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Pathogenicity of experimental infection with 'pneumotropic' porcine coronavirus

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Virus localisation and lesions were studied in 14 one-week-old piglets following combined intranasal-oral inoculation with a British isolate of 'pneumotropic' porcine coronavirus (PCV) and were compared with the effects of transmissible gastroenteritis virus (TGEV) infection in five piglets. Unlike TGEV-infected piglets, all PCV-inoculated piglets remained clinically healthy. Seroconversion was detected at seven days after inoculation. Mild bronchointerstitial pneumonia involving terminal airways was consistently present at two days after infection and thereafter. Both PCV and TGEV infected bronchiolar epithelium and alveolar macrophages but, unlike TGEV, replication by PCV in villous enterocytes was limited and did not cause villous atrophy.

A NEW coronavirus of pigs which has a close antigenic relationship to transmissible gastroenteritis virus (TGEV) has recently been identified as enzootic in Great Britain, France, Belgium and Holland (reviewed by Jestin et al 1987). Conventional serological tests do not distinguish between 'pneumotropic' porcine coronavirus (PCV)-infected and TGEV-infected pigs although a blocking ELISA developed by Garwes et al (1988) shows promise. The significance of this PCV as a pathogen is unclear. Studies in Belgium indicated that PCV was non-pathogenic and infected respiratory but not enteric tracts (Pensaert et al 1986). Several field reports have however linked PCV with outbreaks of respiratory disease (Jestin et al 1987, Veterinary Investigation Service 1987).

The aim of this study was to establish whether or not PCV caused lesions in susceptible piglets with particular attention to respiratory and digestive tracts, and to map the distribution of the virus in selected tissues using immunocytochemistry and virus isolation. Owing to its similarity to TGEV, the changes in PCV-infected piglets were compared with those in piglets following TGEV infection.

Materials and methods

Experimental animals

Twenty-four piglets from three litters of conventional breeds were used. They were hysterotomy derived one or two days before their due date and subsequently colostrum-deprived using a modification of the methods described by Tavernor et al (1971). The three parent sows were seronegative to PCV/TGEV and originated in herds with no previous history of transmissible gastroenteritis. Piglets were reared individually in sterile aluminium anodised cages in plastic isolators kept at 20 to 25°C with positive pressure ventilation. They were fed on a diet of sterilised evaporated milk (Carnation Foods) diluted with a mineral solution containing ferrous sulphate three, four or five times daily according to a schedule based on age. Piglets were also given an oral vitamin supplement (Adibec Drops; Parke, Davis).

Experimental design

As only 12 single isolators with individual cages were available, the study was done in two periods one month apart. The two batches of piglets were maintained under identical conditions. The first batch consisted of 12 Monarch piglets aged six days, all from one litter. They were inoculated with PCV (nine piglets) or were uninoculated controls (three). Following sterilisation of cages and isolators, a second batch of 12 piglets (Large White cross Landrace) aged seven days from two litters were inoculated with PCV (five) or TGEV (five) or were uninoculated controls (two).

Before use, empty cages and isolators were sterilised with ethylene oxide and formaldehyde. Microbiological checks were carried out by swabbing the interior of isolators and placing swabs into tryptose broth at 37°C.

Clinical signs, feed intake and rectal temperatures were monitored daily. Blood samples were collected for serology from the anterior vena cava of all piglets before inoculation and before euthanasia from the heart of piglets six days after inoculation and thereafter. Apart from the one TGEV-inoculated piglet which died as a result of transmissible gastroenteritis, piglets were killed in isolators by intracardiac sodium pentobarbitone at one to 11 days after infection (Table 1).

TABLE 1: Isolation of TGEV/PCV from the tissues of experimentally infected piglets on porcine kidney primary cultures

Piglet	Time after infection (days)	Material			
		Turbinate	Right lung	Ileum	Ileal contents
Controls					
1	2	0	0	0	0
2	4	0	0	0	0
3	6	0	0	0	0
4	11	0	0	0	0
5	11	0	0	0	0
PCV-inoculated					
6	1	0	+	0	0
7	2	+	+	+ (40)	+ (25)
8	3	+	+	0	0
9	3	0	+	0	0
10	5	+	0	+ (160)	+ (160)
11	5	+	+	+ (40)	+
12	6	+	0	+ (40)	+ (160)
13	6	+	0	+ (160)	+ (160)
14	7	+	+	+ (40)	+ (160)
15	7	+	+	0	+ (100)
16	8	+	0	+ (10)	+ (10)
17	9	0	+	0	0
18	11	0	0	+ (10)	+ (10)
19	11	0	0	0	0
TGEV-inoculated*					
20	2	0	+	+ (1600)	+ (6400)
21	3	0	0	+ (640)	+ (640)

0 TGEV/PCV absent, + TGEV/PCV present
Reciprocal value of viral titres in brackets
* No virus isolation attempted from three of five TGEV-infected piglets

Challenge viruses

The PCV isolate (Stopps) was isolated from a nasal swab taken from one of several pigs with respiratory disease. This isolate was twice passaged in porcine kidney primary cultures and the resultant virus stock was titrated before use. Piglets received $10^{3.4}$ TCID50 (50 per cent tissue culture infective dose) of PCV in tissue culture fluid (1 ml intranasally and 2 ml orally).

The TGEV isolate (Miller) was a gut homogenate prepared from experimentally infected piglets. Piglets received $10^{4.0}$ TCID50 of TGEV in filtered gut homogenate (1 ml intranasally and 2 ml orally).

To confirm the specificity of the PCV and TGEV inocula, extracts of each were inoculated on to porcine kidney primary cultures and stained at 48 hours using a conventional direct fluorescent antibody test and an indirect test employing a differentiating monoclonal antibody (4B1) (Garwes et al 1988) which reacts with TGEV alone using a murine fluorochrome conjugate.

Necropsy

Following euthanasia in isolators, piglets were removed and portions of turbinate, right cranial lobe of the lung, terminal ileum and ileal contents were taken aseptically for virus isolation. The extent of pneumonia, where present, was estimated subjectively. No tissues were taken for virus isolation from three of the five TGEV-inoculated piglets.

The following tissues were collected for immersion fixation in 10 per cent neutral, phosphate buffered formalin and processed for light microscopy: oesophagus, stomach, small intestine (six levels), caecum, colon, liver, pancreas, snout with nasal conchae, larynx, trachea, lymph nodes (left tracheobronchial, cranial mediastinal and mesenteric), thymus, spleen, tonsil, kidney, bladder, gonad, thyroid, adrenal, skin, M. semintendinosus, brain (seven levels) and spinal cord (three levels). The primary bronchus of the left lung was cannulated and lobes were inflated with Sorensen's phosphate buffered 3 per cent glutaraldehyde at room temperature. Cranial, middle and caudal lobes were processed for light microscopy. Only a limited range of tissues, chiefly from respiratory and digestive tracts, was taken from three of the five TGEV-inoculated piglets.

Immunocytochemistry

Additional samples of nasal conchae, mid-trachea, right middle lung lobe and distal ileum were collected fresh and immersion fixed for 24 hours in 10 per cent

neutral phosphate buffered formalin. Tissues were dehydrated in ethanol, cleared in xylene, embedded in paraffin wax and sectioned at 4 μm. Sections were stained using a peroxidase-antiperoxidase (PAP) technique following overnight incubation with a 1 in 12,500 dilution of monoclonal antibody (DA3) which reacts with nucleoprotein of TGEV and PCV (Garwes et al 1988). Sections were counterstained with Mayer's haemalum. Selected samples of liver, spleen and thymus fixed for varying lengths of time from control, TGEV- and PCV-inoculated piglets were similarly stained. Test sections were accompanied by three controls: a duplicate of each section stained as above but with the primary antibody step omitted; ileum from a piglet infected 20 hours earlier with TGEV (positive TGEV control); and lung from a piglet infected four days earlier with PCV (positive PCV control).

Electron microscopy

For ultrastructure, 1 mm^3 pieces of consolidated, glutaraldehyde-fixed lung were taken from one PCV-inoculated piglet and one TGEV-inoculated piglet, both at three days after infection. Tissue was rinsed in buffer, post-fixed in 2 per cent osmium tetroxide, dehydrated in ethanol followed by propylene oxide, and embedded in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate.

Virus isolation

Virus was reisolated using porcine kidney cells in a Hanks' medium containing 10 per cent bovine serum, 0·0375 per cent sodium bicarbonate solution, 100 units penicillin ml^{-1}, 100 μg streptomycin ml^{-1} and 20 units mycostatin ml^{-1}. The maintenance medium was Earle's containing 1 per cent fetal bovine serum, 0·15 per cent bicarbonate solution and antibiotics. Tissue collected at necropsy was prepared as 10 per cent homogenates in phosphate buffered saline with antibiotics, incubated for 30 minutes at room temperature, clarified at 1500 *g* for 10 minutes and the resulting supernatant inoculated on to porcine kidney primary cultures. Tissue culture fluid was passed on to fresh cell sheets at seven days and virus growth was tested by the TGEV direct fluorescent antibody test. Positive controls were PCV-infected cultures and negative controls were uninfected cultures.

Serology

Serum antibody was detected using the standard TGEV microserum neutralisation test using A 72 canine tumour cells (modified from Witte 1971).

Results

Clinical signs

All five TGEV-inoculated piglets developed typical signs of transmissible gastroenteritis. Anorexia, diarrhoea, dehydration and hypothermia began at two days after infection and resulted in the death of one piglet at three and a half days after infection. The two remaining TGEV-inoculated piglets at that time were killed as they were moribund. No clinical signs of pneumonia were evident. By contrast, PCV-inoculated and uninoculated control piglets remained clinically healthy over the 11 days of the study.

Gross and histopathological lesions

For convenience and as there were no differences between the two batches of PCV-inoculated piglets, the descriptions of lesions in these animals are combined.

All but one of the PCV-inoculated piglets developed a cranioventral bronchointerstitial pneumonia involving from less than 5 per cent to approximately 20 per cent of lung parenchyma. Grossly, red consolidated areas formed a sublobular or lobular mosaic in cranial and middle lobes. Piglets killed at seven to 11 days after infection also had moderately enlarged tracheobronchial lymph nodes. Histologically, pulmonary lesions were mild or moderate and involved small calibre bronchioles, alveolar ducts and peribronchiolar alveoli. Between two and six days after infection, acute changes were characterised by individual bronchiolar cells bulging into the lumen,

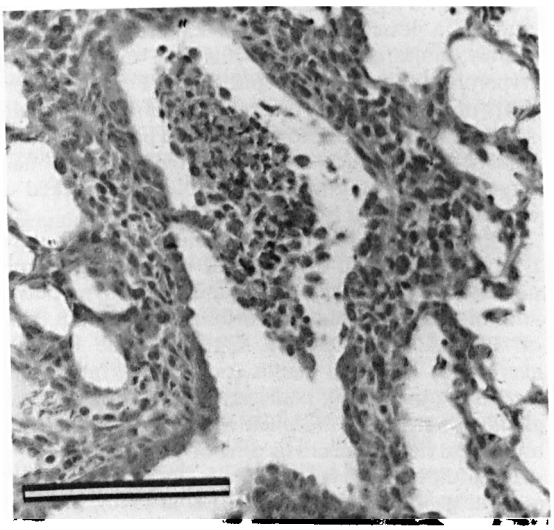

FIG 1: Focal attenuation of bronchiolar epithelium with cellular debris in lumen. PCV-infected piglet at three days after infection. Haematoxylin and eosin. Bar = 100 μm

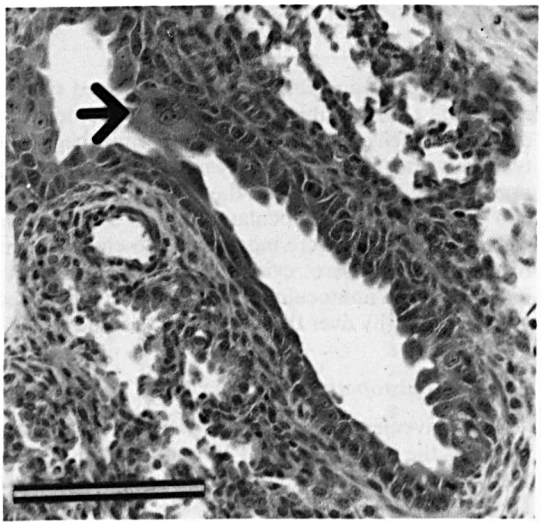

FIG 2: Hyperplasia of bronchiolar epithelium indicating repair in the lung of PCV-infected piglet at 11 days after infection. Solitary multinucleated epithelial cell (arrow). Haematoxylin and eosin, bar = 100 μm

followed by degeneration and detachment. Macrophages, fibrin and cellular debris accumulated in bronchioles, alveolar ducts and alveoli (Fig 1a). The naked basement membrane of bronchioles was covered by attenuated epithelial cells. Interalveolar septa were thickened.

Evidence of repair was present at seven days after infection and was a characteristic feature up to 11 days after infection. There was increased mitotic activity with hyperplasia in the epithelium of medium and to a lesser extent small calibre bronchioles. Rarely, multinucleated epithelial cells were present in hyperplastic bronchiolar epithelium (Fig 2). Modest cuffs of lymphoblasts, macrophages and plasma cells accumulated around bronchioles and peribronchiolar vessels. By 11 days after infection, alveolitis had largely resolved. Some bronchioles still lacked a complete epithelial lining at this time.

Other changes in the PCV-inoculated piglets were lymphoid follicular hyperplasia in thoracic lymph nodes, mild rhinitis with disorganisation, hyperplasia and microcystic change in turbinate epithelium (five of 14 piglets), ulcerative laryngitis (one of 14), necrosis of laryngeal glands (one of 14), multifocal tracheitis (one of 14), multifocal necrosis of tracheal glands (one of 14), mild ileitis (one of 14) and mild multifocal hepatic necrosis (one of 14).

In addition to typical enteric lesions of transmissible gastroenteritis (Pensaert et al 1970), three of the five TGEV-inoculated piglets had gross evidence of mild cranioventral pneumonia. Other gross lesions were dehydration, congestion of mesenteric vessels, thymic atrophy and multifocal pulmonary congestion. Four of the TGEV-inoculated piglets had bronchointerstitial pneumonia similar in histological character to the PCV-infected piglets. Other lesions detected histologically were moderate thymic atrophy in four piglets, depletion of splenic periarteriolar sheaths in four piglets, mild multifocal hepatic necrosis in five piglets, mild rhinitis in two piglets, moderate ulcerative laryngitis in one piglet and multifocal renal tubular necrosis with dilatation in one piglet.

No gross or microscopic lesions developed in the control group. Two of the five piglets had occasional intra-alveolar syncytial cells. Similar cells were present in some of the PCV and TGEV-infected piglets in otherwise normal areas of lung and were assumed to be an incidental finding (Castleman et al 1985).

Immunocytochemistry

PCV antigen was identified in the epithelial cytoplasm of small and medium bronchioles in eight of 14 piglets and its distribution was closely correlated with areas of pneumonia. Typically, solitary cells or small groups of cells at different stages of detachment from the basement membrane were stained (Fig 3). Viral antigen was also present in degenerated intraluminal cells and in alveolar macrophages. Three PCV-infected piglets had widely separated positively stained villous enterocytes (Fig 4) and one had positively stained turbinate epithelial cells. Antigen was absent in spleen, thymus and liver.

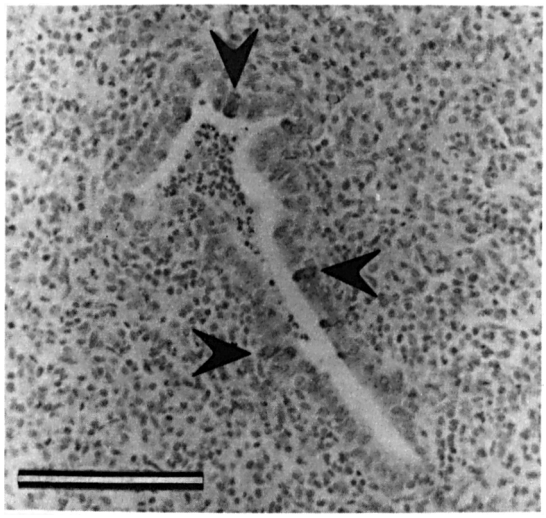

FIG 3: Darkly stained bronchiolar epithelial cells in small calibre bronchiole (arrowheads). PCV-infected piglet six days after infection. PAP, bar = 100 μm

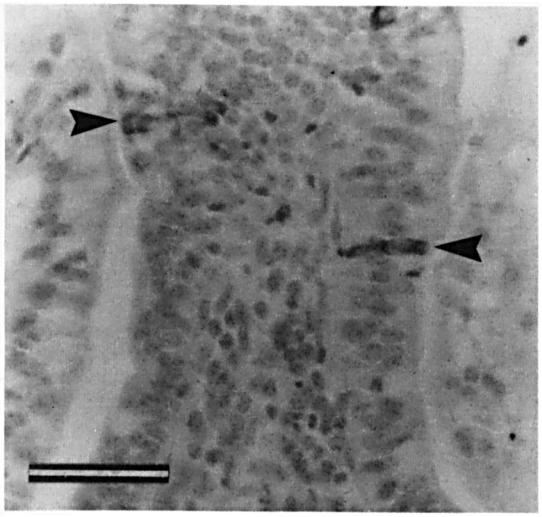

FIG 4: Darkly stained villous enterocytes (arrowheads). No villous atrophy present. PVC-infected piglet at five days after infection. PAP, bar = 50 μm

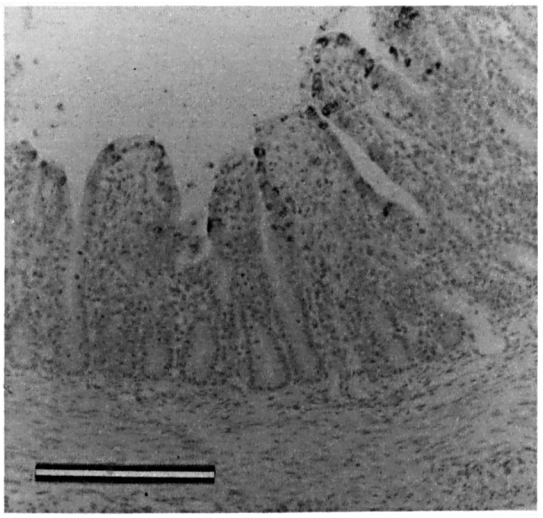

FIG 5: Darkly stained villous enterocytes distributed widely and associated with atrophic villi. TGEV-infected piglet at three days after infection. PAP, bar = 200 μm

All five TGEV-inoculated piglets had viral antigen in villous enterocytes associated with atrophic villi (Fig 5). TGEV antigen was detected in bronchiolar epithelial cells and in alveolar macrophages in areas of pneumonia in three piglets, and hepatocytes in necrotic foci also contained intracytoplasmic viral antigen in three piglets. No positive staining was found in thymus or spleen.

Tissues from the five uninoculated piglets were uniformly negative for PCV/TGEV antigen.

Transmission electron microscopy

The range of cell types which contained coronavirus-like particles in the lung of the PCV-infected piglet was identical to that in the TGEV-infected piglet. Viral particles were most commonly found near the apical plasmalemma of non-ciliated cuboidal bronchiolar cells. Virus occurred in the apical cytoplasm of these cells in endoplasmic reticulum or in membrane bound vesicles and followed a maturation sequence similar to that of TGEV in villous enterocytes (Wagner et al 1973) (Fig 6). Some degenerating cells contained intracytoplasmic virus. Virus also occurred on the surface of alveolar macrophages and free in alveoli. Some type I pneumocytes were degenerative but none contained virus.

Virus isolation

Virus isolation results are summarised in Table 1. PCV was recovered from the turbinates of nine piglets at two to eight days after infection, from the lungs of eight piglets at one to nine days after infection, from distal small intestine of eight piglets and intestinal contents of nine piglets at two to 11 days after infection. The titre of virus in the intestine and intestinal contents of PCV-infected piglets was less than in TGEV-infected piglets. Virus was isolated from the lung of one of two TGEV-inoculated piglets.

Serology

All piglets were seronegative before virus inoculation. PCV-inoculated piglets seroconverted at seven days after infection, with titres rising up to 128 at 11 days after infection. The uninoculated, control piglets remained seronegative throughout the study period.

Discussion

This study confirms a previous report (Pensaert et al 1986) and many observations from the field that PCV is a TGEV-like coronavirus which replicates extensively in the respiratory tract but does not cause clinical disease.

The bronchointerstitial pneumonia observed in the PCV-infected piglets was similar in character to that in the TGEV-infected piglets. The possibility of dual infection is unlikely as individual piglets were maintained in isolation and as neither control nor PCV-inoculated piglets had clinical signs or intestinal lesions of transmissible gastroenteritis. The close similarity between pneumonia caused by each corona-

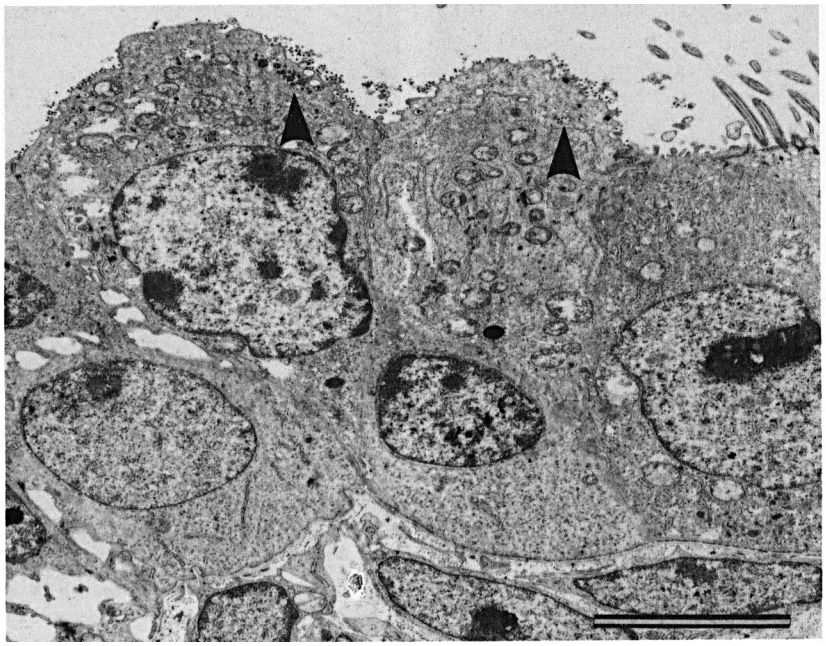

FIG 6: Electron micrograph of bronchiolar epithelium. Viral particles (arrowheads) on the apical plasmalemma and in the apical cytoplasm of non-ciliated cells. TGEV-infected piglet at three days after infection. Uranyl acetate/lead citrate. Bar = 5 μm

virus is not surprising given the similarity of PCV to TGEV and suggests pneumonia in each group had a similar pathogenesis. In the lung, replication of PCV and TGEV in non-ciliated cuboidal bronchiolar cells which are most numerous in terminal airways (Baskerville 1970) accounts for pneumonia involving small calibre bronchioles and adjacent parenchyma. Previous studies of the lungs of TGEV-infected piglets found virus in bronchial and bronchiolar cells (Underdahl et al 1974) and in pneumocytes (Underdahl et al 1974, Mocsari and Horvath 1980). Alveolar macrophages were proposed as a target cell population following infection in vitro by cell adapted but not wild-type TGEV strains (Laude et al 1984). In the present study no virus was detected in alveolar epithelium. Degeneration of these cells was either the result of a low concentration of virus in affected cells, unproductive infection or inadvertent destruction by the acute inflammatory process in terminal airways.

The absence of clinical signs following PCV infection of piglets confirms the results of a previous study that this coronavirus dies not cause diarrhoea or enteric lesions (Pensaert et al 1986). Unlike that study, however, in this study PCV was isolated from small intestine suggesting a limited capacity by this isolate to replicate in enterocytes. Immunocytochemistry confirmed the presence of antigen-positive enterocytes. The paucity of these cells explains the absence of villous atrophy and the low titre of virus in gut compared with TGEV. In many respects PCV behaved like a stable, low pathogenicity strain of TGEV. Non-enteropathogenic TGEV strains retaining their capacity to infect respiratory tract tissues were produced in attempts to find a safe, effective vaccine to transmissible gastroenteritis (Furuuchi et al 1979). Belgian isolates of PCV do not, however, possess the characteristics of vaccinal strains of TGEV (Jestin et al 1987).

Other differences between PCV and TGEV infection were the absence of renal tubular nephrosis (Goodwin and Jennings 1959) and the paucity of hepatic necrosis in PCV-infected piglets. There was no evidence of thymic atrophy or splenic lymphoid depletion in PCV-infected piglets, changes present in the TGEV-infected group.

The correlation between the results of PAP staining of fixed tissue and virus isolation was good, although isolation was more sensitive perhaps because of its greater ability to detect small quantities of virus. The PAP method was a reliable technique for identifying PCV/TGEV antigen in paraffin wax-embedded tissue and was compatible with formalin fixation unlike an earlier PAP technique using polyclonal lapine anti-TGEV serum which worked on acetone-fixed but not

formalin-fixed tissues (Chu et al 1982). Fixation time in formalin was not critical for our PAP method and we have successfully stained TGEV-infected porcine gut fixed in a variety of solutions (periodate-lysine-paraformaldehyde-dichromate, formal sublimate, acetone and Carnoy's). Given that the pneumonia caused by PCV is nondescript with no morphological hallmarks other than the occasional syncytial bronchiolar cell to facilitate aetiological diagnosis, the PAP method may have an application when PCV is suspected of playing a role in field investigations of porcine pneumonia. To date conclusive evidence of such a role has not been established.

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