Detection of Rice Ragged Stunt Virus in Insect Vectors by Enzyme-Linked Immunosorbent Assay

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ABSTRACT

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Rice ragged stunt virus (RRSV) was purified from diseased rice plants, and antiserum against RRSV was prepared and used for virus detection by enzyme-linked immunosorbent assay (ELISA). RRSV was detected by ELISA in extracts of RRSV-containing rice leaves and newly emerged plant hopper vectors, *Nilaparvata lugens*, diluted up to 320 and 5,120 times respectively, with phosphate buffer, pH 7.4. However, strong nonspecific reactions occurred in extracts of virus-free female plant hoppers carrying eggs. Polyvinylpyrrolidone reduced the intensity of the nonspecific reaction. The intensity of the nonspecific reaction varied remarkably with pH of the extraction buffer and was lowest at pH 6.5 or 6.0. RRSV was detected in extracts of viruliferous insects diluted up to 10,240 times with phosphate buffer, pH 6.5, containing 2% polyvinylpyrrolidone. Plant hopper extracts in this buffer showed negligible nonspecific reactions. RRSV was detected efficiently from single plant hoppers by ELISA. In tests of plant leafhoppers fed on RRSV-infected plants, 27% transmitted the virus and gave a positive ELISA; 25% did not transmit the virus but gave a positive ELISA; the remainder gave negative ELISA and transmission tests. RRSV was detected in killed insects stored for 1 day at −80 C, 6 C or at room temperature.

Rice ragged stunt virus (RRSV) of the family Reoviridae was first observed in 1976–1977 in Indonesia and the Philippines (4,8) and soon afterwards became epidemic in several countries in South, Southeast, and East Asia (3). RRSV is transmitted by a plant hopper, *Nilaparvata lugens*, in a persistent manner (4,9) and is propagative in the plant hopper (5). *N. lugens* is distributed widely in rice (*Oryza sativa*) production areas of Asia and is known to migrate long distances even across the ocean borne by seasonal winds (6). The migrating plant hoppers presumably carry RRSV and rice grassy stunt virus (7), and might be a source of these viruses in areas where rice and plant hoppers do not survive winters or dry seasons. It is valuable for disease forecasting and in studies of the epidemiology of the virus to test the frequency of virus-containing plant hoppers among migrating populations. This paper describes a technique for detecting RRSV in the plant hopper vector by enzyme-linked immunosorbent assay (ELISA).

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MATERIALS AND METHODS

Virus, insects, and plants. RRSV was collected at Bangkok, Thailand, in 1980 and maintained on rice seedlings by successive transfers using *N. lugens*. Virus-free *N. lugens* was supplied by A. Sugimoto, Tropical Agriculture Research Center, Japan, and reared on rice seedlings in a plastic cage. Viruliferous plant hoppers were obtained by rearing virus-free nymphs at first or second instar on a RRSV diseased plant for 2 wk. Rice seedlings (cultivar Taichung Native 1) were inoculated with RRSV by viruliferous plant hoppers and grown in a greenhouse for 5–6 wk. Rice leaves and plant hoppers were homogenized with extraction buffer (described below) in a glass homogenizer and the extracts were tested by ELISA. For infectivity tests, individual viruliferous plant hoppers were allowed an inoculation access period of 2 days on three rice seedlings at first or second leaf stage in a test tube. Then, each plant hopper was crushed with 0.5 ml of extraction buffer in a small test tube with a glass rod. The extract was tested directly by ELISA. Inoculated seedlings were grown in a greenhouse. Symptoms appeared about 2 wk after inoculation.

Purification of RRSV and production of antiserum. Fresh roots and leaf sheaths (400 g) were collected from RRSV-infected rice

plants, and were homogenized with 1,200 ml of 0.2 M glycine buffer, pH 7.6, containing 5 mM EDTA and 0.5% sodium ascorbate. The homogenate was emulsified with one-third volume of carbon tetrachloride and the emulsion was centifuged for 5 min at 9,000 *g*. The supernatant fluid was further clarified by adding fluorocarbon (Difron S3) to 25% by volume. Polyethylene glycol 6000 (avg mol wt 7,500) was added to 7% (w/v) to the clarified extracts and the mixture was allowed to stand for 3 hr at 4 C. The pellet obtained after centrifuging for 20 min at 9,000 *g* was suspended in 0.1 M tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and 1 mM EDTA (STE buffer). The suspension was layered onto density gradients of 30, 40, and 50% sucrose solutions (5 ml each) and centrifuged for 60 min at 52,000 *g*. A virus-containing zone in 30 and 40% sucrose layers was collected and layered again onto 40–60% sucrose gradients and centrifuged for 16 hr at 42,000 *g*. Two light-scattering zones appeared in the centrifuged column. The upper zone contained RRSV particles and cellular components, and the lower zone contained mainly RRSV. The lower zone was diluted with STE buffer and centrifuged for 80 min at 81,000 *g*. The pellet was suspended in 1 ml of STE buffer. This purified virus suspension (Fig. 1) was injected into abdomen of virus-free nymphs of *N. lugens* with a glass capillary tube. About 70% of injected plant hoppers became infective. Purified virus suspensions were also emulsified with complete adjuvant (1:1, v/v) and the emulsion was injected into muscles of domestic rabbits. Injection was repeated three times at 3-wk intervals. Antiserum derived from the rabbits was tested by the precipitin ring test and its titer against purified RRSV was 1,024.

Enzyme-linked immunosorbent assay. The procedure described by Clark and Adams (1) was followed for purification of γ-globulin, conjugation of alkaline phosphatase with γ-globulin, and coating and washing of polystyrene microtiter plates (Linbro Sci. Co. Inc). The appropriate concentration of coating γ-globulin was 2.5 μg/ml, as purified RRSV gave reactions almost at the same intensity in ELISA when coating γ-globulin >2.5 μg/ml was applied. Conjugate was diluted at a 1/300 dilution. Each sample was homogenized with 0.02 M phosphate buffer containing 0.15 M NaCl, 0.05% Tween 20 and 0.02% sodium azide (PBS-Tween), and 0.2 ml of homogenate was added to each well directly or after appropriate dilution with buffer. Freshly prepared *p*-nitrophenyl phosphate substrate (1) was added for 1 hr at room temperature and the reaction was stopped by adding 0.05 ml of 3 M NaOH. Reactions were assessed visually and also measured at 405 nm by using a Hitachi Model 200-20 double-beam spectrophotometer after diluting four times with distilled water. Two wells were used for each sample and the average A_{405} values were calculated. The reaction of extracts considered to be positive visually gave A_{405} values >0.07.

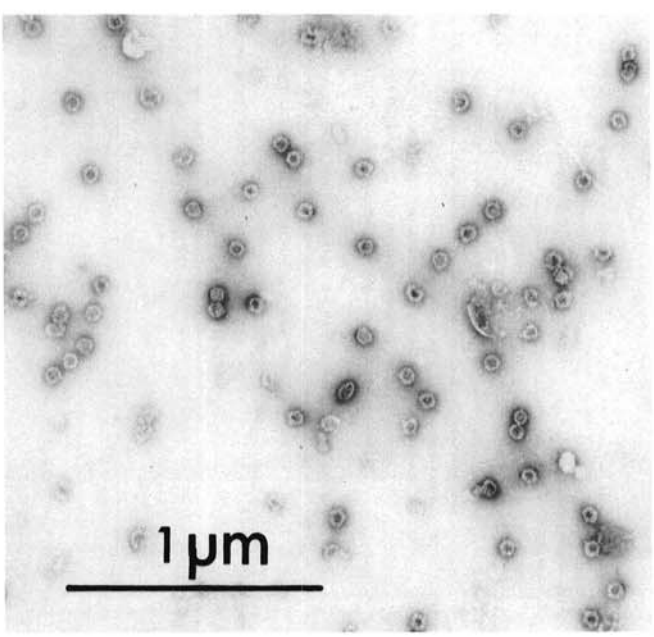

Fig. 1. Purified rice ragged stunt virus stained with 1% uranyl acetate.

RESULTS

Application of ELISA to detect RRSV. Diseased or virus-free rice leaves and newly emerged viruliferous or virus-free plant hoppers were homogenized separately in PBS-Tween (pH 7.4), and each homogenate was tested at various dilutions by ELISA. RRSV was detected in extracts of diseased leaves and viruliferous plant hoppers diluted up to 320 and 5,120 times, respectively (Fig. 2). An A_{405} value of viruliferous plant hopper extract at dilution 1/20 was smaller than that at 1/40. Extracts of virus-free leaves and plant hoppers did not give positive reactions at greater than 1/40.

Infectivity of viruliferous and virus-free plant hoppers of various ages was tested and then each plant hopper was homogenized in 0.5 ml of PBS-Tween (pH 7.4) for ELISA. Extracts of all infective plant hoppers fed on RRSV-infected rice plants gave positive ELISA tests, but extracts of more than half of noninfective plant hoppers also gave positive reactions. Extracts of all nymphs, male adults, and newly emerged females which had not fed on RRSV-infected plants gave negative reactions in ELISA. However, adult females carrying eggs gave positive reactions. The intense nonspecific reactions of adult female masked the RRSV-specific reactions. The nonspecific reaction was intense for dilutions of 1/40–1/600, and was strongest at 1/80 (Fig. 3).

Modification of extraction buffer. The effects of polyvinylpyrrolidone (PVP, avg mol wt 10,000) and bovine serum albumin on the nonspecific reaction associated with female plant hoppers carrying eggs were tested. Twenty viruliferous and virus-free plant hoppers were homogenized separately in 10 ml of PBS-Tween (pH 7.4) containing additives to be tested, and each homogenate was tested directly by ELISA. PVP reduced the intensity of the nonspecific reaction, while not appreciably

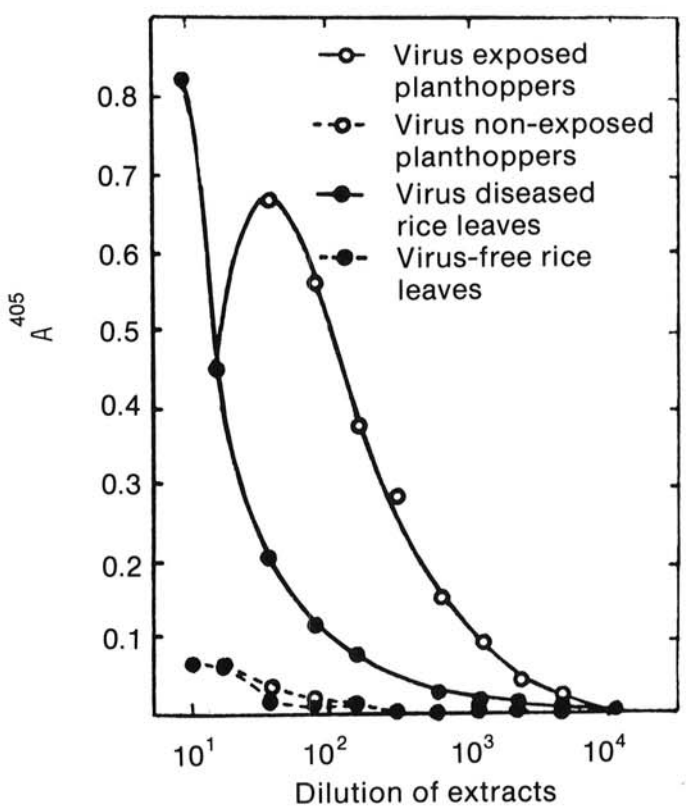

Fig. 2. Reactions of extracts of rice ragged stunt virus exposed or unexposed plant hoppers, and virus-diseased or virus-free rice leaves in ELISA. Newly emerged plant hoppers and rice leaves were homogenized and diluted with PBS-Tween (pH 7.4).

affecting the specific reaction of viruliferous male plant hoppers (Table 1). Serum albumin increased the intensity of the nonspecific reaction, but had little effect on the specific reaction. The effects of the pH of extraction buffer on the nonspecific reaction were similarly tested. The intensity of nonspecific reaction varied remarkably with buffer pH and was least at 6.5 or 6.0 in pH range between 6.0 and 8.5 (Fig. 4). This range of pH did not significantly affect the specific reaction of viruliferous male plant hoppers. Tests for the effects of additives and pH on the reactions were repeated twice and three times, respectively, and results were reproduced, although the intensity of reactions varied somewhat in each test.

Viruliferous or virus-free male and female adults were separately homogenized in PBS-Tween (pH 6.5) containing 2% PVP and each homogenate was tested at various dilutions by ELISA. Homogenates of viruliferous plant hoppers reacted at dilutions up to 1/5,120 or 1/10,240, while those of virus-free plant hoppers gave only weak reactions even at a 1/10 (Fig. 5). RRSV diseased and virus-free leaves were also separately homogenized in the same buffer for ELISA. RRSV was detected in extracts of diseased leaves diluted up to 1/320 or 1/640, and extracts of virus-free leaves gave only weak reactions at 1/10.

Detection of RRSV in individual plant hoppers. Infectivity of viruliferous plant hoppers was tested and then each plant hopper was homogenized with 0.5 ml of PBS-Tween (pH 6.5) containing PVP for ELISA. Tests were repeated three times using 137 female adults carrying eggs and 74 males. Extracts of all infective plant hoppers gave A_{405} values >0.09. Extracts of many noninfective plant hoppers gave A_{405} values <0.1, while some gave values >0.1 to 0.9 (Table 2). ELISA reactions were also assessed visually with 50% being positive of which only 27% were infective. None of the infective plant hoppers gave a negative reaction in ELISA. None of

TABLE 1. Effects of polyvinylpyrrolidone (PVP) and bovine serum albumin (BSA) on the reaction of extracts of plant hopper vectors exposed and unexposed to rice ragged stunt virus in enzyme-linked immunosorbent assay (ELISA)

Additive	A_{405} of extracts in ELISA[a]		
	Unexposed female	Exposed female	Exposed male
None	0.21	0.58	0.38
PVP	0.09	0.38	0.34
BSA	0.45	0.89	0.34
PVP + BSA	0.41	0.79	0.42

[a] Twenty adult plant hoppers were homogenized in 10 ml of PBS-Tween (pH 7.4) containing 2% PVP or 0.2% BSA, and homogenate was tested directly by ELISA.

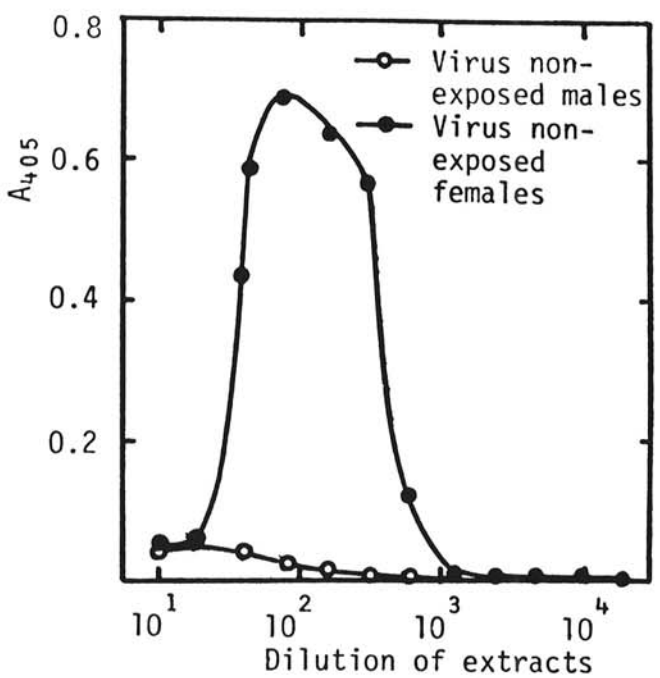

Fig. 3. Nonspecific reaction of extracts of virus-free female plant hoppers carrying eggs and male plant hoppers in various dilutions in ELISA. Plant hoppers were homogenized and diluted with PBS-Tween (pH 7.4).

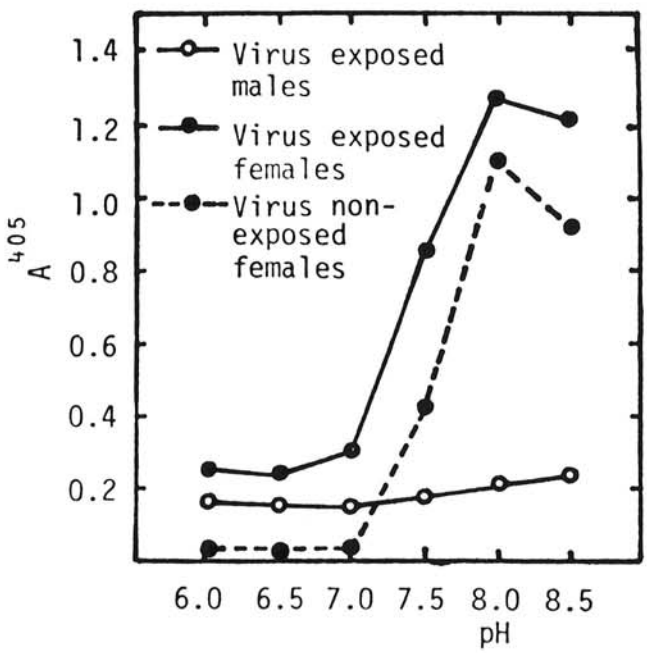

Fig. 4. Effects of pH of phosphate buffer on reaction of extracts of ged stunt virus exposed and unexposed plant hoppers in ELISA. Twenty male or female plant hoppers carrying eggs were homogenized in 10 ml of PBS-Tween (pH 6.0–8.5), and each extract was tested directly by ELISA.

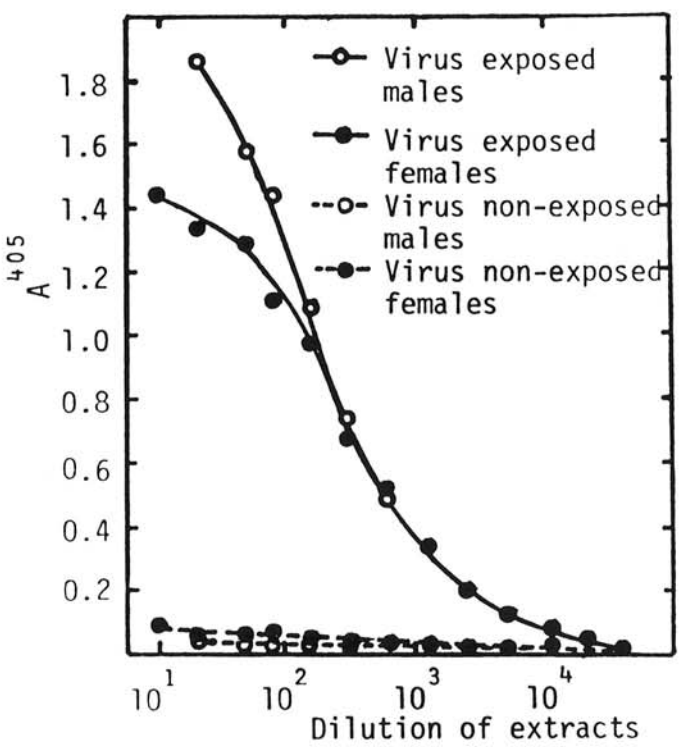

Fig. 5. Reactions of extracts of rice ragged stunt virus exposed and unexposed plant hoppers in ELISA. Male and female plant hoppers carrying eggs were homogenized separately and diluted with PBS-Tween (pH 6.5) containing 2% polyvinylpyrrolidone for ELISA.

TABLE 2. Relations between infectivity of plant hopper vectors and the intensity of reactions of their extracts in enzyme-linked immunosorbent assay (ELISA)[a]

A_{405} values in ELISA	Number of plant hoppers			
	Infective male	Noninfective male	Infective female	Noninfective female
0.00–0.05	0	43	0	42
0.05–0.1	0	4	1	26
0.1–0.2	2	3	4	10
0.2–0.4	5	2	10	6
0.4–0.6	4	3	12	4
0.6–0.8	5	1	11	4
0.8–	1	···	1	1

[a] Individual plant hoppers exposed to rice ragged stunt virus were tested for infectivity and then homogenized in 0.5 ml of PBS-Tween (pH 6.5) containing 2% polyvinylpyrrolidone for ELISA.

virus-free plant hoppers gave a positive reaction.

Effects of storage on RRSV detection. A container of plant hoppers was vigorously shaken several times to kill plant hoppers. Killed plant hoppers were kept for 1 day in a freezer at −80 C, or in dry or moist chamber at 6 C or at room temperature, and their extracts in PBS-Tween (pH 6.5) containing PVP were tested by ELISA. RRSV was detected consistently in plant hoppers stored under these conditions. Extracts of plant hoppers that were kept at −80 C or in cold moist chamber produced as intense a color as those of living plant hoppers (Table 3).

TABLE 3. Effects of 1-day storage conditions for dead plant hoppers on detection of rice ragged stunt virus in extracts from virus exposed plant hopper vectors by enzyme-linked immunosorbent assay (ELISA)

Condition	A_{405} of extracts in ELISA[a]	
	Male	Female
Control[b]	0.49	0.52
Frozen at −80 C	0.57	0.47
Dry chamber at 6 C	0.36	0.39
Moist chamber at 6 C	0.44	0.60
Dry chamber at room temperature	0.25	0.41
Moist chamber at room temperature	0.25	0.46

[a] Ten plant hoppers were homogenized in 5 ml of PBS-Tween containing 2% polyvinylpyrrolidone and homogenate was tested directly by ELISA.
[b] Living plant hoppers were used.

DISCUSSION

ELISA provides a highly sensitive detection method for a number of plant viruses in purified preparations or plant extracts (1). Recently, Gera et al (2) reported the detection of cucumber mosaic virus in single viruliferous aphids by ELISA and suggested the value of ELISA to epidemiological studies of aphidborne viruses. Our study is the first report of the detection of virus in a plant hopper vector by ELISA. Further, we found that ELISA detected RRSV even in extracts of single plant hopper vectors.

Although the purified RRSV preparations used to produce antiserum contained some cellular components, the serum was satisfactory in ELISA. Further purification may cause degradation of virus and loss of virus yield. Because of low concentration in diseased rice tissues, RRSV is difficult to detect in the electron microscope by the leaf-dip method using rice tissues other than leaf galls.

Both electron microscopy and infectivity tests are able to detect RRSV in the plant hopper vector. Electron microscopy efficiently detects RRSV in single plant hoppers (5). However, this method is inadequate for samples in large quantity and is suitable only for laboratory tests in well-equipped institutions. Further, presence of a native insect virus in *N. lugens* tissues (5) makes the electron microscopical detection of RRSV more difficult. Infectivity tests for RRSV take about 1 mo and require much greenhouse space when many samples are assayed. ELISA takes 2 days to perform and many samples can be handled at once. Further, ELISA was more sensitive than the infectivity test for detecting RRSV. It is interesting that some plant hoppers contained large amounts of RRSV but did not transmit the virus. The incubation period of RRSV in the plant hopper is at least 5 days and some plant hoppers became infective only after a long incubation period (>10 days) (4,9). In the case of plant hoppers that contained RRSV as ascertained by ELISA but were not able to transmit virus, the incubation period may have been incomplete or the plant hoppers may have merely failed to transmit virus in the infectivity test because it is an intermittent occurrence among a population of transmitting plant hoppers (4,9). So far, ELISA is highly efficient compared to other methods for detection of RRSV in single plant hopper vectors.

The tissues and organs responsible for nonspecific reaction of egg carrying females is uncertain. Egg-carrying females weighed 2.5–5.5 mg; males weighed 1–1.4 mg. The nonspecific reaction was probably due to the eggs and not the weight difference because nonspecific reactions were not obtained with newly emerged females.

Light traps, airborne insect net traps, or yellow-pan water traps are used to collect migrating insects. The insects usually are dead before they are retrieved from these traps. Therefore, the infectivity test is difficult to apply to trapped insects, but ELISA could be applied efficiently. More than one plant hopper may be homogenized in 0.5 ml buffer for ELISA; nonspecific reactions of extracts were negligible even when 10 plant hoppers were homogenized together and RRSV could be detected in an extract of 10 plant hoppers including one virus-containing and nine virus-free plant hoppers.

LITERATURE CITED

1. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
2. Gera, A., Loebenstein, G., and Raccah, B. 1978. Detection of cucumber mosaic virus in viruliferous aphids by enzyme-linked immunosorbent assay. Virology 86:542-545.
3. Hibino, H. 1979. Rice ragged stunt, a new virus disease occurring in tropical Asia. Rev. Plant Protect. Res. 12:98-110.
4. Hibino, H., Roechan, M., Sudarisman, S., and Tantera, D. M. 1977. A virus disease of rice (kerdil hampa) transmitted by brown plant hopper, *Nilaparvata lugens* Stål, in Indonesia. Contrib. Centr. Res. Inst. Agric. Bogor No. 35, 15 pp.
5. Hibino, H., Saleh, N., and Roechan, M. 1979. Reovirus-like particles associated with rice ragged stunt diseased rice and insect vector cells. Ann. Phytopathol. Soc. Jpn. 45:228-239.
6. Kishimoto, R. 1971. Long distance migration of plant hoppers, *Sogatella furcifera* and *Nilaparvata lugens*. Trop. Agric. Res. Ser. 5:201-216.
7. Ling, K. C. 1972. Rice virus diseases. International Rice Research Institute, Los Baños, Philippines. 142 pp.
8. Ling, K. C., Tiongco, E. R., and Auguiero, V. M. 1978. Rice ragged stunt, a new virus disease. Plant Dis. Rep. 62:701-705.
9. Shikata, E., Senboku, T., Kamjaipai, K., Chou, T. G., Tiongco, E. R., and Ling, K. C. 1979. Rice ragged stunt virus, a new member of plant reovirus group. Ann. Phytopathol. Soc. Jpn. 45:436-443.