Respiratory tract protection upon challenge of pigs vaccinated with attenuated porcine reproductive and respiratory syndrome virus vaccines

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Abstract

In this study, the efficacy of two attenuated porcine reproductive and respiratory syndrome virus (PRRSV) vaccines was assessed. The virological protection in the lungs of vaccinated pigs upon challenge was studied. Also, challenged pigs were exposed to lipopolysaccharide (LPS) to evaluate clinical protection. Six-week-old pigs were immunized intramuscularly with commercial vaccines based on either an attenuated American or an attenuated European virus strain. Non-immunized pigs and pigs intramuscularly inoculated with the virulent Lelystad strain were included as controls. Six weeks after immunization, pigs were challenged either intratracheally or intranasally with the Lelystad strain, and 3 and 6 days later intratracheally exposed to Escherichia coli LPS. After LPS administration, pigs were monitored for clinical signs. At 4 and 7 days after challenge, pigs were euthanized to determine virus quantities in broncho-alveolar lavage (BAL) fluids and in lungs. Challenge virus was recovered from three out of eight pigs that had been primo-inoculated with the Lelystad strain with titers ranging between 0.3 and 3.1 log_{10}. Fifteen out of sixteen pigs vaccinated with the attenuated American strain were positive for challenge virus and their mean virus titers were similar to those of non-immunized challenge controls. Eleven out of 16 pigs vaccinated with the attenuated European strain were positive for challenge virus and their mean virus titers were 2.0–2.5 log_{10} lower than those of non-immunized challenge controls. Thus, the virological protection in the lungs of vaccinated pigs upon challenge was incomplete, but was more pronounced in the homologous situation. Clinical signs upon LPS exposure in both vaccinated groups were not reproducible in two experiments.

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1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), an arterivirus, is widespread in the pig population. The virus replicates highly in the respiratory tract (Duan et al., 1997; Labarque et al., 2000a) and is claimed to play an important role in multi-factorial respiratory disease problems in the field (Done and Paton, 1995). The high economical impact of these problems has stimulated the development of PRRSV vaccines. Currently, several attenuated PRRSV vaccines are commercially available for use in feeder pigs. Since experimental infections with European PRRSV isolates fail to induce overt respiratory signs (Plana-Durán et al., 1992; Ramos et al., 1992; Van Reeth et al., 1996; Labarque et al., 2002), the efficacy of PRRSV vaccines is usually assessed by determining the degree of reduction in viraemia after challenge with a virulent virus (Christopher-Hennings et al., 1997; Nielsen et al., 1997; van Woensel et al., 1998; Labarque et al., 2000b). At present, no information is available about the degree of reduction in challenge virus replication in the respiratory tract. This information would be useful since replication of wild-type virus in the lungs of vaccinated pigs might still predispose for multi-factorial respiratory disease problems.

Since a single PRRSV infection does not induce overt respiratory signs, especially under experimental circumstances and with European isolates (Plana-Durán et al., 1992; Ramos et al., 1992; Van Reeth et al., 1996; Labarque et al., 2002), the clinical efficacy of attenuated PRRSV vaccines cannot be assessed. This problem may be solved by a combined challenge of PRRSV-vaccinated pigs with PRRSV and lipopolysaccharide (LPS). We have previously demonstrated that infection with the virulent Lelystad strain of PRRSV sensitizes the lungs of pigs for respiratory signs when they are, 5 days later, exposed to LPS (Labarque et al., 2002). Clinical signs consisted of fever, depression, tachypnoea, abdominal breathing and dyspnoea, whereas control pigs, which had been exposed to PRRSV or LPS only, experienced a transient fever and mild or no respiratory signs. The clinical synergy was observed in 87% of the pigs.

It was the purpose of the present study to assess the virological protection in the lungs of vaccinated pigs and to examine if the challenge virus replication in the lungs of these pigs may still predispose for respiratory signs upon LPS exposure. To this purpose, two commercial attenuated PRRSV vaccines, one based on an American virus strain and one based on a European virus strain, were tested using a challenge with the European Lelystad strain followed by LPS.

2. Materials and methods

2.1. Vaccines, virus and LPS

Two commercial attenuated PRRSV vaccines and one virulent PRRSV strain were used for active immunization of the pigs. One PRRSV vaccine was based on an American virus strain and the other was based on a European virus strain. A fifth passage on pulmonary alveolar macrophages (PAMs) of the Lelystad strain of PRRSV (Wensvoort et al., 1991) was used as virulent virus. The inoculation dose of each vaccine or virus strain was $10^{6.0} \times TCID_{50}$ per pig.
The virulent Lelystad strain of PRRSV was used as challenge virus. The inoculation dose was $10^{6.0} \times \text{TCID}_{50}$ per pig.

*Escherichia coli* LPS (O111:B4) (Difco Laboratories; Sigma) was used at a dose of 20 μg/kg body weight. This dose was based on data from previous studies and selected to cause mild or no respiratory signs (Van Reeth et al., 2000; Labarque et al., 2002).

2.2. Pigs and experimental design

A total of 58 pigs, originating from eight PRRSV-seronegative sows, were used. Two experiments were performed. In each experiment, pigs were divided into four groups and housed in isolation units. The designation of the groups is shown in Table 1.

At 6 weeks of age, pigs of groups A1 and B1, and of groups A2 and B2, were immunized intramuscularly with one of the two commercial PRRSV vaccines according to the manufacturer’s instructions. Pigs of groups A3 and B3 were inoculated intramuscularly with the virulent Lelystad strain. Pigs of groups A4 and B4 were not immunized and served as challenge control pigs. Six weeks after immunization, all pigs were challenged with the virulent Lelystad strain. The challenge was performed intratracheally in experiment A and intranasally in experiment B. At 3 and 6 days after challenge, all pigs were challenged with the virulent Lelystad strain. The challenge was performed intratracheally in experiment A and intranasally in experiment B. At 3 and 6 days after challenge, pigs of all groups were exposed intratraceally to LPS. After LPS administration, clinical signs were recorded and at 24 h after LPS administration (i.e. at 4 and 7 days after challenge), two to five pigs of each group were euthanized for virological examinations of broncho-alveolar lavage (BAL) fluids, lung samples and serum samples.

2.3. Serological examinations

Blood samples of all pigs were collected at the time of immunization and immediately before challenge. Serum samples were examined for PRRSV-specific antibodies using immunoperoxidase monolayer assays (IPMAs). MARC-145 cells infected with a European PRRSV isolate (94V360) were used. To ensure the maximum sensitivity, serum samples of the pigs vaccinated with the attenuated American strain were also tested in an IPMA with MARC-145 cells infected with an American PRRSV isolate (US5). It had been demonstrated that serum samples from pigs vaccinated with the American serotype, incubated on cells infected with a European serotype, had significantly lower titers than serum samples from pigs vaccinated with the European serotype, and vice versa (van Woensel et al., 1998).

2.4. Clinical monitoring

Clinical signs were recorded every 2 h from 0 until 12 h after LPS administration. Pigs were monitored for general signs, notably fever and depression, and for respiratory signs, notably tachypnoea, abdominal breathing and dyspnoea. Scores were given for these five clinical parameters according to a previously described system (Labarque et al., 2002). Briefly, body temperatures of $\leq 39.9 ^\circ C$ were scored as 0, temperatures between $\geq 40.0$ and $\leq 40.9 ^\circ C$ as 1 and temperatures of $\geq 41.0 ^\circ C$ as 2. Depression was scored as 0 (absent) or 1 (present). Respiration rates of $\leq 45$ were scored as 0, rates between $\geq 46$ and $\leq 59$ as 1.
Table 1
Cumulative clinical scores after LPS administrations at 3 and 6 days after challenge with the virulent Lelystad strain

| Experiment | Group | Strain used for intramuscular immunization/inoculation | Route of challenge with Lelystad strain | Time interval challenge-LPS exposure | No. of pigs | Mean ± S.D. of the cumulative scores after LPS
<table>
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<tbody>
<tr>
<td>A</td>
<td>A1</td>
<td>attAm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Intratracheal</td>
<td>3 days</td>
<td>6</td>
<td>14.5 ± 1.6 A 10.8 ± 4.3 A</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>attEur</td>
<td>Intratracheal</td>
<td>6</td>
<td>6</td>
<td>3.7 ± 0.8 B 0.0 ± 0.0 B</td>
</tr>
<tr>
<td></td>
<td>A3</td>
<td>virEur</td>
<td>Intratracheal</td>
<td>4</td>
<td>4</td>
<td>5.0 ± 1.4 B 0.0 ± 0.0 B</td>
</tr>
<tr>
<td></td>
<td>A4</td>
<td>none</td>
<td>Intratracheal</td>
<td>10</td>
<td>10</td>
<td>12.9 ± 4.0 A 9.8 ± 7.2 A</td>
</tr>
<tr>
<td></td>
<td>A1</td>
<td>attAm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Intratracheal</td>
<td>6 days</td>
<td>3</td>
<td>13.3 ± 2.3 A 12.3 ± 7.1 A</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>attEur</td>
<td>Intratracheal</td>
<td>3</td>
<td>3</td>
<td>0.7 ± 0.6 B 0.3 ± 0.6 B</td>
</tr>
<tr>
<td></td>
<td>A3</td>
<td>virEur</td>
<td>Intratracheal</td>
<td>2</td>
<td>2</td>
<td>1.0 ± 1.4 B 0.0 ± 0.0 B</td>
</tr>
<tr>
<td></td>
<td>A4</td>
<td>none</td>
<td>Intratracheal</td>
<td>5</td>
<td>5</td>
<td>9.8 ± 3.6 A 9.5 ± 3.1 A</td>
</tr>
<tr>
<td>B</td>
<td>B1</td>
<td>attAm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Intranasal</td>
<td>3 days</td>
<td>10</td>
<td>7.3 ± 1.6 B 4.0 ± 3.3 B</td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>attEur</td>
<td>Intranasal</td>
<td>10</td>
<td>10</td>
<td>6.6 ± 1.5 B 8.7 ± 4.9 A</td>
</tr>
<tr>
<td></td>
<td>B3</td>
<td>virEur</td>
<td>Intranasal</td>
<td>4</td>
<td>4</td>
<td>4.3 ± 4.9 B 3.3 ± 3.3 B</td>
</tr>
<tr>
<td></td>
<td>B4</td>
<td>none</td>
<td>Intranasal</td>
<td>8</td>
<td>8</td>
<td>12.1 ± 2.6 A 14.8 ± 7.4 A</td>
</tr>
<tr>
<td></td>
<td>B1</td>
<td>attAm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Intranasal</td>
<td>6 days</td>
<td>5</td>
<td>6.0 ± 2.1 B 7.6 ± 5.0 A</td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>attEur</td>
<td>Intranasal</td>
<td>5</td>
<td>5</td>
<td>5.0 ± 4.5 BC 6.6 ± 4.5 A</td>
</tr>
<tr>
<td></td>
<td>B3</td>
<td>virEur</td>
<td>Intranasal</td>
<td>2</td>
<td>2</td>
<td>0.5 ± 0.7 BC 1.0 ± 0.0 A</td>
</tr>
<tr>
<td></td>
<td>B4</td>
<td>none</td>
<td>Intranasal</td>
<td>4</td>
<td>4</td>
<td>10.0 ± 0.8 AC 13.0 ± 9.1 A</td>
</tr>
</tbody>
</table>

<sup>a</sup> attAm: attenuated American strain; attEur: attenuated European strain; virEur: virulent European strain (Lelystad strain).

<sup>b</sup> Within each experiment, values with different letters in the same column are significantly different by standard two-sample Mann–Whitney test (P < 0.05).

<sup>c</sup> Body temperature (0: ≤39.9 °C; 1: ≥40.0 to ≤40.9 °C; 2: ≥41.0 °C) and depression (0: absent; 1: present).

<sup>d</sup> Respiration rate/minute (0: ≤45; 1: ≥46 to ≤59; 2: ≥60), abdominal breathing (0: absent; 1: present) and dyspnoea (0: absent; 1: present).
and rates of $\geq 60$ as 2. Abdominal breathing and dyspnoea were each scored as 0 (absent) or 1 (present). Scores were added up and a mean of the cumulative general and respiratory scores per group was calculated.

2.5. Virological examinations

Blood samples of all pigs were collected immediately before challenge. Serum samples were examined for virus. Three-day cultivated MARC-145 cells were used for the pigs immunized with the attenuated strains (Botner et al., 1999; Stadejek et al., 1999) and 1-day cultivated PAMs were used for the pigs inoculated with the Lelystad strain (Labarque et al., 2000a).

At necropsy, blood and lungs were collected. The right lung was lavaged using a previously described method (Van Reeth et al., 1998). The BAL fluid was centrifuged ($400 \times g$, 10 min, $4^\circ C$) to separate the cells and the cell-free lavage fluid. Twenty percent suspensions of the left lung lobes were made in phosphate-buffered saline, clarified by centrifugation and the supernatant was used for PRRSV titration. Virus titrations of the cell-free BAL fluids, the supernatants of the lung suspensions and the serum samples were performed on PAMs, according to standard procedures (Labarque et al., 2000a).

2.6. Statistical analysis

Standard two-sample Mann–Whitney tests were used to compare clinical scores between groups. Differences in PRRSV titers were analyzed using an analysis of variance (ANOVA). Least significant differences (LSD) were used to compare the groups. Samples which tested negative for virus were given a numeric value of 0.2 log$_{10}$ TCID$_{50}$ per ml BAL fluid or per ml serum (detection limit 0.3 log$_{10}$) and 0.9 log$_{10}$ TCID$_{50}$ per gram lungs (detection limit 1.0 log$_{10}$). $P < 0.05$ was taken as the level of statistical significance. Statistical analyses were performed using SPSS 6.1.

3. Results

3.1. Serological response to immunization

All pigs were negative for PRRSV-specific antibodies at the start of the experiments. At challenge, all immunized pigs had developed antibodies against PRRSV. Pigs vaccinated with the attenuated American strain had mean antibody titers of 5.3 log$_{2}$ (group A1) and 5.7 log$_{2}$ (group B1), when a European serotype virus was used in the IPMA. When an American serotype virus was used in the IPMA, these pigs had mean PRRSV-specific antibody titers of 8.3 log$_{2}$ (group A1) and 10.5 log$_{2}$ (group B1). When a European serotype virus was used in the IPMA, pigs vaccinated with the attenuated European strain had mean antibody titers of 11.7 log$_{2}$ (group A2) and 11.1 log$_{2}$ (group B2). Pigs inoculated with the virulent Lelystad strain had mean antibody titers of 11.8 log$_{2}$ (group A3) and 13.3 log$_{2}$ (group B3). Non-immunized pigs (groups A4 and B4) were seronegative at challenge.
3.2. Clinical signs upon LPS exposure

Table 1 shows the mean cumulative clinical scores upon LPS exposure at 3 and 6 days after intratracheal or intranasal challenge with the virulent Lelystad strain. In all non-immunized challenged pigs, LPS induced severe general (mean scores from 9.8 to 12.9) and respiratory (mean scores from 9.5 to 14.8) signs at both times examined. Pigs primo-inoculated and challenged with the Lelystad strain showed only general signs in experiment A, and both general and respiratory signs in experiment B. However, clinical signs were clearly milder than in the non-immunized challenged pigs.

The clinical outcome in the vaccinated groups varied between the two experiments. Pigs vaccinated with the attenuated American strain had prominent general and respiratory signs in experiment A, and relatively mild signs in experiment B. In experiment A, clinical signs were similar to those of the non-immunized challenged pigs and more severe than those of any immunized group. In experiment B, in contrast, disease was consistently milder than in non-immunized challenged pigs and comparable to that of the other immunized groups. Pigs vaccinated with the attenuated European strain had negligible clinical signs in experiment A and more severe disease in experiment B. However, clinical signs were milder than in non-immunized challenged pigs in both experiments.

3.3. Virus titration of BAL fluids and lung samples

Fig. 1 shows the results of virus titrations of the individual pigs of each group at 4 and 7 days after intratracheal or intranasal challenge with the virulent Lelystad strain. Challenge virus was isolated from BAL fluids and lungs of all non-immunized challenge controls with peak virus titers after intratracheal challenge at 4 days, and after intranasal challenge at 7 days. The pigs primo-inoculated and challenged with the Lelystad strain tested negative for virus after intranasal challenge, whereas after intratracheal challenge, virus was isolated from lungs of three out of four pigs (2/2 pigs at 4 days after challenge and 1/2 pigs at 7 days after challenge), and from BAL fluids of one pig at 4 days after challenge. However, virus titers were clearly lower than those of the non-immune challenge controls \( (P < 0.05) \).

The two vaccines reduced the number of virus-positive pigs and the virus titers to different degrees. All pigs vaccinated with the attenuated American strain had PRRSV-positive BAL fluids and lungs after intratracheal challenge, whereas 1 out of 10 pigs tested virus-negative after intranasal challenge. Mean virus titers were similar to those of the non-immunized challenge controls \( (P > 0.05) \), except for BAL fluids and lungs at 7 days after intratracheal challenge \( (P < 0.05) \). Pigs vaccinated with the attenuated European strain and challenged intratracheally were more frequently virus-positive (6/6) than those challenged intranasally (5/10). Mean virus titers were significantly lower than those of the non-immune challenge controls \( (P < 0.05) \), except for lungs at 4 days after intranasal challenge \( (P > 0.05) \).

3.4. Virus titration of serum samples

At the time of challenge, neither vaccine nor virulent virus was detected in serum of any of the pigs.

Challenge virus was isolated from the sera of all non-immunized challenge controls, except for one pig euthanized at 7 days after intranasal challenge (Fig. 1). All pigs
Fig. 1. Virus titers in broncho-alveolar lavage (BAL) fluids, lung samples and serum samples at 4 and 7 days after intratracheal (experiment A) or intranasal (experiment B) challenge with the virulent Lelystad strain. Triangles represent individual virus titers in BAL fluids (\(10^9/TCD_50/mL\)) and in lung samples (\(10^9/TCD_50/gm\) tissue or \(10^9/TCD_50/ml\) serum). Bullets represent individual virus titers in serum samples (\(10^9/TCD_50/mL\)). Dashes represent group means at each time point. attAm: attenuated American strain; attEur: attenuated European strain.

Primo-inoculated and challenged with the Lelystad strain were virus-negative in their serum. Fourteen of the total of 16 pigs vaccinated with the attenuated American strain were virus-positive in their serum. Their mean virus titers were similar to those of the non-immune challenge controls \((P > 0.05)\), except at 7 days after intratracheal challenge.
In contrast, only 2 of the total of 16 pigs vaccinated with the attenuated European strain were virus-positive in their serum.

4. Discussion

To our knowledge, this is the first study on virological protection of the respiratory tract of pigs vaccinated with attenuated PRRSV vaccines. Under the given experimental circumstances, vaccination against PRRSV provides only partial virological protection of the lungs. Protection against infection with the European Lelystad strain was better after vaccination with a European strain than with an antigenically and genetically more distant American strain. One of the most surprising results was that PRRSV was isolated from lungs of pigs, which were primo-inoculated with the virulent Lelystad strain and were challenged with the same virulent Lelystad strain 42 days later. Since virulent PRRSV strains may persist for 40 (Labarque et al., 2000a) to 49 (Mengeling et al., 1995) days after inoculation, some virus of the primo-inoculation may still have been present at the time of challenge. So, the detected virus in lungs may be remaining virus of the primo-inoculation, challenge virus, or a mixture of both. The high virus titers are, however, in favor of the replication of challenge virus. Thus, it seems likely that even in a fully homologous situation, PRRSV is still able to replicate in the lungs.

The present experimental study shows that vaccines, whether based on American or European virus strains, are not able to afford a complete virological protection in the lungs. Thus, it is not likely that vaccination, even if extensively applied, will be able to drastically reduce or eliminate PRRSV circulation in the swine population. This may be particularly true if field strains are genetically and antigenically divergent from the vaccine strains. Such genetic diversity exists between American and European PRRSV isolates (Mardassi et al., 1995; Meng et al., 1995; Kapur et al., 1996; Suaréz et al., 1996), but also appears to exist among European field isolates. A recent study has demonstrated that, in Western Europe, PRRSV field isolates show so much genetic diversity in their open reading frames 5 and 7 that three clusters were identified: a Lelystad-like cluster, a purely Danish cluster and a highly diverse Italian-like cluster (Forsberg et al., 2002). These findings may have important consequences with regard to vaccine efficacy and the selection of virus strains for vaccine purposes. It cannot be excluded that a continuous update of vaccine strains may be necessary to reach an acceptable level of protection in the field, even within geographical areas of limited size. Possibly, PRRSV vaccines containing different genotypes may be needed in the future to compensate for such genetic diversity.

We compared intranasal and intratracheal challenge methods because we hypothesized that the challenge virus replication might be lower after intranasal than after intratracheal inoculation. However, the route of inoculation apparently did not affect the degree of challenge virus replication in the lungs. Our data indicate that replication of the challenge virus may be delayed after intranasal inoculation, but certainly not reduced, when compared with an intratracheal challenge.

Correlations between virus titers in the lungs and those in serum were good at the group level, but not in the individual pigs. Eleven of the total of 32 vaccinated pigs were negative

\[ P < 0.05. \]
for challenge virus in their sera, whereas their BAL fluids and/or lungs were positive. Duan et al. (1997) obtained similar results in naïve pigs inoculated with PRRSV. In their study, 5 of 12 pigs examined at ≥21 days post-inoculation had PRRSV-negative sera, while they were still virus-positive in the lungs. All these results demonstrate that efficacy tests of attenuated PRRSV vaccines should not be based on virus titers in serum alone, but should also focus on the degree of virus replication in the lungs upon challenge. This is in accordance with the findings of Nodelijk et al. (2001) who demonstrated that in spite of the fact that vaccinated pigs disseminated significantly less virus in serum than non-vaccinated challenge control pigs, transmission of PRRSV was not significantly reduced by vaccination. Based on the present data, we recommend to perform virus titrations on both BAL fluids and lungs. Pigs with high PRRSV titers had both positive BAL fluids and lungs, but this was not always the case for pigs with low titers (≤10^3.3 × TCID<sub>50</sub>). Lung samples of 6 of the total of 24 pigs immunized with a European strain were negative for challenge virus, whereas BAL fluids were virus-positive, or vice versa.

The present experiments were also undertaken to examine if the challenge virus replication in the lungs of vaccinated pigs predisposes for respiratory signs upon LPS exposure. Singly LPS-exposed pigs were not included because of the limited number of pigs available for these experiments. Earlier experiments had demonstrated that singly LPS-exposed pigs developed transient general signs and no, or only mild respiratory signs (Labarque et al., 2002). Respiratory signs were mild or absent in pigs primo-inoculated and challenged with the Lelystad strain. Non-immunized challenged pigs, on the other hand, suffered from severe respiratory signs upon LPS exposure, such as tachypnoea, abdominal breathing and dyspnoea. In contrast, the clinical outcome upon LPS exposure of pigs that had been vaccinated and 6 weeks later challenged with PRRSV was highly variable within and between experiments. Pigs vaccinated with the attenuated European strain experienced no respiratory signs in experiment A, whereas they developed clear respiratory signs in experiment B. Similarly, all pigs vaccinated with the attenuated American strain developed severe respiratory signs upon LPS exposure in experiment A, whereas in experiment B, respiratory signs were milder and similar to those of the pigs vaccinated with the attenuated European strain. Thus, we were unable to reproduce the clinical results in two subsequent experiments. The severity of the clinical signs upon LPS exposure was not correlated with the degree of virus replication in the lungs of individual pigs. Thus, it remains unclear why clinical synergy occurs reproducibly after PRRSV inoculation in naïve pigs, but not in vaccinated pigs even though challenge virus replication occurs.

Taken together, the present data show that the virological protection in the lungs of pigs immune after infection or vaccination upon challenge is incomplete, but is more pronounced in the homologous situation. The variable clinical outcome upon LPS exposure of vaccinated pigs hampers the use of the combined PRRSV-LPS exposure for testing of the clinical efficacy of attenuated PRRSV vaccines.

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