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Clinical Effects of Experimental Dual Infections with Porcine Reproductive and Respiratory Syndrome Virus Followed by Swine Influenza Virus in Conventional and Colostrum-deprived Pigs

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With 3 figures

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Summary

Previous studies demonstrated that experimental dual infections of pigs with porcine reproductive and respiratory syndrome virus (PRRSV) followed by H1N1 influenza virus cause more severe disease and growth retardation than the respective single virus infections. Here three experiments were undertaken to better define the clinical impact of combined PRRSV-H1N1 infections in conventional and caesarean-derived colostrum-deprived (CDCD) pigs. Groups of pigs were inoculated by aerosol with PRRSV followed by H1N1 at 3-, 7- or 14-day intervals. During the post-H1N1 period, mean body temperatures, respiratory signs and mean weight gains in the PRRSV-H1N1 inoculated groups were recorded and compared with those in uninoculated controls (experiments 1 and 2) or in singly virus-inoculated pigs (experiment 3). In a first experiment with conventional pigs, the PRRSV-3d-H1N1 and PRRSV-7d-H1N1 infections induced mean body temperatures >40.5°C during 8 days (peaks 41.1 and 41.6°C, respectively) and mean growth reductions of 3.4 and 4.8 kg, respectively, during the 2 weeks after H1N1, along with marked depression and respiratory disease. The PRRSV-14d-H1N1 infection, on the contrary, was largely subclinical. In a second experiment with conventional pigs, PRRSV-3d-H1N1 and PRRSV-7d-H1N1 infections were clinically milder, with smaller increases in mean body temperatures (peak 40.5°C in both groups) and growth reductions (1.4 and 1.6 kg, respectively). In both groups, only one pig showed prominent general and respiratory signs. In a final experiment with CDCD pigs, PRRSV-7d-H1N1 infection had minimal effects on mean clinical performances and growth and, except for one pig that was severely affected, differences with the single virus inoculations were negligible. Thus, both the time interval between infections and the sanitary status of pigs can affect the clinical outcome of dual PRRSV-H1N1 infections. However, factors so far unknown seem to cause large variations in the clinical response between individual pigs.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is ubiquitous in swine-producing areas of the world. Virus transmission readily occurs by the respiratory route, and multiplication in the respiratory tract is followed by viraemia and replication in several organs. The virus has a predilection for pulmonary alveolar macrophages (AMs) and the lungs are a major target organ (Duan et al., 1997; Labarque et al., 2000). Experimental infections with European PRRSV isolates consistently result in microscopic

lesions of interstitial pneumonia (Paton et al., 1992; Plana et al., 1992; Halbur et al., 1995; Van Reeth et al., 1999), but overt respiratory signs are more difficult to produce. In most experimental studies, the infection is largely asymptomatic and a transient fever is the most prominent clinical change (Paton et al., 1992; Plana et al., 1992; Albina et al., 1994; Van Reeth et al., 1999). Furthermore, in the field, many PRRSV infections are subclinical, particularly if uncomplicated.

However, it cannot be denied that there has been a general increase in respiratory disease and poor productivity since PRRSV has become enzootic. Respiratory infections which have been very common for many years seem to have increased in severity since the appearance of PRRSV. In problem herds, PRRSV has been demonstrated together with various bacteria and with porcine respiratory coronavirus and swine influenza virus (Keffaber et al., 1992; Morrison et al., 1992; Halbur et al., 1993; Kamogawa et al., 1996). Combined infections with PRRSV and other agents may therefore play an important part in respiratory disease problems. It has previously shown that experimental dual infections with PRRSV followed by H1N1 influenza virus can result in enhanced fever, respiratory disease and growth retardation in comparison with the respective single virus infections (Van Reeth et al., 1996). In these earlier studies, conventional PRRSV-seronegative pigs were used and a fixed 3-day time interval between PRRSV and influenza virus inoculations. Yet, PRRSV replicates in the lungs during 1 month and longer (Duan et al., 1997; Labarque et al., 2000), and there is thus a much longer time period for a second infection to occur in the PRRSV-infected pig. Under field conditions, secondary infections may occur with an interval of a few days or several weeks, and the exact timing and chronology of infections will differ from case to case. The present experiments compare PRRSV-H1N1 inoculations at 3-, 7- and 14-day intervals in conventional pigs. Next to clinical evaluations, this study includes bacteriologic examinations of the lungs of selected pigs. In addition, the clinical effects of the PRRSV-7 day-H1N1 inoculation were studied for the first time in caesarean-derived colostrum-deprived (CDCD) pigs.

Materials and Methods

Pigs and virus inoculation

The Lelystad virus strain of PRRSV (Wensvoort et al., 1991) was used at the fifth passage in primary porcine AMs. The A/Sw/Belgium/1/83 (H1N1) strain of influenza virus was used at the third passage in embryonated eggs. Pig inoculation doses were $10^{5.0}$ 50 % tissue culture infectious doses ($TCID_{50}$) for PRRSV and $10^{7.5}$ 50 % egg infectious doses (EID_{50}) for influenza virus. Virus inoculations were performed by an aerosol method, using a 'Wright nebulizer' (particle size < 8 μg) (Aerosol Products Ltd, London, UK). The inoculum consisted of virus in 8 ml phosphate-buffered saline and was administered during a 35-min period to each pig individually.

Conventional pigs were purchased from a herd without antibodies to PRRSV and influenza virus. Each experimental group was housed in a single isolation room that received HEPA-filtered air. The pigs had free access to water and to unmedicated commercial feed. They were used in experiments 1 week after they were moved to isolation facilities, i.e. at the age of 10 and 12 weeks in experiments 1 and 2, respectively.

CDCD pigs were reared individually in Horsefall-type isolation units until the age of 3 weeks and transferred to flat decks thereafter. From the age of 5 weeks on, housing and nutrition were the same as for the conventional pigs. CDCD pigs were used in experiments at the age of 7 weeks.

Conventional pig studies (experiments 1 and 2)

In experiment 1, 19 pigs from two sows were assigned to four groups, on the basis of weight and litter. One group of four pigs (control group) was left uninoculated. Three groups of five pigs were inoculated with PRRSV followed by H1N1 influenza virus at a 3- (PRRSV-3d-H1N1 group),

7- (PRRSV-7d-H1N1 group) or 14-day (PRRSV-14d-H1N1 group) interval. The dually inoculated groups were monitored daily for fever (body temperature $\geq 40°C$), tachypnoea (respiration rate > 45/min), dyspnoea and coughing until 17 days after H1N1 inoculation. In the control group, body temperatures and clinical signs were recorded at least every other day. All pigs were weighed twice a week from the time of PRRSV inoculation until 1 month after H1N1 inoculation or during a corresponding period in the control group.

In experiment 2, 11 littermate pigs were divided among three groups. One group of three pigs (control group) was left uninoculated. Two groups of four pigs were inoculated with PRRSV followed by H1N1 influenza virus at a 3- (PRRSV-3d-H1N1 group) or 7-day (PRRSV-7d-H1N1 group) interval. Clinical monitoring was performed as in experiment 1. When severe clinical signs were seen in the dually inoculated groups, one pig from each group was killed to examine the lungs for bacteria and *Mycoplasma hyopneumoniae*. Briefly, lung tissue samples were plated on bovine blood agar and cultured aerobically. A nurse colony of coagulase negative *Staphylococcus* spp. was streaked diagonally on each plate. Plates were inspected for bacterial growth after 48 and 72 h. Colonies were then identified by standard techniques. For *M. hyopneumoniae* examination, frozen tissue sections were stained by a fluorescent antibody test.

CDCD pig study (experiment 3)

Fifteen pigs from two litters were randomly assigned to three groups. Two groups of five pigs were inoculated exclusively with either PRRSV (PRRSV group) or H1N1 influenza virus (H1N1 group). The remaining five pigs were inoculated with PRRSV and 7 days later with H1N1 influenza virus (PRRSV-7d-H1N1 group). Body temperatures and clinical signs were recorded from the time of PRRSV inoculation until 14 days after H1N1 inoculation in the PRRSV-7d-H1N1 group, and during corresponding time periods in the singly virus-inoculated groups. All pigs were weighed three times a week during this time period.

Serology

Blood samples were collected for serology at the start of the experiments and 14 days after each virus inoculation. Sera were tested for antibodies against PRRSV by an immunoperoxidase monolayer assay (Wensvoort et al., 1991) and against H1N1 influenza virus by haemagglutination inhibition (Palmer et al., 1975).

Statistics

Standard two-sample *t*-tests were used to compare body temperatures and weight gains within each experiment. Pair-wise comparisons were made at selected times between each of the PRRSV-H1N1 inoculated groups and the uninoculated control group in experiments 1 and 2, and between the PRSSV-H1N1 inoculated group and each of the singly virus-inoculated groups in experiment 3. $P < 0.05$ was considered significant.

Results

All pigs were free of antibodies against PRRSV and H1N1 influenza virus at the time of inoculation with these viruses and they had seroconverted 2 weeks after the respective virus inoculations.

Conventional pig studies (experiments 1 and 2)

At the start of both experiments, no differences in mean body temperatures or body weights were recorded between the experimental groups ($P > 0.05$). Uninoculated controls had normal body temperature and weight gain profiles and remained clinically

healthy throughout the experiments. The symptoms in the dually inoculated pigs are described below.

In experiment 1, the PRRSV inoculation produced a substantial increase in mean body temperatures (Fig. 1a) in all three groups. The PRRSV-3d-H1N1 and PRRSV-7d-H1N1 groups maintained a temperature in excess of 40.0°C until exposure to H1N1 and showed a second, higher increase in body temperatures after the H1N1 inoculation ($P < 0.05$ at 4 days after H1N1). Mean body temperatures exceeded 40.5°C during a total of 8 days and reached peak values of 41.1°C in the PRRSV-3d-H1N1 group and 41.6°C in the PRRSV-7d-H1N1 group. Despite some individual variation, all pigs experienced fever over 41.0°C, along with depression and anorexia. At the same time, they showed tachypnoea, an abdominal 'thumping' respiration and a productive cough. Clinical recovery started 9 days post-inoculation of H1N1. By contrast, the PRRSV-14d-H1N1 group showed normal mean body temperatures at the time of H1N1 inoculation and developed only mild fever thereafter (maximal mean body temperature 40.3°C; $P > 0.05$ at any time). Only one of five individual pigs had body temperatures reaching up to 41.0°C, a decreased appetite and moderate respiratory signs.
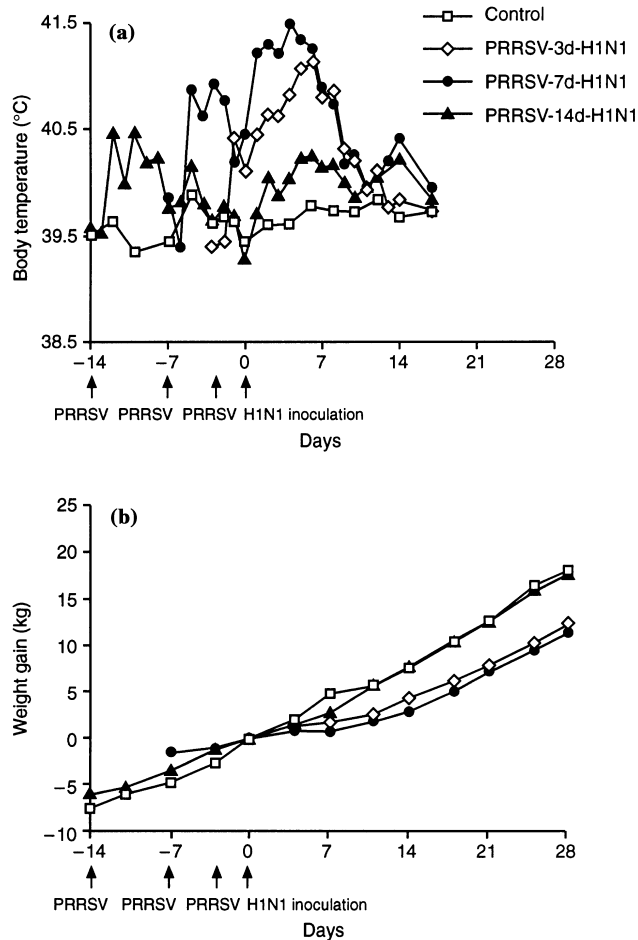

Fig. 1. Mean body temperatures (a) and weight gains (b) in conventional pigs inoculated with PRRSV-HIN1 at 3-, 7- or 14-day time intervals and in uninoculated controls.

Mean weight gains (Fig. 1b) were delayed between the PRRSV and H1N1 inoculations in all three groups. After the H1N1 inoculation, significant growth reductions were noted exclusively in the PRRSV-3d-H1N1 and PRRSV-7d-H1N1 groups. Between 0 and 14 days after H1N1, these pigs grew a mean 3.4 kg (PRRSV-3d-H1N1) and 4.8 kg (PRRSV-7d-H1N1) less than the controls ($P < 0.05$). One month after inoculation of H1N1, growth reduction was still 5.4 kg in the PRRSV-3d-H1N1 ($P > 0.05$) group and 6.1 kg in the PRRSV-7d-H1N1 group ($P < 0.05$). Weight gains in the PRRSV-14d-H1N1 group did not differ significantly from those in the control group ($P > 0.05$). There were, however, wide individual differences and a great deal of overlap between the four groups.

In experiment 2, the pigs to be euthanized were included for the calculation of mean body temperatures and weight gains until 4 days post-inoculation of H1N1. Mean body temperatures (Fig. 2a) were minimally affected by the PRRSV inoculation and showed a slight but insignificant ($P > 0.05$) rise after the H1N1 inoculation (maximal mean body temperature 40.5°C in both groups). Although all individual pigs showed fever during one or more days, only one of four pigs from both groups had temperatures ≥ 41.0°C and marked depression, dyspnoea and tachypnoea. These pigs, and one uninoculated control, were euthanized at 5 days post-inoculation of H1N1 for bacteriologic examination of the lungs. The PRRSV-3d-H1N1 and PRRSV-7d-H1N1 pigs showed gross pneumonia involving 45 and 85 %, respectively, of the total lung surface. No such pneumonic lesions were found in the control pig. Bacterial culture of lung tissue yielded few colonies of *Streptococcus suis* from one of four lung lobes of the PRRSV-7d-H1N1 pig, and was negative in both other pigs. Fluorescent antibody tests for *M. hyopneumoniae* were negative.

Mean weight gains (Fig. 2b) were not affected by the PRRSV inoculation ($P < 0.05$). During the 14-day period after H1N1, the dually infected pigs grew on average 1.4 kg (PRRSV-3d-H1N1) and 1.6 kg (PRRSV-7d-H1N1) less than the controls. The pigs that were euthanized 5 days post-inoculation of H1N1 tended to show the most severe weight losses. One month post-inoculation of H1N1, the dually inoculated pigs had 2.5 kg (PRRSV-3d-H1N1) and 5.1 kg (PRRSV-7d-H1N1) less weight gain than the controls ($P > 0.05$ at any time post-inoculation).

CDCD pig study (experiment 3)

Mean body temperatures and weight gains of the PRRSV-7d-H1N1 group are compared with those of the PRRSV and H1N1 groups in Fig. 3a and b, respectively.

The single PRRSV group showed a transient increase in mean body temperatures 2 days post-inoculation without obvious respiratory disease. Mean weight gains were reduced during the first week post-inoculation ($P < 0.05$ when compared with uninoculated pigs of the H1N1 group) and returned to normal thereafter.

The single H1N1 inoculation failed to induce significant fever. A mild tachypnoea and dyspnoea and occasional coughing were seen at or around 5 days post-inoculation, but mean weight gains were unaffected.

In the PRRSV-7d-H1N1 group, mean body temperatures remained below 40.0°C at any time post-inoculation. Three of five individual pigs, however, had a transient increase in body temperatures 2 and 3 days post-inoculation of PRRSV. After the H1N1 inoculation, one of these pigs had fever (40.1–40.5°C) during 4 days. Another pig was severely depressed and showed fever (40.2–40.9°C) from the fourth to 14th day post-inoculation of H1N1. Prominent respiratory signs were seen exclusively in the latter pig. Mean weight gains during the 14-day period after H1N1 were lower in the PRRSV-7d-H1N1 group (6.44 kg) than in the PRRSV (7.68 kg) or H1N1 (8.26 kg) group, but this difference was not significant ($P > 0.05$). The one severely affected pig grew only 4.30 kg during this period.
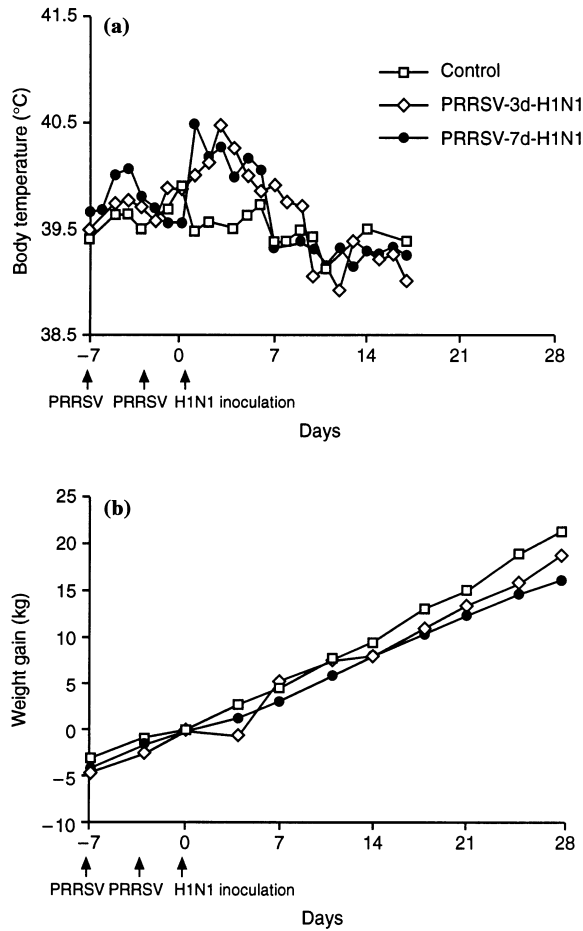
Fig. 2. Mean body temperatures (a) and weight gains (b) in conventional pigs inoculated with PRRSV-HIN1 at 3- or 7-day time intervals and in uninoculated controls.

Discussion

These studies were undertaken to better define the clinical impact of dual viral infections with PRRSV followed by swine influenza virus. In particular, an attempt was made to investigate the effects of the time interval between infections and of the sanitary status of pigs. The design of each of the three experiments differed depending on the principal study objectives and on the numbers of pigs available. As such, a first experiment with conventional feeder pigs aimed at comparing PRRSV-H1N1 infections at 3-, 7- and 14-day intervals. A second conventional pig experiment was designed to confirm the clinical effects of the PRRSV-3d-H1N1 and PRRSV-7d-H1N1 inoculations and, in addition, to judge on the possible contribution of bacterial or mycoplasmal infections. Uninoculated littermates served as controls in both experiments. Singly PRRSV- or H1N1-inoculated pigs were not included, because of the limited number of pigs available for this study. Furthermore, the single virus inoculations were repeatedly shown to be subclinical under the conditions used here (Van Reeth and Pensaert, 1994; Van Reeth et al., 1996). A final CDCD pig experiment was conducted to compare the clinical effects of the PRRSV-7d-H1N1 and the respective single virus inoculations in pigs of the highest possible health status.
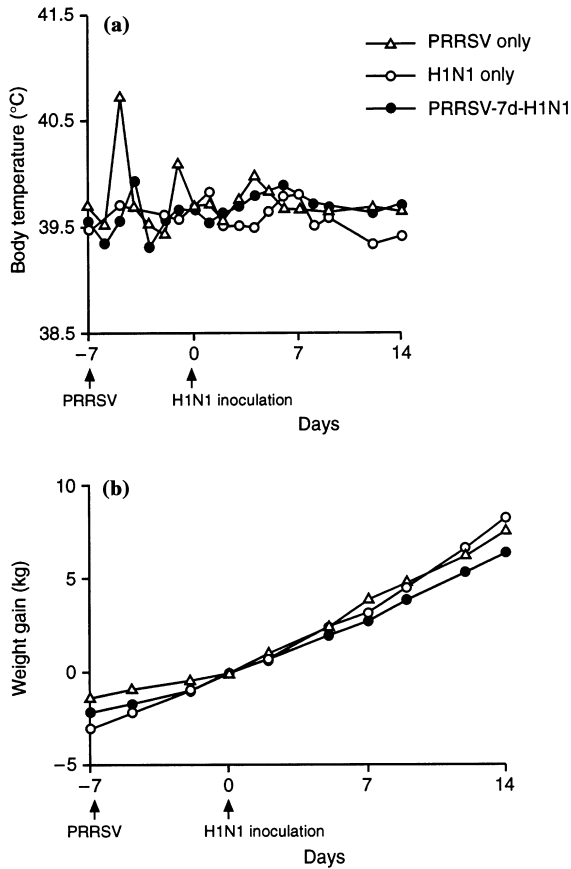
Fig. 3. Mean body temperatures (a) and weight gains (b) in CDCD pigs inoculated with PRRSV only, HIN1 only or PRRSV-7d-HIN1.

The present data suggest that the time interval between a PRRSV and a subsequent influenza virus infection can affect the clinical outcome. That is, pigs of the same herd and/or litter developed disease after PRRSV-H1N1 inoculations at 3- and 7-day intervals, but not with 14-day intervals. The reasons for the limited duration of the interaction between PRRSV and influenza virus are unknown. PRRSV replicates in the lungs during at least 4–6 weeks, and microscopic lung changes have been reported to persist until 28 days after experimental infection (Halbur et al., 1995). However, PRRSV specific immunity starts to develop and virus titres gradually decrease from 14 days post-inoculation onwards (Nelson et al., 1994; Labarque et al., 2000). Although knowledge of the cytokine profile during PRRSV infection remains incomplete, cytokines such as IL-4, IL-10 and transforming growth factor-β have been demonstrated in the lungs during the subacute stages of infection with influenza virus and bovine respiratory syncytial virus (Hennet et al., 1992; McInnes et al., 1998). These 'late' cytokines are known to limit the (pro)inflammatory actions of the early cytokines produced during the acute stage. It has therefore been postulated that a secondary infection occurring during this period will cause few pathological and clinical effects (Bielefeldt-Ohmann, 1995). It is thus an attractive hypothesis that the mild clinical course of the PRRSV-14d-H1N1 infection relates to the production of down-regulatory cytokines.

Conventional pigs, even if clinically healthy, may carry a variety of bacteria in the upper respiratory tract and colonization of the lungs may follow after (experimental) virus infection and thereby enhance disease. *Streptococcus suis* was isolated from the lungs of one of two PRRSV-H1N1 infected pigs in the second conventional pig experiment. The role of this streptococcal infection in the extensive lung pathology and disease seen here is uncertain. On the one hand, the fact that just a few bacterial colonies were isolated from only one of four lung lobes minimizes their clinical significance. On the other hand, *S. suis* has been associated with disease and bronchopneumonia, particularly in pigs with concomitant virus infections of the lungs. Galina et al. (1994) have demonstrated that PRRSV can predispose pigs to infection and disease caused by *S. suis*. Furthermore, it is remarkable that a variety of bacteria – *Haemophilus parasuis*, *Actinobacillus suis* and *Bordetella bronchiseptica* – have also been isolated from pneumonic lung tissue of pigs during experimental infections with PRRSV and *M. hyopneumoniae* (Thacker et al., 2000). Thus, although conventional pig experiments have the advantage of mimicking the field situation closely, it is assumed that bacterial infections often contribute to the clinical picture in such experiments and that PRRSV perhaps predisposes to bacterial colonization of the lungs.

The CDCD pig experiment demonstrates the overall mild clinical course of the PRRSV-H1N1 infection as such. For unknown reasons, only one out of five pigs showed severe clinical signs upon the dual virus inoculation. Although PRRSV is recognized as one of the most important aetiologic agents in multi-infectious respiratory disease of pigs, it has proved to be extremely difficult to reproduce such disease syndrome experimentally. Dual infections with PRRSV followed by *Salmonella choleraesuis*, *Pasteurella multocida* or *Haemophilus parasuis* were all very mild or subclinical (Cooper et al., 1995; Carvalho et al., 1997; Solano et al., 1997). The combination of PRRSV with either influenza virus, *M hyopneumoniae* or *S suis* produced disease in experiments by some researchers (Galina et al., 1994; Van Reeth et al., 1996; Thacker et al., 1999), but remained subclinical in the hands of others (Albina et al., 1995; Cooper et al., 1995; Van Alstine et al., 1996; Pol et al., 1997). The present study supports the suggestions that differences in the time interval between infections or in the sanitary status of pigs can be in part responsible for these discrepancies. However, a large variation in clinical disease between individual pigs is a hallmark of the PRRSV-influenza virus infection, irrespective of the time interval between infections or the sanitary status of pigs. Finally, the important question as to which factors determine the clinical outcome of a PRRSV infection remains unanswered. Further experimental studies will be carried out to find out why severe disease develops in some PRRSV-H1N1 infected pigs and not in others.

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