Experimental Infection of SPF Piglets with Porcine Reproductive and Respiratory Syndrome (PRRS) Viruses Isolated from Two Farms

Osamu HIROSE, Isao SHIBATA[1)], Hiroshi KUDOU, Yasuo SAMEGAI[1)], Shigekatsu YOSHIZAWA, Masaaki ONO[1)], Masaaki NISHIMURA[1)], Tadao HIROIKE, Kiyoshi KAGEYAMA, and Tetuya SAKANO[1)]

Institute of Animal Health, Chiba Prefecture, 497 Iwatomi-cho, Sakura, Chiba 285 and [1)]Zen-noh Institute of Animal Health, 7 Ohja-machi, Sakura, Chiba 285, Japan

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ABSTRACT. Porcine reproductive and respiratory syndrome (PRRS) viruses were isolated from pig samples obtained from two farms characterized by an increased number of stillbirth and high mortality in new-born piglets (farm A), and respiratory distress with high mortality in weaning and growing pigs (farm B) in 1993, respectively. When primary specific pathogen-free piglets, 5-day-old or 13-day-old, were experimentally inoculated with the isolates, they showed clinical signs of depress, anorexia, pyrexia, diarrhea, sitting posture and periocular edema. Rate of the weight gain was reduced in the inoculated piglets compared with the non-inoculated pig. There were no apparent differences in clinical signs between the piglets inoculated with the virus samples derived from farms A and B. Microscopically, the most prominent changes observed in experimentally inoculated piglets were interstitial pneumonia, nonpurulent myocarditis and catarrhal lymphnoditis post inoculation day (PID) 28. Viruses were recovered from tissues collected from the inoculated piglets on PID 7 or 28. Furthermore, the viruses were continuously recovered from the sera from PID 7 to PID 28. Antibodies measured by indirect immunofluorescence assay to PRRS virus were first detected in sera on PID 14, and the antibody titer rose to 1:1280 on PID 28. — KEY WORDS: experimental infection, isolation, PRRS, swine.

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Porcine reproductive and respiratory syndrome (PRRS) was first recognized in 1987 in the United State of America [3] and spread through Western Europe in the early 1990s [5, 15]. The main clinical signs of this disease are reproductive disturbances in sows and respiratory distress in piglets. The causative virus of PRRS was first isolated and designated as Lelystad virus by Wensvoort *et al.* [14], and the disease was experimentally reproduced by Terpstra *et al.* [13]. Thereafter similar viruses to Lelystad virus were isolated in the United State of America and also in European countries. In Japan, similar clinical cases to PRRS were recognized around in 1987 and PRRS viruses were first isolated in 1993 [6, 11]. This paper describes the isolation of PRRS virus from natural cases and experimental infection with the isolates using primary specific pathogen-free piglets to confirm the pathogenicity of the isolates.

MATERIALS AND METHODS

Field samples: Samples for virus isolation were collected from two affected farms (farms A and B) in Chiba prefecture. Farm A, a farrow-to-finish farm with 250 sows, had an outbreak of disease characterized by an increased number of stillbirth (20–30%) and high mortality in new-born piglets in April 1993. Farm B, also a farrow-to-finish farm with 100 sows, had an outbreak of disease characterized respiratory distress with high mortality in weaning and growing pigs in June 1993. Lung samples of the affected cases from 2-day-old piglets in farm A and from 2-month-old pigs in farm B were taken and homogenized with Eagle's minimal essential medium (MEM) supplemented with L-glutamine, 0.3% tryptose broth, 5% fetal bovine serum and antibiotics. The 10% tissue homogenates were centrifuged for 10 min at 3,000 rpm and the resulting supernatants were filtered through at a 450 nm filter. They were used for inoculation of cell cultures and piglets.

Cell cultures: Swine alveolar macrophage (SAM) cells obtained from 4- to 6-week-old specific pathogen-free (SPF) pigs were used for virus isolation. A line cells derived from rhesus monkey kidney, MARC-145 cells [4] which kindly provided by Dr. Murakami, National Institute of Animal Health, Japan, was also used for serological examination. Preparation for the SAM cells was performed according to the method described by Wensvoort *et al.* [14], with some modification. The SAM cells stored in liquid nitrogen were washed twice with Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS-MEM) and resuspended in FBS-MEM to a final concentration of 1.5×10^6 cells/m*l*.

Isolation and titration of virus: The SAM cells suspended in FBS-MEM were seeded into 96-well plates in aliquots of 0.1 m*l* per well. Two hr after seeding, the cell cultures were inoculated with 10% tissue homogenates or undiluted sera in a volume of 0.05 m*l*. After adsorption at 37°C for 1 hr, each well was washed twice with FBS-MEM and then fed with 0.1 m*l* of fresh FBS-MEM. The plates were incubated at 37°C in a 5% CO_2-incubator for 6 days. The supernatant was inoculated onto fresh cultures of the SAM cells if cytopathic effects (CPE) was not observed during the first passage. Virus titration were performed by the microtiter method using SAM cell cultures prepared as mentioned above.

Experimental infection: Experimental design was showed

Table 1. Experimental design

Group	Pig No.	Age at inoculation (days)	Observation period (days)	Inoculum	Virus titer ($TCID_{50}$/m*l*)
A	1	5	7	Lung homogenate from field pig in farm A	$10^{4.5}$
	2	13	7	Lung homogenate from pig No. 1	$10^{2.5}$
	3	13	28	Lung homogenate from pig No. 1	$10^{2.5}$
	4	13	28	Cell culture fluid of the isolate from farm A	$10^{5.5}$
B	5	5	7	Lung homogenate from field pig in farm B	$10^{1.5}$
	6	13	7	Lung homogenate from pig No. 5	$10^{2.0}$
	7	13	28	Lung homogenate from pig No. 5	$10^{2.0}$
	8	13	28	Cell culture fluid of the isolate from farm B	$10^{3.5}$
Cont.	9	13	28	Not inoculated	

in Table 1. Nine primary SPF piglets delivered by Cesarean section were used for experimental infection. Pig Nos. 1 to 4 (group A) and 5 to 8 (group B) were inoculated intranasally with 0.5 m*l* of following virus samples derived from farms A and B, respectively. The remaining one piglet, No. 9 was served as an non-inoculated control. Pig Nos. 1 and 5 were inoculated with 10% lung homogenates from field affected pigs at 5 days old. Pig Nos. 2 and 3, and pig Nos. 6 and 7 at 13 days old were inoculated with 10% lung homogenates from pig Nos. 1 and 5, respectively, on post-inoculation days (PID) 7. Pig Nos. 4 and 8 were respectively inoculated at 13 days old with SAM cell culture fluid of the isolates in the third passage which derived from farms A and B, respectively. Pig Nos. 1, 2, 5 and 6 were sacrificed on PID 7, and the other pigs on PID 28.

Pathological examination: Tissue samples collected from lungs, heart, liver, kidneys, spleen, tonsil, lung lymph node and cerebrum were fixed in 20% formalin, embedded in paraffin and cut at 5 μm. All samples were stained with hematoxylin and eosin.

Serological examination: Indirect immunofluorescence assay (IIF) test was carried out according to the methods reported by Murakami *et al.* [6]. Fixed MARC-145 cell cultures infected with EDRD-1 strain [6] were used for IIF antigen. Test sera were diluted two-fold serially from 1:20 to 1:2560.

Virus neutralization (VN) test was carried out with microtiter method [7] using isolates of PRRS virus in the second or third passages on MARC-145 cells. The antibody titer was expressed as the reciprocal of the highest serum dilution inhibiting CPE in at least one of the 2 wells.

RESULTS

Isolation of PRRS viruses from field cases: PRRS viruses were isolated from lung samples submitted from the affected two farms. CPE was observed 3 days after inoculation on SAM cell cultures in the first passage. One of the isolate from farm A with reproductive disturbance was designated as 5–39 and the other isolate from farm B with respiratory distress was done as 5–53. These isolates reacted with anti-EDRD-1 strain sera and were hence identified as PRRS virus. Mycoplasma and bacteria were not cultured from the lung samples and also no virus was isolated using primary pig thyroid cells, or CPK cells derived from pig kidney cell cultured.

Table 2. Rate of weight gain of pigs experimentally inoculated with PRRS virus samples

Pig No.	Days post inoculation	
	7	28
1	131[a)]	NT
3	NT[b)]	326
4	NT	365
5	133	NT
7	NT	285
8	NT	363
9	164	439

No. 2 and 6 were not tested.
a) weight at 7 or 28 days post inoculation/weight at inoculation × 100 (%).
b) Not tested.

Experimental infection with PRRS virus samples in SPF pigs: Pig Nos. 1 and 5 which respectively inoculated at 5 days old with lung homogenates containing PRRS virus in titers of $10^{4.5}$ and $10^{1.5}$ $TCID_{50}$/m*l* showed depress, anorexia, diarrhea and periocular edema from PID 2 to 7. Clinical signs of pig Nos. 2 and 3, and 6 and 7 which respectively inoculated at 13 days old with lung homogenates from pig Nos. 1 and 5 containing the viruses in titers of $10^{2.5}$ and $10^{2.0}$ $TCID_{50}$/m*l* were the same as those of Nos. 1 and 5. Pig Nos. 4 and 8 which respectively inoculated at 13 days old with cell culture fluid containing the viruses in titers of $10^{5.5}$ and $10^{3.5}$ $TCID_{50}$/m*l* showed periocular edema for 3 days from PID 7. A pyrexia, more than 40.0°C with a peak of 41.2°C (No. 1), was observed from PID 2 to 3 and sustained for 1 to 4 days. The rate of weight gain was significantly reduced in the infected pigs compared with the uninfected pigs ($P<0.05$) (Table 2). There were no apparent

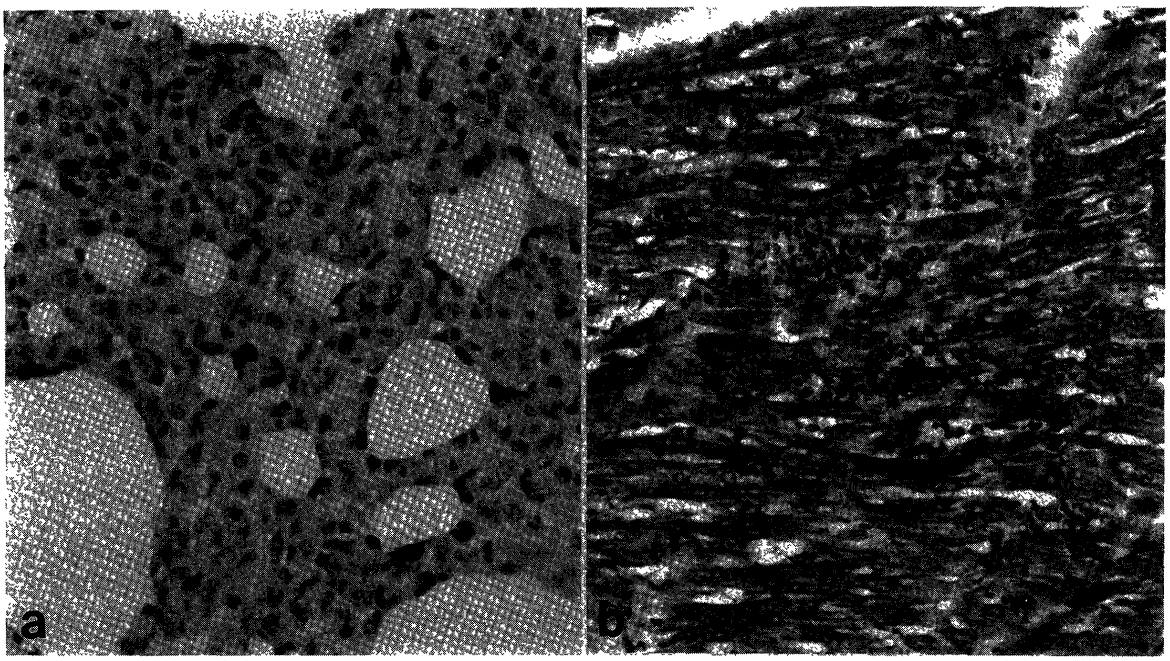

Fig. 1. a: Lung of pig No. 3 on PID 28. Interstitial pneumonia with thickening of alveolar septa with infiltration of mononuclear cells. HE stain. × 200. b: Heart of pig No. 7 on PID 28. Nonpurulent myocarditis with infiltration of mononuclear cells. HE stain. × 100.

Table 3. Virus isolation from tissues and sera collected from pigs experimentally inoculated with PRRS virus samples

Experiment group	Pig No.	Day post Inoculation	Tissues specimens								
			Cerebrum	Tonsil	Heart	Lung	Liver	Spleen	Pulmonary L.N.[a]	Mesenteric L.N.	Serum
A	1	7	–[b]	4.0[c]	3.25	4.25	2.75	2.75	NT[d]	NT	3.75
	2	7	–	1.57	2.2	4.25	–	1.8	NT	NT	0.8
	3	28	–	0.5	0.5	4.5	–	–	–	–	1.5
	4	28	–	3.75	1.0	1.25	–	–	0.75	–	1.25
B	5	7	–	–	–	1.25	–	–	–	NT	+[e]
	6	7	–	–	–	0.75	–	–	1.5	NT	0.75
	7	28	–	–	+	+	+	–	+	–	+
	8	28	–	1.75	–	0.75	–	–	–	–	+
Cont.	9		–	–	–	–	–	–	–	–	–

a) L.N.: Lymph nodes
b) –: Virus not detected.
c) log $TCID_{50}$/0.05 m*l*.
d) Not tested.
e) +: CPE positive in at least one of the four wells inoculated with undiluted sample.

differences in clinical signs between the pigs inoculated with these virus samples from different origins.

Grossly, pig Nos. 3, 4, 7 and 8 sacrificed on PID 28 had swelling in various lymph nodes including the mandibular, tracheobronchial and medial iliac lymph nodes. Pig Nos. 1, 2, 5 and 6 sacrificed on PID 7 had no gross pathological lesions. Microscopically, the lungs from 6 out of 8 pigs inoculated with virus samples showed interstitial pneumonia with thickening of alveolar septa with infiltration of mononuclear cells (Fig. 1). Nonpurulent myocarditis and catarrhal lymphnoditis were also observed in tissues from 4 out of 8 pigs (Fig. 1). These lesions were mainly observed in pigs sacrificed on PID 28. Aggregates of mononuclear cells in the liver and lymphoid hyperplasia and hypertrophy in the tonsil and spleen were seen from 5 out of 8 pigs. Tissues of a control pig did not have any lesions.

PID 7 and 28, viruses were recovered from the tonsil, heart, lung, liver, spleen and lymph nodes of the pigs inoculated with samples derived from farm A or B (Table 3). No virus was isolated from the cerebrum. Furthermore, viruses were isolated from the sera of inoculated pigs from PID 7 to PID 28 (Table 4).

Table 4. Antibody titers to PRRS virus by indirect immunofluorescence assay (IIF) and virus isolation from sera collected from pigs experimentally inoculated with PRRS virus samples

Experimental group	Pig No.	Days post inoculation									
		0		7		14		21		28	
		IIF[a)]	VI[b)]	IIF	VI	IIF	VI	IIF	VI	IIF	VI
A	3	<20	–[c)]	<20	+	320	+	640	+	1280	+
	4	<20	–	<20	+	160	+	640	+	1280	+
B	7	<20	–	<20	+	320	+	640	+	1280	+
	8	<20	–	<20	+	320	+	640	+	1280	+
Cont.	9	<20	–	<20	–	<20	–	<20	–	<20	–

a) Antibody titer by IIF.
b) VI: Virus isolation.
c) –: No virus detected, +: Virus detected.

IIF antibodies to PRRS virus were first detected in the sera from pig Nos. 3, 4, 7 and 8 on PID 14 with antibody titers of 1:160 or 1:320 (Table 4). On PID 28, IIF antibody titers rose to 1:1280. Neutralization antibody against the 5–39 or 5–53 isolates was not detected throughout the examination.

DISCUSSION

In the present study, we isolated PRRS viruses from the affected pigs derived from two farms either with reproductive disturbance or with respiratory distress. These isolates were reacted with antiserum to American type PRRS virus but weakly reacted with antiserum to European type PRRS virus in immunoperoxidase monolyer assay (personal communication from Dr. Murakami). These results indicated that antigenicity of the isolates was more closely related to that of American than European PRRS viruses.

SPF piglets experimentally inoculated intranasally with the isolates of PRRS virus consistently showed clinical signs of mild febrile response, anorexia, diarrhea and depress, but did not conspicuous respiratory signs. These results were similar to those in the previous reports using gnotobiotic piglets [2], SPF piglets [9] and conventional pigs [10]. Although we used two samples for pig inoculation; one derived from farm A with reproductive disturbance and the other from farm B with respiratory distress in weaning and growing pigs, no apparent differences in clinical signs were observed between pigs experimentally inoculated with virus samples from farms A and B. These results indicates that these two isolates of PRRS virus, derived from two farms with distinct symptoms, have almost the same pathogenicity in piglets. The symptoms of PRRS virus infection in these two pig farms might be related to feeding and management, environment or secondary infections. Furthermore, no differences in clinical signs were recognized between the piglets inoculated at 5 and 13 days old. Consequently piglets within 13 days old might have almost the same susceptibility to PRRS virus infection.

In the present experimental infection, the isolates of PRRS virus produced clinical signs and lung lesions in piglets but no piglet died. In the field, secondary infection by bacteria and/or mycoplasma is common among pigs from herds with PRRS [11, 12, 16]. Consequently the high mortality in pigs in the field must be associated with secondary infection in addition to the direct pathogenicity come from PRRS virus infection.

In the experimental infection, viruses were isolated from several tissues of the piglets inoculated with the isolates on PID 7 or 28. However, amount of viruses in the tissues of piglets in group A was higher than that in group B. These results might be due to a difference in the exposured dose of virus or the variation of virus infectivities among PRRS virus isolates. Further detail studies are now needed to compare the infectivities of these two isolates for pigs.

IIF antibody was first detected at PID 14 and rose to a titer of 1:1280 by PID 28. Antibody and virus could be detected simultaneously in the serum samples up to 28. As reported in the previous paper [8, 10], circulating antibodies produced early stage of the infection were apparently not effective in virus clearance from infected host animals. Neutralization antibody was not detected throughout this examination period. Christianson *et al.* [1] described that 6 out of the 9 sows inoculated with PRRS virus developed neutralizing antibodies to PRRS virus on 3 weeks post inoculation. On the other hand, Nelson *et al.* [7] reported that the neutralizing antibodies appeared by day 51 to 70 post inoculation. To elucidate the immune responses to PRRS virus infection, it would be important to investigate development and actual function of the neutralizing antibody on PRRS virus.

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