PURIFICATION AND MORPHOLOGY OF RICE RAGGED STUNT VIRUS

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Introduction

Rice ragged stunt virus (RRSV), found in Indonesia in 1976[3] and in the Philippines in 1977[5], is persistently transmitted to Gramineae plants by brown planthoppers, *Nilaparvata lugens* STÅL.[3,4,5,7,8]

Our previous report mentioned that purified preparations contained only smooth 36-40 nm particles associated with relatively low RRSV-infectivity.[8] MILNE[6] pointed out that they could not find such small particles in the partially purified preparations, and the RRSV virions are 63 nm in diameter, which corresponded with those found in ultrathinsections and dip preparations.[2,8] In our further investigation, we detected two kinds of particles after sucrose density-gradient centrifugation of the preparations from RRSV infected rice plant materials. They were smooth smaller spherical particles with low infectivity and larger spherical particles associated with high RRSV-infectivity.

In this paper, we described the purification procedures of RRSV, and also smooth smaller particles, which seemed to be a contaminated virus of unknown origin.

Materials and Methods

Virus source:

Original RRSV* infected rice plants were supplied by the late K. C. LING, International Rice Research Institute, Philippines, and the virus was

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* Yokohama Quarantine Office, Ministry of Agriculture, Fisheries and Foresty, Japan, permission No. 1043, Aug. 11, 1983.

maintained on rice plants (*Oryza sativa*, cv. Mihonishiki and Kinmaze), maize (*Zea mays*, cv. Goden Cross Bantam) or barley (*Hordeum vulgare*, cv. Hokutohadaka and Rokkakuozeki) inoculated by viruliferous brown planthoppers in a greenhouse.

Virus purification:

Leaf and sheath tissues of the RRSV infected plants were pulverized at 4°C with a meat chopper in 2-3 vols (w/v) of 0.1 M phosphate buffer (P. B.), pH 6.0-6.1, containing 1% driselase (Kyowa Hakko Kogyo Co. Ltd, Tokyo) and incubated at 20-23°C for 1 hour, according to TAKANAMI and KUBO[9]. The debris was removed by squeezing through two layers of cheesecloth. The extract was homoginized with 1/3 vol (v/v) carbon tetrachloride for 3-4 min, and centrifuged at 3,000 rpm for 10 min. Solid 6% polyethylene glycol #6,000, 0.3 M NaCl and 3% triton X-100 were added to the supernatant fluid and stirred for 1 hour with a magnetic stirrer. After centrifugation at 5,000 rpm for 20 min, the precipitate was suspended in 0.01 M P. B., pH 7.0, containing 0.005 M disodium ethylenediaminetetraacetate (EDTA). The suspension was homogenized with 1/3 vol (v/v) fluorocarbon (Daifron solvent S 3, Daikin Kogyo, Osaka) and centrifuged at 3,000 rpm for 5 min. This procedure was repeated twice. The clarified aqueous phase was concentrated by one cycle of differential centrifugation at 8,000 rpm for 10 min and 22,000 rpm for 1 hour in a Hitachi RP-30 rotor. The final pellet was resuspended in a small volume of 0.01 M P. B., pH 7.0, containing 0.005 M EDTA, layered on a linear gradient of 20-50% (w/v) sucrose in 0.01 M P. B., pH 7.0 and centrifuged at 20,000 rpm for 2-2.5 hours in a Hitachi RPS-25 rotor. The virus fraction was collected by an ISCO model 180 fractionator, and centrifuged at 36,000 rpm for 1.5 hours in a Hitachi RP-40 rotor to remove sucrose. Ultraviolet spectra were taken by a Hitachi model 624 digital double-beam spectrophotometer.

Infectivity assay:

The purified preparations were injected into vector insects, *Nilaparvata lugens*, at 8°C after anesthetized with CO_2. The injected insects were confined on healthy rice plants for 10 days, and the survivors were transfered to rice seedlings in test tubes at 2-3 insects per seedling. After 3 days, the inoculated seedling were transplanted to pots in the greenhouse.

Electron microscopy:

The purified virus preparations were diried onto a carbon formvar coated grids, which were treated by glow discharge prior to use. The grids were

washed with several drops of distilled water and negatively stained with 2% uranyl acetate (UA) or 2% potassium phosphotungstic acid (PTA) at pH 5 or 7, and drained with a filter paper. Some virus preparations were fixed with 1% glutaraldehyde (GA) before staining. The preparations were examined by a JEM 100 B electron microscope.

Results

i) *Purification*: Two light scattering zones, designated as top and bottom fractions, were always obtained in the concentrated preparations from the infected rice plants after sucrose density-gradient centrifugation (Fig. 1). The bottom fraction contained numerous spherical particles about 63 nm in diameter (63 nm particle) and a few smaller spherical particles of 35 nm in diameter (35 nm particle). While the top fraction consisted of mostly 35 nm particles contaminating with a few 63 nm particles (Fig. 2).

The UV absorption spectra of the top and bottom fractions, indicated that both fractions contained the particles of virus nature (Fig. 3).

Fresh tissues yielded more the bottom fraction which mostly consisted of the 63 nm particles than those from the frozen tissues, whereas the top

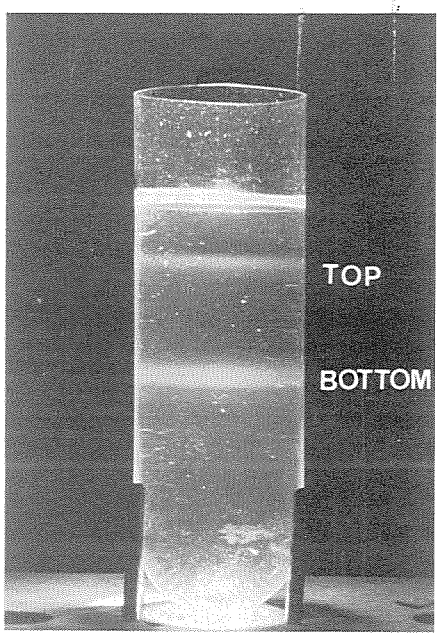

Fig. 1. Two light scattering zones obtained by sucrose density-gradient centrifugation at 22,000 rpm for 2 hours.

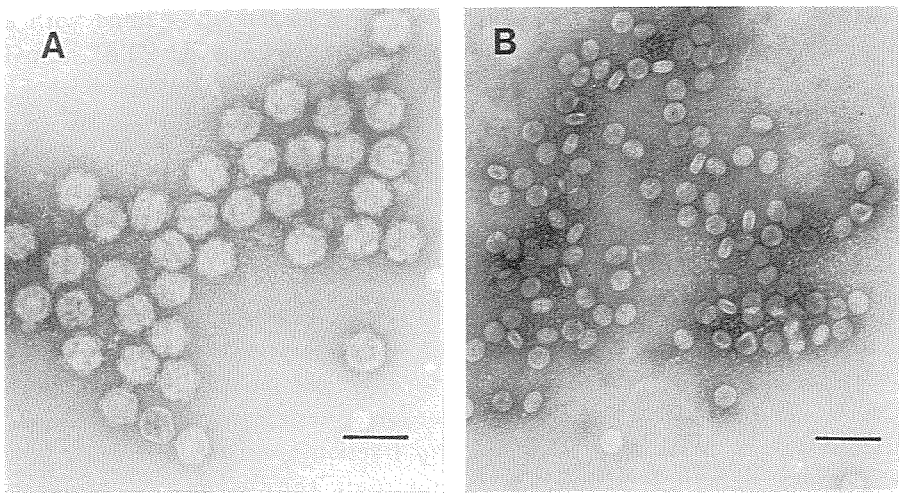

Fig. 2. Electron micrographs of the 63 nm particles (A) and 35 nm particles (B). 2% UA stained. Bar represents 100 nm

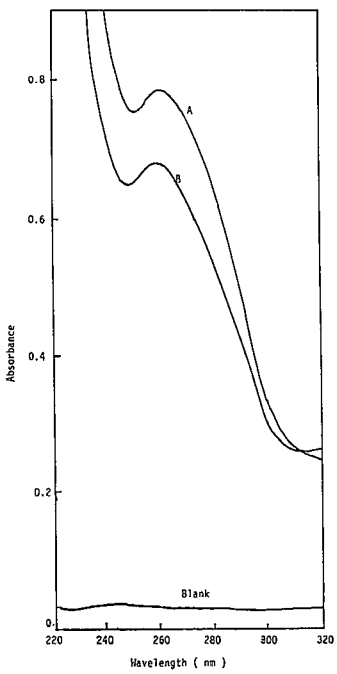

Fig. 3. UV absorption spectrum of the top (A) and bottom (B) fractions.

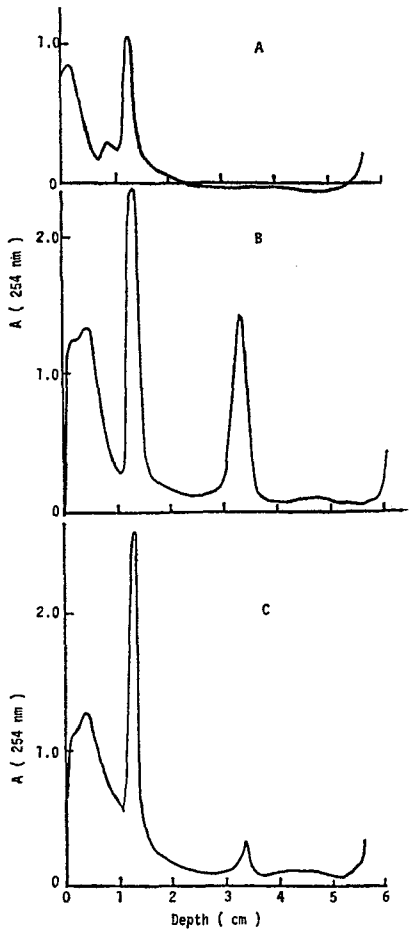

Fig. 4. Ultraviolet absorption profiles of purified materials after sucrose density-gradient centrifugation from healthy and RRSV infected rice plants. A; healthy, B; fresh diseased materials. C; frozen (−40°C) diseased materials.

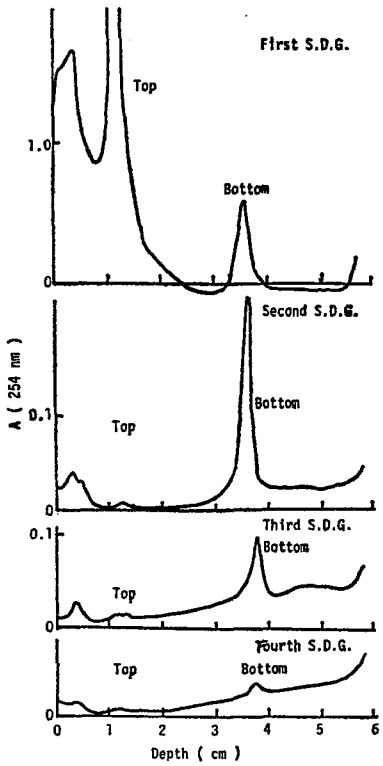

Fig. 5. Ultraviolet absorption profiles of the tubes from repeated sucrose density-gradient centrifugation of bottom fractions.

fraction consisted of the 35 nm particles did not influenced by freezing (Fig. 4 B and C).

ii) *Repeated sucrose density-gradient centrifugation*: The bottom fraction obtained in the purified preparation after sucrose density-gradient centrifugation (first S. D. G.) were subjected to repeated sucrose density-gradient centrifugations for 3 times (second, third and fourth S. D. G., repectively).

As shown in Fig. 5, the amount of the particles in the top fractions

did not increase as that of the bottom fractions decreased by the repeated sucrose density-gradient centrifugations. Top and bottom fractions from each S. D. G. were examined by electron microscopy. The bottom fraction in the first S. D. G. consisted of mixture of the 35 and 63 nm particles. After repeated S. D. G., as indicated in Table 1 the bottom fractions predominantly contained large particles of 63 nm in diameter, but the amount of small 35 nm particles in the same fractions rapidly eliminated.

TABLE 1. Result of repetition of sucrose density-gradient centrifugation of the bottom fractions*

Materials	Size of particles	Fractions		
		Top	Bottom	Pellet
1st S. D. G. from diseased rice materials	35 nm particles	346**	10.67	0
	63 nm particles	10.4	243.3	15.3
2nd S. D. G. from bottom fraction of 1st S. D. G.	35 nm particles	2.9	0	0
	63 nm particles	2.4	48.5	1.8
3rd S. D. G. from bottom fraction of 2nd S. D. G.	35 nm particles	0	0	0
	63 nm particles	1.2	41.5	0.01
4th S. D. G. from bottom fraction of 3rd S. D. G.	35 nm particles	0	0	0
	63 nm particles	0	5	0

* Average number of particles per photograph (3 or 4 photographs); Three grids were examined at magnification of 20,000.
** See Fig. 5.

TABLE 2. Result of RRSV-infectivity of purified preparations

Source	No. of insect injected*	No. of insect survived after 10 days	No. of insect per plant	Plant infected /plant inoculated**
Rice 1st S. D. G.				
Top fraction	57	29	3	1/10 (10%)
Bottom fraction	56	21	3	7/7 (100%)
Maize	94	62	2	24/33 (73%)
Barley				
Hokutohadaka	52	46	2	23/23 (100%)
Rokkakuozeki	44	28	2	14/14 (100%)

* The preparations were injected into healthy brown planthoppers, *Nilaparvata lugens*.
** The inoculated plants were rice, *Oryza sativa* cv. Kinmaze.

iii) *Infectivity assay*: The top and bottom fractions, obtained from first S. D. G. (Table 1 first S. D. G.), were injected into healthy vector insects. As shown in Table 2, the bottom fraction was highly infectious, while the top fraction showed little infectivity.

iv) *Purification from RRSV infected maize and barley plants*: Purified preparations from infected maize and barley plants appeared only the bottom fractions (Fig. 6), and the 63 nm particles were abundant in these fractions, associated with high RRSV-infectivity (Table 2). The top fraction was not seen in these preparations. There was no 35 nm particles in the bottom fractions.

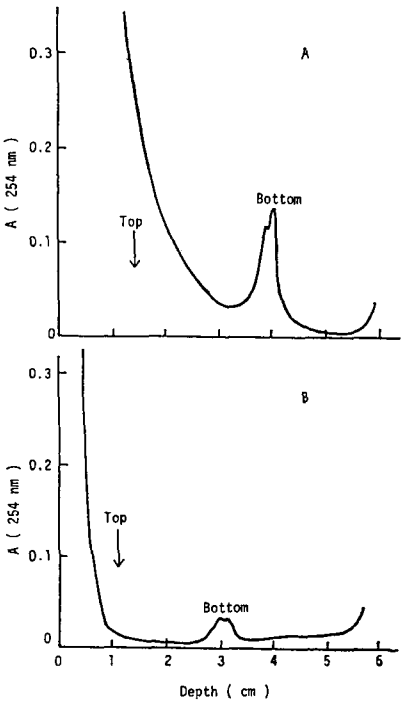

Fig. 6. Ultraviolet absorption spectra of the purified preparations by sucrose density-gradient centrifugation from RRSV infected barley (A) and maize (B).

v) *Isolation of the 35 nm particles from old grown healthy rice plants*: The healthy rice plants which had been grown for about 6 months after transplanting, and were not inoculated with RRSV by the vector insects, were subjected to the same procedures as RRSV purification. The ISCO scanning pattern of the healthy purified preparations showed, as shown in Fig. 2 A, only one peak corresponding to the top fraction of the virus infected rice materials. The preparation of such a peak revealed only the 35 nm particles, but no 63 nm particles were found by electron microscopy.

ELISA tests were performed to investigate the location of the 35 nm particles within the rice plants grown in our greenhouse using the antiserum against the 35 nm particles. The positive reaction was detected in the preparation from leaf sheaths, and upper culms of old grown healthy rice plants. Extremely high reaction was found in the leaf sheath materials.

By immune electron microscopy of dip preparations from the leaf sheaths, numerous 35 nm particles were detected (Fig. 7).

vi) *Morphology of RRSV*: The RRSV particles stained with UA revealed spherical structure 57–65 nm in diameter with spikes 8–10 nm in length (Fig. 8 C and D). However, when those particles were stained by

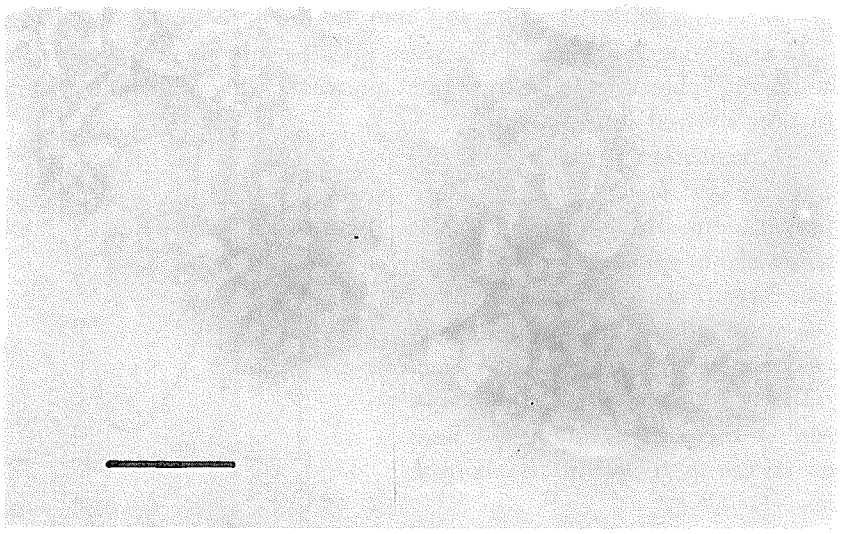

Fig. 7. Immuno electron microscopy of the 35 nm particles. Bar represents 200 nm.

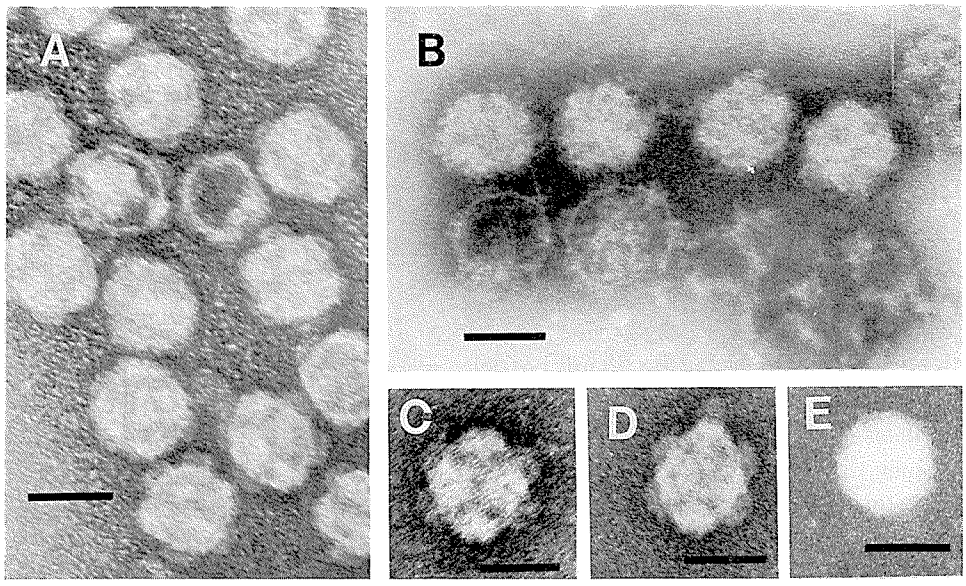

Fig. 8. Electron micrographs of purified RRSV. Bar represents 50 nm. A; The virus particles prefixed by 1% GA and stained by 2% UA. B; The same as A, but stained by 2% PTA, pH 5. C and D; The virus particles stained by 2% UA only. E; The virus particles stained by 2% PTA pH 7.

PTA, the surface structure looks smooth and the spikes over the particles could not be detected (Fig. 8 E). The size of the smooth particles is about 61 nm in diameter. When the virions were prefixed by 1% glutaraldehyde and stained by PTA and UA, the fine structure having broad-based spikes were preserved well (Fig. 8 A and B). But the spikes on the PTA stained particles were less clear than those stained by UA.

Discussion

When RRSV was purified from rice plants grown in our greenhouse, we found two (top and bottom) UV absorbing fractions. The top fraction mostly consisted of 35 nm particles, but contaminated with a small amount of large 63 nm particles. The bottom fraction predominantly contained the 63 nm particles, but a little of the 35 nm particles. The preparations purified from infected maize and barley plants appeared only bottom fractions containing only the 63 nm particles, but no 35 nm particles were found. After repeated sucrose density-gradient centrifugations of the bottom fractions from rice, the amount of the top fraction did not increase as that of the bottom fraction decreased. The result suggested that the 63 nm particles did not convert into the 35 nm particles during the purification procedures.

The top fractions isolated from RRSV infected rice plants showed very low infectivity, and were always obtained from preparations of both fresh and frozen rice materials. Whereas the 63 nm particles could not be obtained from frozen materials but only from fresh materials, associating with high RRSV-infectivity. The result strongly suggests that the 63 nm particles are RRSV. Low infectivity appeared in the top fraction is probably due to contamination by the 63 nm particles. Based on their size, low sedimentation velocity and low RRSV-infectivity, the 35 nm particles appeared in our rice preparations are probably the same as the purified RRSV particles previously reported.[8]

The facts that the 35 nm particles were also obtained from the old grown healthy rice materials but not from maize and barley, and that they were detected mostly within leaf sheaths from both the diseased and healthy rice plants by ELISA and immune electron microscopy, but not in the healthy upper leaves, suggest that they are not associated with RRSV and thus are supposedly a contaminant as suggested by MILNE.[6] But the origin of the source of the 35 nm particles is not certain yet.

Summary

Rice ragged stunt virus (RRSV) was purified from fresh tissues of infected

rice, maize and barley plants by carbon tetrachloride and fluorocarbon treatments, followed by differential centrifugations and a rate zonal sucrose density-gradient centrifugation. The top and bottom zones were obtained in extracts from the disease rice plants, which were grown in our greenhouse. But only a bottom zone was obtained from maize and barley. The bottom zone was associated with high RRSV-infectivity, contained numerous spherical particles of 63 nm in diameter (63 nm particles) and was contaminated with small spherical particles of 35 nm in diameter (35 nm particles). The top zone was associated with low RRSV-infectivity, contained numerous spherical particles of 35 nm in diameter and a few amount of the 63 nm particles. The repeated cycles of sucrose density-gradient centrifugation revealed that the 63 nm particles did not convert into the 35 nm particles. The result suggested that the 63 nm particles were RRSV virions. The 35 nm particles which appeared in the diseased rice plant as well as in old grown healthy rice plants, seemed to be a contaminaed virus of unknown origin.

Literature Cited

1. HIBINO, H.: Rice ragged stunt, a new virus disease occurring in tropical Asia, *Rev. Plant Portec. Res.*, **12**: 98-110. 1979
2. HIBINO, H., SALEH, N. and ROECHAN, M.: Reovirus-like particles associated with rice ragged stunt diseased rice and insect vector cells, *Ann. Phytopath. Soc. Japan*, **45**: 228-239. 1979
3. HIBINO, H., ROECHAN, M., SUDARISMAN, S. and TANTERA, D. M.: A virus disease of rice (Kerdil hampa) transmitted by brown planthopper, *Hilaparvata lugens*, in Indonesia, *Contr. Centr. Res. Inst. Agric. Bogor.*, **35**: 1-15. 1977
4. KAWANO, S., SENBOKU, T. and SHIKATA, E.: Host range of rice ragged stunt virus, *Ann. Phytopath. Soc. Japan*, **47**: 697-699. 1981
5. LING, K. C., TIONGCO, E. R. and AGUIERO, V. M.: Rice ragged stunt, a new virus disease, *Plant Dis. Rep.*, **62**: 701-705. 1978
6. MILNE, R. G.: Dose rice ragged stunt virus lack the typical double shell of the Reoviridae?, *Intervirology*, **14**: 331-336. 1980
7. MILNE, R. G., BOCCARDO, G. and LING, K. C.: Rice ragged stunt virus, *CMI/AAB Descriptions of Plant Viruses* No. **248**. 1982
8. SHIKATA, E., SENBOKU, T., KAMJAIPAI, K., CHOU, T. G., TIOGCO, E. R. and LING, K. C.: Rice ragged stunt virus, a new member of plant reovirus group, *Ann. Phytopath. Soc. Japan*, **45**: 436-443. 1979
9. TAKANAMI, Y. and KUBO, S.: Enzyme-assisted purification of two phloem-limited plant virus: Tobacco necrotic dwarf and potato leafroll, *J. gen. Virol.*, **44**: 153-159. 1979