FULL PAPER *Pathology*

Evidence of Porcine Circovirus Infection in Pigs with Wasting Disease Syndrome from 1985 to 1999 in Hokkaido, Japan

Kenshi SATO[1,2], Tomoyuki SHIBAHARA[1]*, Yoshiharu ISHIKAWA[1], Hiroshi KONDO[3], Masanori KUBO[4] and Koichi KADOTA[1]

[1]*Hokkaido Research Station, National Institute of Animal Health, 4 Hitsujigaoka, Toyohira, Sapporo 062–0045,* [2]*Tokachi Livestock Hygiene Service Center, 59–6 Kisen, Kawanishi, Obihiro 089–1182,* [3]*Ishikari Livestock Hygiene Service Center, 3 Hitsujigaoka, Toyohira, Sapporo 062–0045 and* [4]*National Institute of Animal Health, 3–1–1 Kannondai, Tsukuba, Ibaraki 305–0856, Japan*

(Received 24 November 1999/Accepted 23 February 2000)

ABSTRACT. An epizootiological survey with histopathological methods was conducted for porcine circovirus in 220 diseased pigs (1–200 days old) in 49 farms from 1985 to 1999. Histopathological lesions containing PCV antigen were detected mainly in the lymphoid tissues from 42 of 189 diseased pigs (22.2%) in 4 of 45 farms (8.9%) from 1990 to 1999. The rate of positive pigs gradually increased from 1997 onward and PCV infection was found in 50% of diseased pigs in 1999. Histopathologically, the lesions in the lymphoid tissues (including lymph nodes, Peyer's patches, tonsil and spleen) were highly correlated with the presence of numerous spherical basophilic intracytoplasmic inclusion bodies with PCV antigen, and consisted of lymphocellular depletion and infiltration of macrophages. Although most affected cells showed cytoplasmic reactivity for PCV, intranuclear antigen was also seen in the lymphocytes, macrophages and ileal epithelial cells. Ultrastructurally, macrophages and giant cells contained electron-dense, round to ovoid lysosomal bodies, in which there were concentric circle or paracrystalline arrays of small nonenveloped icosahedral viral particles, approximately 15–17 nm in diameter. Other consistent infectious agents were present in 90.5% of cases, and porcine reproductive and respiratory syndrome virus infection was in 52.4% of the cases with PCV. The histopathological findings suggested that PCV induced systemic immunosuppression in the infected pigs and made them more susceptible to infection of the organisms. Because of the presence of PCV antigens in the intestinal epithelium, feces may play a significant role in dissemination of PCV.—KEY WORDS: epizootiological survey, porcine, porcine circovirus, postweaning multisystemic wasting syndrome.

J. Vet. Med. Sci. 62(6): 627–633, 2000

Porcine circovirus (PCV) was first detected as a contaminant of the pig kidney cell line PK/15 [26]. Several surveys have documented a high seroprevalence of antibodies to PCV in swine populations in several parts of the world [9, 15, 27]. It is suggested that transplacental infection with PCV does occur, possibly prior to fetal immunocompetance [3]. However, experimental infection of pigs with this virus has produced no clinical signs [3, 28].

Postweaning multisystemic wasting syndrome (PMWS) is a recently described disease that affects pigs shortly after weaning and fattening. This syndrome, first described in Canada in 1991 [7, 13], is characterized by progressive weight loss, respiratory stress and jaundice. Gross lesions include lymphadenopathy, interstitial pneumonia, hepatopathy and nephritis. Histopathologically, histiocytic infiltrates are detected in the lymphoid organs, liver and kidney, and characteristic intracytoplasmic inclusion bodies are found in the lymphoid organs [23]. Similar syndromes have been described in U.S.A. [8], France [20], Northern Ireland [16] and Spain [24]. PCV PK/15-like antigen and nucleic acid were demonstrated in tissues of pigs with PMWS in Canada [10], and in tissues from pigs exhibiting similar syndromes in U.S.A. [8] and Spain [24]. This has led to the speculation that a new or modified pathogenic PCV may have emerged in the pig population of several countries. In 1998, circovirus-like virus was isolated from lesions of pigs in Canada, U.S.A. and Europe with cases of PMWS [5, 10]. This virus had a limited antigenic cross-reactivity with the PCV contaminant of PK/15 cell cultures [5], and exhibited 68% nucleotide sequence homology with the PCV [12]. On the basis of the antigenic and genomic analyses, the new circovirus isolates were referred to as PCV2 and the original PCV as PCV1 [6]. A strong correlation between PMWS and the presence of PCV2 antigen and nucleic acid in tissues has been reported in North America and Europe [5, 10, 17, 23], and recently, clinical disease and histopathological lesions were reproduced in piglets inoculated with PCV2 cell culture isolates [2, 11].

There has been no epizootiological study in Japan, except report of a case of PCV infection in Chiba in 1996 [18]. Here, we investigate 220 diseased pigs which had died or been euthanized from 1985 to 1999 by using histopathological, immunohistochemical and electron microscopical examinations, and report the evidence, histopathological features, and mixed-infections of PCV.

MATERIALS AND METHODS

Samples of the liver, spleen, kidneys, heart, lungs, small

* CORRESPONDENCE TO: SHIBAHARA, T., Hokkaido Research Station, National Institute of Animal Health, Ministry of Agriculture, Forestry and Fisheries, 4 Hitsujigaoka, Toyohira, Sapporo 062–0045, Japan.

intestine, tonsil and bronchial or mesenteric lymph nodes were collected from 220 diseased pigs (aged 1 to 200 days) in 49 farms of Hokkaido from 1985 to 1999. Tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin wax, and routinely processed for histological examination. Sections were stained with hematoxylin and eosin (HE). Paraffin sections of the lymphoid organs (including lymph nodes, tonsil, Peyer's patches and spleen), liver, kidney, lungs and small intestine were also stained by the streptavidin-biotin-peroxidase complex immunoperoxidase techniques (SAB) using hyperimmune rabbit antiserum to PCV1 (CCL-33) at 1/256 dilution (kindly provided by Dr. T. Imada, National Institute of Animal Health, Japan). Subsequent procedures were carried out by means of an immunoperoxidase staining system (Nichirei, Tokyo, Japan). For electron microscopical examination, small blocks taken from the lymph nodes demonstrable to be positive for PCV1 were post-fixed in 1% osmium tetroxide, embedded in epoxy resin, stained with 1% uranyl acetate and lead citrate, and examined with a transmission electron microscope (TEM, JEM-1010, JEOL, Tokyo, Japan).

To clarify the mixed-infections (including PRRSV, *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae*, *Streptococcus suis*, *Pasteurella multocida*, *Actinobacillus suis*, and *Pneumocystis carinii*) with PCV, all pigs were also examined with routine histopathological, immunohistochemical and serological methods or isolation techniques.

RESULTS

Evidence of PCV infection in Hokkaido: Microscopical lesions containing PCV antigen were first found in 1990 (Table 1). PCV antigen was detected in 42 of 189 cases (22.2%) from 1990 to 1999. The evidence of PCV infection increased to 30.8% (4/13) in 1997, and to 50.0% (8/16) in 1999. On the other hand, the number of farms with PCV infection was only 4 of 45 (8.9%) from 1990 to 1999. In a farm where PCV infection was first found in 1993, the infection occurred every year up to 1999. The age of PCV infected pigs ranged from 39 to 84 days (63.4 ± 12 days) and there was no apparent sex predilection.

Microscopical Lesions: Microscopical lesions attributable to PCV infection were found mainly in the lymphoid organs (Table 2). In the lymph nodes, lymphoid follicles were unclear, and lymphocyte depletion was seen in follicles. In the germinal center of follicles, infiltration of macrophages was prominent, occasionally with multinuclear giant cells (Fig. 1), and lymphocytic apoptosis was markedly frequent. The most characteristic histological feature was the presence of spherical, basophilic, intracytoplasmic inclusion bodies, and they were found chiefly in macrophages (Fig. 2) and occasionally in giant cells. Such inclusions were either large and single or smaller and multiple. Infiltration of macrophages with or without inclusions was also detected in the T-cell-dependent zones, but its degree was milder than those in follicles. The changes in the spleen, tonsils and Peyer's patches were similar to those in the lymph nodes. Intensity of lesions in the lymphoid organs were more severe in the cases in which many inclusions were presented.

Table 1. Evidence of PCV infection in Hokkaido, Japan

	PCV positive/examined (%)
1985–1989	0/31 (0.0)
1990	3/24 (12.5)
1991	4/19 (21.1)
1992	0/14 (0.0)
1993	4/28 (14.3)
1994	5/18 (27.8)
1995	1/13 (7.7)
1996	4/21 (19.0)
1997	4/13 (30.8)
1998	9/23 (39.1)
1999*	8/16 (50.0)
1990–1999	42/189 (22.2)

*; From January to June.

The frequency of interstitial pneumonia and / or bronchopneumonia was high. Of the 27 cases exhibiting interstitial pneumonia, 22 (81.5%) had porcine reproductive and respiratory syndrome virus (PRRSV) antigens in the lungs. Histological changes including inclusion bodies in the lungs were mainly attributable to PCV infection (10/35: 28.6%). These 10 cases showed mild bronchointerstitial pneumonia, characterized by filling of bronchi and bronchiole with macrophages, neutrophils and sloughed epithelial cells, and macrophages containing inclusions were distributed in alveolar septa, peribronchi, peribronchiolar and hyperplastic bronchus-associated lymphoid tissues. Proliferation of type II pneumocytes was also apparent. In the kidney, focal accumulations of macrophages and lymphocytes were seen mainly in the renal pelvis, and inclusions were in some macrophages. The number of inclusions in the lungs, kidney and liver was relatively small in comparison with that in the lymphoid organs.

Immunohistochemical detection of PCV antigen: PCV antigen was detected in the lymphoid organs, small intestine, lungs, kidney and liver (Table 3). In all tissues examined, immunolabeling was seen mainly in the cytoplasmic inclusions of macrophages (Fig. 3). In the vicinity of the positive cells, apoptosis of lymphocytes was frequently seen in the lymphoid organs, and nuclear immunolabeling of macrophages and lymphocytes was occasionally found (Fig. 4). Noteworthy evidence was the positive nuclear staining of intestinal epithelial cells (Fig. 5). The cases in which PCV antigen was detected in various tissues had a tendency to show more severe histological lesions and more abundant antigen than the cases with PCV antigen only in the lymph nodes or tonsils.

Electron microscopical evidence: In the lymph nodes

Table 2. Frequency and type of histopathological lesions from PCV infected pigs

Tissues	Lesions	Number of pigs with lesion/examined (%)
Lymph node[a)]	Indistinctness of follicles	36/41 (87.8)
	Depletion of lymphocytes	37/41 (90.2)
	Apoptosis of lymphocytes	31/41 (75.6)
	Infiltration of macrophages	34/41 (82.9)
	Presence of cytoplasmic inclusions	35/41 (85.4)
	Presence of multinuclear giant cells	12/41 (29.3)
	Granulomatous inflammation	4/41 (9.8)
	Suppurative lymphadenitis	24/41 (58.5)
Tonsil	Indistinctness of follicles	12/20 (60.0)
	Depletion of lymphocytes	17/20 (85.0)
	Apoptosis of lymphocytes	16/20 (80.0)
	Infiltration of macrophages	17/20 (85.0)
	Presence of cytoplasmic inclusions	14/20 (70.0)
	Presence of multinuclear giant cells	3/20 (15.0)
Small intestine (Peyer's patches)	Indistinctness of follicles	10/13 (76.9)
	Depletion of lymphocytes	12/13 (92.3)
	Apoptosis of lymphocytes	10/13 (76.9)
	Infiltration of macrophages	11/13 (84.6)
	Presence of cytoplasmic inclusions	11/13 (84.6)
	Presence of multinuclear giant cells	3/13 (23.1)
(Lamina propria)	Presence of cytoplasmic inclusions	7/17 (41.2)
Spleen	Depletion of lymphocytes	30/36 (83.3)
	Apoptosis of lymphocytes	20/36 (55.6)
	Infiltration of macrophages	29/36 (80.6)
	Presence of cytoplasmic inclusions	18/36 (50.0)
	Presence of multinuclear giant cells	5/36 (13.9)
Lung	Interstitial pneumonia	27/35 (77.1)
	Bronchopneumonia	31/35 (88.6)
	Fibrinous pleurisy	12/35 (34.3)
	Fibrinous pneumonia	5/35 (14.3)
	Presence of cytoplasmic inclusions	10/35 (28.6)
Liver	Infiltration of macrophages in portal zones	21/31 (67.7)
	Focal necrosis of hepatocytes	16/31 (51.6)
	Accumulation of mononuclear cells in sinusoid	11/31 (35.5)
	Presence of cytoplasmic inclusions	1/31 (3.2)
Kidney	Interstitial nephritis and pyelitis	17/31 (54.8)
	Presence of cytoplasmic inclusions	6/31 (19.4)

a) Lesions in at least one of the lymph nodes examined (bronchial and mesenteric).

with PCV antigen, macrophages and giant cells contained electron-dense, round to ovoid lysosomal bodies with sharp margins (Fig. 6), in which there were granular small (15–17 nm size range) naked viral particles with concentric circular (Fig. 7) or paracrystalline arrays.

Mixed-infections with PCV: Other consistent infectious agents were noticed in 38 of 42 PCV infected cases (90.5%) (Table 4), and PRRSV was most frequently seen (22/42: 52.4%). As for bacteria, *Streptococcus suis* (7/42: 16.7%), *Haemophilus parasuis* (5/42: 11.9%), *Actinobacillus pleuropneumoniae* (4/42: 9.5%), *Pasteurella multocida* (2/42: 4.8%) and A*ctinobacillus suis* (1/42: 2.4%) were isolated. *Pneumocystis carinii* was present in the lungs in 8 cases (19.0%).

DISCUSSION

We investigated 220 diseased pigs which had been necropsied from 1985 to 1999 in Hokkaido, and found PCV infection in 42 cases. The age of affected pigs, histopathological lesions, immunohistochemical findings and electron microscopical evidence in these cases were similar to those of pigs with PMWS [11, 23]. These lesions attributable to PCV infection in 1990 were first findings in the world literature. This epizootiological survey indicates that PCV infection has been present in Japan since 1990 at

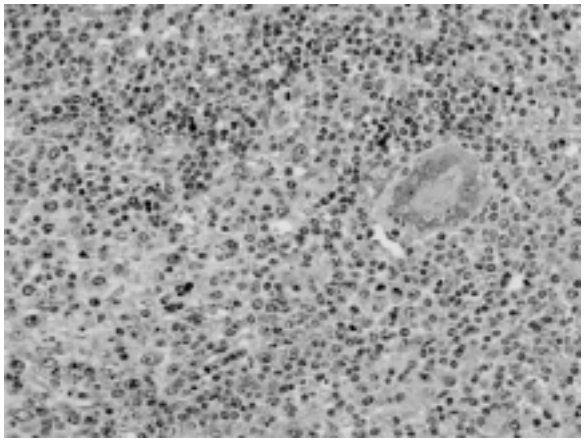

Fig. 1. Mesenteric lymph node. Infiltration of macrophages in the follicular zone, with depletion of lymphocytes. Lymphoid follicles are unclear, and multinuclear giant cell is present within the follicle. HE. × 200.

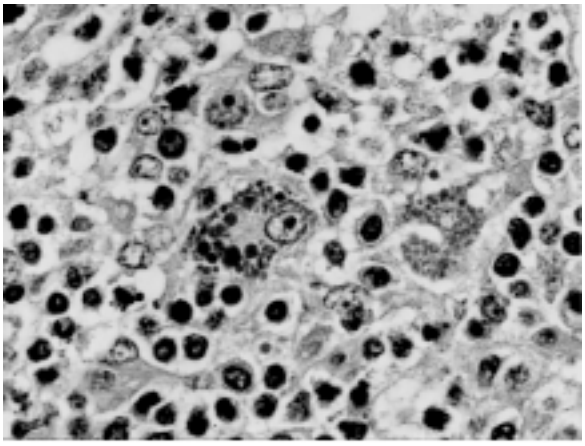

Fig. 2. Bronchial lymph node. Many spherical, basophilic, cytoplasmic inclusion bodies are present within a large macrophage. HE. × 600.

least. The evidence of PCV infection had risen since 1997, and seemed to coincide with the year when PMWS-like syndromes began to be reported in several countries [8, 16, 19, 24]. In one farm, PCV infection was found every year from 1993 to 1999. This evidence indicated difficulty in clearing PCV infection from contaminated farms. However, the evidence of PCV infection in the farms remained at 8.9%, and prevention of the introduction of pigs with PCV in contaminated farms to free farms will makes it possible to prevent extensive infection among farms.

Histopathological lesions of PCV infection were found mainly in the lymphoid organs. Lymphocellular depletion, infiltration of macrophages and presence of characteristic inclusions were the predominant lesions, as observed by others [16, 17, 25].

In addition to the cytoplasmic inclusions of macrophages and giant cells, PCV antigen was observable in the nucleus of lymphocytes and macrophages. This finding was similar to the results that Rosell *et al*. [23] reported. The nuclear staining of lymphocytes suggested that PCV could infect lymphocytes, and that some cytoplasmic inclusions of macrophages were phagolysosomes whose contents were derived from apoptotic lymphocytes. However, previous studies failed to demonstrate infection of lymphocytes in culture, and showed PCV replication in monocytes and macrophages [4, 21]. Future immunohistochemical studies about cell markers and PCV strains are necessary to determine the target cells of the pathogenic PCV strains.

Except for lymphoid organs, histopathological lesions attributable to PCV infection and evidence of PCV antigen existed in the lungs, liver, kidney and small intestine. The lungs, liver and kidney have been described as major organs affected in PMWS [10, 14, 23], and PCV antigen have been observed in infiltrating macrophages, epithelial cells of the lung, hepatocytes, biliary epithelial cells and renal tubular epithelial cells [7, 10, 14, 23]. In the present study, PCV antigen was present exclusively in infiltrating macrophages, and the rate of positive organs was lower than that in the previous reports [7, 10, 14, 23]. These may be associated

Table 3. Immunohistochemical detection of PCV antigen in tissues from PCV infected pigs

Tissues	Number of pigs with PCV antigen/examined (%)	Distribution of PCV antigen			
		Macrophages	Lymphocytes	MGC[b)]	Epithelial cells
Lymph node[a)]	35/41 (85.4)	C, N[c)]	N	C	
Small intestine					
(Peyer's patches)	11/13 (84.6)	C	N	C	
(Lamina propria)	7/17 (41.2)	C			
(Epithelial layer)	2/17 (11.8)				N
Tonsil	14/20 (70.0)	C, N	N	C	
Spleen	18/36 (50.0)	C	N	C	
Lung	10/35 (28.6)	C			
Kidney	6/31 (19.4)	C			
Liver	1/31 (3.2)	C			

a) Detections in at least one of the lymph nodes examined (bronchial and mesenteric).
b) Multinuclear giant cells.
c) C; Cytoplasmic, N; Nuclear.

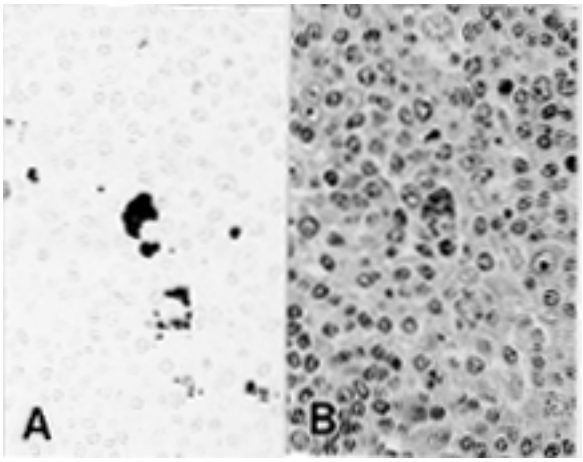

Fig. 3. Mesenteric lymph node. A) Note the presence of PCV antigen in the cytoplasm of macrophages. It is mostly conform to cytoplasmic inclusion bodies in B). A). SAB. B). HE. × 400.

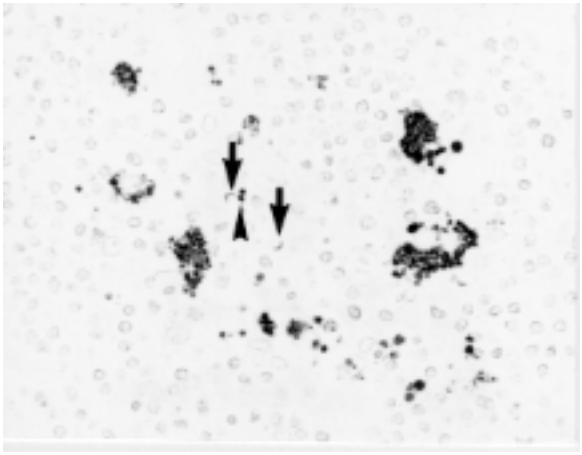

Fig. 4. Bronchial lymph node. The presence of PCV antigen in macrophages is apparent, and apoptosis (arrows) and nuclear immunolabeling of apoptotic lymphocytes (arrowhead) are also visible. SAB. × 400.

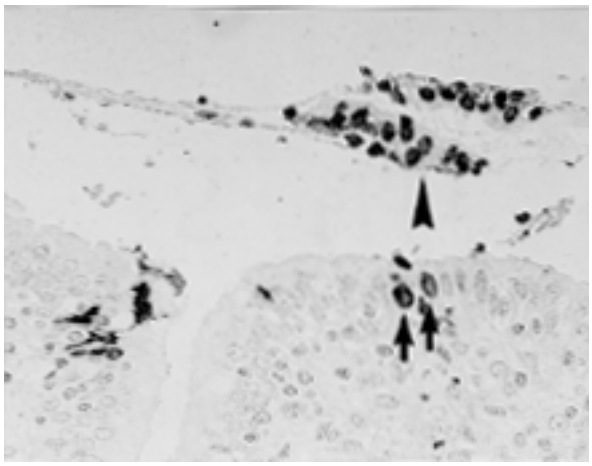

Fig. 5. Small intestine. Note the presence of PCV antigen in the nucleus of epithelial cells (arrows) and sloughed epithelial cells (arrowhead). SAB. × 400.

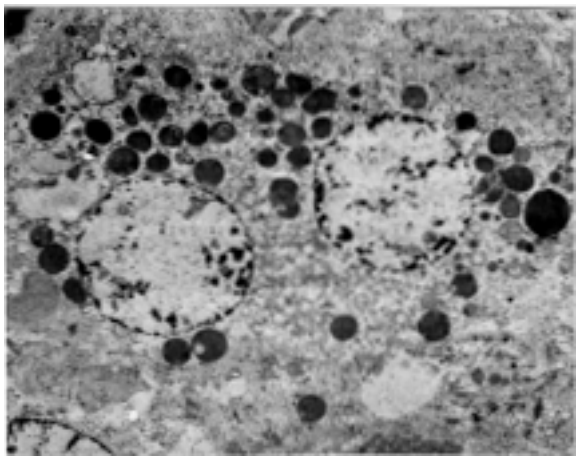

Fig. 6. Bronchial lymph node. A multinuclear giant cell with multiple electron-dense, round to ovoid cytoplasmic inclusion bodies. TEM. × 4,000.

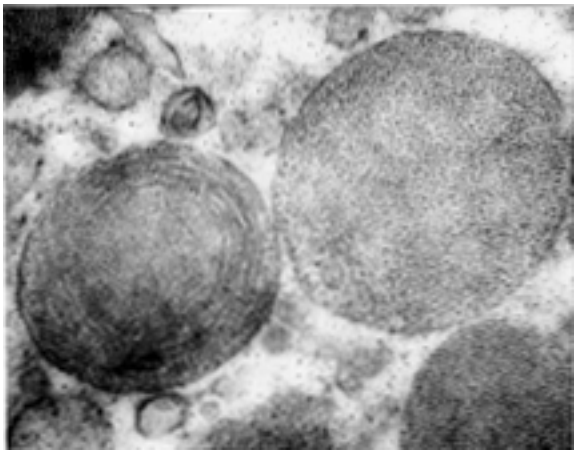

Fig. 7. Bronchial lymph node. There are concentric circular arrays of small naked viral particles in cytoplasmic inclusion bodies. TEM. × 50,000.

with stages of the disease and virus strain. In our 2 cases, PCV antigen was detectable in the nucleus of epithelial cells of the small intestine. On the basis of this fact, PCV was considered to be spread by feces.

Chicken anemia virus, belonging to the same virus family as PCV, is known to cause immunosuppression [1, 22]. Similarly, some previous studies suggested that PMWS caused immunosuppression based on the histology (lymphocytic depletion) and detection of *Pneumocystis carinii* in affected pigs [7, 10, 23]. In this study, depletion and apoptosis of lymphocytes were conspicuous, and

Table 4. Pathogens mix-infected with PCV

Pathogens	Number of pigs with pathogens/examined (%)
PRRSV, *Haemophilus parasuis, Pneumocystis carinii*	1/42 (2.4)
PRRSV, *Pneumocystis carinii*	7/42 (16.7)
PRRSV, *Haemophilus parasuis*	1/42 (2.4)
PRRSV, *Actinobacillus pleuropneumoniae*	1/42 (2.4)
PRRSV	12/42 (28.6)
Streptococcus suis	7/42 (16.7)
Haemophilus parasuis	3/42 (7.1)
Actinobacillus pleuropneumoniae	3/42 (7.1)
Pasteurella multocida	2/42 (4.8)
Actinobacillus suis	1/42 (2.4)
Total	38/42 (90.5)

nuclear positivity for PCV was found in lymphocytes. These findings were suggestive of activation of apoptosis by PCV. Our results may suggest that PCV lead to apoptosis of lymphocytes. There was *Pneumocystis carinii* infection in 8 cases (19.0%), and this proportion was higher than in the previous reports [7, 10].

In our study, other consistent infectious agents were present in 90.5% of cases, and PRRSV infection was in more than 50.0% of the cases with PCV. These two viruses can be present together in swine herds as previously described [19], and those results suggested that PCV may not be the only factor required for development PMWS. Therefore their possible association or synergism should be investigated further. It is of paramount importance to control mixed-infections in PCV-positive farms, because there is no effective measure of PCV infection to date.

ACKNOWLEDGMENTS. We would like to thank Ms. M. Hikita for her advice, and we also acknowledge Dr. Y. Ando and Mr. T. Fujisawa for preparation of photomicrographs.

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