

# 感染耐過ウシ血清を用いたSRID法による口蹄疫ウイルス抗原の定量

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## Quantitative Single Radial Immunodiffusion of Foot-and-Mouth Disease Viral Antigens Using Convalescent Cattle Sera

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**ABSTRACT.** Single radial immunodiffusion (SRID) using convalescent cattle sera was developed to evaluate the potency of foot-and-mouth disease inactivated vaccine. The complete virion (140S antigen) was collected by sucrose-gradient centrifugation of inactivated virus. The 12S subunit antigen was prepared by heat treatment of purified 140S antigen. Convalescent cattle sera produced diffusion ring with 140S antigen alone, while hyperimmunized guinea pig sera produced with both 140S and 12S antigens. In SRID of mixed viral antigen (140S and 12S) tested against hyperimmunized guinea pig sera, diffusion rings of the former was affected by contamination of the latter. When the same samples were tested in SRID using convalescent cattle sera, 12S antigen did not affect the quantitation of 140S antigen. Area of diffusion rings of purified 140S antigen clearly correlated with concentration of proteins measured by optical density, as well as by complement fixation tests using hyperimmunized guinea pig sera. SRID using convalescent cattle sera is more practical than conventional SRID using immunized guinea pig sera, because cattle sera react with effective component (140S antigen) alone, and not with ineffective 12S antigen, in the vaccine, and because infected cattle is more suitable to make large amount of pooled sera for the routine assay.—**KEY WORDS:** foot-and-mouth disease, single radial immunodiffusion, vaccine potency, vaccine quality, 140S antigen.

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Foot-and-mouth disease (FMD) virus induced vesicular lesions on the feet and in the mouth of cloven-footed animals in highly communicable disease. In endemic area the full control and eventual eradication of this disease are matters of paramount importance in animal husbandry. To prevent the disease large amount of FMD vaccines are prepared by inactivating the virus grown in cell culture.

Cultured cells infected with viruses contain a number of antigenic components. Major antigens are 140S (complete virion) antigen, 75S (empty capsid) antigen, 12S (12S subunit) antigen, and virus-infection-associated (VIA) antigen. Complete virion and natural precursor empty capsid are responsible for the induction of neutralizing antibodies in vaccinated animals,

although 75S antigen is less potent as the vaccine [9]. VIA antigen and 12S antigen do not induce any neutralizing antibodies in cattles or guinea pigs [6, 9].

Protection assay is conventionally performed on cattle or pigs to evaluate the potency of inactivated vaccine. However, this method is costly and risky of spreading virulent virus at the time of challenge, and is unpractical because of virulent virus at the time of challenge, and because of difficulty of seronegative animal procurement in prevailing countries. Alternative methods, such as determination of viral protein mass, or utilizing laboratory animals such as guinea pig can be used to replace the challenge methods.

More precise *in vitro* evaluation assays have been developed to quantitate the

protein mass in complement fixation (CF) test [8], single radial hemolysis [13], enzyme-linked immunosorbent assay (ELISA) [12], or quantitation of 140S antigen by sucrose gradient procedure and optical density [9]. Guinea pig IgM antibodies are used for single radial immunodiffusion (SRID) to 140S antigen [7]. This SRID method was not widely applied to evaluate the potency, because of difficulty in purification and concentration of viral antigen, and because of difficulty in developing large amount of antibodies in guinea pigs. In this report we developed SRID using infected cattle sera to evaluate the potency. Methods for 140S antigen isolation were simplified for the practical use of large scale of evaluation assay.

#### MATERIALS AND METHODS

*Virus production and purification:* Vaccine strains of FMD virus (O type Bangkok/60 and Asia-1 type Bangkok/65) were used in all experiments. Virus grown in BHK-21 cell was inactivated in 0.01 M bromoethyleimine (BEI) for 18 hr at 37°C. In most experiments, virus titer before inactivation was approximately  $10^8$  TCID<sub>50</sub>/ml. Inactivated virus was concentrated first by ultrafiltration using Hollow-fiber system (Amicon Hollow-fiber, DC-2, M.W. 100,000 cut off) and further concentrated by precipitation with 10% polyethylene glycol (PEG 6000). Five hundred times concentrated virus solutions were layered onto 15% to 45% sucrose-gradients and centrifuged at 80,000 G for 2.5hr at 4°C [9]. Complete virion (140S) were collected in peak fraction monitored by optical density (OD) at 259 nm. Total amount of purified viral protein was determined spectrophotometrically according to Bachrach's method [1] using extinction  $E^{1\%}_{1\text{cm}}$ , 259nm of 76.0, and relative purity was estimated by  $OD_{259}/OD_{238}$  and  $OD_{259}/OD_{280}$  value [2].

The purified preparations contained 1 to 2 mg/ml of viral proteins. For the preparation of 12S antigen, 140S antigen was dissociated by heating at 56°C for 30 min. Total amount of 12S antigen was regarded the same as the original amount of 140S antigen.

*Antiserum:* Guinea pigs were inoculated in the plantar pads with a guinea pig-adapted virus (10% homogenate of vesicular epithelium of plantar pad). They were given an additional intramuscular inoculation of the virus at 4 weeks later. Blood samples were obtained at 10 days postinfection (DPI). The sera were collected and pooled. Cattles were inoculated into tongue epithelium with virulent FMD virus containing  $10^4$  ID<sub>50</sub>/0.1 ml in vesicular epithelium homogenates. Infected cattles were sacrificed at 21 DPI. And the sera collected were pooled. The antisera were heated for 30 min at 56°C and kept in -20°C.

*Ouchterlony's agar diffusion test:* Purified FMD viral antigens and serially diluted antisera were placed in separate wells in the agar as described previously [11].

*SRID test:* Agarose (Miles, type LSL) was suspended in Tris-HCl buffer (pH 7.6) at the concentration of 2%, melted at 50°C and stored at 4°C. Sera were diluted with 0.15 M NaCl in distilled water or phosphate buffered saline (PBS), and stored at -20°C. The SRID plates were prepared as follows. 1) Ten ml of the agarose solution was pipetted into screw-cap vials and held at 50°C. 2) Equal volume of serum suitably diluted with 0.15 M NaCl in distilled water was added. 3) 250  $\mu$ l of the mixture was poured into a well of plastic plates (kindly supplied by Dr. Tomiaki Morimoto, Eisai Pharmaceutical Co., Tokyo) to form layer of 1 mm in thickness and 25 mm in diameter. The plates were kept at 4°C in a humidified condition until use. 4) Wells of 4 mm or 3.4 mm in diameter were cut and plugs were removed by aspiration. Antigens were added to each of the wells in duplicates

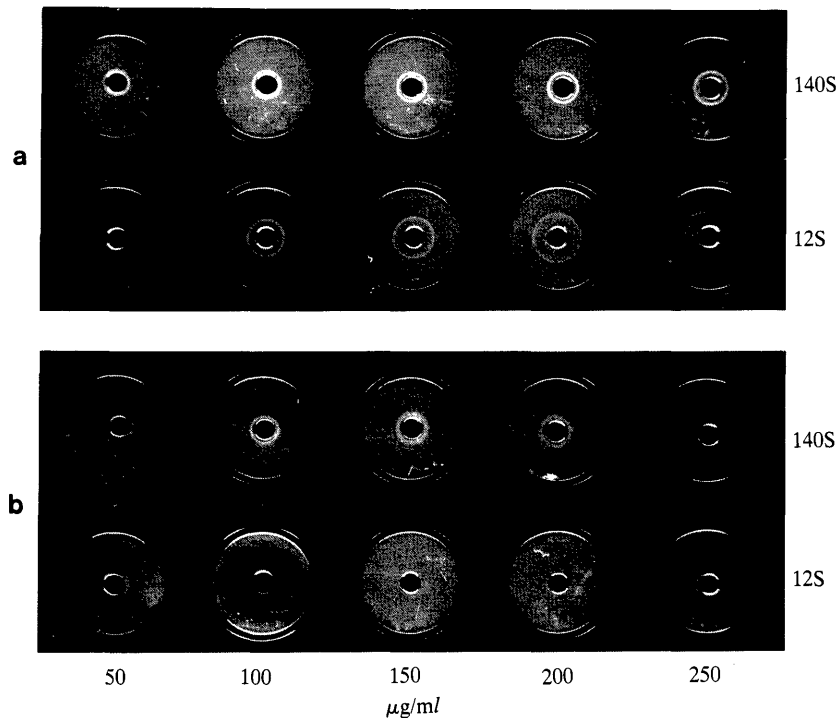


Fig. 1. Single radial immunodiffusion reaction of various concentration of 140S (upper row) and 12S (lower row of each plate) antigen of O type FMD virus tested against diluted sera (1:40) from hyperimmunized guinea pigs (a) and from convalescent cattles (b). The quantity of viral antigens estimated by OD were indicated under the photograph.

using microliter syringes; 15  $\mu$ l for 4 mm wells, 12  $\mu$ l for 3.4 mm wells. 5) The plates were incubated at room temperature for 2 days in a humidified condition. The diameter of the diffusion rings formed around the wells was determined with a measuring magnifier.

*CF test:* The basic procedure was described previously [8,14]. The hemolytic indicator system consisted of a 1.5 % suspension of washed sheep erythrocytes sensitized with 4 minimum hemolytic dose of horse serum.

Serially diluted FMD virus antigen (25  $\mu$ l), hyperimmune guinea pig serum (25  $\mu$ l) and three 50%-hemolytic units of complement (25  $\mu$ l) were incubated in microtiter plate wells at 37°C for 1 hr. After incubation, 50  $\mu$ l of hemolytic indicator system was

added to each well and incubated for 30 min at 37°C. The sheep erythrocytes which had not been hemolysed in the plates were then sedimented by centrifugation and the degree of hemolysis in each wells was determined with a simple colorimeter. Antigen titer was indicated by reciprocal of highest dilution which showed 50% hemolysis. Complement fixation unit (c.f.u.) of FMD antigens was estimated by Bradish's method [3].

## RESULTS

*Ouchterlony's agar diffusion for O type FMD virus:* Immuno-precipitation to 140S antigen and 12S antigen were tested in Ouchterlony's agar diffusion using anti-O type sera. Convalescent cattle sera or hyper-

immunized guinea pig sera distinguished 140S antigen from 12S antigen in different bands of precipitation. Cattle sera showed precipitin bands to 140S antigen and 12S antigen until 1:64 and 1:4 dilution, respectively. Hyperimmune guinea pig sera showed bands to 140S antigen and 12S antigen until 1:128 and 1:16 dilution, respectively. Immuno-precipitation was more sensitive to 140S antigen.

**SRID tests for O type FMD virus:** Cattle or guinea pig sera were utilized in SRID to estimate the amount of FMD virus antigen in the samples. Convalescent cattle sera or hyperimmune guinea pig sera were incorporated in agarose plates in a final dilution of 1:40. Both 140S and 12S antigens were diluted in PBS to 50, 100, 150, 200 and 250  $\mu\text{g/ml}$ . Twelve  $\mu\text{l}$  of samples were put into 3.4 mm diameter wells (Fig. 1). Hyperimmune guinea pig sera reacted to diluted antigens showing clear diffusion rings. The rings of 12S antigen were larger in diameter than those of 140S antigen (Fig. 1a). Convalescent cattle sera showed clear diffusion rings with 140S antigen but not with 12S antigen. Diffusion rings with cattle sera

were less intense than those with hyperimmune guinea pig sera. (Fig. 1b). Table 1 showed the diameter of diffusion rings using convalescent cattle sera or hyperimmune guinea pig sera.

In the next experiments, mixtures of 140S antigen (100  $\mu\text{g/ml}$ ) and 12S antigen (50, 100, 150  $\mu\text{g/ml}$ ) were put into 3.4 mm wells

Table 1. Single radial immunodiffusion to 140S antigen and 12S antigen of O type virus using convalescent cattle sera or hyperimmune guinea pig sera

Virus subunit	Amount of antigens measured by optical density ( $\mu\text{g/ml}$ )					Reacted serum
	50	100	150	200	250	
140S antigen	4.2 <sup>a)</sup> 4.2	4.7 4.6	5.5 4.9	6.0 5.2	6.7 5.6	CS <sup>b)</sup> GPS <sup>c)</sup>
12S antigen	N <sup>d)</sup> 5.1	N 6.1	N 6.7	N 7.8	N 8.6	CS GPS

- a) Diameter (mm) of diffusion rings in SRID plates. 12  $\mu\text{l}$  samples were put in 3.4 mm diameter wells.  
 b) Convalescent sera from cattles infected with O type FMD virus. Sera were diluted in 1:40 in agarose.  
 c) Hyperimmune sera from guinea pigs infected with O type FMD virus. Sera were diluted in 1:80 in agarose.  
 d) Not detected.

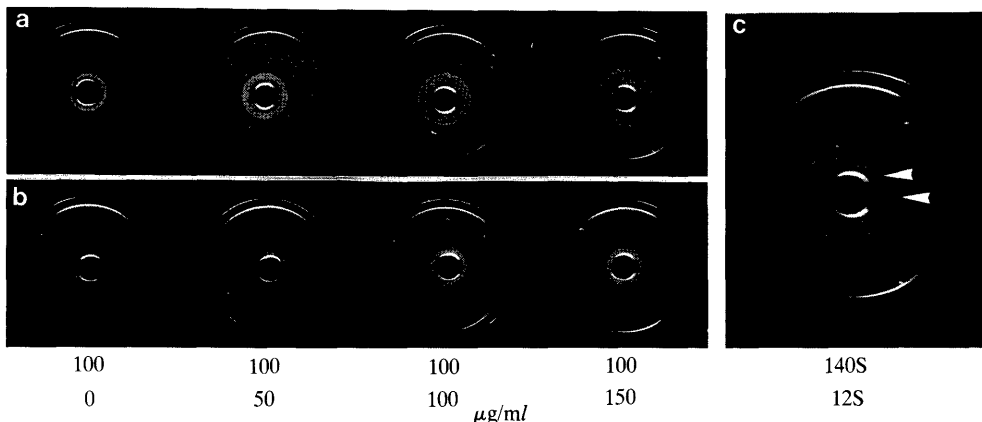


Fig. 2. Single radial immunodiffusion reaction with mixture of 140S and 12S antigens, and diluted sera from hyperimmunized guinea pigs (a) and from convalescent cattles (b). Antibodies used were the same as Fig. 1. The quantity of viral antigens estimated by optical density were indicated under the photograph. c; Higher magnification of SRID of 140S antigen (100  $\mu\text{g/ml}$ ) and 12S antigen (150  $\mu\text{g/ml}$ ) using hyperimmune guinea pig sera. Arrows indicate inner ring of 140S antigen and outer ring of 12S antigen.

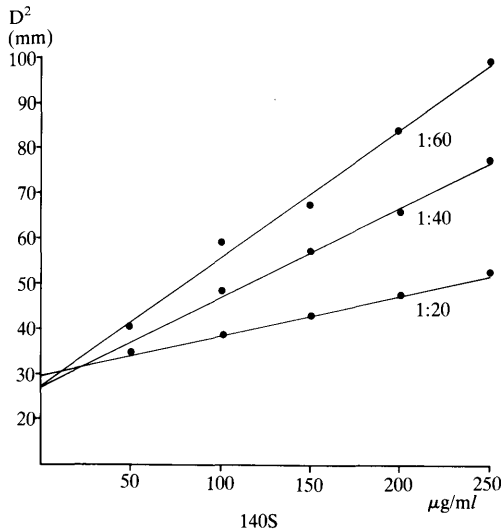


Fig. 3. The relation between the square of diffusion ring diameter and the quantity of 140S antigen estimated by OD. Convalescent cattle sera were diluted in 1:20, 1:40 and 1:60.

to see the coexistence effect of two antigens on diffusion rings (Fig. 2). In guinea pig serum-agarose plates these mixture produced distinct double rings. Outer rings were reaction of 12S antigen, and inner rings were of 140S antigen. Diameter of outer rings were the same as in the control wells of 12S antigen alone, while diameter of inner rings were larger than the control wells of 140S antigen alone. This indicated that 12S antigen affected the diffusion rings of 140S antigen. When the same antigen samples were tested against convalescent cattle sera, 12S antigen did not produce outer diffusion rings and did not affect the diffusion rings of 140S antigen.

When convalescent cattle sera were serially diluted in serum-agarose, and reacted against 140S antigen in varying dilution, area of diffusion rings statistically correlated with concentration of antigen measured by optical density as shown in Figure 3. 12S antigen did not produce any diffusion ring in any dilutions of antigen or antibody.

Table 2. Single radial immunodiffusion to 140S antigen and 12S antigen of Asia-1 type virus using convalescent cattle sera or hyperimmune guinea pig sera

Virus subunit	Amount of antigens measured by optical density ( $\mu\text{g/ml}$ )					Reacted serum
	50	100	150	200	250	
140S antigen	5.4 <sup>a)</sup>	6.0	6.5	6.8	7.2	1:40 CS <sup>b)</sup>
	5.5	6.5	7.5	8.0	8.9	1:80 CS
	5.8	6.3	7.0	7.3	7.9	1:40 GPS <sup>c)</sup>
	6.2	7.5	8.8	9.4	—	1:80 GPS
12S antigen	N <sup>d)</sup>	N	N	N	N	1:40 CS
	N	N	N	N	N	1:80 CS
	6.9	9.1	10.8	— <sup>e)</sup>	—	1:40 GPS
	8.1	—	—	—	—	1:80 GPS

- Diameter (mm) of diffusion rings in SRID plates. 15  $\mu\text{l}$  samples were put in 4.0 mm diameter wells.
- Convalescent sera from cattles infected with Asia-1 type FMD virus. Sera were diluted in 1:40 and 1:80 in agarose.
- Hyperimmune sera from guinea pigs infected with Asia-1 type FMD virus. Sera were diluted in 1:40 and 1:80 in agarose.
- Not detected.
- Widely diffused and unable to measure in SRID plates.

*SRID test for Asia-1 type FMD virus:* 140S or 12S antigen was diluted in 50, 100, 150 and 200  $\mu\text{g/ml}$ . Fifteen  $\mu\text{l}$  of each sample was put in 4 mm well, and incubated. As shown in Table 2, hyperimmune guinea pig sera reacted to 140S antigen and 12S antigen, producing diffusion rings. Smaller concentration of 12S antigen produced more distinct rings in more diluted sera. Convalescent cattle sera produced diffusion rings to 140S antigen alone, and did not produce diffusion rings to 12S antigen. In another experiment 140S antigens (100  $\mu\text{g/ml}$ ) were mixed with 12S antigens (50, 100, 150, 200  $\mu\text{g/ml}$ ), and reacted in cattle serum-agarose plates. 12S antigen of Asia-1 type FMD virus did not affect diffusion rings of 140S antigen in any dilutions of antigens.

*CF tests for O type FMD virus:* Figure 4 shows the SRID and CF test of each fraction of 15% to 45% sucrose-gradient centrifugation. In CF test hyperimmune guinea

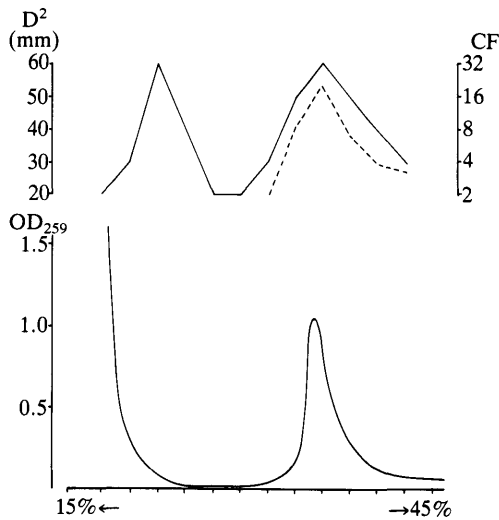


Fig. 4. Fractions separated by sucrose-gradient centrifugation of O type FMD virus. Quantity of antigens estimated by OD, CF test and SRID ( $D^2$ ) were indicated in the right and left hand of photograph. CF test was with hyperimmune sera from guinea pigs. SRID was with convalescent sera from cattles infected with the same virus. In the upper figure, solid line indicates CF antigen titer, while broken line indicates quantitation by SRID. Lower figure showed OD of each fraction.

pig sera reacted to two fractions consisted of 140S antigen and 12S antigen, while convalescent cattle sera produced diffusion rings to 140S antigen alone.

Figure 5 shows the results of CF tests using isolated and purified 140S antigen and 12S antigen. Guinea pig sera reacted against 140S antigen and 12S antigen in CF test. 140S antigen showed more efficient CF reaction than 12S antigen.

#### DISCUSSION

SRID for FMD virus antigen was previously reported by Cowan and Wagner [7]. They detected and quantitated the viral antigen using three different kinds of guinea pig sera of 1) 7 DPI, 2) repeatedly inoculated, 3) 85 days after immunized with purified inactivated FMD virus and Freund's incomplete adjuvant. Immunoglo-

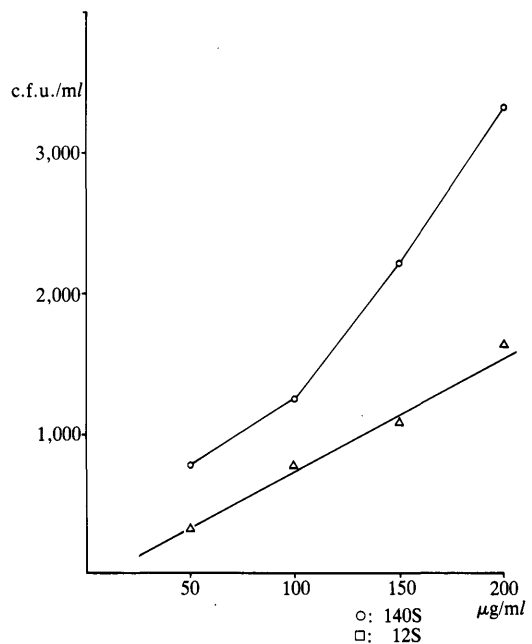


Fig. 5. Quantitation of 140S antigen and 12S antigen by quantitative CF test and by OD. Complement fixation unit (c.f.u.) (vertical line) was with hyperimmune sera from guinea pigs infected with O type Bangkok FMD virus. Quantitation by OD was shown in horizontal line.

bulin (Ig)G antibodies against FMD virus were induced in guinea pigs which were infected repeatedly or were immunized with virus and adjuvant. These IgG antibodies produced diffusion rings to both 140S antigen and 12S antigen.

However using guinea pig IgG antibodies 12S-140S antigen mixture gave larger rings than obtained with 140S alone. This was due to the fact that 12S antigen diffused out faster than 140S antigen and adsorbed out some of the antibody reactive with 140S antigen. Guinea pig IgM antibodies collected at 7 DPI produced diffusion rings to 140S antigen alone [7]. In our experiment hyperimmune guinea pig sera produced diffusion rings to 140S antigen and 12S antigen, as reported previously [7]. 12S antigen affected the diffusion rings of 140S antigen.

Convalescent cattle sera produced diffu-

sion ring to 140S antigen alone, although less diluted sera reacted to both 140S antigen and 12S antigen in Ouchterlony's agar diffusion. 12S antigen did not affect the diffusion rings of 140S antigen even in less diluted cattle sera. These observations suggested that bovine antibodies to 140S antigen did not or rarely cross-react to 12S antigen.

In previous reports guinea pig IgG antibodies were less specific to FMD virus [4], and did not react to 140S antigen alone, Guinea pig IgG antibodies reacted to 12S and 140S antigens of FMD virus, while IgM antibodies specifically reacted to trypsin-sensitive and cell-attachment site of 140S antigen. IgM antibodies were more suitable for SRID in guinea pig sera. However, guinea pig sera were not utilized routinely for the large scale assay to evaluate the potency of FMD inactivated vaccine, because of the difficulty to produce large amount of antibodies in guinea pigs.

Cattle infected with FMD virus produced IgM antibodies from 7 DPI [5], then produced IgG antibodies from 14 DPI [4,7]. Bovine IgM antibodies reacted to common antigen in several different types of FMD virus, while bovine IgG antibodies reacted to type specific antigens [10]. Present results indicated that convalescent cattle sera specifically reacted to 140S antigen of FMD virus alone. These previous results and our results suggested that bovine IgG antibodies specifically reacted to 140S antigen of FMD virus sub-type. Ongoing experiments showed that SRID plates using O type FMD virus immunized cattle sera did not produce diffusion rings to Asia-1 type or A type FMD virus (unpublished observations).

Our method for SRID is more practical than conventional SRID using guinea pig sera. Type specific antibodies were produced in infected cattle to make large amount of pooled sera. To evaluate the potency of FMD vaccine, cattle sera were

suitable for standardized large scale assay. Using Hollow-fiber filter system and polyethylene glycol we developed simplified and accurate concentration method for FMD virus. After these concentration we could estimate the amount of 140S antigen in FMD vaccine using SRID instead of sucrose-gradients and optical density. Besides, our method is more advanced than conventional CF test to evaluate the potency, because our method could detect type specific 140S antigen alone, which is the effective component of inactivated vaccine. Therefore this SRID method is practical for the *in vitro* quality control test of FMD inactivated vaccine.

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#### 要 約

感染耐過ウシ血清を用いた SRID 法による口蹄疫ウイルス抗原の定量：津田知幸，難波功一<sup>1)</sup>，山崎康人，S. ニルチャベ<sup>2)</sup>，N.バタナブラシット<sup>2)</sup>，W.シンチョンスボンコイ<sup>2)</sup>，小野寺節（家畜衛生試験場海外病研究部免疫研究室，<sup>1)</sup> 製剤研究部製剤工学研究室，<sup>2)</sup> タイ国口蹄疫ワクチン製造センター）——口蹄疫ウイルス蛋白のサブユニット中，中和抗体産生抗原で，ワクチンの有効物質である140S粒子を，口蹄疫ウイルス不活化抗原から分離・精製し，単純放射免疫拡散 (SRID) 法により定量した。高度免疫モルモット血清を用いると，140S粒子とともにワクチンに含まれる12S蛋白により，140S粒子の反応が影響された。これに対して感染耐過ウシ血清を用いた SRID 法では，140S粒子のみが定量され，12S蛋白の混入があっても影響はなかった。140S粒子の SRID 値は，光電比色測定値および補体結合反応測定値と相関した。この方法は口蹄疫不活化ワクチンの有効性の測定や力価検定に有用と思われた。