Localization of classical swine fever virus in male gonads during subclinical infection

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In an experiment using ten boars, the distribution of classical swine fever virus (CSFV) was determined in the male reproductive tract by in situ hybridization over a period of 120 days after intranasal inoculation. CSFV was detected in the testicular tissue of infected boars. Viral nucleic acid was localized to spermatogonia, spermatocytes and spermatids but was not detected in the epithelia of the prostate, epididymis or bulbourethral gland. Sections from control, CSFV-negative, pigs showed no hybridization signals for CSFV. The demonstration that CSFV infects the spermatogonia (and their progeny) suggests that this may serve as a primary reservoir for the venereal spread of CSFV.

Classical swine fever virus (CSFV) is the causative agent of classical swine fever (CSF), otherwise known as hog cholera, a disease of swine that has economically important consequences. CSFV is an RNA virus classified within the genus Pestivirus, family Flaviviridae, which also includes bovine viral diarrhoea virus (BVDV) and border disease virus (Wengler et al., 1995). The CSFV genome is a single-stranded, positive-sense, non-polyadenylated RNA of about 12.3 kb in length (Meyers et al., 1989; Moormann et al., 1990).

CSFV is a devastating disease of swine; for this reason, many countries pursue surveillance and/or eradication programs to limit infections (Moennig, 1992; Pearson, 1992). The detection of CSFV in semen (Floegel et al., 2000) suggested that transmission via this medium is possible. Transmission of CSFV via semen to offspring has been reported previously by de Smit et al. (1990). This primer set resulted in amplified fragments of 300 bp. Sequencing was performed on the purified PCR products before being labelled by random priming with DIG–dUTP (Boehringer Mannheim), according to the manufacturer's instructions. The porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus (PCV) probes were used as a negative control (Cheon & Chae, 1999; Choi & Chae, 1999). In situ hybridization and virus isolation was performed as described previously (Cheon & Chae, 1999; Choi & Chae, 1999; Ahrens et al., 2000).

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CSFV strain SNUVR2345 used in this study was isolated from a 58-day-old pig from a herd of 200 sows located in Chungcheong Province, South Korea, in 1997. This pig presented with severe respiratory disease and growth retardation and was diagnosed with chronic CSFV infection on the basis of clinical signs, virus isolation, immunohistochemistry and in situ hybridization. CSFV strain SNUVR2345 was considered to be a low virulence strain.

Ten 1-year-old boars were randomly allocated between an infected (n = 5) or control (n = 5) group. Serum samples from all boars were tested by enzyme-linked immunosorbent assays (Idexx) for antibodies against CSFV and virus neutralization assays for antibodies against BVDV before experimental infection. Five boars were inoculated intranasally with 3 ml of CSFV strain SNUVR2345 (2nd passage) at a concentration of 10^5 TCID_{50} per ml. Five control boars were inoculated with 3 ml of the supernatant of non-infected PK-15 cells. The boars were housed individually in isolation facilities. Serum samples were collected every 10 days following experimental inoculation. Two boars, one infected and one control, were humanely killed at 60, 80, 90, 110, 120 days post-inoculation (p.i.). Tissues were collected from each pig at necropsy and virus infection was performed on the tissues (Table 1).

The primer sequence specific to the CSFV genome was used, as previously described (Liu et al., 1991). The forward and reverse primers were 5’ AGTGAACAAGCGCACAATAAGG 3’ (nt 1198–1217) and 5’ CTTATCTGGAGGGCCTTCTG 3’ (nt 1467–1478), respectively (Liu et al., 1991). This primer set resulted in amplified fragments of 300 bp. Sequencing was performed on the purified PCR products before being labelled by random priming with DIG–dUTP (Boehringer Mannheim), according to the manufacturer's instructions. The porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus (PCV) probes were used as a negative control (Cheon & Chae, 1999; Choi & Chae, 1999). In situ hybridization and virus isolation was performed as described previously (Cheon & Chae, 1999; Choi & Chae, 1999; Ahrens et al., 2000).

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Table 1. Detection of CSFV in male genital tracts by *in situ* hybridization

Virus isolation and positive *in situ* hybridization results are indicated as ‘+’.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Virus isolation/in situ hybridization in boars (days p.i.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60</td>
</tr>
<tr>
<td>Testicle</td>
<td>+ / +</td>
</tr>
<tr>
<td>Epididymis</td>
<td>+ / +</td>
</tr>
<tr>
<td>Ductus deferens</td>
<td>+ / +</td>
</tr>
<tr>
<td>Total</td>
<td>3 / 4</td>
</tr>
</tbody>
</table>

Table 2. Morphometric analysis of testicular tissues from CSFV-infected boars

<table>
<thead>
<tr>
<th>Boar</th>
<th>Day p.i.</th>
<th>Positive seminiferous tubules (%)</th>
<th>Number of positive germ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Spermatogonia</td>
<td>Spermatocytes</td>
</tr>
<tr>
<td>1</td>
<td>60</td>
<td>30</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>23</td>
<td>13</td>
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<tr>
<td>3</td>
<td>90</td>
<td>27</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>110</td>
<td>43</td>
<td>22</td>
</tr>
<tr>
<td>5</td>
<td>120</td>
<td>37</td>
<td>6</td>
</tr>
</tbody>
</table>

Five sections of formalin-fixed testes were taken from each virus-infected boar for morphometric analysis. Only well-oriented sections were measured. Numbers of positive seminiferous tubules, as determined by *in situ* hybridization, were estimated by measuring 100 seminiferous tubules per section. Numbers of positive spermatogonia, spermatocytes and spermatids, as determined by *in situ* hybridization, were also measured in seminiferous tubules.

Boars became febrile between 4 and 11 days p.i. After the onset of fever, clinical signs of disease were not observed in the boars. Negative control boars remained clinically normal. Serologically, all boars were negative for CSFV and BVDV prior to inoculation and all control boars remained CSFV seronegative throughout the experimental study period. Antibodies to CSFV could be detected as early as 20 days p.i. in one boar (no. 2) and all five infected boars were found to be seropositive by 30 days p.i. Thereafter, all infected boars remained seropositive for CSFV. Boars were humanely killed at 60, 80, 90, 110 and 120 days p.i., respectively. Of the tissues tested, 26% (8 of 30) from the five infected boars were positive for CSFV by virus isolation (Table 1).

The results of *in situ* hybridization are summarized in Table 1. The male reproductive tracts from boars with CSFV infection were histologically normal. The morphology of host cells was preserved despite the relatively high temperature required in parts of the hybridization procedure. Viral nucleic acid-positive cells typically exhibited a dark brown reaction product in the cytoplasm, without background staining. The signal intensity varied within and between histological structures in any one section, and also between pigs.

CSFV-infected positive cells were found in the testes of all five of the CSFV-infected pigs examined. Of the testes tissue examined, 32% were infected with CSFV (Table 2). The distribution of positive cells was focal, found in single or small clusters of germ cells (Fig. 1a) with the virus localized to the spermatogonia, spermatocytes and spermatids (Fig. 1b). Hybridization signals for CSFV RNA were frequently detected in spermatocytes, followed by spermatids and spermatogonia (the average number of CSFV RNA-positive cells was 55, 50-8 and 15-2, respectively) (Table 2). The virus was not detected in Sertoli, Leydig or endothelial cells. Infected cells were noted rarely in stromal cells that appeared, cytologically, to be macrophages, with large oval nuclei and abundant cytoplasm. Small numbers of CSFV RNA-positive cells with distinctly round morphology and oval nuclei, resembling monocytes, were also observed in the blood vessels (Fig. 1c).
Fig. 1. *In situ* hybridization of reproductive tract tissues from male boars experimentally infected with CSFV. CSFV nucleic acids were detected in germ cells (a), spermatids (b), monocytes in a testicular blood vessel (c) and non-sperm cells in ductus deferens (d).
Hybridization signals were also detected in epididymal tissues from CSFV-infected boars. Most of the virus-infected cells were in the lumen of efferent ducts. Non-sperm cells contained viral nucleic acid. Occasionally, positive cells were also seen in the stromal connective tissue. CSFV-infected cells were not detected in the epithelium of efferent ducts. CSFV-infected non-sperm cells were seen in the lumen of ductus deferens (Fig. 1d). Spermatogonia were consistently negative for in situ hybridization of CSFV in the lumen of ductus deferens. Lymphoid tissues in the propria submucosa of the penis from three boars at 60, 80 and 90 days p.i. contained macrophages with CSFV nucleic acid. No hybridization signals were detected in the prostate or bulbourethral glands from CSFV-infected boars.

A consistent hybridization signal was not seen in tissue sections treated with RNase A prior to in situ hybridization. Sections from CSFV-negative control pigs showed no hybridization signals for CSFV. Probes for PRRSV and PCV gave consistently negative results in all tissues tested.

This study demonstrated the target cells for CSFV in the male genital tracts of experimentally infected boars. There were few or no microscopic lesions within reproductive tissues of these boars. CSFV infected neither the epithelial cells lining the ducts and glands of the male reproductive tracts nor the sperm heads from the boars examined. There was a prominent localization of the CSFV nucleic acid to the spermatogonia and their progeny. Spermatogonia infection is probably secondary to the haematogenous spread of the virus. Direct infection from infectious virions in the peripheral blood is a distinct possibility given the highly vascular nature of the testes.

CSFV nucleic acid was detected using in situ hybridization on post-mortem samples until 120 days p.i., whereas infectious virus has not been isolated in boars at 90 and 120 days p.i., although the number of seminiferous tubules with a positive hybridization signal did not decrease until 120 days p.i. Because of the limited numbers of animals used in this study, it is not possible to explain the difference in results between in situ hybridization and virus isolation. One possibility is that CSFV is not well adapted to PK-15 cells. Another possibility is that cytotoxic factors in seminal fluid have made laboratory isolation of viruses from semen on continuous cell lines difficult or impossible (van Engelenburg et al., 1993). Detection of CSFV in male gonads has important implications for disease control strategies because it suggests that apparently healthy adult boars may act as carriers of CSFV. Contact transmission of the virus from older to younger boars may also be an important means of CSFV spread.

The observation that the spermatogonia and their progeny are actively infected by CSFV may explain a mechanism whereby the virus is transmitted by artificial insemination. Spermatogonia infection clearly offers an advantage for the veneral distribution of virus, especially in the early stages of the disease when spermatogenesis is robust and many spermatogonia and their progeny are present. The transmission of CSFV via semen to offspring by artificial insemination has been also reported (de Smit et al., 1999). Most importantly, this study has identified a reservoir for CSFV and a putative mode of transmission, the combination of which could be responsible for the widespread dissemination of CSFV in the swine industry. The transmission of pestiviruses via semen to offspring has been reported previously in cattle and sheep, both by natural mating and by artificial insemination (Gardiner & Barlow, 1981; Meyling & Jensen, 1988). This, therefore, appears to be a particular feature of pestivirus infection.

CSFV has also been detected in the non-sperm cells of infected boars in the lumen of efferent ducts. These cells were leukocytes, as determined by their morphological appearance and labelling with SWC3a (Thacker et al., 2001), a pan-myeloid marker (data not shown). Evidence suggests that seminal fluid may enhance virus transmission by facilitating contact between infected leukocytes and epithelial cells of the mucosa (Pearce-Pratt & Phillips, 1993). Since the semen samples of the boars were not tested for infectious virus, more information is needed, however, before the pathogenetic relationship between CSFV infection and the male reproductive tract can be completely understood.

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References


Summary of workshop findings for porcine myelomonocytic markers. Veterinary Immunology and Immunopathology 80, 93–109.


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