

Slow-Reacting and Complement-Requiring Neutralizing Antibody in Swine Infected with Porcine Reproductive and Respiratory Syndrome (PRRS) Virus

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ABSTRACT. Various conditions were evaluated and modified to enhance the sensitivity of the neutralization (NT) test for detecting antibody in swine infected with porcine reproductive and respiratory syndrome (PRRS) virus. Higher NT antibody titers were consistently obtained by the addition of 10% (v/v) complement, fresh guinea pig serum, to the virus diluent and by the incubation of serum-virus mixture at 4°C for 24 hr. The appearance and persistence of antibodies detected by the modified NT test showed a similar pattern to those of antibodies detected by the indirect fluorescent antibody (IFA) assay, although the antibody titers obtained by the former method were consistently lower than those obtained by the latter method. Slow-reacting complement-requiring NT antibody was detected in sera from pig 2 weeks after infection with PRRS virus. The slow-reacting complement-requiring NT antibody in the early serum samples was sensitive to 2-mercaptoethanol (2-ME), whereas the slow-reacting complement-requiring NT antibody in the late serum samples was resistant to 2-ME. The initial phase may represent the IgM response and the later phase a change to IgG. A NT test was developed in which serum-virus mixtures were incubated at 4°C for 24 hr with complement; this gave an improved sensitivity over the previous incubation at 37°C for 60 min. — **KEY WORDS:** complement, indirect immunofluorescent assay, 2-mercaptoethanol, neutralization test, PRRS virus.

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Porcine reproductive and respiratory syndrome (PRRS) is mainly characterized by reproductive failure in sows and respiratory problems in pigs of all ages [2, 3, 5, 6, 11, 13, 17, 18]. The causative virus, PRRS virus, is a member of the genus *Arterivirus* [12, 14].

The neutralization (NT) test [2, 3, 11], hemagglutination-inhibition (HI) test [8], indirect fluorescent antibody (IFA) assay [2, 5, 13, 19], immunoperoxidase monolayer assay (IPMA) [18], and enzyme-linked immunosorbent assay [1] have been used to assay PRRS virus antibodies in swine sera. The NT test, however, gives relatively low titers in pigs experimentally or naturally infected with PRRS virus [11]. Wensvoort *et al.* [17] also reported that NT antibodies directed against PRRS virus, Lelystad virus, were not detected in any of the paired sera from sows infected with PRRS virus, but an overwhelming antibody response directed against the virus was measured in IPMA in which they measured the binding to macrophages infected with the virus.

Recently, Yoon *et al.* [20] reported that the sensitivity of the NT test could be increased by adding fresh normal swine serum to serum being assayed. Furthermore, Hyllseth and Pattersson [7], Radwan and Burger [15] and Fukunaga *et al.* [4] have increased the sensitivity of the NT test for equine arteritis virus, a member of the genus *Arterivirus* by incubation of serum-virus mixtures with complement.

These observations encouraged us to investigate the presence of complement-requiring NT antibody in serum of swine infected with PRRS virus. This paper describes the observations on slow-reacting and complement-requiring NT antibody against PRRS virus in swine sera.

MATERIALS AND METHODS

Cell culture : MARC-145 cells [10], kindly provided by Dr. Y. Murakami, National Institute of Animal Health, Kodaira, Tokyo, were grown in Eagle's minimum essential medium (MEM) supplemented with 10% tryptose phosphate broth, 5% inactivated fetal bovine serum, 0.12% NaHCO₃, 100 units/ml penicillin, 100 µg/ml Kanamycin and 2 µg/ml fungizone. The maintenance medium (MM) was MEM supplemented with 0.5% lactalbumin hydrolysate, 0.2% BSA (bovine serum albumin; Fraction V), 0.162% NaHCO₃ and antibiotics as above. The cells were used for preparation of virus, determination of infective titer and NT test.

Virus : The PRRS virus strain used was the EDRD-1 strain [13], supplied by Dr. Y. Murakami, National Institute of Animal Health, Kodaira, Tokyo. The virus was firstly isolated on primary porcine alveolar macrophage (PAM) cultures and thereafter propagated on MARC-145 cells. Culture fluid harvested from infected MARC-145 cell cultures was clarified by centrifugation and stored at –80°C.

Infectivity assay : Monolayer cultures of MARC-145 cells grown in flat-bottom 96-well microtiter plate were used. Serial decimal dilutions of the virus suspension were made in MM, and each dilution was inoculated in 0.05 ml amount onto the cell cultures, 4 wells per dilution. After incubation at 37°C for 1 hr, the cultures were fed with 0.5 ml of MM and incubated in CO₂ incubator at 37°C for 5 days. The 50% tissue culture infectious dose (TCID₅₀) was calculated on the basis of cytopathic effect (CPE).

PRRS virus recovery : Undiluted serum samples from the

experimentally infected pigs were inoculated in 0.05 ml amount onto MARC-145 cell cultures. After incubation at 37°C for 1 hr, the cultures were fed with 0.05 ml of MM and incubated in CO₂ incubator at 37°C. The cultures were observed daily for CPE.

Neutralization test : Serial 2-fold dilutions of serum inactivated at 56°C for 30 min were made in MM and 0.05 ml of each dilution was mixed with an equal volume of the virus diluent (see below) containing 200 TCID₅₀/0.05 ml of virus. The serum-virus mixtures were incubated at 37°C for 60 min, or at 4°C for 24 or 48 hr, as indicated in each experiment. At the end of incubation each mixture was inoculated in 0.05 ml volumes into wells of flat-bottom microplate cultures; 2 wells were used for each serum dilution. After virus was adsorbed at 37°C for 1 hr, the cultures were washed once with PBS (0.15 M NaCl, 0.01 M phosphate buffer, pH 7.2), and fed with 0.05 ml of MM, incubated in a CO₂ incubator at 37°C for 5 days, and examined for CPE. The antibody titer was expressed as the reciprocal of the highest serum dilution which showed complete inhibition of CPE in at least one of the two wells. The virus diluent used was MM containing 10% (v/v) of the complement, unheated normal guinea pig serum, unless otherwise stated, and MMs containing PBS or heat-inactivated (56°C, 30 min) guinea pig serum instead of unheated complement were served as control virus diluents. Back titration of the virus was carried out each time to confirm that 200 TCID₅₀ of virus were used. Each samples were run in duplicate, and the mean titer was calculated.

Complement: Sera from healthy guinea pigs having no NT antibody to PRRS virus, as a source of complement [9] were collected, pooled and stored at -80°C until use.

Indirect fluorescent antibody (IFA) assay : This test was carried out according to the method described in the previous paper [5], using fixed MARC-145 cells infected with the EDRD-1 strain of PRRS virus [13]. The test sera were diluted 2-fold serially from 1:20 to 1:2,560.

Serum samples: Field serum samples — On 21st December 1994, pigs showing depression, emaciation, hyperpnea, coughing and severe abdominal breathing were observed on a 300-sow farrow-to-feeder pig farm in the Chiba Prefecture in Japan. Mortality in young pigs reached up to 20%. The causative agent, PRRS virus, was isolated in PAM cultures from sera of these affected pigs [6], and serum samples were collected from 8 sows on the farm, weekly up to 8 weeks after the onset of the disease. Antibodies were monitored by the NT test and IFA assay over a 8-week period in these sows.

Experimental serum samples — Serum samples were collected from pigs infected with PRRS virus. Three specific-pathogen-free (SPF) pigs, 40-day-old, were inoculated intranasally with 10^{5.25} TCID₅₀ of PRRS virus, and serum samples for antibody assay and virus recovery were collected weakly up to 6 weeks after inoculation.

Effect of 2-mercaptoethanol (2-ME) treatment : For 2-ME treatment, 0.05 ml of the serum was mixed with an equal volume of 0.2 M 2-ME (Wako Junyaku Co., Tokyo) solution

in PBS and incubated at 4°C for 24 hr. The mixture was dialyzed against PBS at 4°C for 2 days, and used for the modified NT test after centrifugation at 1,500 × g for 5 min.

RESULTS

Effect of different concentrations of complement in virus diluent on NT antibody titer : In order to confirm an optical complement concentration to enhance NT antibody titer, NT antibody titers in serum samples, collected weekly from one (sow No. 4) of 8 sows on the farm where PRRS virus was prevalent, were determined in the presence of various concentrations of complement. As shown in Table 1, an enhancing effect was observed, depending upon the concentration of the complement added to the virus diluent; the enhancement was proportionally related to concentrations up to 5%. Complement at the concentrations between 5% and 40% resulted consistently in a 16-fold enhancement of NT antibody titer.

Effect of incubation time and complement on NT antibody titer : NT antibody titers of serum samples collected on 25th January 1995 from 8 sows, naturally infected with PRRS virus, were determined by incubation of serum-virus mixture at 37°C for 60 min or at 4°C for 24 or 48 hr in the presence or absence of complement. As shown in Table 2, the results indicated the presence of slow-reacting and complement-requiring NT antibody in all the serum samples. The greatest sensitivity of the NT test was obtained by incubating serum-virus mixture at 4°C for 24 or 48 hr with unheated complement.

Antibody responses in experimentally infected pigs : The serum samples obtained from experimentally infected SPF pigs (Nos. 17, 18 and 19) were tested for NT antibody titer by incubation of serum-virus mixture at 4°C for 24 hr with complement or without complement and for antibody titer by the IFA assay. As shown in Table 3, all of the pigs were negative (NT antibody titer : <4 ; IFA titer : <20) for antibody by both NT test and IFA assay before infection. The NT antibody in these animals was first detected 2 weeks after infection, increased and reached a peak titer of 128 or 256 at 4 or 5 weeks after infection. Marked enhancement (8- to 16-fold) in NT antibody titer was demonstrated in all the animals by incubating serum-virus mixture at 4°C for 24 hr with complement. Similar titer rises were observed for antibody by the IFA assay, although the titers obtained by the IFA assay were much higher than those obtained by the modified NT test at all stages tested after infection.

No virus was recovered from all the serum samples which were collected from experimentally infected pigs weekly up to 6 weeks after inoculation.

Effect of 2-ME treatment on NT antibody titer : The serum samples from 2 pigs (Nos. 17 and 18) infected experimentally with PRRS virus were tested for NT antibody titer by incubation at 4°C for 24 hr with complement, before and after 2-ME treatment. As shown in Table 4, the slow-reacting, complement-requiring NT antibody contained in the early sera was sensitive to 2-ME treatment, while the

Table 1. Neutralizing (NT) antibody titers^{a)} of sera from a sow^{b)} in the presence of various concentrations of guinea pig complement

Weeks after the onset of the disease	Concentration of complement in virus diluent (%)								
	40	20	10	5	2.5	1.3	0.6	0.3	0.0
0	<4 ^{c)}	<4	<4	<4	<4	<4	<4	<4	<4
1	<4	<4	<4	<4	<4	<4	<4	<4	<4
2	<4	<4	<4	<4	<4	<4	<4	<4	<4
3	16	16	16	16	5	4	<4	<4	<4
4	32	32	32	32	16	16	4	<4	<4
5	64	64	64	64	32	16	4	4	4
6	128	128	128	128	64	32	16	16	8
7	256	256	256	256	128	64	64	32	16
8	512	512	512	512	256	128	128	64	32

- a) NT antibody titers were determined by incubating serum-virus mixture at 4°C for 24 hr with or without complement.
- b) Serum samples were collected from a sow on the farm where PRRS virus was prevalent.
- c) NT antibody titer.

Table 2. Effect of incubation time and complement on neutralizing (NT) antibody titer of sera from sows^{a)}

Incubation	Complement ^{b)}	NT antibody titer							
		No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8
37°C, 1 hr	C'	32	4	32	32	64	16	8	32
	HC'	8	<4	16	8	64	8	8	32
	PBS	8	<4	16	8	64	8	8	32
4°C, 24 hr	C'	256	32	512	256	256	128	64	256
	HC'	8	<4	16	16	64	8	8	32
	PBS	8	<4	16	8	64	8	8	32
4°C, 48 hr	C'	128	32	256	128	256	64	64	1028
	HC'	8	<4	16	16	64	8	8	32
	PBS	8	<4	16	8	64	8	8	32

- a) Serum samples were collected on 25th January 1995 from sows on the farm where PRRS virus was prevalent.
- b) C': unheated guinea pig serum; HC': heated guinea pig serum; PBS: phosphate buffered saline.

Table 3. Antibody response of the experimentally infected pigs measured by the neutralization (NT) tests and the indirect fluorescent antibody (IFA) assay

Pig No.	Method ^{a)}	Weeks after inoculation						
		0	1	2	3	4	5	6
17	A	<4 ^{b)}	<4	<4	<4	<4	16	16
	B	<4	<4	8	16	32	128	64
	C	<20	<20	320	640	640	1280	640
18	A	<4	<4	<4	16	128	64	16
	B	<4	<4	32	128	512	256	128
	C	<20	<20	320	640	1280	640	640
19	A	<4	<4	8	32	32	16	16
	B	<4	<4	64	128	512	256	128
	C	<20	<20	160	160	1280	640	640

- a) A: NT test without complement; B: NT test with complement; C: IFA assay.
- b) Antibody titer.

Table 4. Effect of 2-mercaptoethanol (2-ME) on neutralizing (NT) antibody titers of sera from pigs infected experimentally with PRRS virus

Pig No.	2-ME treatment	Weeks after virus inoculation						
		0	1	2	3	4	5	6
17	No	<4 ^{a)}	<4	4	16	16	64	32
	Yes	<4	<4	<4	<4	8	32	32
18	No	<4	<4	16	64	256	128	128
	Yes	<4	<4	<4	8	64	128	128

a) NT antibody titer was determined by incubation of serum-virus mixture at 4°C for 24 hr with complement.

slow-reacting, complement-requiring NT antibody in the late sera was resistant to 2-ME treatment.

DISCUSSION

For the purpose of this study it was assumed that the enhancing effect of fresh guinea pig serum on virus neutralization by specific antibody was due to complement since previous work has shown that this enhancing effect is due to the complement system [9]. As we expected, the present investigation demonstrated that the sensitivity of the NT test for antibody against PRRS virus was significantly improved by incubating serum-virus mixture at 4°C for 24 hr with complement (modified NT test), as compared with incubating at 37°C for 60 min (standard method) with or without complement.

It was suggested that 10% (v/v) complement in the virus diluent would be a sufficient dose for the modified NT test, because no difference was found in NT antibody titers at complement concentrations between 5% and 40%. An excessive amount of complement did not further enhance NT antibody titers. These observations were in agreement with those of studies on equine arteritis virus [4, 7, 15]. Similar increased sensitivity of NT test for antibodies against PRRS virus has been obtained by the addition of 20% fresh swine serum to the virus diluent and by the use of MARC-145 cell culture [20].

The appearance and persistence of antibodies detected by the modified NT test showed a similar pattern to those of antibodies obtained by the IFA assay, although the antibody titers obtained by the former method were consistently lower than those obtained by the latter method. The modified NT test, however, will be used as a tool for the differentiation of serologic groups of PRRS virus isolates as described by Yoon *et al.* [20].

In this study, our failure of virus recovery from the sera of experimentally infected pigs at all stages examined after infection is attributable to use the MARC-145 cell cultures instead of PAM cultures used commonly for PRRS virus isolation. Because this virus was continuously recovered on PAM cultures from the sera of infected pigs from 7 to 28 days after infection in our previous study [5].

The slow-reacting complement-requiring NT antibody

against PRRS virus was sensitive to 2-ME treatment in the early sera, while the slow-reacting complement-requiring NT antibody was resistant to 2-ME treatment the later sera. These findings suggest that the slow-reacting complement-requiring NT antibody may represent the IgM response and the later slow-reacting complement-requiring NT antibody may represent a change to IgG. These findings were in agreement with those obtained with antibodies to PRRS virus [20].

The results obtained in this study with PRRS virus corroborate the findings reported in the previous paper [16], using Akabane virus, that the prolonged incubation of serum-virus mixture and the addition of complement to the mixture caused enhancement of NT activity. Mechanisms of this phenomenon are not known, but the slow-reacting complement-requiring NT antibody may have lower binding affinity to the virus or a decreased ability to neutralize viral infectivity.

In the present study, a NT test by prolonged incubation of serum-virus mixture with complement at 4°C was developed and proven to be useful in detecting earlier and higher PRRS virus antibody in sera from swine as well as IFA assay.

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