Isolation and Serological Characterization of Porcine Reproductive and Respiratory Syndrome (PRRS) Viruses from Pigs with Reproductive and Respiratory Disorders in Japan

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ABSTRACT. Three porcine reproductive and respiratory syndrome viruses (PRRSVs) were successfully isolated from stillborn piglets and fattening pigs derived from different herds affected either with an epizootic reproductive failure or a severe chronic respiratory distress in Japan. The isolates were reacted with antisera against both European (Lelystad virus) and American (strain 46448) PRRSVs in indirect immunofluorescence (IIF). However, cross immunoperoxidase monolayer assay revealed the close serological relationships between the Japanese isolates and American PRRSV, but not between the Japanese isolates and the European PRRSV. The data of preliminary serological survey with IIF technique showed a high frequency of antibody positive pigs against both Japanese isolate and the American PRRSV, whereas a low frequency of antibody positive pigs against the European PRRSV, on herds which have had clinical episodes of PRRS-like diseases. These results indicate that PRRSVs are prevalent in Japan, and suggest that the antigenicities of the prevalent PRRSVs are more closely related to those of the American than the European PRRSVs.—KEY WORDS: antigenicity, isolation, PRRS virus, swine.

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Porcine reproductive and respiratory syndromes (PRRS), a new pig disease causing reproductive disturbances in sows and respiratory distress in piglets, was first recognized in 1987 in North America [5] and spread rapidly through Western Europe in early 1990s [2, 10]. The disease was initially called wide variety of names, for example, 'mystery swine disease' in the United States of America, 'Seuchenhafter Spätabort der Schweine' in Germany, 'abortus blauw' in the Netherlands or 'blue-eared pig disease' in the United Kingdom [2, 3, 7, 11, 12]. After the establishment of the etiology of the disease in 1991, the term, PRRS is widely used in the world [1, 2].

The causative agent of the disease, Lelystad virus (LV), was first isolated by G. Wensvoort *et al.* in 1991 in the Netherlands and thereafter similar viruses to LV were isolated in many countries [2, 8, 10, 12]. Antibodies against LV were also detected in pig sera from a number of countries indicating the world wide distribution of the disease [10]. The virus has been currently classified into a member of the Arterivirus group of the Family *Togaviridae*, though a new proposal as a member of the *Arteriviridae* has been submitted according to the morphogenetical properties [6]. Serological variation, however, exists between European and North American isolates [10]. Consequently, serological characterization of the prevalent virus is decisively of important not only for the establishment of serodiagnosis, but for the epidemiology or prophylaxis of PRRS in once affected country.

In this report, we present the isolation of PRRS viruses (PRRSVs) from pigs in herds affected with either reproductive or respiratory disorders and the serological characterization of the isolates in Japan.

MATERIALS AND METHODS

Field samples: For virus isolation, seven lung homogenates of each fattening pigs with pneumonia and three tissue homogenates (pools of brain, liver, spleen and kidney) of stillborn piglets, were collected during a period from July 1992 to April 1993 and sent to our laboratory for diagnosis. The fattening pigs were derived from two herds affected with a severe chronic respiratory disorder, and the stillborn piglets were derived from different sows in a herd affected with epizootic reproductive disturbances (abortion, stillbirth and farrowing weak piglets). The homogenates were stored at -80°C until virus isolation. For serological examination, serum samples of breeding and growing pigs were obtained from six herds (Herds A to F). These herds had experienced epizootics of undiagnosed, reproductive and respiratory diseases within previous 12 months. Sera were stored at -20°C until examination for antibodies.

Cells: Swine alveolar macrophage (SAM) cells, obtained from lungs of 4-week-old specific pathogen-free pigs purchased from a commercial farm, were used throughout this study. Preparation and storage in liquid nitrogen for SAM cells were performed by the same method as previously described [12]. The SAM cells from liquid nitrogen were prepared for cell culture by washing once with Eagle's minimum essential medium (MEM) supplemented with 10% of fetal calf serum (FCS-MEM) and resuspended in FCS-MEM. The cell suspension was seeded in 96-well microplates and 25 cm^2 plastic flasks (Falcon, U.S.A.) to give final concentrations of 10^5 cells per well and 10^7 cells per flask, respectively. The cultures were incubated at 37°C for 1 hr to allow the cells to attach

to the culture bottom, and then used for virus isolation or propagation of viruses after washing the cultures twice with MEM.

Reference viruses and sera: LV and anti-LV pig serum, and PRRSV, strain 46448, and anti-46448 pig serum were obtained by the courtesy of Dr. G. Wensvoort (Central Veterinary Institute, Lelystad, the Netherlands) and Dr. M. Frey (National Veterinary Services Laboratories, Ames, U.S.A.), respectively. The 46448 virus is antigenically identical to VR-2332, the prototype of American PRRSV [1, 2 and personal communication from Dr. M. Frey]. Stock viruses of them were propagated in SAM cells by the same method described elsewhere [12].

Antiserum against one of the Japanese isolates was prepared in 2-month-old pigs which have been shown to be free of antibodies to LV and 46448 virus by indirect immunofluorescence (IIF). The animals were inoculated intranasally with infected culture fluid of the isolate in a titer of 10^5 TCID₅₀. The serum sample was collected at 6 weeks after the inoculation.

Virus isolation: Virus isolation was carried out according to the methods reported by Wensvoort *et al.* [12]. Briefly, after centrifugation and filtration ($0.45\ \mu\text{m}$) of 10% tissue homogenates, the filtrates were inoculated into SAM cell cultures in 96-well microplates ($50\ \mu\text{l}$ per well). After adsorption at 37°C for 1 hr, the inoculum was replaced with $100\ \mu\text{l}$ of FCS-MEM and the cultures were continued for incubation at 37°C in CO_2 incubator. The cultures were observed daily for cytopathic effects (CPE), and were frozen at -80°C when CPE reached at 50–70%. A single passage was made on each sample if CPE was not observed at a primary virus isolation.

Serology: Immunoperoxidase monolayer assay (IPMA) was also performed according to the established technique [12], using anti-swine immunoglobulin conjugated with horseradish peroxidase (Dako, Denmark) and 3-amino-9-ethyl-carbazol (Sigma, U.S.A.) as a substrate. For IIF, SAM cells were seeded in 96-well microplates, and optimal dilution of virus was inoculated except rows A, C, E and G which served as uninfected control. The plates were incubated at 37°C for 24–72 hr. After washing twice with $0.15\ \text{M}$ NaCl, the plates were air-dried and fixed with $100\ \mu\text{l}$ of cold ethanol for 10 min. They were stored at -25°C until use. The test sera were diluted 4-fold serially from 1:10 to 1:10,240, and a $50\ \mu\text{l}$ of each dilution was transferred to both infected and uninfected wells. The plates were incubated at 37°C for 1 hr, washed 5 times with $100\ \mu\text{l}$ of phosphate buffered saline (PBS) and incubated again at 37°C for 40 min with optimal dilution of rabbit anti-swine IgG conjugated with fluorescein isothiocyanate (affinity-purified, Zymed, U.S.A.). The plates were washed 5 times with PBS and examined under a fluorescent microscope. Antibody titer was expressed as a reciprocal of the highest serum dilution.

RESULTS

Isolation and identification of PRRSV: Seven lung

Fig. 1. Swine alveolar macrophage (SAM) cells uninfected (a) or infected (b, c and d) with EDRD-1 virus of porcine reproductive and respiratory syndrome virus isolate. Cytopathic effect (b), specific antigens detected by indirect immunofluorescence (c) and immunoperoxidase monolayer assay (d) are observed in cytoplasm on SAM cells.

homogenates of each fattening pig with severe chronic respiratory disease, and three tissue homogenates of each stillborn piglet were examined for virus isolation using SAM cell culture. Of the 10 homogenate samples, a total of three cytopathic isolates were detected in a single subculture (Fig. 1b). Of the isolates, two from herd with respiratory disorders were designated as EDRD-1 and 2, one from herd with reproductive disturbances was done as EDRD-8, respectively. All of the isolates reacted positively in IIF with both anti-LV and anti-46448 virus pig sera (Fig. 1c), whereas uninfected control cultures did not. One of the isolates, EDRD-1 was examined further. Its replication was not affected with 5-iodo-2-deoxyuridine. It was sensitive to treatment with chloroform, and furthermore reacted negatively in IIF with a set of sera against 10 enveloped RNA animal viruses; equine arteritis virus, Japanese encephalitis virus, bovine viral diarrhoea virus, swine fever virus, transmissible gastroenteritis virus, bovine coronavirus, swine influenza virus (HON1), bovine parainfluenza virus III, bovine respiratory syncytial virus and rinderpest virus (data not shown). Thus, the isolates were successfully confirmed as PRRSV.

Serological characterization of PRRSV isolates: Three Japanese PRRSV isolates were serologically characterized by IIF and IPMA using pig antisera against European (LV), American (46448 virus), Japanese (one of the isolates, EDRD-1 virus) PRRSVs and a convalescent sow

Table 1. Serological relationships among porcine reproductive and respiratory syndrome viruses by indirect immunofluorescence (IIF) and immunoperoxidase monolayer assay (IPMA)

Virus ^{a)}	Assay ^{b)}	Antibody titers of pig sera to following viruses			
		LV	46448	EDRD-1	G258 ^{c)}
LV	IIF	2,560	640	40	40
	IPMA	10,240	40	10	10
46448	IIF	160	2,560	2,560	2,560
	IPMA	160	10,240	10,240	10,240
EDRD-1	IIF	160	2,560	2,560	2,560
	IPMA	40	10,240	10,240	10,240
EDRD-2	IIF	640	2,560	2,560	2,560
	IPMA	160	10,240	10,240	10,240
EDRD-8	IIF	640	2,560	2,560	10,240
	IPMA	160	10,240	10,240	10,240

a) LV: Lelystad virus of European PRRS virus, 46448: 46448 virus of American PRRS virus and EDRD-1, 2 and 8: Japanese PRRS isolates.

b) IIF: indirect immunofluorescence and IPMA: immunoperoxidase monolayer assay.

c) G258: convalescent serum from a sow with reproductive failure in a herd where EDRD-8 virus was isolated.

serum (G258) obtained from a herd where EDRD-8 virus was isolated (Table 1). All the sera reacted with infected SAM cells with the PRRSVs used in this experiment, but not with uninfected control SAM cells in both IIF and IPMA. In cross IPMA, serological differences were clearly observed between LV and 46448 viruses. Furthermore, three Japanese isolates reacted equally with antisera to 46448 and EDRD-1 viruses, and with the convalescent sow serum in high titers (Fig. 1d), whereas they weakly reacted with antiserum to LV in a low titer compared with the homologous titer. Similar reactivities between the PRRSVs and pig antisera used in this experiment were also observed in IIF, though the serological differences among PRRSVs were less obvious than in IPMA.

Detection of antibodies to different PRRSVs in field serum samples: Preliminary antibody survey was conducted by IIF to compare differences in frequency of antibody detection and antibody titers against distinct PRRSVs using field serum samples (Table 2 and 3). The serum samples were obtained from six herds (Herds A to F) which had or suspected to have experiences of an epizootics of undiagnosed reproductive and/or respiratory diseases previously.

Antibodies against Japanese (EDRD-1 virus) and American (46448 virus) PRRSVs were equally detected with high frequency in all six herds examined. In contrast, antibodies against European PRRSV (LV) were detected with low frequency in these herds (Table 2). In Herd D, none of the sera possessed an antibody against LV though antibodies were detected in high frequency against EDRD-1 and 46448 viruses. On the other hand, six sets of

Table 2. Detection of antibodies against different PRRS viruses in pigs by IIF from herds having had a clinical episodes of PRRS-like diseases

Herd	Pigs examined	Clinical disorders	Number of antibody positive pigs per number of pigs examined to following PRRS viruses ^{a)}		
			LV	46448	EDRD-1
A	Breeding	Reproductive	4/11	11/11	11/11
B	Growing	Respiratory	4/5	5/5	5/5
C	Breeding	Reproductive	6/12	12/12	12/12
D	Breeding	Reproductive	0/6	5/6	6/6
E	Breeding	Reproductive	6/10	10/10	10/10
F	Breeding	Reproductive	2/10	9/10	9/10

a) LV:46448 and EDRD-1: the same viruses represented in footnotes in Table 1. Serum samples were estimated positive at 1:20 dilution.

Table 3. Seroconversion against different PRRS viruses by IIF in a set of paired sera from Herd A affected with an epizootic of reproductive disturbances

Sow	Antibody titers of sow sera against following PRRS viruses ^{a)} at acute (A) and convalescent (C) phases.					
	LV		46448		EDRD-1	
	A	C	A	C	A	C
1	<10	<10	<10	2,560	<10	2,560
2	<10	<10	<10	10,240	<10	10,240
3	10	40	160	10,240	160	10,240
4	<10	<10	40	10,240	40	10,240
5	<10	<10	40	2,560	<10	10,240
6	<10	40	<10	10,240	<10	10,240

a) LV, 46448 and EDRD-1: the same viruses represented in footnotes in Table 1.

paired sera which were obtained from sows at a interval of 3-4 weeks in Herd A where epizootic reproductive failures were prevalent, were examined for seroconversion against these PRRSVs. Significant seroconversions were observed in all sows against EDRD-1 and 46448 viruses and the antibodies reached at high titers in the convalescent sera of them (Table 3). However, only two sows showed seroconversions at low titers and remaining four sows did not at all against LV.

DISCUSSION

According to virological and serological evidences, PRRSVs have been considered to be prevalent in the whole world [2, 10, 12]. However, current situation of PRRS in Japan is still obscure though there is a report on the isolation of PRRSV from pigs affected with respiratory disorders [8]. In this study, we successfully demon-

strated the existence of PRRSV in pigs from herds affected with either an epizootic reproductive disturbance or a severe chronic respiratory distress in Japan. Preliminary serological survey also revealed the prevalence of PRRSV in Japanese pig herds which have had clinical episodes of PRRS-like diseases. These results suggest that PRRS may act as a part of causes of both reproductive and respiratory disorders in pigs in Japan since the etiology of PRRSV has fully established [2, 3, 7, 9, 11].

In the present study, serological properties of PRRSVs isolated in Japan were examined in comparison with those of American 46448 virus which is antigenically identical to VR-2332 virus [personal communication from Dr. M. Frey], and European LV of PRRSVs. Wensvoort *et al.* reported that serological differences among PRRSVs were observed with IPMA, in particular, between American and European PRRSV isolates [10]. In addition, Dea *et al.* reported that field serum samples reacted with VR-2332 virus, American prototype of PRRSV [1, 2], failed to react with LV [4]. These findings indicated the existence of antigenic variation among PRRSVs. In this study, serological difference was also demonstrated between 46448 virus and LV by IPMA. Furthermore, the results obtained from cross IPMA revealed that the antigenicity of Japanese isolates were closely related to 46448 virus, but not to LV. The data of serological survey with IIF also supported this evidence since sera negative for antibody to LV were positive to both Japanese (EDRD-1) and 46448 PRRSVs. These findings suggest that the serological properties of Japanese PRRSVs are more close to those of the American than those of the European PRRSVs in so far as these isolates were examined. Although further studies are needed in order to clarify the serological relationships among PRRSV isolates, the serological properties of the isolates are, at least, of importance for the establishment of serodiagnosis on PRRS in Japan.

The results of preliminary serological survey showed the possibility of high frequency of antibody positive pigs in some herds having experienced clinical episodes of PRRS-like diseases. Consequently, nationwide survey is needed to clarify the current situation of PRRS in Japan including retrospective investigation.

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