Pathologic Observations of Pigs Intranasally Inoculated with Serovar 1, 4 and 5 of *Haemophilus parasuis* Using Immunoperoxidase Method

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ABSTRACT. Nineteen, 7- to 13-week-old pigs were inoculated intranasally with different strains of *Haemophilus parasuis* (serovar 1, 4 and 5), and the pathological lesions induced by each strain were compared. Eleven of thirteen pigs inoculated with either strain Nagasaki (serovar 5) or No. 4 (serovar 1) died between days 1 to 6 after inoculation, and had septicemic lesions, meningitis, or polyserositis. One of six pigs inoculated with strain SW124 (serovar 4) died with polyserositis, another one recovered after illness, and the remaining four pigs remained in good health. Five of the septicemic pigs had thrombi at many organs. Endotoxin was detected in the plasma of 10 pigs in the acute stage of infection. Using the immunoperoxidase technique, *H. parasuis* antigen was detected in lesions of infected pigs. In the serosal lesions the bacterial antigen was found mainly in the cytoplasm of infiltrating neutrophils and macrophages and appeared as degenerated bacteria and/or lytic bacterial material in dilated phagosomes. Many of the bacteria in the blood vessels of pigs with septicemic lesions were also degenerated. Although *H. parasuis* was reisolated from nasal secretions of infected pigs, the bacterial antigen could not be detected in the nasal cavities of these pigs. No lesions were observed in the parenchyma of the lung. However, *H. parasuis* antigen was detected in the tonsil of infected pigs.—KEY WORDS: endotoxin, Glasser's disease, *Haemophilus parasuis*, immunoperoxidase method, swine.

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Glasser's disease caused by *Haemophilus parasuis* is pathologically characterized by polyserositis, polyarthritis and meningitis [11]. *H. parasuis* also produces acute septicemia [1, 15, 16]. Isolation rate of *H. parasuis* was rather low from lesions of both naturally [1, 12, 15, 16] and experimentally [6, 9] infected animals. However, *H. parasuis* antigen was demonstrable by the indirect immunofluorescent antibody technique [9].

It has been reported that bacterial endotoxin induces disseminated intravascular coagulation [8]. In the acute septicemic stage of *H. parasuis* infection, microthrombi were often observed in many organs [1, 15, 16]. However, there are few reports on the relationship between *H. parasuis* infection and production of endotoxin in infected pigs.

The main purpose of this study was to demonstrate the relationship between pathological lesions and the distribution of bacterial antigens, using the immunoperoxidase technique, in SPF pigs intranasally inoculated with 3 different strains of *H. parasuis*. In addition, the presence of endotoxin in the plasma of the infected pigs was also demonstrated.

MATERIALS AND METHODS

Animals: Twenty-one, 7- to 13- week-old, specific pathogen-free (SPF) large white pigs were derived from a SPF herd. They were negative for the isolation of *H. parasuis* from the nasal cavities and for complement-fixing antibodies against *H. parasuis*.

Preparation of inocula : Strains No.4, SW124 and Nagasaki of *H. parasuis* were used as inocula [7]. Bacterial inocula were prepared from 18 hr cultures in chocolate agar. The chocolate agar was prepared from brain heart infusion agar (Difco) with addition of 7% defibrinated sheep blood, heated at 80°C for 20 min, supplemented with 5% sterilized fresh yeast extract [4]. The cultures on chocolate agar plate were suspended in cold 0.01 M phosphate-buffered saline (PBS, pH 7.2) and washed two times by centrifugation at 7,000 rpm for 10 min. Then the bacterial suspensions in PBS were stored in chilled state up to the time of inoculation [5].

Experimental procedure: Twenty-one SPF pigs were divided into 4 groups; 3, 6, 10 and 2 pigs were assigned to groups 1, 2, 3 and 4, respectively. Pigs in groups 1, 2 and 3 were inoculated intranasally with 10^6 to 10^{10} colony forming unit (CFU) of strain No. 4, SW124 and Nagasaki, respectively. The two pigs in group 4 which served as controls were inoculated with PBS only, and were housed separately from the three experimental groups. Following exposure, all the pigs were observed daily for clinical signs of disease.

Detection of endotoxin and bacterial examination: Blood samples were obtained for the detection of endotoxin before inoculation and at 1, 2, 4, 5, 6 and 7 days post inoculation (dpi). Endotoxin concentration in plasma was determined by an automated turbidimetric time assay instrument (Toxinometer ET-201, Wako) [13, 17]. Samples of nasal swabs, body fluids, and tissues from trachea and other organs were inoculated onto chocolate agars. The cultures were incubated at 37°C for 24 hr in an atmosphere containing 5% carbon dioxide. Identification procedures were followed by the criteria described previously [3].

Pathological examination: Pigs were necropsied after death or euthanasia. Tissues of the liver, spleen, kidney,

lung, heart, brain, intestines, tonsil, main lymph nodes, and the nasal cross section were collected from each pig, fixed in 20% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (H & E), and phosphotungstic acid-hematoxylin (PTAH). Formalinized segments of the brain, spleen and kidney from the infected pigs were washed with 0.1 M PBS and post fixed in 1% osmic acid. Tissue blocks were dehydrated in a graded series ethanol and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with an electron microscope.

Immunopathological examination: Bacterial antigens were of demonstrated by the avidin-biotin complex (ABC) method [14], using a Vectastain ABC kit (Vector Lab., U.S.A.). Briefly, sections were deparaffinized, rehydrated through graded alcohol, quenched with 0.3% H_2O_2 in absolute methanol to block endogenous peroxidase, and incubated with rabbit anti-*H. parasuis* antibodies. Rabbit anti-No. 4, SW124, and Nagasaki of *H. parasuis* hyperimmune sera [7] were used as primary antibodies at a dilution of 1:8192. The slides were successively incubated with ABC and peroxidase activity was demonstrated by treating them with 3,3'-diaminobenzidine tetrahydrochloride. The slides were counterstained with methyl green. A hyperimmune serum against *Actinobacillus pleuropneumoniae* 4074 strain (serovar 1) [10] and pneumonic lung specimens from a pig inoculated with this organism were used as controls. An immunoelectoron microscopical study was also performed on paraffin-embedded brain, spleen and kidney sections. Sections stained by the method described above were fixed with 2% osmic acid in a moisture chamber for 1 hr. After dehydration with ethanol, gelatin capsules filled with Epon were applied to the sections and were embedded. Tissue sections were cut and unstained sections were examined with an electron microscope.

RESULTS

Clinical observation: Results of the clinical examination in the infected pigs are shown in Table 1. Five pigs (Nos. 10, 12, 14, 16 and 17) inoculated with the Nagasaki strain (group 3) died within 48 hr after inoculation. Clinically, there was pyrexia, recumbency and cyanosis. Seven pigs (Nos. 1, 2, 3, 5, 6, 18 and 19), which either died or were killed between 3 to 14 dpi, developed nervous distress including lameness, recumbency and paddling. In contrast, 5 pigs (Nos. 4, 7, 8, 9 and 15) exhibited no clinical signs and were killed at 13 dpi. The two non-infected control pigs (Nos. 20 and 21) showed no abnormality during the observation period.

Endotoxin was detected in the plasma of 10 pigs and its concentration ranged from 0.15 to 432.0 endotoxin units (EU) per m*l*. The volume of endotoxin from 2 pigs (Nos.

Table 1. Results of clinical and bacterial examination of pigs inoculated with *H. parasuis*

Group	Pig No.	Infecting dose	Clinical sign	Course	Endotoxin in plasma ($EU^{f)}$/m*l*)	Recovery of *H. parasuis*			
						Organs[m)]	Brain	Fluid of cavity	Synovial
1[a)]	1	10^{10}	+	K-14 days[d)]	+(6.05)[h)]	−	−	−	−
	2	10^8	+	D- 4 days[e)]	+(0.58)[h)]	−	+	−	−
	3	10^6	+	D- 4 days	+(7.45)[h)]	−	+	+	−
2[b)]	4	10^{10}	−	K-13 days	−	−	−	−	−
	5	10^{10}	+	D-12 days	+(0.31)[i)]	+[n)]	+	+	+
	6	10^8	+	K-13 days	+(0.15)[i)]	−	−	−	−
	7	10^8	−	K-13 days	−	−	−	−	−
	8	10^6	−	K-13 days	−	−	−	−	−
	9	10^6	−	K-13 days	−	−	−	−	−
3[c)]	10	10^{10}	+	D-48 hr	+(0.26)[j)]	+[o)]	+	+	+
	11	10^{10}	+	D-5 days	+(17.06)[k)]	−	+	+	−
	12	10^8	+	D-40 hr	ND[g)]	+[o)]	+	+	+
	13	10^8	+	D- 6 days	+(1.91)[k)]	−	+	−	−
	14	10^6	+	D-40 hr	ND	+[o)]	+	+	+
	15	10^6	−	K-13 days	−	−	−	−	−
	16	10^{10}	+	D-36 hr	+(413.0)[j)]	+[o)]	+	+	+
	17	10^{10}	+	D-36 hr	+(13.55)[j)]	+[o)]	+	+	+
	18	10^8	+	D- 3 days	ND	+[n)]	+	+	+
	19	10^8	+	D- 4 days	ND	+[n)]	+	+	+
4	20	0	−	K-13 days	−	−	−	−	−
	21	0	−	K-13 days	−	−	−	−	−

a) Strain No. 4. b) Strain SW 124. c) Strain Nagasaki. d) K=Killed. e) D=Dead. f) EU=endotoxin unit. g) ND=Not done. h-k) concentration was determined on 4[h)], 7[i)], 2[j)], and 5[k)] dpi, respectively. Figures in parenthesis indicate the peeks of observed values. m) Parenchymal organs. n) Organs with serositis. o) Many organs.

16 and 17) which died within 48 hr and a pig (No. 11) which died on at 5 dpi was higher than the other pigs.

Bacterial examination: Results of the re-isolation of *H. parasuis* from the infected pigs is shown in Table 1. *H. parasuis* was mostly recovered from multiple organ sites of affected pigs. However, *H. parasuis* was isolated only from the nasal cavities in 2 pigs that recovered (Nos. 1 and 6) and 5 apparently healthy pigs (Nos. 4, 7, 8, 9 and 15). Both the control pigs were negative for *H. parasuis* (Nos. 20 and 21).

Pathological examination: The necropsy findings consisted principally of fibrinous or serofibrinous meningitis, polyserositis, and arthritis occurring in various combinations (Table 2). Seven pigs (Nos. 2, 3, 5, 11, 13, 18 and 19) which either died or were killed between 3 to 14 dpi had meningitis, serositis and/or arthritis. The 5 pigs (Nos. 10, 12, 14, 16 and 17) which died within 48 hr after inoculation had septicemic lesions consisting of petechiae or ecchymoses in the liver, kidney and meninges.

Microscopically, these 5 pigs (Nos. 10, 12, 14, 16 and 17) had fibrinous thrombi in the renal glomeruli (Fig. 1), sinusoids of the liver and capillaries of the pulmonary alveolar walls. There was necrosis in the liver and lymph nodes, and edema in the lung. Seven pigs (Nos. 2, 3, 10, 11, 13, 18 and 19) had fibrinopurulent meningitis. In addition, there were regressive change of lymphatic tissues in Nos. 5, 10, 11, 12, 14 and 16–18, mild hepatic cell necrosis in Nos. 2, 5, 10–12 and 18, pericarditis in No. 3 and mild pleuritis in Nos. 11 and 12. Four pigs (Nos. 1, 5, 18 and 19) had fibrinopurulent polyserositis and arthritis. There were no remarkable changes in other organs. No lesions were observed in the controls.

Table 2. Histopathologic findings and detection of *H. parasuis* antigen using immunoperoxidase method

Group	Pig No.	Prevalent lesions	Detection of *H. parasuis* antigen
1	1	Polyserositis, arthritis	+
	2	Meningitis	+
	3	Meningitis, pericarditis	+
2	4	None	−
	5	Polyserositis, arthritis	+
	6	None	−
	7	None	−
	8	None	−
	9	None	−
3	10	Septicemia, meningitis	+
	11	Meningitis, mild pleuritis	+
	12	Septicemia, mild pleuritis	+
	13	Meningitis	+
	14	Septicemia	+
	15	None	−
	16	Septicemia	+
	17	Septicemia	+
	18	Polyserositis, meningitis, arthritis	+
	19	Polyserositis, meningitis, arthritis	+
4	20	None	−
	21	None	−

Ultrastructurally, many bacteria were found in the thrombi of septicemic pigs (Fig. 2). The cell walls of some bacteria were smooth and had Gram-negative features, and the others showed degenerative appearance. Many macrophages and neutrophils found in the inflammatory foci had dilated phagosomes, which included electron-dense and -opaque structures and/or were filled with transparent substance. Bacteria were found in dilated phagosomes of some macrophages and neutrophils. They were degenerative and/or lytic (Figs. 3 and 4).

Immunopathological examination: In the septicemic cases, *H. parasuis* antigen was found on the reticular cells of the spleenic red pulp, sinusoids of the liver, and renal glomerular capillaries. The antigen was also detected in the lumen and endothelial cells of the small blood vessels in the heart, lung, brain, lymph nodes and tonsils. In the pigs with meningitis and/or polyserositis, bacterial antigen was detected in these lesions (Fig. 5). The antigen was found in the cytoplasm of infiltrating macrophages and neutrophils, and in bacteria among these cells. Bacterial antigen was not detected in the nasal cavities of infected pigs. However, small to large quantities of bacterial antigen were observed in the crypt epithelium (Fig. 6) and in the lymphoid follicle of the tonsil of pig No.1 and all the dead pigs. Bacterial antigen was not observed in any tissues of the controls. The *A. pleuropneumoniae*-induced pneumonic lesions did not react with any of the *H. parasuis* antisera evaluated, but only with *A. pleuropneumoniae* antisera.

Ultrastructurally, the bacterial antigen reacted positively with *H. parasuis* antiserum. The antigen was detected in infiltrating macrophages and neutrophils within the lesions of infected pigs. The bacterial antigen was found on the surface of the degenerated bacteria and/or lytic material in the dilated phagosomes (Fig. 7).

DISCUSSION

Fifteen distinct serovars of *H. parasuis* have been distinguished on the basis of immunodiffusion using heat stable antigens [2, 7]. Kielstein and Rapp-Garbrielson [2] demonstrated differences in pathogenicity among strains representing serovars. In their report [2], No. 4 strain (serovar 1) was the most virulent, Nagasaki strain (serovar 5) was moderately virulent and SW124 (serovar 4) was mildly virulent for SPF pigs after intraperitoneal inoculation of this organism. In this study, eleven of thirteen SPF pigs inoculated intranasally with strain Nagasaki or No.4 died between 1 to 6 dpi and had septicemic lesions, polyserositis, pericarditis and meningitis. One of the six SPF pigs inoculated with the SW124 strain died of severe polyserositis, but the remaining five pigs had no lesions. There seems to be some difference in the pathogenicity of these strains for pigs in the present cases as compared to the findings of the earlier report [2]. This could be explained by the difference in the method of preparation of inoculum, inoculation route, and pigs used.

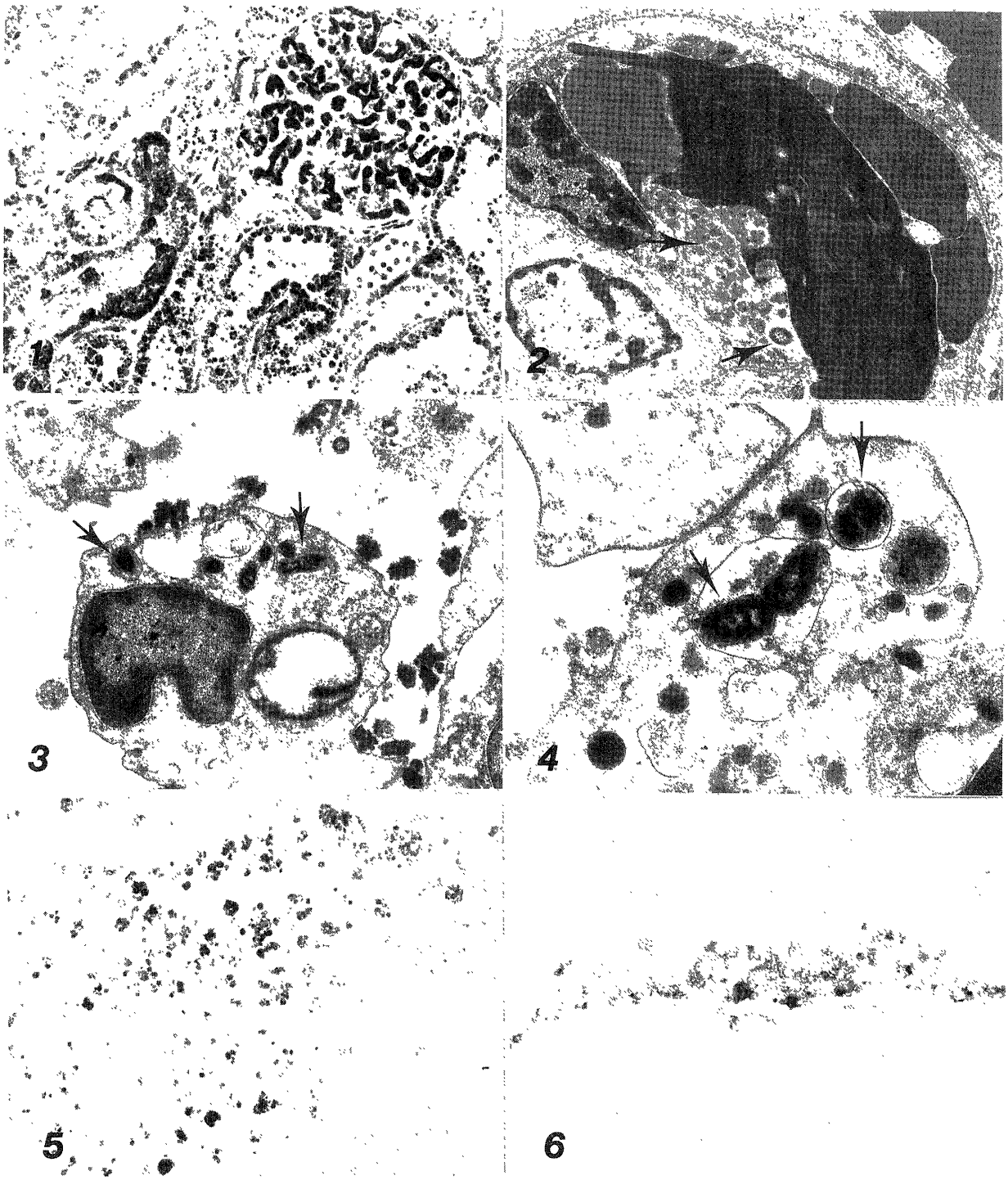

Fig. 1. Multiple fibrin thrombi are found in a glomerulus in pig No. 14 inoculated with strain Nagasaki. Phosphotungstic acid-hematoxylin staining. × 200.

Fig. 2. Many bacteria are found in fibrin thrombi in a glomerulus of pig No. 12 inoculated with strain Nagasaki. Bacteria are intact (arrow) or degenerative (arrow). × 5,400.

Fig. 3. Macrophage infiltrated into the lesion of meningitis in pig No. 11 inoculated with strain Nagasaki. Bacteria (arrows) are seen within dilated phagosomes, which include electron-dense and — opaque substances. × 13,000.

Fig. 4. Macrophage infiltrated into the lesion of meningitis in pig No. 11 inoculated with strain Nagasaki. Bacteria (arrows) seen in the phagosomes are degenerative and lytic. × 26,000.

Fig. 5. Bacterial antigen is seen in the cytoplasm of many infiltrated cells in lesions of pleuritis of pig No. 1 inoculated with strain No. 4. Immunoperoxidase staining. × 200.

Fig. 6. Bacterial antigen is detected in the crypt epithelium of the tonsil of pig No. 13 inoculated with strain Nagasaki. Immunoperoxidase staining. × 160.

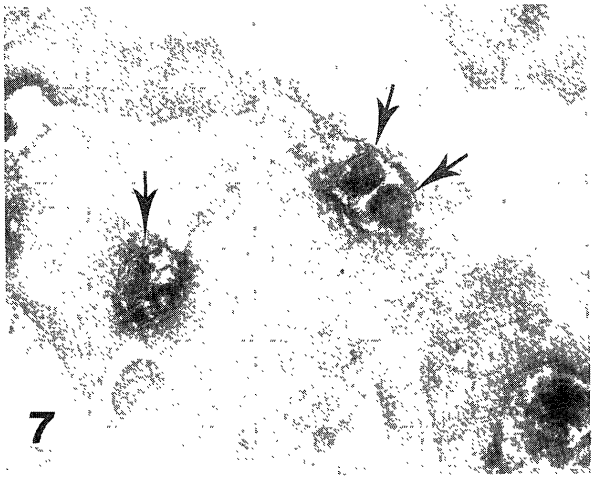

Fig. 7. Bacterial antigen is detected in the dilated phagosomes (arrows) of a macrophage infiltrated in the lesion of meningitis in pig No. 11 inoculated with strain Nagasaki. Immunoperoxidase staining. × 9,400.

Bacterial endotoxin causes various kinds of host responses including fibrin deposition in capillaries and intravascular coagulation [8]. Extensive vascular lesions have often been observed in infected pigs, especially in the cases having no classical lesions of Glasser's disease [1, 15, 16]. Therefore, *H. parasuis* may release the endotoxin into the blood circulation of infected pigs. In this study, endotoxin was detected in the plasma of all the pigs showing clinical signs. Five of them had thrombi in many organs. The present data indicates the septicemia due to *H. parasuis* and endotoxin may play an important role in the pathogenesis of the septicemic lesions.

Numerous round bodies and a few rod bodies were demonstrated in frozen sections of tissues and impression smears from experimentally *H. parasuis* infected pigs by the indirect immunoflourescent antibody technique [9]. In this study, *H. parasuis* antigen was detected mainly in the cytoplasm of macrophages and neutrophils in lesions of polyserositis. These phagocytosed bacteria seemed to be degenerated and digested in the infiltrating cells. Immunoelectron microscopically, many infiltrating neutrophils and macrophages contained degenerating bacteria in the cytoplasm. Bacterial antigen was detected in degenerated and/or lytic bacteria within dilated phagosomes. These results suggest that *H. parasuis* are phagocytosed, killed, and digested by inflammatory cells in the early stage of infection. On the other hand, intact bacteria were found in lesions of septicemic pigs, but many of them seen in the blood vessels of pigs with septicemic lesions were degenerated. These findings support the reports describing difficulty in isolation of *H. parasuis* from lesions in the post-febrile stage of this disease; organisms may disappear soon from the visceral organs [6, 9]. We could not demonstrate the round bodies that have been reported by Neil *et al.* [9]. This may be due to differences in the sensitivity of detection between the flourescent antibody technique and the ABC method. However, the distribution of *H. parasuis* antigen by the latter method was correlated with the results of isolation of this organism. In addition, *H. parasuis* antigen was detected in lesions of pig No. 1 from which the bacteria were not isolated. These data suggest that the ABC method is a useful tool in the diagnosis of this disease.

It was considered that the area where the inoculated bacteria first colonized was the respiratory tract. Although *H. parasuis* was reisolated from nasal secretions of infected pigs, bacterial antigen was not detected in their nasal cavities. Neither lesion nor bacterial antigen was observed in the lung. On the other hand, *H. parasuis* antigen was detected both in the crypt epithelium and the blood vessels of the tonsil of infected pigs. These findings imply that the pig's tonsil must be considered as the site for harboring this organism. The primary site of infection of this organism in naturally occurring Glasser's disease has not been precisely decided. Further investigation is required to elucidate this.

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