Experimental Dual Infection of Cesarean-Derived, Colostrum-Deprived Pigs with *Mycoplasma hyopneumoniae* and Pseudorabies Virus

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ABSTRACT. To determine whether pseudorabies virus (PRV) infection increases the severity of pneumonia by *Mycoplasma hyopneumoniae*, 18, 10-week-old Cesarean-derived, colostrum-deprived pigs were randomly assigned to 3 groups of 6 pigs each. Pigs in groups A and C were inoculated intranasally with *M. hyopneumoniae* at 10-week-old. At 11-week-old, pigs in groups B and C were inoculated intranasally with PRV. All pigs were initially seronegative for *M. hyopneumoniae* and PRV. Three pigs of each group were euthanized at 12-week-old, and remaining pigs at 14-week-old. At necropsy, gross lesions in the lung were observed in the pigs of groups A and C. On post-inoculation-week (PIW) 2 with *M. hyopneumoniae* (at 12-week-old), lung lesions were recognized in one of the 3 pigs in group A and all the pigs in group C. The mean percentage of the lung lesions were 0.1% in group A and 9.8% in group C. *M. hyopneumoniae* was isolated from broncho-alveolar lavage fluids (BALF) of pigs in group A with titer of 10^2 to 10^3 CCU/0.2 m*l* and in group C with titer of 10^5 to 10^6 CCU/0.2 m*l*. On PIW 4 (at 14-week-old), lung lesions were observed in all the pigs in groups A and C, and the mean percentage of the lung lesions were 8.3% in group A and 17.2% in group C. *M. hyopneumoniae* was isolated from BALF in group A with titer of 10^4 to 10^7 CCU/0.2 m*l* and in group C with titer of 10^6 to 10^7 CCU/0.2 m*l*. PRVs were isolated from nasal swab and tissue samples in groups B and C. After inoculation, antibody against *M. hyopneumoniae* was detected in groups A and C, and against PRV in groups B and C. Under the present experimental conditions, PRV infection appear to have effect on the severity of experimentally induced acute mycoplasmal pneumonia in young pigs. — KEY WORDS: BALF, dual infection, *Mycoplasma hyopneumoniae*, pseudorabies virus, swine.

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Mycoplasmal pneumonia of swine, caused by *Mycoplasma hyopneumoniae* is a widespread, chronic respiratory disease of growing pigs. *M. hyopneumoniae* infection in pigs reflects a considerable economic problem owing to reduced feed efficiency and weight gain. The disease is often aggravated by secondary bacterial infection in common in field pig herds [3].

On the other hand, pseudorabies virus (PRV) infection causes nervous disorders and mortality in young piglets, and abortion in pregnant sows. Furthermore, PRV infection in growing pigs is sometimes associated with respiratory disease [12, 25]. It has been reported that pig alveolar macrophages (AM) which play an important role in defense against infectious agents for the lung are highly susceptible to PRV [9, 24], and PRV infection in AM effect on the cell function [4, 10, 11]. PRV infection in AM may induce immunosuppression and interfere with bacterial clearance in the lung. The pigs infected with PRV plus *Pasteurella multocida* [8], *Streptococcus suis* [13] or *Haemophilus parasuis* [20] caused severe pneumonia. Recently, Sakano *et al.* [22] reported that the pigs inoculated with PRV plus *Actinobacillus pleuropneumoniae* type 1 developed severe pneumonia than *A. pleuropneumoniae* alone and with PRV alone developed no pneumonia. Up to date, however, influence of PRV infection on the development of mycoplasmal pneumonia is not known.

The purpose of this study was to determine whether PRV infection increases the severity of experimentally induced *M. hyopneumoniae* infection in young pigs.

MATERIALS AND METHODS

Pigs: Eighteen 10-week-old Cesarean-derived, colostrum-deprived (CDCD) pigs were used for experimental examination. Each group of pigs was housed separately in barrier-maintained room.

Virus and mycoplasma strains: PRV strain Yamagata S-81 [6] was obtained from Dr. Imada (National Institute of Animal Health, Japan). *M. hyopneumoniae* strain E-1 [16] supplied by Dr. Mori (National Institute of Animal Health, Japan) was used for inoculum. The strain had been passaged in CDCD pigs and cultivated once in BHL medium (see below). Culture fluid containing *M. hyopneumoniae* was shown to be pathogenic for pigs and free of culturable virus and bacteria.

Cell cultures: CPK cells derived from pig kidney [14] were used for a virus isolation and neutralization test. The growth and maintenance media (MM) used were described previously [23].

Experimental design: The pigs were randomly assigned to 3 groups of 6 pigs each. At 10-week-old, pigs in groups A (*M. hyopneumoniae* alone) and C (*M. hyopneumoniae* plus PRV) were inoculated intranasally with 10^6 color changing units (CCU) of *M. hyopneumoniae*. Next day, they were inoculated intranasally again with 10^4 CCU. A week later, pigs in groups B (PRV alone) and C were inoculated intranasally with $10^{5.5}$ $TCID_{50}$ of PRV. Three pigs of each group were euthanized and necropsied on post-inoculation-week (PIW) 2 with *M. hyopneumoniae* at

12-week-old, and the remaining pigs were done on PIW 4 at 14-week-old.

After inoculation, the pigs were observed for clinical signs of disease and the rectal temperature was taken daily. Nasal swabs were collected every other day for virus isolation during the period of observation. Blood samples for serological test were collected weekly until slaughter. At necropsy, tissue samples (the cerebrum, cerebellum, olfactory bulb, medulla oblongata, trigeminal ganglia, mandibular lymph node, tonsil, lung, heart, liver, spleen, kidney and adrenal gland) were collected for virus isolation.

Broncho-alveolar lavage fluids (BALF) was harvested as follows. Lungs and the trachea were collected at necropsy. A silicone rubber balloon catheter (Terumo Inc., Japan) was inserted into the bronchus of right frontal pulmonary lobe, and 20 m*l* of phosphate-buffered saline (PBS) were infused and aspirated three times. Collected BALF was used for *M. hyopneumoniae* isolation.

Mycoplasma and bacteria isolation: Mycoplasma culture was done from BALF samples in BHL medium contained 200 μg/m*l* ampicillin and 3% anti *M. hyorhinis* rabbit serum to suppress the growth of *M. hyorhinis* were diluted in a series of 10-fold dilution with the same medium. BHL medium was prepared as described previously [27], except that swine serum was used instead of horse serum. Incubation was carried out at 37°C. Cultures with acid shift and/or turbidity were subcultured 2 times in BHL medium after filtration through a 0.45 μm filter. If no color change was observed after 2 months of inoculation, the cultures were judged as negative.

For isolation of bacteria, the remainder of each sample was inoculated on 5% sheep blood agar plate. Suspect colonies were identified by standard microbiological methods.

Virus isolation from nasal swabs and tissues: Virus isolation was carried out by the microtiter method described previously [23].

Complement fixation (CF) test: The serum antibody against *M. hyopneumoniae* was measured by CF test. The CF test was performed as described previously [16] using SEP-CF antigen (Synthetic Feed Laboratory Co., Ltd., Japan).

Virus neutralization (VN) test: The test was carried out by the microtiter method described previously [23] with a slight modification. The serum-virus mixtures were incubated at 37°C for 24 hr.

Pathological examination: To determine the extent of gross pneumonia, the ratio of gross lesions was calculated using an image processor (Nexus, 600, Tokyo, Japan) [1]. Histopathological examination was performed according to routine procedures. In brief, lung tissue samples were fixed in 20% neutral phosphate-buffered formalin. Thin sections of paraffin embedded samples were stained by hematoxylin and eosin.

RESULTS

No clinical sign was recognized in the pigs after inoculation with *M. hyopneumoniae*. After inoculation with PRV, depression, nasal mucus and anorexia were observed in groups B and C. A febrile response was observed from post-inoculation-day (PID) 3 with PRV in all the pigs inoculated with PRV (Fig. 1). A slight coughing was transiently observed in several pigs in groups B and C on PID 4 to 14 with PRV. Difference in severity of clinical sign was not recognized between groups B and C. In group A, clinical signs were inapparent throughout the examination. Mean weight gains at necropsy between 10- and 12-week-old were 4.7 ± 2.4, 2.2 ± 1.6 and 3.4 ± 1.0 kg and between 10- and 14-week-old were 15.2 ± 1.9, 13.6 ± 0.7 and 10.8 ± 4.5 kg in groups A, B and C, respectively.

The main gross lesion recorded at necropsy was consolidation in the lungs in groups A and C. On PIW 2 with *M. hyopneumoniae*, gross lung lesions were observed in one of the 3 pigs in group A and all the pigs in group C (Table 1). The mean percentage of the lung lesions were 0.1% in group A and 9.8% in group C. On PIW 4, lung lesions were observed in all the pigs in groups A and C and the mean percentage of the lesions were 8.3% in group A and 17.2% in group C. Lung lesions were recognized mainly at the cranial, middle and accessory lobe. In all the pigs in group B, gross lung lesions were undetectable.

Histopathological lung lesions of groups A and C were characterized by peribronchiolar and perivascular cuffings consisting of lymphocytes. Macrophages, polymorphonuclear lymphocytes and plasma cells accumulated in alveoli and lumina of airways. Apparent differences in histopathological findings were not recognized between in groups A and C. In group B, mild peribronchiolar lymphocyte infiltration was observed on

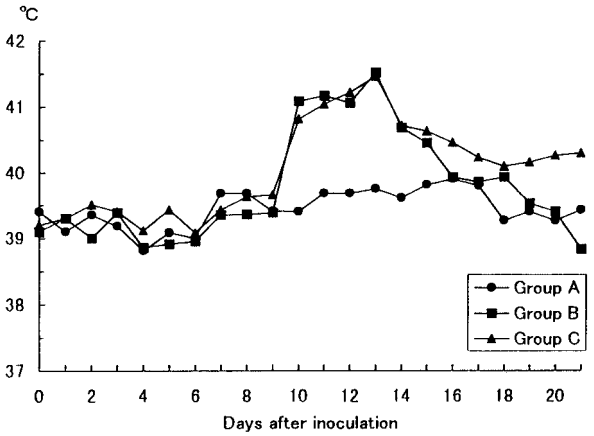

Fig. 1. Mean body temperature of experimental pig group. Pigs in groups A and C were inoculated with *M. hyopneumoniae* on day 0 and 1, and in groups B and C were inoculated with PRV on day 7.

Table 1. Pneumonic lesions and isolation of *M. hyopneumoniae* from BALF collected from pigs inoculated with *M. hyopneumoniae* and/or PRV

Experiment group	Inoculation with		Pig No.	Observation period (weeks)	Pneumonic lesions in lung (%)	*M. hyopneumoniae* isolation from BALF[a)]
	M. hyopneumoniae (CCU)	PRV ($TCID_{50}$)				
A	10^6, 10^4	None	1	2	0	3[b)]
			2	2	0	2
			3	2	0.3	2
			4	4	19.2	6
			5	4	4.2	7
			6	4	1.4	4
B	None	$10^{5.5}$	7–9	2	0	–[c)]
			10–12	4	0	–
C	10^6, 10^4	$10^{5.5}$	13	2	3.7	5
			14	2	7.8	5
			15	2	17.9	6
			16	4	12.2	6
			17	4	9.0	6
			18	4	30.5	7

a) Broncho-alveolar lavage fluids. b) log CCU/0.2 m*l*. c) *M. hyopneumoniae* not detected.

Table 2. Virus isolation from nasal swabs and tissues from pigs inoculated

Experiment group	Pig No.	Nasal swabs: days after inoculation									Tissues specimens				
		0	1	3	5	7	9	11	13	14	Olfactory bulb	Trigeminal ganglia	Mandibular lymph node	Tonsil	Lung
A	1–6	–[a)]	–	–	–	–	–	–	–	–	–	–	–	–	–
B	7	–	3.5[b)]	5.75	4.75	4.0					–	1.0	–	1.0	–
	8	–	3.5	7.0	6.0	5.25					–	1.25	–	4.25	–
	9	–	3.75	6.0	6.25	5.5					1.75	2.75	2.75	4.5	2.25
	10	–	1.0	6.0	6.25	6.0	3.25	0.75	1.0	1.0	–	–	–	2.5	–
	11	–	4.75	5.75	6.0	5.75	3.25	–	0.75	1.25	–	–	–	4.25	–
	12	–	3.0	5.0	5.0	4.25	2.0	3.0	–	–	–	–	–	–	–
C	13	–	4.25	4.75	4.75	2.5					–	–	–	0.75	–
	14	–	3.0	6.5	5.0	2.5					–	1.0	–	2.25	–
	15	–	1.75	5.75	5.0	2.5					–	1.75	–	3.5	–
	16	–	–	4.75	7.0	5.25	2.0	–	–	–	–	–	–	0.75	–
	17	–	4.25	5.25	5.0	4.75	0.75	1.25	–	–	–	–	–	0.75	–
	18	–	–	6.0	5.25	4.25	4.0	4.5	4.25	3.75	–	–	–	2.0	–

a) Virus not detected. b) log $TCID_{50}$/0.05 m*l*.

PIW 1 with PRV.

M. hyopneumoniae was isolated from all BALF samples collected from pigs in groups A and C (Table 1). In group B, *M. hyopneumoniae* was not isolated. The amounts of *M. hyopneumoniae* in BALF collected on PIW 2 were 10^2 to 10^3 CCU/0.2 m*l* in group A and 10^5 to 10^6 CCU/0.2 m*l* in group C. On PIW 4, the amounts were 10^4 to 10^7 CCU/0.2 m*l* in group A and 10^6 to 10^7 CCU/0.2 m*l* in group C. From one lung sample of No. 14, *Streptococcus* spp was isolated.

PRV was isolated from nasal swab samples of pigs in groups B and C between PID 1 and 14 (Table 2). At necropsy, PRV was isolated from tonsils of all the inoculated pigs except No. 12, and the olfactory bulb, trigeminal ganglia, mandibular lymph node and lung of some pigs in these groups (Table 2).

CF antibody against *M. hyopneumoniae* was not detected at the inoculation with the organism. CF antibody was first detected on PIW 2 to 4 in groups A and C (Table 3). On PIW 4, its titers ranged between 1:4 and 1:32. All the pigs in group B were negative for CF antibody through the examination. NT antibody against PRV was first detected on PIW 1 to 3 with PRV in groups B and C (Table 3), and the titers ranged between 1:8 and 1:1,024 on PIW 3. In group A, all the pigs were negative for NT antibody throughout the examination.

Table 3. Serum antibody titers against *M. hyopneumoniae* or PRV

Experimental group	Pig No.	Weeks after inoculation with *M. hyopneumoniae*[a)]					PRV[b)]			
		0	1	2	3	4	0	1	2	3
A	1	<4	<4	<4			<2	<2	<2	
	2	<4	<4	<4			<2	<2	<2	
	3	<4	<4	<4			<2	<2	<2	
	4	<4	<4	8	16	32	<2	<2	<2	<2
	5	<4	<4	<4	4	8	<2	<2	<2	<2
	6	<4	<4	<4	4	8	<2	<2	<2	<2
B	7	<4	<4	<4			<2	<2		
	8	<4	<4	<4			<2	<2		
	9	<4	<4	<4			<2	<2		
	10	<4	<4	<4	<4	<4	<2	<2	64	256
	11	<4	<4	<4	<4	<4	<2	<2	64	256
	12	<4	<4	<4	<4	<4	<2	<2	128	256
C	13	<4	<4	<4			<2	64		
	14	<4	<4	<4			<2	<2		
	15	<4	<4	<4			<2	<2		
	16	<4	<4	<4	<4	16	<2	<2	128	1024
	17	<4	<4	<4	16	32	<2	<2	128	512
	18	<4	<4	<4	<4	4	<2	<2	<2	8

a) Complement fixation antibody titer. b) Neutralizing antibody titer.

DISCUSSION

The present experimental conditions using CDCD pigs caused pneumonia in all the infected pigs in groups A and C inoculated with *M. hyopneumoniae*. The pigs in group C inoculated with *M. hyopneumoniae* followed by the inoculation with PRV developed more severe lung lesions than those in group A inoculated with *M. hyopneumoniae* alone. The pigs in group B inoculated with PRV alone did not have any gross lung lesion. Development of clinical features observed in groups B and C almost depended on the PRV infection, not on *M. hyopneumoniae* infection. On PIW2, the amount of *M. hyopneumoniae* in BALF collected from pigs in group C was considerably higher than that in group A. Consequently, PRV infection might have influence on the growth of *M. hyopneumoniae* in the lung and development of lung lesions at the early stage of mycoplasma infection.

Little is known about the association between amount of *M. hyopneumoniae* in BALF and the ratio of gross lung lesion. In our previous experimental studies, there were no correlation between these values, however, BALF collected from the lung with lesions was usually had more than 10^4 or 10^5 CCU of *M. hyopneumoniae* (unpublished date). The present date was coincident with the previous results. Amount of *M. hyopneumoniae* in the lung may be associated to the stage of infection and each lung lobe or region may be not on the same stage. BALF is collected from restricted area of the lung and *M. hyopneumoniae* titer in BALF dose not reflect the mean titer of all over the tissue.

The amount of PRV in nasal swab and tissues was not influenced by the dual infection. Dual infection also might not effect on the antibody production against *M. hyopneumoniae* and PRV. In swine mycoplasmosis, the humoral responses against *M. hyopneumoniae* correlate poorly with recovery or protection from mycoplasmosis [17].

PRV infection has low mortality, but high morbidity in growing finishing pigs, and is sometimes associated with respiratory tract disease. It is reported that some PRV strains produce pulmonary lesions in addition to lesion in the central nervous system, and pigs inoculated with PRV develop mild respiratory disease [12, 25]. On the other hand, pneumonic lesions were not observed in pigs inoculated intranasally with the Yamagata S-81 strain which was same strain used in the present study [18]. In the present study, pneumonic lesions except lymphocyte infiltration were not observed in group B infected with PRV alone. At early stage of the infection within PID 7, pneumonic lesion by PRV infection might be observed. In groups A and C, pigs had lung lesions characterized by peribronchiolar and perivascular cuffing of infiltrated cells. These lesions resembled those already described in *M. hyopneumoniae* infection of pigs [26].

PRV is thought to assist bacterial proliferation [19, 24]. Pigs inoculated with a virulent strain of PRV plus *P. multocida* developed severe pneumonia, whereas pigs given PRV alone did not develop pneumonia [5]. Likewise, clinical disease associated with *S. suis* type 2 was enhanced by dual infection with PRV [13]. Furthermore, we recently indicated that pigs inoculated with PRV plus *A. pleuropneumoniae* developed more severe clinical symptoms and pneumonia than that with PRV or *A. pleuropneumoniae* alone [22]. Our present study indicated that PRV infection

had apparent effect on the severity of experimentally induced acute mycoplasmal pneumonia. However, at this time, the exact mechanism to promote mycoplasmal pneumonia by the dual infection is not known.

The AM that has many important functions, such as phagocytosis and killing of organisms, cytotoxicity against infected cells and production of cytokines are the most important defense line in the respiratory tract [15]. Clearance of organisms by AM is clearly a primary host defense mechanism against infection of bacteria, viruses and fungi [15]. *In vitro* examinations have demonstrated that swine AM are susceptible to PRV [9, 24], and PRV infection in AM effects on the cell function, such as phagocytosis of bacteria, phagosome-lysosome function and O_2 release [4, 10]. In addition, PRV infection of AM had a deleterious effect upon cytotoxicity and production of cytokine [11]. It has been reported that PRV was isolated from swine AM collected from postmortal pigs infected with PRV between on PID 2 and 7 [12], and PRV antigens were detected in BALF cells between on PID 3 and 10 [20]. Furthermore we recently demonstrated that PRV was isolated from AM collected from pigs experimentally infected with the Yamagata S-81 strain used in the present study between PID 2 and 22 [24]. At least during that period, PRV infection in AM might reduced its function and render the lung more susceptible to bacterial infection. It has been reported that phagocytosis of *Mycoplasma dispar, Mycoplasma agalactiae* and *Mycoplasma bovis* by bovine AM were enhanced by the presence of specific antibody [7, 8], and opsonization by complement induced efficient phagocytosis by macrophages of *Mycoplasma pneumonia* [2]. Although there is little information in published literature describing the *in vivo* function of AM against *M. hyopneumoniae* infection, it is possible to speculate that damage of AM function by PRV infection induce an impairment of the defense mechanism in the lung and promote development of mycoplasmal pneumonia, especially at acute stage of infection.

The mucociliary escalator plays important role in the lung's physical defense against organisms. It was reported that the pigs which the mucociliary escalator was suppressed showed severe pleuropneumonia after inoculation with a small amount of *Actinobacillus pleuropneumoniae* [21]. In PRV infection, ciliary activity in the respiratory mucosa is disrupted and mucociliary clearance is significantly reduced [19]. Consequently, suppression of mucociliary clearance by PRV infection may also influence the severity of mycoplasmal pneumonia. Further extensive studies are needed to investigate the mechanism to enhance mycoplasmal pneumonia following PRV infection.

PRV is relatively common infection of pigs in the infected area. The PRV infection may become chronic and subclinical in closed herds with none of apparent clinical signs. In such herd, it might be assumed that PRV infection had some influence on the spread of the mycoplasma or bacteria present in the respiratory tract and the development of clinical feature and lung lesion.

Under the present experimental conditions, PRV infection appear to have an effect on the severity of experimentally induced acute mycoplasma pneumonia in young pigs.

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REFERENCES

1. Asai, T., Okada, M., Ono, M., Irisawa, T., Mori, Y., Yokomizo, Y. and Sato, S. 1993. Increased levels of tumor necrosis factor and interleukin 1 in bronchoalveolar lavage fluids from pigs infected with *Mycoplasma hyopneumoniae*. *Vet. Immunol. Immunopathol.* 38: 253–260.
2. Bredt, W. 1975. Phagocytosis by macrophages of *Mycoplasma pneumoniae* after opsonization by complement. *Infect. Immun.* 12: 694–695.
3. Christensen, G. and Mousing, J. 1992. Respiratory system. pp. 138–162. *In*: Disease of Swine (Leman, A. D., Straw, B. E., Mengeling, W. L., D'Allaire, S. and Taylor, D. J. eds.), Iowa State University Press, Iowa, U.S.A.
4. Fuentes, M. and Pijoan, C. 1986. Phagocytosis and intracellular killing of *Pasteurella multocida* by porcine alveolar macrophages after infection with pseudorabies virus. *Vet. Immunol. Immunopathol.* 13: 165–172.
5. Fuentes, M. and Pijoan, C. 1987. Pneumonia in pigs induced by intranasal challenge exposure with pseudorabies virus and *Pasteurella multocida*. *Am. J. Vet. Res.* 48: 1446–1448.
6. Fukusho, A., Shimizu, M., Kudo, M., Nanba, K., Shimizu, Y., Konno, S., Suzuki, K. and Otaki, T. 1981. The outbreak of Aujeszky's disease in swine in Japan. II. Virus isolation. *Bull. Natl. Inst. Anim. Health 82*: 5–11 (in Japanese).
7. Howard, C. J. 1984. Comparison of bovine IgG1, IgG2 and IgM for ability to promote killing of *Mycoplasma bovis* by bovine alveolar macrophages and neutrophils. *Vet. Immunol. Immunopathol.* 6: 321–326.
8. Howard, C. J., Taylor, G., Collins, J. and Gourlay, R. N. 1976. Interaction of *Mycoplasma dispar* and *Mycoplasma agalactiae* subsp. *bovis* with bovine alveolar macrophages and bovine lacteal polymorphonuclear leukocytes. *Infect. Immun.* 14: 11–17.
9. Iglesias, G., Pijoan, C. and Molitor, T. 1989. Interactions of pseudorabies virus with swine alveolar macrophages I: virus replication. *Arch. Virol.* 104: 107–115.
10. Iglesias, G., Pijoan, C. and Molitor, T. 1989b. Interactions of pseudorabies virus with swine alveolar macrophages: Effects of virus infection on cell function. *J. Leukocyte Biol.* 45: 410–415.
11. Iglesias, G., Pijoan, C. and Molitor, T. 1992. Effects of pseudorabies virus infection upon cytotoxicity and antiviral activities of porcine alveolar macrophages. *Comp. Immun. Microbiol. Infect. Dis.* 15: 249–259.
12. Iglesias, G. J., Trujano, M., Lokensgard, J. and Molitor, T. 1992. Study of the potential involvement of pseudorabies virus in swine respiratory disease. *Can. J. Vet. Res.* 56: 74–77.
13. Iglesias, G. J., Trujano, M. and Xu, J. 1992. Inoculation of pigs with *Streptococcus suis* type 2 alone or in combination with pseudorabies virus. *Am. J. Vet. Res.* 53: 364–367.
14. Komaniwa, H., Fukusho, A. and Shimizu, Y. 1981. Micro method for performing titration and neutralization test of hog

cholera virus using established porcine kidney cell strain. *Natl. Inst. Anim. Health Q*. 21: 153–158.
15. Lohmann-Matthes, M-L., Steinmuller, C. and Franke-Ullmann, G. 1994. Pulmonary macrophages. *Eur. Respir. J*. 7: 1678–1689.
16. Mori, Y., Yoshida, Y., Kuniyasu, C. and Hashimoto, K. 1983. Improvement of complement fixation test antigen for the diagnosis of *Mycoplasma hyopneumoniae* infection. *Natl. Inst. Anim. Health Q*. 23: 111–116.
17. Murphy, D. A., Van Alstine, W. G., Clark, L. K., Albregts, S. R. and Knox, K. E. 1993. Aerosol vaccination of pigs against *Mycoplasma hyopneumoniae* infection. *J. Am. Vet. Res*. 11: 1874–1880.
18. Narita, M., Imada, T. and Haritani, M. 1990. Comparative pathology of HPCD pigs infected with wild-type and ara-T-resistant strains of Aujeszky's disease virus. *J. Comp. Pathol*. 102: 63–69.
19. Narita, M., Imada, T. and Kobayashi, M. 1991. Pathogenesis of Aujeszky's disease virus infection in swine tracheal organ culture. *J. Comp. Pathol*. 105: 439–444.
20. Narita, M., Kawashima, K., Matsuura, S., Uchimura, A. and Miura, Y. 1994. Pneumonia in pigs infected with pseudorabies virus and *Haemophilus parasuis* serovar 4. *J. Comp. Pathol*. 110: 329–339.
21. Narita, M., Kawashima, K., Morozumi, T. and Takashima, H. 1995. Effect of physical defenses of the respiratory tract on the development of pneumonia in pigs inoculated endobronchially with *Actinobacillus pleuropneumoniae*. *J. Vet. Med. Sci*. 57: 839–844.
22. Sakano, T., Shibata, I., Samegai, Y., Taneda, A., Okada, M., Irisawa, T. and Sato, S. 1993. Experimental pneumonia of pigs infected with Aujeszky's disease virus and *Actinobacillus pleuropneumoniae*. *J. Vet. Med. Sci*. 55: 575–579.
23. Shibata, I., Hatano, Y. and Inaba, Y. 1992. Multiplication and immunogenicity of a temperature-sensitive and thymidine kinase-deficient strain of Aujeszky's disease virus in pigs. *J. Vet. Med. Sci*. 54: 1111–1115.
24. Shibata, I., Uruno, K., Samegai, Y., Okada, M. and Inaba, Y. 1994. Replication of virulent and attenuated strains of Aujeszky's disease virus in swine alveolar macrophages. *J. Vet. Med. Sci*. 56: 465–468.
25. Vannier, P. 1987. Aujeszky's disease virus and respiratory disease in pig. *Rec. Med. Vet*. 163: 407–417.
26. Yagihashi, T., Nunoya, T., Mitui, T. and Tajima, M. 1984. Effect of *Mycoplasma hyopneumoniae* infection on the development of *Haemophilus pleuropneumoniae* pneumonia in pigs. *Jpn. J. Vet. Sci*. 46: 705–713.
27. Yamamoto, K., Koshimizu, K. and Ogata, M. 1986. *In vitro* susceptibility of *Mycoplasma hyopneumoniae* to antibiotics. *Jpn. J. Vet. Sci*. 48: 1–5.