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Detection of Porcine Reproductive and Respiratory Syndrome Virus and *Mycoplasma hyorhinis* Antigens in Pulmonary Lesions of Pigs Suffering from Respiratory Distress

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Summary

Eleven field cases of a disease characterized by severe dyspnoea or abdominal breathing were examined *post mortem*. The affected pigs had antibody against porcine reproductive and respiratory syndrome virus (PRRSV). The predominant lung lesions were severe proliferative and interstitial pneumonia, and slight suppurative bronchopneumonia. The lesions were closely associated with the sites at which PRRSV and *Mycoplasma hyorhinis* antigens were detected. Four of five pigs inoculated with PRRSV developed slight pneumonitis. The fifth animal, which died of severe pneumonitis, yielded a heavy culture of *M. hyorhinis*. These findings demonstrate that dual infection with *M. hyorhinis* and PRRSV caused severe pulmonary lesions.

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Introduction

Pneumonia is a worldwide problem for the pig industry. A recent outbreak of pneumonia in Japan was aetiologically linked to porcine reproductive and respiratory virus (PRRSV) (Murakami *et al.*, 1994; Shimizu *et al.*, 1994), which was first isolated from pigs with reproductive failure or respiratory disease in the Netherlands (Terpstra *et al.*, 1991; Wensvoort *et al.*, 1991). The pneumonic features may vary with the presence of other organisms. In the USA and Great Britain, organisms isolated from PRRSV-seropositive herds with severe endemic respiratory disease have included porcine respiratory coronavirus (Halbur *et al.*, 1993), swine influenza virus (Kay *et al.*, 1994), *Pasteurella multocida* (Hopper *et al.*, 1992) and *Salmonella choleraesuis* (Stevenson *et al.*, 1993). However, there has been no description of a relationship between PRRSV and *Mycoplasma hyorhinis* in pig pneumonia.

This paper describes an immunohistopathological and microbiological study of the lung lesions in 11 field cases of severe respiratory disease and five experimentally infected pigs, and throws light on the effects of dual infection with *M. hyorhinis* and PRRSV.

Materials and Methods

Field Cases

Eleven pigs aged 1 to 2 months and showing severe respiratory distress (dyspnoea or abdominal breathing) were collected from three pig farms in Chiba prefecture, an

area in which PRRSV had already been demonstrated by virological and serological examinations (Murakami *et al.*, 1994; Shimizu *et al.*, 1994). The pigs were humanely killed and subjected to post mortem examination.

Experimental Cases

Five specific pathogen-free pigs, aged 2 months and seronegative for PRRSV, were housed in isolation units. Each pig was inoculated intranasally with 1 ml of a suspension containing $10^{6.0}$ TCID₅₀ of the Chiba 92-1 strain of PRRSV (Shimizu *et al.*, 1994). The inoculated pigs were observed for clinical signs, killed 35 days later, necropsied, and examined by the same methods as those (see below) used for the field cases.

Viral, Bacterial and Serological Examinations

Viral isolation was carried out as described by Wensvoort *et al.* (1991). Briefly, after centrifugation of 10% lung homogenates, the supernates were inoculated into porcine alveolar macrophage cultures. The cells were observed daily for cytopathogenic effects and infection was confirmed by indirect immunofluorescence. Bacteria (*Haemophilus* sp., *Actinomyces* sp., *Pasteurella* sp., *Streptococcus* sp. and others) were isolated on YTB agar, consisting of trypticase soy agar medium (Difco, Detroit, USA) containing fresh yeast extract 1% (Gibco, Paisley, Scotland) and horse blood 5%. Isolates were identified by biochemical tests (Holt *et al.*, 1994). *M. hyorhinis* and *M. hyosynoviae* were isolated in M-broth (5% mucin [Difco] -supplemented PPLO broth [Difco]) and on M-agar (Noble agar [Difco] 1% in M-broth), and *Mycoplasma hyopneumoniae* in BHL broth (Difco) and on BHL agar (Difco). The isolates were identified by biochemical properties (Aluotto *et al.*, 1970) and metabolism inhibition tests. Antibody to PRRSV was detected by an enzyme-linked immunosorbent assay (IDEXX Laboratories, Westbrook, Maine, USA).

Pathological Examination

At necropsy, the lungs were examined visually and each cranial lobe was fixed in 10% buffered formalin and embedded in paraffin wax. Sections 4 to 5 µm thick were cut and stained with haematoxylin and eosin (HE) or by an immunohistochemical technique.

Immunohistochemical Examination

The PRRSV and selected bacterial antigens were demonstrated with an avidin-biotin-complex immunoperoxidase kit (Vector Laboratories, Burlingame, CA, USA). Embedded tissues were dewaxed, rehydrated through graded alcohols into phosphate-buffered saline pH 7.4, and then treated with actinase E (Kaken Pharmaceutical Co. Ltd, Tokyo) 0.1% in phosphate buffered saline for 5 min at 37°C. The sections were incubated for 1 h with anti-PRRSV (Chiba 92-1 strain) rabbit serum at a dilution of 1 in 8192, or with one of the following antibacterial rabbit sera: mixed anti-*Actinobacillus pleuropneumoniae* (serovars 1-6) serum at a dilution of 1 in 8192 (provided by Drs K. Yamamoto and T. Morozumi, National Institute of Animal Health, Japan), mixed anti-*Haemophilus parasuis* (serovars 1-4) serum at a dilution of 1 in 16 384 (provided by Dr T. Morozumi), mixed anti-*P. multocida* (serovars A, B, D and E) serum at a dilution of 1 in 8192 (provided by Drs K. Hashimoto and S. Takeuchi, National Institute of Animal Health), mixed anti-*Escherichia coli* (serovars O-8, 9, 15, 45, 78, 88, 101, 117, 153) serum at a dilution of 1 in 4096 (provided by Dr M. Nakazawa, National Institute of Animal Health), anti-*M. hyopneumoniae* serum at a dilution of 1 in 8192 (provided by Dr Y. Imada, National Institute of Animal Health) and anti-*M. hyorhinis* serum at a dilution of 1 in 4096. Sections were counterstained with methyl

green. Tissue sections from non-infected gnotobiotic pigs and serum from a non-immunized rabbit were used for control purposes.

Results

Field Cases

The macroscopical changes, distributed throughout the lungs, were a failure to collapse, consolidation and moderate interlobular oedema. The lung lesions were of two histopathological types. One type was characterized by severe proliferative and interstitial pneumonia, with marked infiltration of mononuclear cells; the alveolar spaces contained proteinaceous debris, degenerating cells and macrophages (Fig. 1a). In the other type, proliferative and interstitial pneumonia was complicated by suppurative bronchopneumonia, the alveolar septa being moderately thickened by cellular proliferation and most alveolar spaces being occupied by neutrophils and macrophages (Fig. 1b); occasionally, the lumina of the bronchioles contained neutrophils and proteinaceous debris. In both types of lesion there was slight peribronchiolar lymphocytic hyperplasia.

Immunohistochemically, a moderate to large amount of PRRSV antigen was demonstrated in the lungs of 10 pigs. In the interstitial pneumonic lesion, PRRSV antigen was detected in the cytoplasm of macrophages, degenerating cells or cell debris in the alveolar spaces (Figs 1c and d). It was found occasionally in the mononuclear cells within the alveolar septa. *M. hyorhinis* antigen was observed frequently on the bronchiolar epithelium and in degenerated neutrophils in the alveolar spaces of seven pigs (Figs 1e and f). Small to moderate amounts of *P. multocida*, *H. parasuis* and *E. coli* antigens were also found in the degenerated cells, corresponding to the suppurative bronchopneumonia (Table 1).

Experimentally Infected Pigs

All five pigs inoculated with PRRSV showed mild depression, anorexia and periocular oedema 2 to 3 days after inoculation. However, all except one (see below) of the pigs had recovered clinically by 14 days after inoculation. The lungs of those that recovered had no gross lesions at necropsy but showed mild interstitial pneumonia, characterized by slight thickening of the alveolar septa and hyperplasia of type II pneumocytes. A small amount of PRRSV antigen was found *post mortem* within the cytoplasm of macrophages in the alveolar spaces and septa in two of the four recovered pigs, but *M. hyorhinis* antigen was not detected.

One pig (no. 5) showed abdominal breathing and died 22 days after inoculation. At necropsy it showed severe proliferative and interstitial pneumonia complicated by suppurative bronchopneumonia. A large amount of PRRSV antigen was observed within macrophages in the alveolar spaces (Fig. 2a). Moreover, *M. hyorhinis* antigen was found on the epithelial cells of the bronchioles and in the alveolar spaces (Fig. 2b). A small amount of *E. coli* antigen was also found on the surface of epithelial cells of the bronchioles (Table 1).

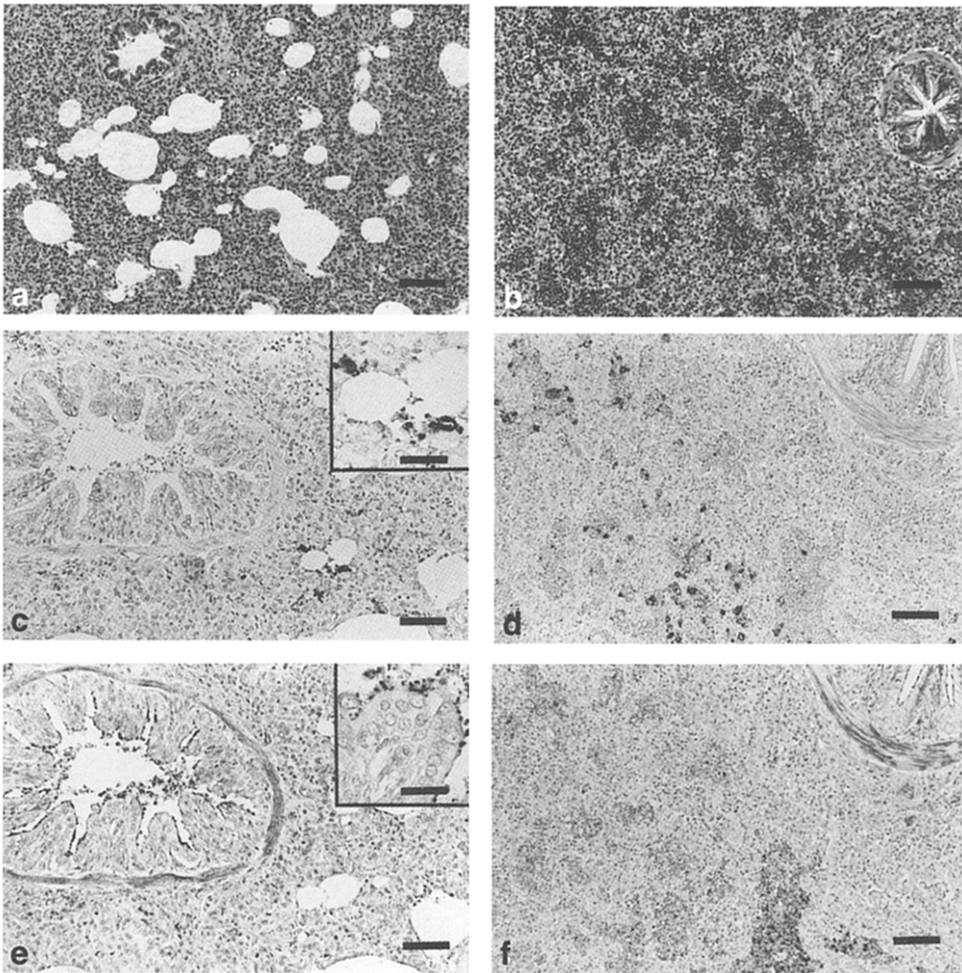


Fig. 1. Histological and immunohistochemical findings in lung lesions of field cases of PRRSV infection. a, c and e: Proliferative and interstitial pneumonia in pig 3. b, d and f: Proliferative and interstitial pneumonia complicated by bronchopneumonia in pig 7. a: Marked infiltration of mononuclear cells in alveolar septa and accumulation of proteinaceous debris, degenerating cells and macrophages in alveolar spaces. HE. Bar = 100 μ m. b: Moderately diffuse thickening of alveolar septa and infiltration of inflammatory cells in alveolar spaces and bronchioles. HE. Bar = 100 μ m. c: PRRSV antigen in the cytoplasm of macrophages, and in degenerating cells or cellular debris in the alveolar spaces. Immunohistochemical staining. Bar = 62.5 μ m. Inset: High magnification of Fig. 1c. Bar = 25 μ m. d: PRRSV antigen in the cytoplasm of macrophages in the alveolar spaces, with infiltration of inflammatory cells. Immunohistochemical staining. Bar = 62.5 μ m. e: *M. hyorhinitis* antigen in the bronchiolar epithelium. Immunohistochemical staining. Bar = 62.5 μ m. Inset: High magnification of Fig. 1e. Bar = 25 μ m. f: *M. hyorhinitis* antigen on the bronchiolar epithelium and in degenerating neutrophils in the alveolar spaces. Immunohistochemical staining. Bar = 62.5 μ m.

Viral, Bacterial and Serological Examinations

The serum samples from the 11 field cases and from four of the experimentally infected pigs contained antibody to PRRSV. This virus was isolated from three field and three experimental cases, and heavy cultures of *M. hyorhinitis* were obtained from all field cases and from the single experimental pig that

Table 1
Findings in 11 field cases and five experimental cases

Type of case	Pig no.	Immunohistochemical detection*							PRRSV antibody	Viral isolation†	Bacterial isolation‡						
		PRRSV	M.hy	P.mu	H.pa	E.co	A.pl	M.hp			M.hy	P.mu	P.sp	E.co	Others		
Field	1	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-
	2	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-
	3	+	+	+	-	-	-	-	+	+	+	-	-	-	-	-	-
	4	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-
	5	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
	6	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-
	7	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-
	8	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-
	9	+	+	-	+	-	-	-	+	-	-	-	-	-	-	-	-
	10	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-
	11	+	-	-	+	+	-	-	+	-	-	-	-	-	-	-	-
Experimental	1	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
	2	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
	5§	+	+	+	-	+	-	+	nd	+	+	+	+	+	+	+	+

* Number of positive reactions in lung tissue by ABC method: - = none; + = small; ++ = moderate; +++ = large.

† Detection of anti-PRRSV antibody by ELISA: + = positive; - = negative; nd = not done.

‡ Isolation from serum in alveolar macrophage cultures: - = negative; + = positive.

§ Number of bacteria isolated from lung (colony forming units/g): + = <10⁵; ++ = 10⁵ to 10⁶; +++ = >10⁶.

¶ Pig that died 22 days after inoculation.

M.hy = *Mycoplasma hyorhinis*, P.mu = *Pasteurella multocida*, H.pa = *Haemophilus parasuis*, E.co = *Escherichia coli*, A.pl = *Actinobacillus pleuropneumoniae*, M.hp = *Mycoplasma hyopneumoniae*, P.sp = *Pasteurella* sp.

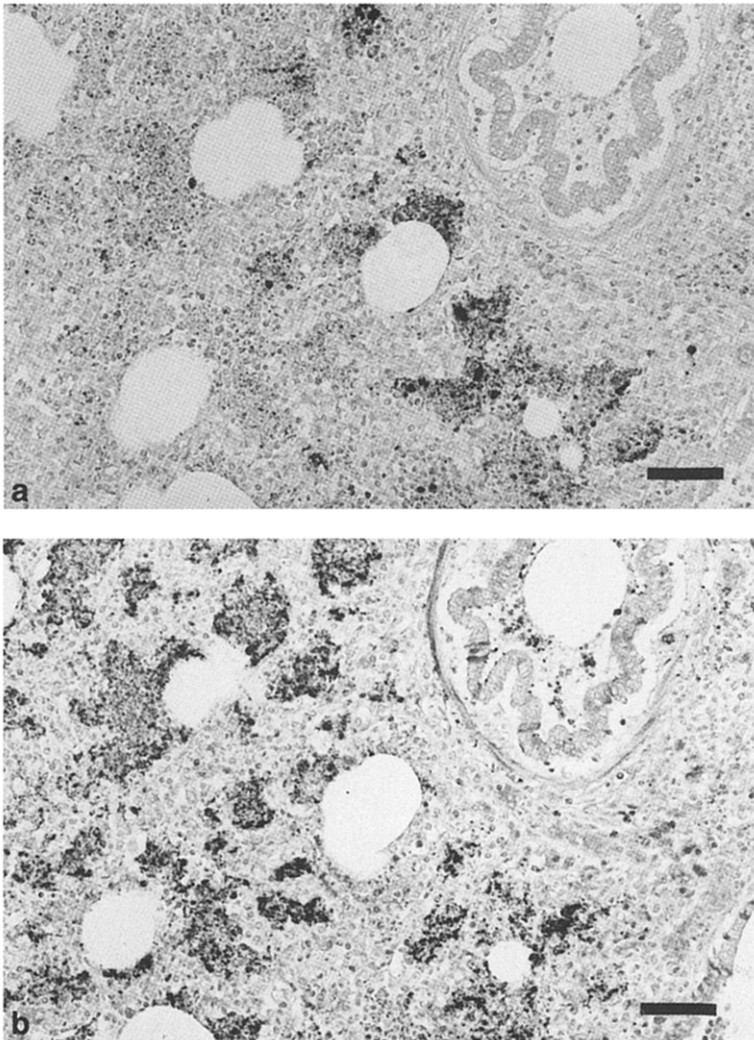


Fig. 2. Immunohistochemical findings in lung lesions in pig no. 5, which died 22 days after inoculation with PRRSV. a: PRRSV antigen within macrophages, degenerating cells and cell debris in the alveolar spaces. Immunohistochemical staining. Bar = 62.5 μ m. b: *M. hyorhinis* antigen on the bronchiolar epithelium and in neutrophils and cell debris in the alveolar spaces. Immunohistochemical staining. Bar = 62.5 μ m.

died. Light cultures of *P. multocida*, *Pasteurella* spp. and *E. coli* were isolated from the lungs of four field cases and one experimental pig. These results are summarized in Table 1.

Discussion

In the field, clinical signs of PRRS vary considerably. After investigating the clinical signs of 3612 sows in 16 herds, De Jong *et al.* (1991) reported that 50% of the pigs had anorexia, up to 10% had fever, and up to 30% had

respiratory distress. Shimizu *et al.* (1994) also pointed out that PRRSV-infected pigs showed abdominal breathing at the late stage of the disease. In the present study, 11 field cases with severe dyspnoea or abdominal breathing had pneumonic lesions throughout the lungs and antibody against PRRSV. Clinically and virologically, they resembled pigs in previously reported outbreaks of porcine reproductive and respiratory syndrome.

Pathologically, the predominant lung lesion in the field cases was moderate to severe proliferative and interstitial pneumonia, with slight suppurative bronchopneumonia. The lesion was composed of interstitial infiltration of mononuclear cells, accumulations of macrophages and cell debris in the alveolar spaces. A large amount of PRRSV antigen was detected in the cytoplasm of macrophages and in cell debris in the lesions. *M. hyorhinis* was also found on the surface of broncho-alveolar epithelial cells and degenerated neutrophils. The bacteria were closely associated with the pneumonic lesions. The pathological changes in four of five pigs experimentally infected with PRRSV resembled those described by investigators in Canada (Dea *et al.*, 1992) and the USA (Yoon *et al.*, 1992; Rossow *et al.*, 1994). These infected pigs were kept in isolation houses, but one of them died 22 days after inoculation with virus. At necropsy it had severe pneumonitis and a heavy culture of *M. hyorhinis* was isolated from the lung lesion, together with PRRSV. The pathological findings in the pig that died, including the detection of PRRSV and *M. hyorhinis* antigens, were almost the same as those in the 11 field cases. Therefore, if there had been no enhancement of *M. hyorhinis* proliferation in the lung of experimental pig no. 5, the pneumonitis caused by PRRSV infection might have resolved, as in the other four experimentally infected pigs.

Infection by other pathogens, such as *Streptococcus suis*, *Salmonella choleraesuis*, *P. multocida* and *H. parasuis*, increases in incidence and severity in PRRSV-seropositive pig herds (Hopper *et al.*, 1992; Stevenson *et al.*, 1993; Done and Paton, 1995). Galina *et al.* (1994) stated that infection by PRRSV predisposed to disease caused by *Streptococcus suis*. *M. hyorhinis* is frequently isolated from pneumonic lesions of slaughtered pigs (L'Ecuyer *et al.*, 1961; Gois *et al.*, 1975), but its ability to cause pneumonia is unclear. Experimentally, some strains of *M. hyorhinis* produced slight bronchopneumonia or interstitial pneumonia in gnotobiotic pigs (Friis, 1971; Gois *et al.*, 1971) and specific fluorescence for *M. hyorhinis* antigen was detected on the bronchial epithelium by indirect fluorescent-antibody staining (Gois *et al.*, 1971). In the present study, isolation of PRRSV was not always successful, but PRRSV infection was confirmed in all pigs serologically or immunohistochemically.

M. hyorhinis was found in all of 11 field cases and in one pig that died of experimental infection. Such pigs had severe interstitial pneumonia and suppurative bronchopneumonia. These findings suggest that dual infection with PRRSV and *M. hyorhinis* induced severe pulmonary lesions. It is well known that some micro-organisms, such as *Mycoplasma flocculare* and *M. hyorhinis*, can regularly be isolated, not only from the upper respiratory tract but also from the bronchial tree, of conventionally reared pigs (Christensen and Mousing, 1992). Potentially pathogenic micro-organisms in the respiratory

tract may be an important factor in the causation of severe PRRSV infection. The present study suggests that *M. hyorhinis* plays a major role in PRRSV infection in Japan.

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