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Dual infections of feeder pigs with porcine reproductive and respiratory syndrome virus followed by porcine respiratory coronavirus or swine influenza virus: a clinical and virological study

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Abstract

Dual infections of pigs with porcine reproductive and respiratory syndrome virus (PRRSV) followed by a second common respiratory virus, either porcine respiratory coronavirus (PRCV) or swine influenza virus (SIV), were studied. The aim was to determine if dual infections, as compared to single virus infections, result in enhanced clinical manifestations. It was also examined if PRRSV replication affects replication of PRCV or SIV in the respiratory tract. Groups of conventional 10 week old pigs were inoculated with PRRSV-only (3 pigs), PRCV-only (4 pigs) or SIV-only (4 pigs). Dual inoculations with PRRSV-PRCV (4 pigs) and PRRSV-SIV (3 groups of 4, 4 and 5 pigs) were performed at a 3 day interval. A group of uninoculated control pigs (8 pigs) was included.

The infection with PRRSV-only induced a transient fever (40.2°C) at 2 DPI, but no respiratory signs. The PRCV-only infection remained subclinical. The SIV-only infection resulted in a one day fever (40.1°C) with moderate tachypnoea and dyspnoea. Mean weight gain in the virus-inoculated groups was retarded compared with the control group.

The PRRSV-PRCV infection induced a 9 day lasting fever (peak 40.9°C) with tachypnoea, dyspnoea and productive coughing. The PRRSV-SIV infection resulted in fever and respiratory signs in all 3 groups. Clinical signs, however, were more pronounced in group 1 than in groups 2 and 3. Pigs of group 1 showed fever during 10 days (peak 41.4°C), tachypnoea, marked dyspnoea with abdominal breathing, and a productive cough. Pigs of groups 2 and 3 had fever for 5 and 3 days (peaks 40.6 and 40.3°C) respectively and mild respiratory disorders. Mean weight gain during 14 DPI of the 2nd virus was 5.9 kg in the PRRSV-PRCV group and 4.0, 6.8 and 6.7 kg in PRRSV-SIV

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groups 1, 2 and 3 respectively. Mean weight gain during the corresponding period in the PRRSV-only group was 8.6 kg. It was concluded that dual infections with viruses causes more severe disease and growth retardation than single PRRSV infection.

PRCV excretion curves were similar in single and dual virus inoculated groups. Excretion of SIV was delayed by 2 days in the dual inoculated pigs. Thus, replication of the second virus is not (PRCV) or only slightly (SIV) affected by a prior infection with PRRSV.

Keywords: Porcine reproductive and respiratory syndrome virus; Porcine respiratory coronavirus; Swine influenza virus; Dual infections; Pigs

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) appeared for the first time in Europe in the northwestern part of Germany in November 1990 (Ohlinger et al., 1991). During the next months, the virus spread rapidly through the major pig producing areas of Western Europe. The virus has become enzootic in Germany, The Netherlands, Belgium, Spain, United Kingdom, France and Denmark since 1992 (Ohlinger et al., 1991; Wensvoort et al., 1991; Vynckier and Pensaert, 1993; Plana Duran et al., 1992a; Edwards et al., 1992; Albina et al., 1992; and Boetner et al., 1993). Since that time, an increase in respiratory disease in weaners, growing and fattening pigs has been widely reported. The clinical picture is characterized by respiratory disorders, increased mortality and suboptimal performance. The PRRSV is often incriminated as the causative agent, but the signs observed in the field have never been reproduced experimentally.

The pattern of infection with PRRSV on Belgian swine farms has been studied by Houben et al. (1995). Most pigs are born from immune sows and thus have maternal antibodies during their first weeks of life. On breeding farms where the virus persists, pigs may or may not become infected with PRRSV before the age of 10 weeks. When infection occurs, maternal immunity is replaced by active immunity. In the absence of infection, maternal PRRSV antibodies do not persist beyond 10–11 weeks of age. Thus, pigs which are to be transported to industrial fattening farms and which have not previously been infected with PRRSV on the breeding farm are fully susceptible and have a high risk to contract the infection shortly after they are grouped together. In the study mentioned, it was shown that on the average 50% of the pigs entering intensive fattening units were devoid of PRRSV antibodies and that all these pigs had seroconverted to PRRSV 1 month later (Houben et al., 1995). Around that age, the pigs are confronted with several respiratory infectious agents. Indeed, earlier studies have shown that infections with porcine respiratory coronavirus (PRCV) and swine influenza viruses (SIV) are extremely common shortly after grouping together of pigs in intensive fattening units (Van Reeth and Pensaert, 1994b). Combined infections with the latter viruses and with PRRSV are, therefore, likely.

Upon a respiratory infection with PRRSV, primary virus replication takes place in the lungs and there seems to be a predilection for alveolar lung macrophages (Pol et al., 1991). Some researchers suggest that lung defence mechanisms may be temporarily impaired as a result of virus replication in alveolar macrophages. A synergism between PRRSV and other viruses possibly leading to disease has often been proposed (Groschup et al., 1993), but

clinical experiments to document this hypothesis have not been done. It was the purpose of the present study to examine the clinical effects of dual infections with PRRSV followed either by PRCV or by SIV.

In a previous study on dual infections with PRCV followed by SIV, the authors demonstrated that PRCV infection highly interferes with SIV replication (Van Reeth and Pensaert, 1994a). Replication of H1N1-influenza virus in the lungs of pigs was reduced by a factor of 99%. Thus, it was also the purpose of the present study to find out if PRRSV is able to interfere with replication of PRCV or SIV.

2. Materials and methods

2.1. Viruses

The Lelystad virus strain of PRRSV (Wensvoort et al., 1991) was used in this study. It was kindly provided by G. Wensvoort, Institute for Animal Science and Health (ID-DLO), Lelystad, The Netherlands. Virus used for inoculation was at the 5th passage in alveolar macrophages. The PRCV strain 91V44 (Van Reeth and Pensaert, 1994a) was isolated from the lungs of a feeder pig in February 1991. It had been passaged twice in swine testicle (ST) cells. The A/Sw/Belg/1/83 isolate of H1N1-influenza virus was obtained from an outbreak of swine influenza on a breeding farm in January 1983 and used at the third passage in embryonated eggs.

2.2. Pigs and inoculation

Thirty six conventional feeder pigs, free of antibodies against the respective virus(es) with which they were to be inoculated, were used at the age of 10 weeks. The pigs were allocated to 8 groups, each of which was housed in separate isolation facilities. Eight pigs served as uninoculated controls (control group). Three pigs were inoculated with PRRSV only (PRRSV-only group), 4 with PRCV only (PRCV-only group) and 4 with SIV only (SIV-only group). Four pigs were inoculated with PRRSV first and 3 days later with PRCV (PRRSV-PRCV group) and, in 3 separate experiments, 4, 4 and 5 pigs were inoculated with PRRSV first and 3 days later with SIV (PRRSV-SIV group 1, 2 and 3).

All inoculations were performed individually by aerosol, during 35 min with 8 ml phosphate buffered saline (PBS) containing either $10^{4.9}$ median tissue culture infective doses (TCID₅₀) of PRRSV, 10^7 TCID₅₀ of PRCV or $10^{7.5}$ median egg infective doses (EID₅₀) of SIV using the "Wright nebulizer" (particle size $< 8 \mu\text{m}$) (Aerosol Products). Pigs were treated with oxytetracycline (1 ml/20 kg IM) every other day to avoid complications with bacterial infections.

2.3. Clinical studies

The groups were monitored daily for fever (body temperature $\geq 40^\circ\text{C}$), tachypnoea (respiration rate $> 45/\text{min}$), dyspnoea and coughing from 6 days before the first inoculation until 9 (control, PRCV-only and SIV-only groups) or 14 (PRRSV-only, PRRSV-PRCV

and PRRSV-SIV groups) days after the last inoculation. The pigs were weighed daily throughout the observation period.

2.4. *Virological studies*

Nasal swabs were taken before and 3 days post inoculation (DPI) with PRRSV in the respective groups to make sure that PRRSV infection was successful.

Nasal swabs were collected daily for titration of PRCV or SIV during 10 consecutive days PI to follow the excretion curves in the respective groups. The swabs from the individual animals in each group were pooled daily for virus titration.

Cotton swabs were weighed before and after collection to determine virus titers per 100 mg nasal secretions. Swabs from both nostrils were suspended in 1 ml of PBS, supplemented with penicillin (100 IU/ml) and streptomycin (0.1 µg/ml) and mixed vigorously for 1 h. The medium was collected, clarified by centrifugation and used for virus isolation/titration. Isolation of PRRSV was performed in porcine alveolar macrophages seeded in 96 well – microtiter plates and reading occurred at 60 h PI using an immunoperoxidase monolayer assay (IPMA) (Wensvoort et al., 1991). Titration of PRCV and SIV was performed in ST cells (Van Reeth and Pensaert, 1994a) and 10 day old embryonated chicken eggs respectively (Palmer et al., 1975). Two passages in embryonated eggs were made.

2.5. *Serology*

At the end of the experiments, sera from all pigs were tested for antibodies against PRRSV by the IPMA (Wensvoort et al., 1991), against PRCV by virus neutralization (Voets et al., 1980) and against H1N1-influenzavirus by haemagglutination inhibition (Palmer et al., 1975).

2.6. *Statistical analysis*

Multiple regression analysis was used to compare groups on the basis of weight gain. Pair-wise comparisons were made between each of the dual virus inoculated groups and a) the control group, b) the PRRSV-only group, c) the group inoculated with the 2nd virus only.

3. **Results**

All the pigs were clinically healthy before virus inoculation. At the time of the first inoculation, nasal swabs were virus-negative and sera were free of antibodies against the respective viruses. Three DPI with PRRSV, virus excretion was detected in every group inoculated. These results indicate that PRRSV was replicating in the respiratory tract of pigs at the time of inoculation with the second virus. At the end of the experiments, all pigs had seroconverted against the viruses with which they had been inoculated.

Table 1
Summary of body temperatures and weight gain in control and single PRRSV, PRCV and SIV infected groups

Group	Days of fever (DPI)	MWG*
Control	0	6.3
PRRSV-only	1 (2)	4.4
PRCV-only	0	3.9
SIV-only	1 (5)	5.3

*Mean weight gain during the 9 day post inoculation period in kg.

3.1. Control group and single PRRSV, PRCV and SIV infections

Body temperatures and weight gain in the uninoculated control and single PRRSV, PRCV and SIV infected groups are summarized in Table 1.

The control group was clinically healthy and mean body temperatures were between 39.4 and 39.9°C. Mean weight gain during the 9 day observation period was 6.3 kg.

The infection with PRRSV only induced an increase in body temperature (40.2°C) at 2 DPI. From the 3th DPI on, body temperatures returned to normal and the further course of the infection was subclinical. Except for a slight nasal discharge, respiratory signs were not observed. Growth arrest was recorded the first 2 DPI only and mean weight gain between 0 and 9 DPI was 4.4 kg. Between 3 and 17 DPI, mean weight gain was 8.6 kg, as shown in Fig. 2.

Clinical responses were not observed in the PRCV-only group. Growth was retarded between 0 and 3 DPI, but thereafter daily weight gain parallely increased with that of the control group (data not shown). Mean weight gain was 3.9 kg.

Pigs of the SIV-only group showed fever (40.1°C) at 5 DPI. Respiration rates were increased (peak 65 breaths/min) 4, 5 and 6 DPI. Coughing was observed occasionally, particularly when the pigs were forced to move. Mean weight gain was 5.3 kg.

3.2. Dual PRRSV-PRCV and PRRSV-SIV infections

Body temperatures and weight evolution of the dual virus infected groups are compared with those of the PRRSV-only group in Fig. 1 and Fig. 2 respectively. The time of inoculation with PRRSV is represented as day -3, the time of inoculation with the 2nd virus as day 0.

In the PRRSV-PRCV group, pigs developed fever and respiratory disease. A transient rise in body temperature 2 DPI with PRRSV was followed by a 9 day lasting fever after inoculation with PRCV. The peak of fever (40.9°C) was accompanied by increased respiration rates (peak 55 breaths/min). All 4 pigs showed respiratory distress with abdominal respiration and a productive cough between 8 and 11 DPI. They huddled together while showing signs of chilling, listlessness and inappetence. Mean weight gain (5.9 kg) was statistically different ($P < 0.01$) from that of the control and PRRSV-only groups, but not from that of the PRCV-only group.

Pigs of PRRSV-SIV group 1 had severe and long lasting febrile responses, with fever for 10 days peaking at 41.4°C. They were dull and made no effort to rise when disturbed. There

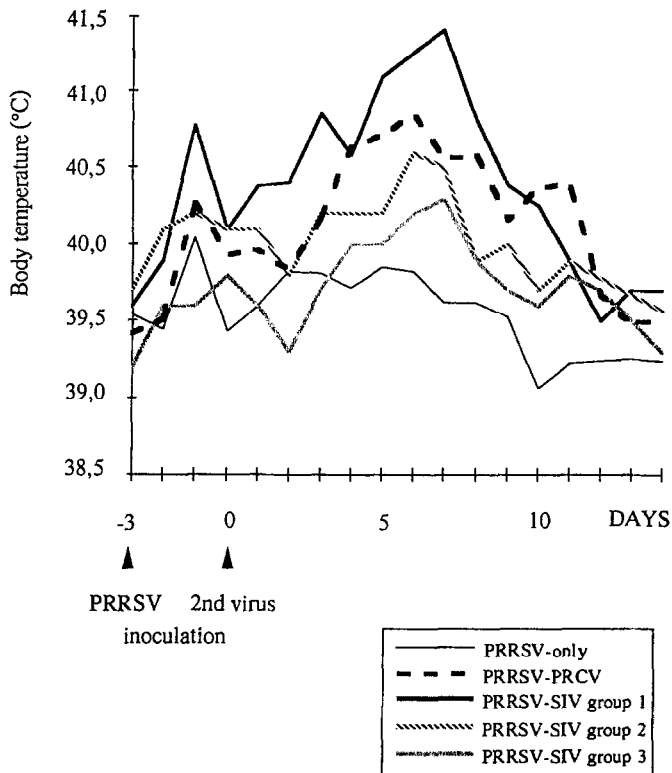


Fig. 1. Body temperatures in single PRRSV and dual PRRSV-PRCV and PRRSV-SIV infected groups.

was a marked anorexia. All 4 pigs exhibited prominent respiratory disease between 4 and 10 DPI. There was tachypnoea (peak 90 breaths/min), labored, jerking, abdominal breathing and severe productive coughing. Mean weight gain at the end of the experiment was 4.0 kg.

Because of the marked clinical disturbances in PRRSV-SIV group 1, the experimental PRRSV-SIV infection was repeated twice (PRRSV-SIV groups 2 and 3). In these 2 additional experiments, pigs exhibited fever for 5 (group 2, peak 40.6°C) and 3 days (group 3, peak 40.3°C). Respiratory disorders were noted between 5 and 8 DPI. These groups were less severely affected than PRRSV-SIV group 1. Pigs were only slightly less alert than before virus inoculations and respiratory rates were moderately increased (peak 69 breaths/min in group 2, 62 breaths/min in group 3). Sneezing and nasal discharge were recorded in all pigs, superficial respiration and coughing were recorded occasionally. Mean weight gains were 6.8 (group 2) and 6.7 kg (group 3). In each of the 3 PRRSV-SIV groups, mean weight gains were statistically different ($P < 0.01$) from that of the control, PRRSV-only and SIV-only groups.

3.3. Excretion of PRCV or SIV after a prior inoculation with PRRSV

Data on excretion of PRCV and SIV in single and dual virus inoculated groups are presented in Table 2 and Table 3 respectively.

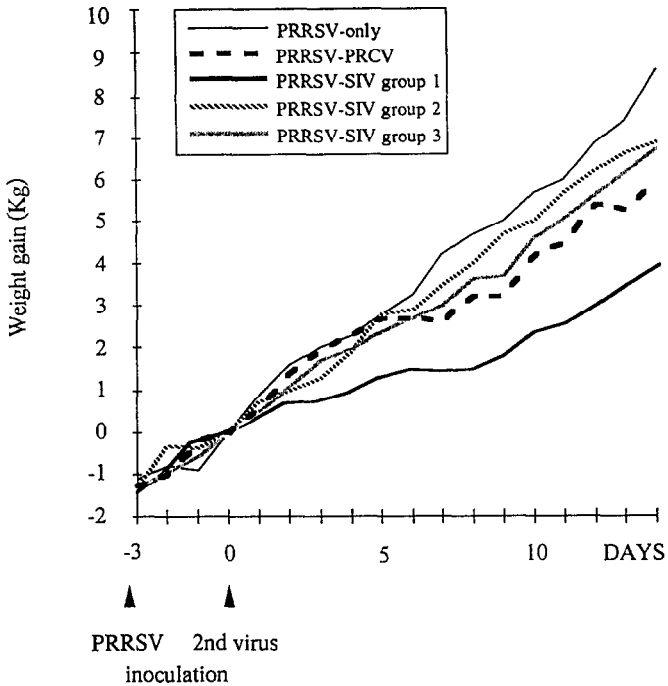


Fig. 2. Weight evolution in single PRRSV and dual PRRSV-PRCV and PRRSV-SIV infected groups.

Table 2
PRCV excretion in PRCV-only and PRRSV-PRCV groups

Group	Virus titer* (log ₁₀ TCID ₅₀ /100 mg nasal secrete) at days after inoculation with PRCV										
	0	1	2	3	4	5	6	7	8	9	10
PRCV-only (n=4)	neg	2.5	3.3	5.0	5.2	5.2	5.2	3.6	2.6	neg	neg
PRRSV-PRCV (n=4)	neg	2.7	3.4	5.6	5.9	5.6	5.0	3.5	2.4	2.7	neg

*virus titer in pooled nasal swabs; TCID₅₀; median tissue culture infective doses

Table 3
SIV excretion in SIV-only group and in PRRSV-SIV group 1

Group	Virus titer* (log ₁₀ EID ₅₀ /100 mg nasal secrete) at days after inoculation with SIV										
	0	1	2	3	4	5	6	7	8	9	10
SIV-only (n=4)	neg	2.9	5.2	6.2	5.6	3.8	4.1	neg	neg	neg	neg
PRRSV-SIV(1) (n=4)	neg	neg	neg	2.6	4.1	6.4	5.2	4.3	neg	neg	neg

*virus titer in pooled nasal swabs; EID₅₀; median egg infective doses

PRCV was detected in nasal swabs from 1 to 8 DPI in the PRCV-only group and from 1 to 9 DPI in the PRRSV-PRCV group (Table 2). Virus excretion curves were similar and highest amounts of virus (\log_{10} 5.0–5.9 TCID₅₀/100 mg nasal secretions) were found between 3 and 6 DPI in both groups.

The excretion of SIV in the PRRSV-SIV group was delayed by 2 days, not only with regard to the presence of virus, but also with regard to the peak amounts (Table 3).

4. Discussion

This study confirms the hypothesis that the clinical effects of a PRRSV infection may be exacerbated when a concurrent infection with common respiratory viruses occurs. As in previous experimental studies (Plana Duran et al., 1992b; Ramos et al., 1992), it was found that the uncomplicated PRRSV infection has no clinical importance in feeder pigs. A short transient fever and inappetence were the only clinical signs observed in the present and other studies. Combined PRRSV-PRCV and PRRSV-SIV infections, on the other hand, consistently resulted in fever, respiratory illness and growth retardation. Growth retardation over a 14 day period was 2.7, 4.6, 1.8 and 1.9 kg in PRRSV-PRCV and PRRSV-SIV groups 1, 2 and 3 respectively. We cannot explain why growth retardation also occurred following inoculation with PRCV-only, because pigs of this group were clinically normal and even lively throughout the observation period. Also, experimental PRCV infections have been performed frequently by the authors (Van Reeth and Pensaert, 1992) and generally had a mild or subclinical course.

In the authors' opinion, secondary infection with PRCV or SIV is one of the factors that may determine whether or not disease results from an infection with PRRSV. There is no doubt that other factors will also play a role. The variable clinical outcome in the pigs inoculated with PRRSV-SIV in the present experiment shows that the effect of the dual infections is not reproducible to the same level in every group. Three separate groups of pigs were inoculated with PRRSV-SIV. Respiratory disease, fever and growth retardation were much more severe in PRRSV-SIV group 1 than in groups 2 and 3. Still, there were no indications of concurrent infections with mycoplasma or other respiratory viruses in any of the groups. All the sera collected 1 month after the virus inoculations scored negative in a blocking ELISA test (Dako *Mycoplasma hyopneumoniae* ELISA; Feld et al., 1992) for detection of *Mycoplasma hyopneumoniae* antibodies. There was no serological evidence of an accidental infection with other viruses such as PRCV, Aujeszky's disease virus or H3N2-influenza virus. Certainly, the variation in disease observed between experimentally inoculated pigs in the present study must also occur under field conditions and may be a reflection of hitherto undefined physiological parameters.

The viruses used for the experimental dual infections were purposely selected on the basis of a serologic study earlier performed in intensive fattening units in Belgium (Van Reeth and Pensaert, 1994b). This study showed that infections with PRCV and H1N1-influenzavirus are very common when 10 week old feeder pigs, derived from several breeding farms, are grouped together in intensive fattening units. Also, it was shown that infections with PRRSV systematically occur in such pigs (Houben et al., 1995). Thus, combined infections with PRRSV and/or PRCV and/or H1N1-influenzavirus are likely to

occur within a short time interval. Experiments in the authors' laboratory have shown that PRRSV can be isolated from the lungs of experimentally infected pigs until at least 1 month PI. Consequently, a primary infection with PRRSV can be easily followed by infection with other respiratory viruses, even when they occur with several weeks intervals. Definite proof is, however, still lacking to show that combined PRRSV-PRSV or PRRSV-SIV infections are the cause of increased respiratory problems in the field. There is an urgent need to study the true economical significance of dual virus infections under field circumstances. To obtain more clarity on this point, we intend to study the prevalence as well as the chronology of respiratory virus infections in young pigs and fatteners and the relationship between the infection patterns obtained and the disease observed.

At this time, the exact mechanism of disease development in dual virus infections is not known. It is believed that direct structural damage following replication of 2 viruses in the lungs is at least partly responsible for respiratory disease. Mild to moderate degeneration and necrosis of the airway epithelia have been reported in experimental infections with PRRSV (Pol et al., 1991), as well as with PRCV (Cox et al., 1990) and H1N1- influenza virus (Haesebrouck and Pensaert, 1986). Because of the apparent difference in lung cell tropism between PRRSV on the one side and PRCV and SIV on the other side, it seems only logical that a greater amount of lung tissue is damaged in dual virus infections than in single infections. Certainly, pathological changes will also occur indirectly as a result of the host's inflammatory response. Interstitial pneumonia with cellular infiltration in bronchial lumina and alveolar spaces, in alveolar septae and in blood vessels occurs with all 3 virus infections (Pol et al., 1991; Cox et al., 1990; Haesebrouck and Pensaert, 1986). In the experimental PRRSV-PRCV and PRRSV-SIV infections, the peak of disease occurs later than the peak of replication of the second virus. This observation strongly supports that inflammation can play an important role in the development of clinical disease. In recent years, the importance of cytokines as mediators of virus-induced inflammatory responses has been recognized. Zhou et al. (1992) reported that high levels of interleukin-1 (IL-1), one of the most potent inflammatory cytokines, are expressed in alveolar macrophages of PRRSV-infected pigs. IL-1 is also produced in the lungs of PRCV-infected pigs (Van Reeth and Pensaert, 1994c). Possibly, unregulated large amounts of IL-1 and/or other inflammatory cytokines and their complex interactions contribute to the development of pathology in dual infections in which PRRSV is involved.

Previous studies have shown that a PRCV infection strongly interferes with replication of a subsequent influenza virus infection in the lungs of pigs (Van Reeth and Pensaert, 1994a). Following an infection with PRCV, excretion of influenza virus was undetectable and virus production in the lungs was reduced by a factor of more than 99%. Interferon- α and tumour necrosis factor were shown to be released into the lungs of pigs following a PRCV infection (Van Reeth and Pensaert, 1994c) and these cytokines may mediate the PRCV-induced antiviral activity. Apparently, a similar antiviral effect is not observed after a PRRSV infection. In the current study, the PRRSV showed no interference with replication of PRCV and only minimal interference with replication of SIV.

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