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Pathogenicity of concurrent infection of pigs with porcine respiratory coronavirus and swine influenza virus

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Combinations of porcine respiratory coronavirus (PRCV) and either of two swine influenza viruses (H1N1 or H3N2) were administered intranasally and by aerosol to six- to eight-week-old specific pathogen-free pigs. The clinical responses, gross respiratory lesions and growth performances of these pigs were studied and compared with those of single (PRCV, H1N1 or H3N2) and mock-infected animals. PRCV infection caused fever, growth retardation and lung lesions, but no respiratory symptoms. Infection with swine influenza viruses caused rather similar, mild symptoms of disease, with H1N1 infection being the least severe. Combined infections with influenza viruses and PRCV did not appear to enhance the pathogenicity of these viruses. Furthermore, viruses were isolated more frequently from tissues and nasal swabs taken from 'single' than 'dual' infected animals, suggesting a possible in vivo interference between replication of PRCV and swine influenza virus.

PORCINE respiratory coronavirus (PRCV) is a variant of transmissible gastroenteritis virus (TGEV) (Pensaert et al 1986). Despite complete in vitro cross-neutralisation between these two viruses (Pensaert 1989), PRCV, unlike classical, enteric forms of TGEV, grows poorly if at all in the gut, but grows to high titres in respiratory tissues (Cox et al 1990). Experimentally infected pigs develop pulmonary lesions (O'Toole et al 1989) but rarely show clinical signs of disease. The role of the virus in field outbreaks of respiratory disease is uncertain, although in the majority of cases seroconversion to PRCV occurs asymptomatically. It is, however, suspected that PRCV may be a contributory cause of more severe respiratory disease in combination with other influences such as concurrent infections (Pensaert 1989).

Since both swine influenza viruses and PRCV are circulating in the European pig population, combined infections are likely to occur, particularly at the beginning of the fattening period, when pigs from different origins are mixed. In 1987, an H3N2 swine influenza virus was isolated from a farm in England following an outbreak of severe respiratory disease (Pritchard et al 1987). PRCV seroconversion coincided with the outbreak, but the virus was not isolated. However, the influenza isolate on its own failed to reproduce the clinical disease when inoculated into experimental pigs.

The aim of this study was to establish whether or not a concurrent infection in pigs with PRCV and either of two swine influenza virus strains (H1N1 or H3N2) resulted in the development of respiratory symptoms and more severe pulmonary lesions, as compared to single agent infections.

Materials and methods

Experimental animals

Twenty-eight specific pathogen-free pigs, from a minimal disease herd at the Central Veterinary Laboratory, Weybridge (CVL), were used. This herd was seronegative to TGEV/PRCV and to both swine influenza strains. The animals were fed a commercial diet in predetermined quantities dependent upon age. Each group was housed separately (in 20 m^2 pens) in different blocks to avoid cross-contamination. The air from each block was exhausted through high efficiency (HEPA) sterilising filters.

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Challenge viruses

PRCV. The Stopps strain (135308) of PRCV, isolated in 1986 from an outbreak of respiratory disease was used to infect a colostrum-deprived, two-day-old piglet by the intranasal route. Three days after infection, it was killed and the lungs removed, each lobe being homogenised separately in phosphate buffered saline (PBS) pH 7·2 containing antibiotics. The homogenates were titrated on primary pig kidney monolayers (PPKM). The lobes with the highest viral titre were pooled, passaged once in PPKM cells and used as inocula at a titre of 105 TCID50 ml^{-1}.

Influenza viruses. Two strains of influenza A virus were used: A/SW/Weybridge/86/H1N1 and A/SW/Weybridge/87/H3N2. Challenge inocula for each strain were prepared by intranasal dosing of colostrum-deprived, two-day-old piglets, using virus previously passaged five times in the allantoic cavity of embryonated chicken eggs. Three days after infection the piglets were killed and homogenates of each lung lobe were inoculated into the allantoic cavity of 10-day-old embryonated chicken eggs. After three days incubation at 34°C, the allantoic fluids were clarified by centrifugation and tested for haemagglutinating activity using 1 per cent chicken erythrocytes. Fluids giving the highest titres were pooled and stored at –70°C until used as inocula. The H1N1 inocula had titres of 108·5 egg infective doses (EID)50 ml^{-1} and the H3N2 inocula had titres of 108 EID50 ml^{-1}.

Experimental design

Twenty-eight pigs, six to eight weeks old, were split into six groups (Table 1).

Animals inoculated with a single virus received 4 ml intranasally and 1 ml aerogenically (by nebuliser). Control pigs received equal volumes of Hanks' medium by the same routes. Inocula for dual infected animals were administered intranasally (8 ml) and aerogenically (2 ml) as a mixture of the corresponding single doses.

All animals were monitored twice daily for the first seven days after infection and then daily until 21 days after infection. Monitoring consisted of: clinical assessment, nasal swabbing, rectal temperature taking and observation of food intake. The animals were weighed on alternate days and were bled from the anterior vena cava on days 0, 7, 10, 14 and 21 after infection.

TABLE 1: Groups of experimental animals

Group (infection)	Age (days)	Number of animals
Control	46	2
H3N2	46	6
PRCV	47	6
PRCV+H3N2	47	6
H1N1	61	4
PRCV+H1N1	61	4

To follow the development of lesions, one pig from each infected group was killed (intraperitoneal sodium pentobarbitone) on days 3, 6 and 10 after infection in all groups. One H1N1- and one H1N1+PRCV-infected pig were killed on day 1, while one H3N2-, one PRCV- and one H3N2+PRCV-infected animal were killed on day 21. Both control pigs were killed on day 21.

At post mortem examination, the following tissues were taken for virus isolation: nasal turbinates, tonsil, trachea, tracheobronchial lymph nodes and lung (apical and diaphragmatic lobes).

Virus isolation

Tissues collected at necropsy were prepared as 10 per cent (w/v) homogenates in PBS pH 7·2 containing 1000 iu penicillin, 1000 g streptomycin and 200 iu mycostatin (nystatin) ml^{-1}, incubated for 30 minutes at room temperature, clarified at 1500 *g* for 10 minutes and the resulting supernatant stored at –70°C until tested. Nasal swabs were immediately suspended in PBS pH 7·2 containing antibiotics (as for tissues) and then treated as for tissue homogenates.

PRCV isolation. Virus was isolated using PPKM grown in Hanks' medium containing 10 per cent bovine serum, 0·0375 per cent sodium bicarbonate and antibiotics (1:10 dilution of tissue/swab preparation). The maintenance medium was Earle's, containing 2 per cent bovine serum, 0·15 per cent sodium bicarbonate and antibiotics. If after seven days after infection cytopathic effect was not detected, tissue culture fluid was passed on to fresh cell cultures. Following incubation for 24 hours, the presence of virus was assessed using a TGEV direct fluorescent antibody test.

Influenza isolation. Nine- to ten-day-old specific pathogen-free embryonated chicken eggs were inoculated allantoically with 0·1 ml of sample and incubated for three days at 34°C. The harvested allantoic fluids were tested for haemagglutinating activity, as already described.

Serological tests

All sera were inactivated at 56°C for 30 minutes. PRCV serum antibodies were detected by a TGE virus neutralisation test, using A72 (canine rectal tumour) cells and TGEV strain FS68/216 (Paton 1989).

H1N1 or H3N2 influenza serum antibodies were detected by the haemagglutination inhibition test (Palmer et al 1975). To further destroy non-specific inhibitors, inactivated sera were treated with 25 per cent Kaolin overnight at +4°C. After adsorption with a 10 per cent suspension of chicken erythrocytes for one hour at 37°C, the sera were tested against four haemagglutinating units of H1N1 or H3N2 virus using 1 per cent chicken erythrocytes.

Growth rate changes

A regression line for weight against time was calculated for each group, from the individual weights of all pigs not killed before day 10 after infection. Liveweight gains were compared for the same animals. To find out if growth changes were significantly different between groups, an analysis of variance (ANOVA) was performed with the live weight gain figures for days 4 and 10 after infection.

Results

Clinical assessment

Table 2 shows the mean daily temperatures and the proportion of pigs with fever for each group during the first seven days after infection.

Control group. No clinical signs were observed and their temperature remained within the normal physiological range throughout the experiment (38·4° to 39·6°C).

H3N2-infected group. At 24 hours after infection the pigs were dull and feverish, with an average temperature of 40·2°C (range 39·7° to 40·8°C). Temperatures remained elevated for five days after infection, returning to normal thereafter. A serous nasal discharge was observed in all the animals of the group until four days after infection.

PRCV-infected group. Respiratory symptoms were not observed in any pig. On the first day after infection, rectal temperature ranged between 39·5°C and 40·5°C (average 39·9°C) and remained elevated for three days.

PRCV+H3N2-infected group. On days 4 and 6 after infection, sneezing and coughing were observed. The average temperature of the group rose to 40·4°C (range 40·1° to 40·8°C) on the first days after infection. From the fourth day on, the average temperature returned to normal. One pig died on day 9 from a mesenteric torsion.

TABLE 2: Mean body temperatures and proportion of pigs with fever

Days after infection / Group	Mean temperatures* (proportion of pigs with fever)							
	0	1	2	3	4	5	6	7
Control	38·4	39·4	39·4	39·3	39·4	39·2	39·5	39·7
	(0/2)	(0/2)	(0/2)	(0/2)	(0/2)	(0/2)	(0/2)	(0/2)
H3N2	39·5	40·2	39·8	39·7	39·6	40·1	39·3	39·1
	(0/6)	(5/6)	(3/6)	(2/6)	(0/5)	(5/5)	(0/5)	(0/4)
PRCV	39·5	39·9	39·5	39·6	39·6	39·1	39·4	39·3
	(0/6)	(3/6)	(2/6)	(2/6)	(0/5)	(0/5)	(0/5)	(0/4)
PRCV+H3N2	39·5	40·4	39·7	39·2	40	38·6	39·6	39·5
	(0/6)	(6/6)	(2/6)	(0/6)	(4/5)	(0/5)	(3/5)	(1/4)
H1N1	39·4	39·6	39·5	39·4	39·4	39·3	39·1	39·5
	(0/4)	(1/4)	(0/3)	(0/3)	(0/2)	(0/2)	(0/2)	(0/1)
PRCV+H1N1	39·9	40·5	40·3	40·1	39·7	39·7	39·6	39·7
	(0/4)	(3/4)	(3/3)	(2/3)	(0/2)	(0/2)	(0/2)	(0/1)

* Celsius degrees (fever: over 39·6°C)

H1N1-infected group. There were no respiratory symptoms. The highest average temperature was 39·7°C (range 39·6 to 40°C) on the first days after infection. On that day, a transient anorexia was observed.

H1N1+PRCV-infected group. The pigs in this group were anorexic during the first three days after infection, but showed no respiratory symptoms. The mean temperature on the first day was 40·5°C (range 39·7° to 40·9°C). Fever was present for the first three days.

TABLE 3: Growth rates

Group	Slope*	Mean daily weight change (g) 0–2†	2–4†	4–6†	6–10†
Control	0·56	575	500	375	625
H3N2	0·40	−100	0	500	575
PRCV	0·35	−150	375	400	407
PRCV+H3N2	0·47	250	500	250	735
H1N1	0·50	0	500	−250	1100
PRCV+H1N1	0·57	250	−500	1000	1000

* Slope: gradient of regression line from graphs of weight in kilograms versus time in days
† Period of time (DPI)

Necropsy findings

Control group. Lung lesions were not observed in either pig belonging to this group.

H3N2 and H1N1-infected groups. In both groups, tracheobronchial lymph nodes were moderately enlarged and a few small lung lesions were observed on days 6 and 10.

PRCV-infected group. Red consolidated areas affecting mainly the diaphragmatic lobes were present on days 3 after infection. The lungs of the pigs killed on days 6 and 10 had larger pneumonic lesions scattered throughout the lungs, but most pronounced in the apical and cardiac lobes. Tracheobronchial lymph nodes were enlarged in all the pigs and were haemorrhagic in the pig killed on day 10. On day 21 after infection lung lesions had disappeared.

PRCV+H3N2-infected group. Areas of consolidation were seen on days 3, 6 and 10 spreading from the diaphragmatic to the cardiac and apical lobes. The extent of lung tissue affected was similar or slightly greater than for the PRCV-infected pigs. Tracheobronchial lymph nodes were enlarged. There were no lesions in the pigs necropsied at 21 days.

PRCV+H1N1-infected group. The lesions observed on day 3 were very small, but were more pronounced and widespread in the lungs of the pig killed on day 6, with the diaphragmatic lobes being the worst affected. The extent of pneumonic lung tissue was similar to the corresponding PRCV-infected pig, although on day 10, lesions in the dual infected pig were smaller and less widespread. Tracheobronchial lymph nodes were enlarged in all the pigs.

Growth rates

For each group, the mean daily weight changes at intervals during the first 10 days are shown in Table 3. This table also shows the regression weight-time line for each group. Since a shallow slope indicates slow growth, this indicated poorest growth in the PRCV-infected group.

In all of the infected groups the growth rate slowed down, as compared to the controls, during the first two days after infection. H3N2, PRCV and H1N1-infected animals were the most affected. Concurrent infection did not have a synergistic effect in either of the dual-infected groups, where the liveweight gain was higher than in single-infected animals during the first two days. Although H1N1 and PRCV+H1N1-infected animals had a lower liveweight gain than the controls in the first four days (PRCV+H1N1-infected animals lost weight between two and four days), the slopes of the regression lines over the full 10 day period were similar to the control group.

The ANOVA showed significant differences in the growth rate between the control group and all the infected groups during the first four days after infection, although differences were not significant at 10 days. Differences between single and dual-infected groups were not significant at any stage.

Virus isolation

PRCV. All pigs given PRCV shed this virus in nasal swabs until seven to nine days after infection. In tissues, PRCV persisted slightly longer in pigs given this virus only (Tables 4 and 5).

TABLE 4: Mean duration of virus excretion in the infected groups

Group	Mean number of days (±SD) of viral isolation from nasal swabs PRCV	Influenza
H3N2	0	0·6 ± 0·4
PRCV	8 ± 0·7	0
PRCV+H3N2	8·7 ± 0·4	0·2 ± 0·4
H1N1	0	2·6 ± 0·9
PRCV+H1N1	9 ± 0·2	1·3 ± 0·4

TABLE 5: Viral isolation from tissues

Group	Viral isolation PRCV				PRCV+H3N2				PRCV+H1N1			
Days after infection killed	3	6	10	21	3	6	10	21	1	3	6	10
Turbinates	+	+	−	−	+	+	−	−	+	+	+	−
Tonsil	+	+	+	−	+	+	−	−	+	+	+	−
Trachea	+	+	−	−	+	+	−	−	+	+	+	−
TBLN*	+	+	−	−	+	+	−	−	+	+	−	−
Apical lobe	+	+	−	−	+	+	−	−	+	+	+	−
Diaphragmatic lobe	+	+	−	−	+	+	−	−	+	+	−	−

\+ Virus isolated, − No virus isolated
* Tracheobronchial lymph nodes

Influenza virus H1N1 and H3N2. The mean duration of excretion of influenza virus, as detected by nasal swabs (Table 4), was shorter in dual than in single-infected pigs. Three days after infection, H3N2 virus was detected in five of six tissue samples taken from an H3N2-infected pig, whereas only two samples were positive from the corresponding PRCV+H3N2-infected animal (Table 6).

H1N1 influenza virus was isolated less frequently from tissues than H3N2. Concurrent infection reduced the rate of isolation, since at three days after infection the virus was detected in three out of six tissues taken from an H1N1-infected pig, but not at all from the equivalent PRCV+H1N1-infected animal.

TABLE 6: Influenza virus isolation from tissues

Groups	Viral isolation H3N2/PRCV+H3N2				H1N1/PRCV+H1N1			
Days after infection killed	3	6	10	21	1	3	6	10
Turbinates	+/−	−/−	−/−	−/−	−/+	+/−	−/−	−/−
Tonsil	+/−	−/−	−/−	−/−	+/+	−/−	−/−	−/−
Trachea	+/+	−/−	−/−	−/−	+/+	+/−	−/−	−/−
TBLN*	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Apical lobe	+/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
Diaphragmatic lobe	+/+	−/−	−/−	−/−	−/−	+/−	−/−	−/−

* Tracheobronchial lymph nodes

TABLE 7: Development of serum antibody against influenza virus and, or, PRCV, following single or dual infection

Days after infection	Geometric Mean Antibody Titres 7		10		14		21	
Group	Flu*	PRCV*	Flu	PRCV	Flu	PRCV	Flu	PRCV
H3N2	40	neg	80	neg	63	neg	40	neg
PRCV	neg	2	neg	32	neg	97	neg	101
PRCV+H3N2	28	neg	320	32	56	45	60	64
H1N1	40	neg	80	neg	NT	NT	NT	NT
PRCV+H1N1	20	neg	80	4	NT	NT	NT	NT

*All titres are expressed as reciprocal dilutions of sera able to neutralise TGEV/PRCV or inhibit haemagglutination by the homologous strain of swine influenza virus in 50 per cent of replicates
neg: under 10 (flu) or under 2 (TGEV/PRCV)
NT Not tested

Serology

In each infection group there was seroconversion against the virus or viruses inoculated (Table 7), while the control group remained seronegative against all three viruses throughout the experiment.

The titres of influenza antibodies reached a peak at 10 days while the highest PRCV antibody titres were detected at 21 days. The antibody titres were very variable in the different pigs and there were no significant differences between the single and the dual infected animals.

Discussion

The animals infected with PRCV alone developed lung lesions and fever, and suffered growth retardation, as previously described (O'Toole et al 1989, Cox et al 1990, Vannier 1990). However, respiratory symptoms were not observed, in contrast to the findings of Duret et al (1988) and Van Nieuwstadt and Pol (1989). The prolonged shedding of PRCV from infected animals, together with airborne transmission, probably helps to explain the rapid spread of PRCV across Europe.

Both influenza strains used in this study had been isolated from outbreaks of respiratory disease (Roberts et al 1987, Pritchard et al 1987). However, animals infected with them showed little illness, although fever and decreased growth rate were observed. The H3N2 infected pigs were the worst affected and did show mild respiratory symptoms (nasal discharge). Similar results have been described previously for the H3N2 strain (Wibberley et al 1988). Serial passage in embry-

onated eggs might have reduced the pathogenicity of these viruses for pigs.

Field observations have suggested the possible association of swine influenza virus with porcine respiratory coronavirus in outbreaks of respiratory disease. However, the clinical picture observed in these experimentally induced dual infections was quite similar to that seen when PRCV was given on its own. Body temperatures were slightly higher after dual than after single infections and PRCV+H3N2-infected pigs were the only ones to develop coughing and sneezing. However, no significant differences in growth rates were detected between the single and dual-infected animals. Lesions observed in dual-infected pigs were generally similar to, or in some instances less severe than those found in PRCV-only-infected animals.

Overall, no significant enhancement of the pathogenicity of PRCV by the simultaneous infection with either of two different influenza viruses could be detected. Under field conditions, infections with different viruses are unlikely to be absolutely simultaneous, but it is not possible to predict what if any difference this would make to the severity of disease. Other routes of infection, such as the intratracheal might also have been explored, although intranasal and aerosol routes were selected as being closest to those occurring naturally.

H1N1 and H3N2 viruses were recovered from some tissues and, or, nasal swabs for longer after single than after dual infection. It is thus possible that PRCV may have actually interfered with the replication of influenza virus in vivo. An analogous situation in chickens has been described in vitro for the effect of infectious bronchitis virus on the growth of Newcastle disease virus (Buxton and Fraser 1977).

Although coinfection with influenza virus did not seem to increase the pathogenicity of PRCV, potentiation by other respiratory pathogens of the pig cannot be ruled out. For instance, Marois et al (1989) considered that concurrent infection with TGEV increased the severity and extent of the lesions produced by *Mycoplasma hyopneumoniae* in the lungs of experimentally infected pigs. Further research is needed to see if bacterial respiratory pathogens, such as *Pasteurella multocida* and *Actinobacillus pleuropneumoniae*, can enhance the pathogenicity of viruses such as PRCV.

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