A Study of Serologic Relationships among Non-Cytopathogenic Strains of Bovine Viral Diarrhea-Mucosal Disease Virus by Reverse Plaque Technique

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ABSTRACT. Neutralizing antibody in serial serums from calves infected with non-cytopathogenic (NCP) strain No. 12 of bovine viral diarrhea-mucosal disease (BVD-MD) virus was comparatively titrated by the semi-micro reverse plaque formation (RPF) and the END methods. There was a tendency that the neutralizing antibody titer was estimated eightfold higher in the semi-micro RPF method than in the END method. Antigenic relationships among three Japanese strains and two American strains were examined by the cross-neutralization and kinetic neutralization tests using the RPF technique. In cross-neutralization test, differences in antibody titers were not more than twofold among three Japanese strains. However, there were eightfold differences in some combinations of the Japanese and American strains. Kinetic neutralization test also demonstrated that three NCP strains of BVD-MD virus isolated in Japan were closely related. The American strains were antigenically distinct from three Japanese strains used in this study although some antigenic similarity was recognized in some combinations of the strains and the antisera. Moreover, these two American strains appeared to differ one another slightly.—*Key words:* BVD-MD virus, reverse plaque technique, serology.

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INTRODUCTION

Several *in vitro* techniques for detection and titration of antibody to the non-cytopathogenic (NCP) strains of bovine viral diarrhea-mucosal disease (BVD-MD) virus have been developed [5, 8, 16]. The authors previously described the reverse plaque formation (RPF) method for the titration of the NCP strains [13]. As for the RPF method, it was known that one reverse plaque (RP) was formed by a single infectious unit of the NCP strain. However, no report has been published on a BVD-MD virus neutralization test using the RPF method so far.

The BVD-MD virus strains originating from cattle with varied disease syndromes have been tested for their antigenic relationships by cross-immunity [3], cross-neutralization [12], immunodiffusion [9], complement fixation [10] and immunofluorescence

[5] tests. Gillespie *et al.* [7] showed an antigenic homogeneity of BVD-MD virus strains by a neutralization test [7]. Similar relationships were reported by Omori and coworkers [16], who compared cytopathogenic (CP) and NCP strains of BVD-MD virus by a cross-neutralization test. In 1968, Castrucci *et al.* [2] reported that Italian strains were antigenically different from American strains, and also American strains were antigenically different one another. Subsequently one additional study of a similar nature was carried out. Fernelius *et al.* [6] proposed three serologically different prototype strains of the BVD-MD virus.

In the present paper, an experiment was carried out with antiserums against NCP strains of BVD-MD virus to know whether the RPF method was applicable to the antibody determination. The antigenic relationships of five NCP strains, in which three were

isolated in Japan and two in the United States, were studied by cross-neutralization and kinetic neutralization tests using homologous and heterologous antisera prepared in calves. This is the first report on the antigenic relationships of the NCP strains of the virus investigated by a plaque technique.

MATERIALS AND METHODS

Viruses: The following NCP strains of BVD-MD virus were used in this study: from the United States, strains Indiana-46 and New York-1 (NY-1); and in Japan, strains No. 12, Shizuoka and Kanto Tozan. All the virus strains were kindly supplied by Dr. Y. Inaba (National Institute of Animal Health, Ibaraki) and Dr. K. Kodama (Bien Laboratories, Kyoto), and were purified by at least three terminal dilutions in cell culture before use. The CP strain Nose of BVD-MD virus was used as a challenge virus for the RPF method. The strain was plaque-purified by three consecutive passages.

Cell: Bovine testicle cell cultures at the 2nd to 8th level were used in ordinary tissue culture techniques.

Production of antiserum in calves: Five 6-month-old calves devoid of detectable neutralizing antibody to BVD-MD virus were used. Each of calves was inoculated subcutaneously with 1 ml of the inoculum containing $10^{6.0}$ TCID₅₀/ml of cloned NCP strain. All calves were reared in separate pens. Serum samples were collected at one-week intervals for up to ten weeks after inoculation, heat inactivated (56°C for 30 min) and stored at -20°C until tested.

RPF in 60-mm petri dish: The RPF method was based on the semi-micro plate technique previously described [13]. Briefly, confluent bovine testicle cell monolayers grown in petri dishes (Falcon NO. 3002, U.S.A.) were inoculated with 0.2 ml aliquot of NCP virus dilution and incubated at 37°C for 90 min. The infected cells were then overlayed with 5 ml of Eagle's minimum essential medium

containing 1.2% methyl cellulose (Wako Chem. Japan), 0.29% tryptose phosphate broth, 0.85 g/l of sodiumbicarbonate, 5% calf serum, 100 units/ml of penicillin and 50 µg/ml of amphotericin B. After further incubation for 5 days, the overlay medium was removed and the cell surface was rinsed with Earle's solution. They were reinoculated with 1 ml of Nose strain ($10^{6.5}$ TCID₅₀/ml). After adsorption, each of the petri dishes was added with 5 ml of the overlay medium. After additional 4 or 5 days incubation, the cell cultures were fixed with methanol, stained with 0.2% aqueous crystal violet solution, and then the RP was counted.

RP-neutralization test: Plaque reduction test using a semi-micro RPF method was applied for the determination of antibody against NCP strain of BVD-MD virus. The serum samples were diluted in serial twofold steps in Eagle's minimum essential medium, and each was mixed with an equal volume of the virus suspension containing about 50 plaque forming units (pfu) per well. The test mixtures were held at 37°C for 60 min, and 0.05 ml of the mixture was inoculated onto each of four cell-culture wells by dropper. The plate was subjected to incubate for adsorption of virus at 37°C for 90 min in a humidified 5% CO₂ atmosphere. After adsorption, the plate was treated as described above. Serum neutralization antibody titer was expressed as reciprocal of the highest serum dilution that reduced mean plaque count by 50 per cent.

END-neutralization test: Neutralization test with antiserum against strain No. 12 was performed by the END method described previously [16]. The antibody titer was expressed as reciprocal of the highest dilution of serum that showed cytopathic effect in at least two of four tubes.

Kinetic neutralization test: Kinetic neutralization of the virus was tested by a modification of the procedure reported by McBride [14] as follows. The immune serum was

diluted in Earle's solution to 1:20. Each virus strain was diluted to make an appropriate virus titer of $10^{4.5}$ to $10^{5.0}$ pfu per 0.2 ml. All were held at 37°C during the test. The test was conducted by rapid mixing of 5 ml of virus preparation and an equal volume of serum dilution. At time zero, 0.1 ml of the virus-serum mixture was immediately diluted 100-fold with 9.9 ml of chilled diluent. This and a subsequent 1:10 dilution were assayed for the residual virus, for which 0.2 ml aliquots were inoculated onto 2 plates of 60-mm petri dish. From remainder of the mixtures held at 37°C, samples were successively removed at 5, 10 and 15 minutes, and then assayed as in the zero-time sample. The amount of residual virus in aliquots was quantitated by the RPF method as stated above. The neutralizing activity was calculated by the formula $K = (D/t)2.3 \log_{10}(V_0/V_t)$ according to McBride [14], where K is the neutralization rate constant, D the reciprocal value of the serum dilution used, t the time of reaction at 37°C in minutes, V_0 the infectivity measured at time zero, and V_t the infectivity at the end of reaction time. To determine the K value of each virus-serum mixture, mean plaque counts at various time intervals were calculated as percentages of plaque count at time zero and were plotted semilogarithmically against time. A statistical estimate of the virus neutralization rate was calculated by linear regression. It was calculated by selecting a point on the line and inserting its data into the K value formula. From the K value, normalized K (NK) representing the rate at which serum neutralized heterologous virus was calculated. Homologous NK was considered as 100. The criterion by Munro and Wooley [15] was adapted for appraisal of antigenic relationships, in which the reciprocal of NK value 90 or more meant serologic homogeneity, 60 to 89 showed antigenic similarity, and less than 59 showed antigenic distinction.

Fig. 1. Neutralizing antibody titers in calf inoculated with strain No. 12.

○: Semi-micro RPF method.
△: END method.

RESULTS

Comparison of antibody titers determined by the semi-micro RPF method and the END method: The feasibility of the semi-micro RPF method for virus neutralization test is shown (Fig. 1). Serums from calf inoculated with NCP strain No. 12 were titrated for neutralizing antibody by the END method and the semi-micro RPF method. Pre-inoculation serum was negative by both assay methods. Neutralizing antibody was first detected at 2 weeks after inoculation. And then, antibody titers determined by both methods rose at a similar rate throughout the test period. Titer determined by the END method revealed a maximum of 1:512 to 1:1,024 at 6 weeks after inoculation, while titer determined by the semi-micro RPF method was 1:8,192 or higher at the same stage. There was a tendency for the estimated neutralizing antibody titer to be eightfold higher in the semi-micro RPF

Table 1. Cross-neutralization test of NCP strains of BVD-MD virus

Antiserum	Strain				
	No. 12	Kanto Tozan	Shizuoka	NY-1	Indiana 46
No. 12	4,096 ^{a)}	2,048	2,048	512	512
Kanto Tozan	4,096	4,096	2,048	2,048	1,024
Shizuoka	2,048	2,048	4,096	1,024	256
NY-1	512	1,024	2,048	4,096	1,024
Indiana 46	256	512	512	256	2,048

a) Antibody titer expressed as reciprocal of serum dilution.

Fig. 2. Neutralization kinetics of five NCP strains of BVD-MD virus with homologous or heterologous antiserum.

Virus strains: ○ = No. 12; ● = Kanto Tozan; × = Shizuoka; △ = NY-1; ▲ = Indiana-46.

Abscissa = time in minutes; ordinate = per cent survival.

method than in the END method.

Antigenic relationships among five NCP strains in cross-neutralization test: Antigenic relationships among three Japanese strains

and two American strains were compared by the cross-neutralization test with the semi-micro RPF method. To examine the antibody to each strain, sera were collected from calves

Table 2. Comparison of neutralization rates of five NCP strains of BVD-MD virus

Antiserum ^{a)}	Viral strain									
	No. 12		Kanto Tozan		Shizuoka		NY-1		Indiana 46	
	K ^{b)}	NK ^{c)}	K	NK	K	NK	K	NK	K	NK
No. 12	8.5	100	6.5	75	8.8	104	3.7	43	4.7	55
Kanto Tozan	8.1	104	7.8	100	7.4	95	6.3	81	4.7	60
Shizuoka	6.9	70	7.1	73	9.8	100	5.8	59	2.2	22
NY-1	4.9	49	5.1	50	7.7	76	10.1	100	6.2	61
Indiana 46	2.4	34	3.4	48	4.1	58	3.9	55	7.1	100

a) Antisera were collected from calves at 3 weeks after inoculation.

b) Neutralization rate constant.

c) NK: Normalized constant.

at 3 weeks after inoculation. The results are listed in Table 1. The homologous titers were wholly in the range of 1:2,048 to 1:4,096, and were equivalent to or higher than those obtained with any heterologous strains. More than twofold difference in neutralizing antibody titers was not observed among three Japanese strains examined with respective antisera to the strains. It can be assumed that three Japanese strains were antigenically identical. Strain No. 12 was different eightfold in the titers with the antisera against American strains NY-1 and Indiana-46. And, strain NY-1 was poorly neutralized with antisera against strains No. 12 and Indiana-46. Strain Indiana-46 was poorly neutralized with antisera against strains No. 12 and Shizuoka.

Antigenic relationships among five NCP strains in kinetic neutralization test: To determine antigenic differences among serologically related strains, kinetic neutralization tests have been required. Accordingly, each of the five strains was tested against respective immune sera. These data indicated that this procedure could characterize uniquely each strain of BVD-MD virus (Table 2, Fig. 2).

anti-No. 12 serum. Comparison of NK values indicated that strain No. 12 was antigeni-

cally homologous to strain Shizuoka, and was similar to strain Kanto Tozan. On the other hand, the NK values of remaining two strains with anti-No. 12 serum indicated these strains were antigenically distinct from strain No. 12.

anti-Kanto Tozan serum. A close relationship was demonstrated between homologous strain and heterologous strains No. 12 and Shizuoka. Strain NY-1 was similar to strain Kanto Tozan. In contrast, the rate of neutralization of strain Indiana-46 was considerably low. The higher NK value with strain No. 12 was considered to be caused by some technical error so, it was regarded as similar to the NK value of the homologous combination.

anti-Shizuoka serum. Strains No. 12 and Kanto Tozan were antigenically similar to strain Shizuoka. Lower rate in the neutralization of strain NY-1 evidently suggested some antigenic distinction, while was even more marked with strain Indiana-46.

anti-NY-1 serum. The NK values of strains No. 12 and Kanto Tozan were much lower than that of the homologous strain indicating that the antigenic relationships distinguished between strain NY-1 and each of two strains. The NK values of the remaining two strains (Shizuoka and Indiana-46) with the anti-NY-1 serum indicated that these strains were anti-

genically similar to strain NY-1. anti-Indiana-46 serum. No close relationship was recognized among all the heterologous strains.

DISCUSSION

Plaque assay systems for CP strain of BVD-MD virus have been described by various workers. The reproducibility and sensitivity of the plaque technique have been elucidated [17], which are similar to other virus-cell systems [4]. The authors have developed the RPF method using methyl cellulose overlay for plaque titration of the NCP strains. The RPF method described herein could be applicable for the virus neutralization test. Pre-inoculation serum negative for the antibody to BVD-MD virus by the END method was also negative for that by the RPF method. Following inoculation into calf with the NCP strain, appearance of the antibody detected by the RPF method closely related to the antibody pattern by the END method. However, the antibody titer by the RPF method was almost eightfold higher than that by the END method.

Cross-neutralization test has been generally regarded as one of the most serologically specific methods, and applied to the identification of serotype. The results of the present cross-neutralization test indicated that strains No. 12, Kanto Tozan and Shizuoka were serologically indistinguishable. It could be concluded that this virus had been widespread in Japan, since strain No. 12 was isolated from slaughtered cattle in Tokyo, and strains Kanto Tozan and Shizuoka were from the cattle in Tochigi and Shizuoka prefectures, respectively. These results are in agreement with Omori *et al.* [16] who showed that five NCP strains isolated in Japan were antigenically similar each other by the END-neutralization test. However, in antisera against strains No. 12 and Shizuoka, there was eightfold difference in the antibody titers measured with homologous strain and heter-

ologous strains, either both strains Indiana-46 and NY-1 or the strain Indiana-46. The calf antisera against strains NY-1 and Indiana-46 had neutralizing antibody at low level against either the strain No. 12 or both the strains No. 12 and NY-1. The results seem to indicate that the antigenic differences in the cross-neutralization tests were very slight in most combinations. Moreover, these serologic differences among strains could not be evaluated on statistical analysis.

Kinetic neutralization test has been used to determine a little of antigenic difference among virus strains. McBride [14] demonstrated that kinetic neutralization was a sensitive method for antigenic analysis of poliovirus strains. Buening *et al.* [1] reported that, among four strains of infectious bovine rhinotracheitis virus, minor antigenic difference between ISU-IBR-1 strain and each of the other three strains could be demonstrated by kinetic neutralization test. Hafez and Liess [11] reported on the kinetic neutralization tests with four CP strains of BVD-MD virus, in which antigenic relationships among three strains isolated in Germany and one strain in the United States were described. However, these experiments lacked proof of serologic relationships among NCP strains of BVD-MD virus. Antigenic relationship or divergence among NCP strains could be estimated most clearly from the NK values. There were close relationships among three NCP strains isolated in Japan, which reacted in a similar way to each of the antisera under the test. No close relationship was evident between three Japanese strains and strain Indiana-46 except with anti-Kanto Tozan serum which was unable to distinguish four strains each other. Similar results were demonstrated in the relationships between three Japanese strains and the strain NY-1 except for that between anti-NY-1 serum and strain Shizuoka, and between strain NY-1 and anti-Kanto Tozan serum. It indicated that two American strains were antigenically distinct

from the Japanese strains used in this study, although some antigenic similarities were recognized in some combinations of the virus strains and the antisera. Moreover, the American strains appeared to differ one another slightly.

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要 約

リバースプラーク法による牛ウイルス性下痢-粘膜病ウイルス非細胞病原性株間の血清学的関係に関する検討: 伊藤 治・佐々木英治・花木琢磨¹⁾ (農林水産省動物医薬品検査所, ¹⁾(財) 化学及血清療法研究所)——牛ウイルス性下痢-粘膜病 (BVD-MD) ウイルスの非細胞病原性 (NCP) No. 12 株感染牛から得た一連の血清中の中和抗体価を, セミマイクロのリバースプラーク形成 (RPF) 法と END 法で比較測定した。セミマイクロ RPF 法で測定された中和抗体価は END 法で測定された中和抗体価に比べ 8 倍高い値を示した。日本で分離された 3 株と米国で分離された 2 株間の抗原性の差を交差中和試験で調べると, 日本の 3 株間には各抗血清中和抗体価で 2 倍を超える差は認められなかったが, 米国の株と日本の株間にはいくつかの組み合わせで 8 倍以上の差が認められた。中和反応速度試験においても, 日本で分離された BVD-MD ウイルスの 3 NCP 株間に近縁性が確認され, 米国の 2 株は, 日本の 3 株と多くの組み合わせにおいて抗原性に差が存在した。さらに, 米国の 2 株は相互に多少の差のあることが明らかとなった。