI.

2.4. Virus assay

Virus isolation was conducted on PAMs collected by lung lavage from 4 to 6 week old pigs obtained from a PRRSV-free herd. In preparation for VI, PAMs were placed in 48 well plates (Costar Corporation, Cambridge, MA) at a rate of 10⁶ cells/well with RPMI 1640 media (Sigma Chemical Company, St. Louis, MO) supplemented with 10% fetal calf serum (Sigma Chemical Company, St. Louis, MO), 10 mM HEPES (Sigma Chemical Company, St. Louis, MO) and antibiotic-antimycotics (Sigma Chemical Company, St. Louis, MO), then incubated for 24 h at 37°C in a 5% CO₂ atmosphere.

All samples for VI were processed immediately following collection and each sample was run in duplicate. One day old PAM cultures in 48 well plates were inoculated with 0.25 ml samples of serum, fecal filtrates, oropharyngeal or tissue filtrates and observed daily for cytopathic effect (CPE) for up to 7 days after inoculation. The presence of PRRSV in cultures showing CPE was confirmed by subinoculating onto MA-104 cell monolayers prepared on 8 chambered glass slides (Nunc, Naperville, IL), incubating for 48 h, and staining with PRRSV fluorescent monoclonal antibody conjugate SDOW17. Samples were considered negative after one blind passage.
Table 1
Virus isolation from serum

<table>
<thead>
<tr>
<th>Pig No.</th>
<th>Days post inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 2 4 7 9 11 14 16 18 21 23 25 28 30 32 35 37 39 42</td>
</tr>
<tr>
<td>128</td>
<td>- + + + + - + - + + + - - - - - - - - -</td>
</tr>
<tr>
<td>135</td>
<td>- + + + + - + _ _ + - - - - - - - - -</td>
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<tr>
<td>141</td>
<td>- + + + + + + _ + + - - + + + + + + +</td>
</tr>
<tr>
<td>149</td>
<td>- + + + + + - + + + + + - + + + + + +</td>
</tr>
</tbody>
</table>

* Virus isolation negative (-) or virus isolation positive (+).

2.5. Serology

Serum antibody titers were measured on samples collected on days 0, 7, 11, 14, 21, 28 and then approximately every 14 days until day 213 PI. Serum samples were randomized and assayed as a block by IFA, SVN and ELISA. The IFA test was performed using the protocol described by Swenson et al. (1994). SVN test has been described by Yoon et al. (1995). A commercially available ELISA (HerdChek: PRRS, IDEXX Laboratories, Westbrook, Maine) was performed following the procedures described by the manufacturer. The sample to positive (S/P) ratio was calculated for each sample, with a S/P ratio of 0.4 or greater considered positive.

2.6. Polymerase chain reaction

Tonsil samples collected postmortem and fecal filtrates collected on days 0, 7, 14, 21, 28 and 35 PI were assayed for the presence of PRRSV by PCR. To process tonsil samples for PCR, one gram of frozen tonsil tissue was minced slightly in a sterile petri dish, then 2 ml of HBSS was added to the tissue in a sterile plastic bag and homogenized in a Stomacher 80 (Tekmar, Cincinnati, OH) for 1 min. The supernatant was stored frozen at -80°C. Prior to RNA extraction, the supernatant was thawed and cell debris was removed by centrifugation at 14,000 × g for 15 s. Five hundred μl of the homogenized tonsil tissue supernatant or fecal filtrate was added to an equal volume of lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7, 0.5%

Table 2
Virus isolation from oropharyngeal samples

<table>
<thead>
<tr>
<th>Pig No.</th>
<th>Days post inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>56</td>
<td>70 84 98 115 128 143 157 171 185 199 213</td>
</tr>
<tr>
<td>128</td>
<td>+ _ - - - - - - - - -</td>
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<td>135</td>
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<td>141</td>
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<tr>
<td>149</td>
<td>+ + + + + + + + + - -</td>
</tr>
</tbody>
</table>

* Virus isolation negative (-) or virus isolation positive (+).
Fig. 1. Results of IFA serological test and virus isolation from oropharyngeal samples.

Fig. 2. Results of ELISA serological test and virus isolation from oropharyngeal samples.
sarkosyl, 0.1 M 2-mercaptoethanol). Five hundred μl of the lysate was then added to an equal volume of phenol chloroform-isooamyl alcohol (24:1), vortexed and centrifuged at 10,000 × g for 5 min. Further extractions, reverse transcription and outer and nested PCR reactions are described elsewhere (Christopher-Hennings et al., 1995b).

3. Results

Virus isolation results varied among the tissues sampled. Virus was isolated from all serum samples collected on days 2 through 11 PI, then intermittently for up to 23 days in two of the animals (Table 1). Serum samples collected on days 25 through 220 were negative for VI. All VI attempts on fecal samples were negative, although viral RNA was detected by PCR analysis of fecal samples from pigs 141, 149 and 128 collected on days 7, 14 and 21, respectively. Oropharyngeal samples from all pigs were positive by VI one or more times between 56 and 157 days PI (Table 2). Virus was isolated from 3 of 4 pigs on 56, 70 and 84 days PI. Virus was isolated from one animal 157 days PI, which was 134 days after the last isolation of virus from the serum of this pig. The VI results on oropharyngeal samples are presented in conjunction with serological test results in Figs. 1–3. Virus was isolated from oropharyngeal samples for several weeks after the maximum serum antibody response, as measured by IFA, ELISA and SVN tests. Virus was not isolated from any of the tissue homogenates or serum collected following euthanasia on day 220 PI. Viral RNA was not detected by PCR analysis of tonsil tissue collected post mortem.

![Graph showing SVN titers and virus isolation results](image-url)

**Fig. 3.** Results of SVN serological test and virus isolation from oropharyngeal samples.
4. Discussion and conclusions

Research into the epidemiology of PRRSV is still in its infancy and many of the
dactors involved in transmission of PRRSV have not been defined. Portals of exit and
entrance are important areas of continuing research. In part, the intent of this research
was to study fecal shedding of PRRSV by swine. However, none of 64 samples
collected from 4 pigs was positive by virus isolation. A PCR assay subsequently
performed on a subsample of 24 samples detected viral RNA in 3 samples, one each
from 3 different pigs sampled on days 7, 14 and 21 PI. The infrequency of PCR-positive
samples suggested that PRRSV was shed intermittently and at low levels in feces. It has
recently been reported that PRRSV is rapidly inactivated in fecal slurry (Pirtle and
Beran, 1995). We suggest that the occasional presence of viral RNA and the absence of
infectious virus in feces is compatible with intermittent shedding of virus at low levels
with rapid inactivation of virus, perhaps within the intestinal tract itself. There are 2
previously published reports of isolation of PRRSV from feces. Yoon et al. (1993)
reported isolation of PRRSV from 55 of 154 fecal samples collected from principal and
sentinel pigs. Fecal samples from 4 of 4 experimentally inoculated pigs were VI positive
for up to 35 days. Similar to the results reported here. Rossow et al. (1994) isolated
PRRSV from only 2 of 15 fecal swabs taken 28 days PI, while 105 fecal swabs collected
over days 1, 4, 7, 14 and 21 PI were VI negative. The reason for differences among
these studies is not known. Possibly, alterations in the intestinal tract due to physiologic
or infectious causes may affect either the rate of shedding of PRRSV or the persistence
of intact virus in feces. This is an area which requires further investigation.

A critical feature in the transmission of PRRSV is the duration of infection. Virus
isolation from oropharyngeal samples for up to 157 days after experimental inoculation
provided direct evidence for persistent infection with PRRSV. This evidence can be
added to previous work providing indirect evidence of persistent PRRSV infection in
swine. Zimmerman et al. (1992) reported transmission by direct contact between
susceptible animals and sows infected 99 days earlier. Albina et al. (1994) demonstrated
transmission of PRRSV by pigs infected 15 weeks earlier. Boars have been shown to
shed infectious virus in their semen for up to 43 days (Swenson et al., 1994). Viral RNA
has been detected for up to 92 days in semen (Christopher-Hennings et al., 1995a).

The results of this study suggest that PRRSV resembles lactate dehydrogenase-elevat-
ing virus (LDV), equine arteritis virus (EAV) and simian hemorrhagic fever virus
(SHVF) in its capacity to produce persistent infections (Plagemann and Moennig, 1992).
In contrast to PRRSV, persistent infection in LDV and SHFV is characterized by a
persistent viremia (Gravell et al., 1986; Plagemann and Moennig, 1992). Because of the
sampling process utilized in this work, the oropharyngeal sample potentially consisted of
blood traces, saliva, lacrimae, nasal secretions and respiratory tract secretions. From the
work done to date, the palatine tonsil can not be unequivocally stated to be the site of
the persistent infection. Further research is needed to define the site(s) of infection.

Isolation of virus from oropharyngeal samples for several weeks after peak IFA, SVN
and ELISA antibody titers indicated that the immune responses measured by these tests
were not central to the clearance of the virus from the host. However. negative VI
attempts on tissues collected at necropsy, in conjunction with the negative PCR results
on tonsil tissue collected post mortem, suggested that the immune system of the host
was eventually able to clear PRRSV from the body. The results of this study hold
profound implications for our understanding of the immunology and epidemiology of
PRRSV infections in swine. The development of effective PRRSV prevention and
control strategies will need to be assessed in the context of this new information.

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