リバースプラーク形成法を用いた牛ウイルス性下痢

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Reverse Plaque Formation Method for Titration of Non-Cytopathogenic Bovine Viral Diarrhea-Mucosal Disease Virus

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The reverse plaque formation (RPF) method with a semi-micro plate was applied to the titration of a non-cytopathogenic (non-CP) strain of bovine viral diarrhea-mucosal disease (BVD-MD) virus. All the five non-CP strains used in this experiment formed reverse plaques (RP) on bovine testicle cell culture under methyl cellulose overlay. The RPF was inhibited by the pretreatment of a non-CP virus strain with immune rabbit serum to a reference strain. The specificity of the RPF method was demonstrated by the linear test and Poisson distribution test. Comparative titration of commercial BVD-MD vaccines was carried out by the semi-micro RPF method and the tube method based on the exaltation on Newcastle disease virus. The virus titer obtained by the former was slightly higher than that obtained by the latter. The former was proved to be a method of high sensitivity for determining non-CP virus.

Various methods have been reported for the detection and titration *in vitro* of the non-cytopathogenic (non-CP) strain of bovine viral diarrhea-mucosal disease (BVD-MD) virus. They have been devised on the basis of the immunofluorescence technique,[2] the interference phenomenon with the cytopathogenic (CP) strain,[4] and the END (exaltation of Newcastle disease virus) method.[8] Recently, the reverse plaque formation (RPF) method has been developed for titration of hog cholera virus by Fukusho et al.[3] It is based on the interference phenomenon with vesicular stomatitis virus.

This paper deals with the technical procedure of the RPF method using a semi-micro plate and based on the interference phenomenon observed among the homologous strains of BVD-MD virus.

MATERIALS AND METHODS

Tissure culture: Bovine testicle (BT) cells was suspended in Eagle's minimum essential medium (MEM) containing 10% calf serum and 0.29% tryptose phosphate broth, so that the resulting suspension might contain 2.5×10^5 cells/ml. Then 0.5 ml of the cell suspension was placed in each well of a multi-well plate (Falcon No. 3008, U.S.A.), which was put in an incubator at 37°C for 2 days in an atmosphere containing 5% carbon dioxide.

Viruses: The non-CP strains of BVD-MD virus used in this study were No. 12,[8] Shizuoka,[8] Kanto Tozan,[8] NY-1[1] and

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Indiana-46.[9] Strain No. 12 was used mainly for determination of the optimum condition for the RPF method. The CP strain of BVD–MD virus used was Nose.[6] All the strains were propagated in BT cells at 37°C for 5 days. Three lots of live BVD–MD virus vaccine which had been prepared from the attenuated strain No. 12 were also used.

Reverse plaque formation (RPF) method: The cells in each well were inoculated with 0.05 ml of an appropriate dilution of strain No. 12 by the aid of a dropper. The plate was subjected to incubation at 37°C for 1.5 hours for adsorption of virus. After the virus adsorption the cells in each well were washed once with MEM, and then covered with 0.5 ml of overlay medium. The overlay medium was 2.4% aqueous solution of methyl cellulose mixed with an equal volume of double concentrated MEM contained 0.29% tryptose phosphate broth, 5% calf serum and antibiotics. It has been adjusted to pH 7.2 with 8% sodium bicarbonate solution. After incubation at 37°C for 4 days the methyl cellulose–overlaid medium was removed from the surface of the cell culture with Earle's solution. Then the cells of all the wells were reinoculated with 0.1 ml of strain Nose suspension containing about $10^{6.0}$ $TCID_{50}$/ml. After the virus adsorption the cells of each well were again overlaid with 0.5 ml of methyl cellulose–overlaid medium. After incubation for 3 days the culture was fixed in methanol and stained with 0.2% crystal violet solution to count the number of reverse plaques (RP) formed.

Experiments on specificity: Five-tenth ml of hyperimmune rabbit serum prepared with strain Nose and showing a neutralizing antibody titer of 1 : 2,048 was mixed with 0.5 ml of a suspension containing about $10^{5.0}$ $TCID_{50}$/ml of strain No. 12. The resulting mixture was incubated at 37°C for 1 hour and inoculated into the cell culture.

To every well of the semi-micro plate was inoculated 0.05 ml of a given dilution of the viral suspension of strain No. 12. Observation was made on the degree of fluctuation of the number of RP formed in each well.[7]

The six wells of the plate were inoculated with one of three different dilutions of the viral suspension to examine the relationship between the number of RP formed and the degree of dilutian of the viral suspension.

Exaltation of Newcastle disease virus (END) method: The method was carried out in essentially the same manner as described by Omori et al.[8]

RESULTS

RPF: All the strains of non-CP BVD–MD virus formed RP in BT cells under a methyl cellulose overlay. Neither RP morphology nor rate of development varried

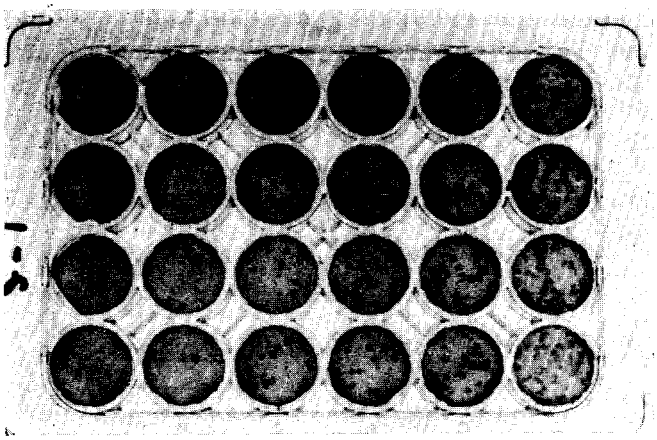

Fig. 1. RPF by strain No. 12 of non-CP BVD-MD virus on semi-micro tissue culture plate

among strains No. 12, Shizuoka and Kanto Tozan. Fig. 1 shows RP of strain No. 12 on the semi-micro plate. These RP had a distinct outline and were 0.7~1.5 mm, or 1.1 mm on the average, in diameter. Strains NY-1 and Indiana-46 produced round plaques and formed a litte larger RP than the former 3 strains. The majority of the RP were 0.9~1.9 mm, or 1.5 mm on the average, in diameter. In some batches of BT cells, RP consistently formed until the 8th passage of the cells. When the period of reincubation was prolonged by 2~3 days after virus inoculation, RP became so distinct and large that they could be counted with ease. RPF was compared among 3 lots of methyl cellulose which had been used to prepare the overlay medium. As a result, there were little differences in the number and size of RP among these.

Specificity of RPF: RPF was completely inhibited when strain No. 12 was mixed with strain Nose antiserum before inoculation, whereas a control mi xture without the antiserum gave to an expected number of clear RP.

The number of RP formed in each well of the semi-micro plate is shown in Table 1. It is 34 to 45 and within a range of 12% of the average. It exhibits a 95% confidence limit of 36.6 to 39.4[7] and a Poisson distribution. These results indicate that the RPF method with a semi-micro plate and BT cells was efficient enough to apply to the virus titration.

Table 1. *Number of reverse plaques formed in each of 24 wells*

45	43	43	42	42	41	41	41
40	40	40	38	38	38	37	37
36	35	35	35	35	35	34	34

The dose response pattern presented 3 different points of dilution rate (Fig. 2). It was demonstrated that the number of RP increased linearly in proportion to the concentration of strain No. 12. From these results it was suggested that one particle of the non-CP strain of BVD-MD virus might form one RP.

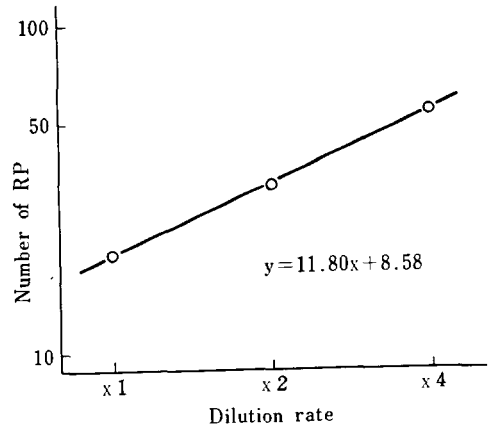

Fig. 2. Regression of mean plaque count on dilution. *Reverse plaque counts were made in 6 wells for each virus dilution.*

Comparison of vaccine virus titer determined between semi-micro RPF method and END method: An experiment was carried out with three lots of live vaccine to determine whether the RPF method was applicable to titration of the virus and whether there was a difference in virus titer estimated by the RPF and the END method. The virus titers of all the lots of vaccine could be measured easily by the semi-micro RPF method. When the infective virus titer was compared between the RPF and the END method, there was a tendency for the titer estimated by the former was a little higher than that estimated by the latter (Table 2).

Table 2. *Comparative virus titration of BVD-MD live virus vaccines by semi-micro RPF and tube END methods*

Vaccine No.	RPF method PFU/ml	END method $TCID_{50}$/ml	RPF method/ END method
1	1.1×10^5	5.6×10^4 (3.86×10^4)	2.9
2	6.4×10^3	2.4×10^3 (1.66×10^3)	3.9
3	1.4×10^4	5.6×10^3 (3.86×10^3)	3.6

In parentheses is shown the number of infective particles. The titer was determined by Poisson formula on condition that 0.69 infective particles are equal to 1 $TCID_{50}$.

DISCUSSION

In the present experiment the RPF method with a semi-micro plate was applied to the titration of non-CP strains of BVD-MD virus. As a result, it was clearly demonstrated to be a method of high reproducibility and exactitude for the titration of these strains.

The plaque formation by a non-CP strain of BVD-MD virus was already reported by Straver[11] in 1971. It is examined by essentially the same method as the plaque formation method of CP strains of this virus.[10] In that method a culture medium containing agar is used as overlay medium. The culture is incubated for 5 days after inoculation, stained with neutral red, and allowed to stand in a dark place for 2~5 days. Then the plaques formed on the culture are counted. It has been reported that plaques formed within several days after staining are too small to be distinguished. It has also been mentioned that no plaques were formed when some batches of bovine embryonic testicle cells were used or when a certain type of agar was contained in the overlay medium.

When the method developed by the authors was applied, the portion of the medium where a non-CP strain of BVD-MD virus multiplied by infection was round and stained well with crystal virus. It was clearly discriminated from cells destroyed by the CP strain, and made it easy to count RP formed. Moreover, methyl cellulose added to the overlay medium solution was efficient enough to obtain satisfactory results, in the 3 lots. The BT cells used were derived from normal cattle.

When they were at about the 8th or younger passage generation, distinct RP could be formed. In the RPF by hog cholera virus,[3] it was verified that RP were formed in cells of passage originated from the swine kidney (SK-H), as well as in primary swine testicle cells. Then it was desired that studies would be made in future on the possibility of using cells of passage of bovine origin for the RPF method of BVD-MD virus.

RP formation was found in all the five non-CP strains of BVD-MD virus. It was inhibited specifically by pretreatment with anti-strain Nose hyperimmune serum prepared from the rabbit. The RP formed by the non-CP strains were 0.7~1.9 mm in diameter. There was a small difference in the average diameter of RP formed between the group of three strains isolated in Japan and that of two strains isolated in other countries than Japan. When strain No. 12 was used as a non-CP strain, it was possible to count about 60 RP per well by the semi-micro RPF method. In the present investigation it was demonstrated from the results of the linear test and the Poisson distribution test that one reverse plaque was formed lug one infective unit of the non-CP strain of virus. The titer of virus contained in vaccine was a little higher when estimated by the semi-micro RPF method than when estimated by the tube END method. This result indicated that the former was more sensitive than the latter. It was the same as the result of comparison made by Fukusho et al.[3] on the titer of hog cholera virus estimated between the RPF and the interference methods.

It was considered that if the RPF method was applicable to the neutralization test with a non-CP strain of BVD-MD virus, it would be such an efficient method of estimation of antibody titer as the known END method and the interference method.

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