# Comparison of Four Serological Methods for Practical Detection of Ten Viruses of Rice in Plants and Insects

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**ABSTRACT**

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Four serological methods (ELISA, simplified ELISA, latex flocculation test [LF], and passive hemagglutination test [PHA]) were compared for practical detection of 10 viruses of rice in plants and viruliferous insects. All of the viruses were detected by ELISA and simplified ELISA. The detection of the viruses in plants with systemic infections was easier than the detection of phloem-restricted viruses. Some viruses in plants could not be detected in LF and PHA because of inhibition or nonspecific reactions. All of the viruses that are transmitted in a persistent manner were easily detected in individual insect vectors by ELISA and simplified ELISA, but some viruses were unable to detect by LF and PHA. Based on the results, the order of virus detection sensitivity of the methods was: ELISA $\geq$ simplified ELISA $\geq$ LF $\geq$ PHA. On the other hand, the order of simplicity of the tests was: LF $\geq$ PHA $\geq$ simplified ELISA $\geq$ ELISA. In mass inspection of viruliferous insects, the smashing method (one push of individual insects with a vinyl chloride rod directly in a well of microplate) provides sufficient extraction of the virus antigen. By adopting this direct smashing method, simplified ELISA has a comparative advantage over LF and PHA. The establishment of the practical serodiagnosis we demonstrated will undoubtedly strengthen integrated control of the virus diseases of rice.

Plant hoppers and leafhoppers that vector viruses of plants are widely distributed in Asia and are able to migrate long distances. Some vectors may spread viruses between countries in Asia, and some vectors even cross the sea (7,12,14). This may account for the difficulty in controlling virus diseases of rice. Ten viruses of rice that are transmitted by insects have been identified in Asia. Of the 10 viruses, seven multiply in the vector and are transmitted persistently throughout the life of the insect. Three of the viruses are transovarially transmitted, which makes control of the diseases more difficult.

Symptom observation is the method currently practiced for the detection of rice viruses in the field. However, the method is not always reliable, because more than one virus can cause similar symptoms in rice plants, and many nonpathogenic disorders, such as nutritional deficiencies, excess water after drought, or insect damage, may cause symptoms similar to virus diseases. Furthermore, one virus produces variable symptoms on different cultivars and depends on the growth stages of host plants. The transmission test with insect vectors takes at least 3 wk for the semipersistent viruses and 5 wk for the persistent viruses to obtain results. Furthermore, the method is labor intensive and requires a large number of vector populations at a similar growth stage. Thus, this method is not practical for routine testing. Detection with an electron microscope (EM) is sensitive, but it is suitable mainly for well-equipped institutions and not for testing many samples.

Serological methods are generally specific and, in most cases, sensitive enough to detect viruses in small pieces of samples. Enzyme-linked immunosorbent assay (ELISA), simplified ELISA, the latex flocculation test (LF), and the passive hemagglutination test (PHA) are suited for the detection of virus diseases of rice. Although PHA, LF, and ELISA are sufficiently sensitive, each has its characteristic shortcomings. The red blood cells for PHA are difficult to preserve, and it is difficult to judge the reaction in LF with the naked eye. ELISA requires a long time and involves several manipulations. Simplified ELISA, in which the manipulation number is reduced by simultaneous incubation of samples and antibody-enzyme conjugate with solid-phase antibody, overcomes the shortcomings of ELISA and is efficient for mass indexing of samples (28). These methods have been used in detecting some rice viruses (1,7,10,12,16, 27,28,31), however, comparative efficiencies and the limitations and/or advantages of these methods have not been determined for each virus.

This study compared ELISA, simplified ELISA, LF, and PHA for the detection of rice viruses in rice plants and viruliferous insects.

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## MATERIALS AND METHODS

**Viruses, insects, and plants.** All insect-borne rice viruses that have been identified in Asia were included in these tests. They include the following: rice dwarf virus (RDV) (9,13), rice gall dwarf virus (RGDV) (17), rice black-streaked dwarf virus (RBSDV) (22), rice ragged stunt virus (RRSV) (15), rice tungro bacilliform virus (RTBV) (19), rice tungro spherical virus (RTSV) (5,19), rice waika virus (RWV) (30), rice transitory yellowing virus (RTYV) (21), rice stripe virus (RSV) (29), and rice grassy stunt virus (RGSV) (6). Viruses were maintained in rice plants at the National Agriculture Research Center, Tsukuba, Japan. RSV and RBSDV were maintained by successive transfers with the small brown plant hopper, *Laodelphax striatellus* Fallén; RRSV and RGSV were maintained by successive transfers with the brown plant hopper, *Nilaparavata lugens* Stål. The green rice leafhopper, *Nephotettix nigropictus* (Stål), was used for transmission of RDV, RGDV, and RTYV. For transmitting these viruses, second to third instar nymphs were fed on diseased plants for 2 days and transferred to healthy rice plants at the three- to five-leaf stages. The semipersistent viruses, RTBV, RTSV, and RWV, were transmitted by the green rice leafhopper species, *N. virescens* (Distant). Adult insects were allowed to feed for 2 days on infected plants and transferred to healthy rice seedlings at the three- to five-leaf stages for 2 days or longer. All inoculated plants were grown in an air-conditioned greenhouse (27 $\pm$ 3 C). Leaf samples were stored at $-70$ C and tested for virus detection. More than 50 insect vectors exposed or unexposed to each virus were examined with respective assays in one experiment.

**Serological procedures.** Antisera against RDV (18), RGDV (18), RBSDV (10), RRSV (7), RTBV (19), RTSV (19), RWV (19), RTYV (27), and RGSV (8) had titers in the precipitin ring interface test of 1:2,000, 1:2,560, 1:4,000, 1:1,024, 1:2,560, 1:320, 1:640, 1:800, and 1:1,600, respectively. Monoclonal antibodies against RSV (20) with a titer of 1:1,600

in the same test were also used. Purification of each γ-globulin and its conjugation with alkaline phosphatase was performed according to Clark and Adams (3). The optimal concentrations of coating γ-globulins and conjugates for each virus were determined for each lot of antisera used. The concentration of coating γ-globulins ranged from 1 to 5 μg/ml and the dilution of conjugate ranged from 1:200 to 1:800.

The double antibody-sandwich ELISA procedure (3) was used as standard ELISA. In the ELISA tests, the incubation period for coating was 3 hr at 37 C or overnight at 4 C and that for trapping the antigen in samples was 3 hr at 37 C. The absorbance at 410 nm was measured by an MR710 microplate autoreader (Dynatech Laboratories, Inc., Alexandria, VA). Infected rice plants were homogenized and diluted in 0.02 M phosphate buffer (PB) containing 0.85% NaCl and 0.05% Tween 20, pH 7.4 (PBS-T). Individual insects (except *N. lugens*) were macerated by glass rods in test tubes with 0.4 ml of PBS-T. Each homogenate of 0.2 ml was added in a well of microplate. *N. lugens* was homogenized and diluted in PBS-T, pH 6.5, containing 2% (w/v) polyvinylpyrrolidone (PVP) to prevent nonspecific reactions (7).

The simplified ELISA followed the method described by Takahashi et al (28). Plates were coated with respective γ-globulins at the same concentrations as those used in the standard ELISA and stored at 4 C until use. After washing with PBS-T, respective conjugate was mixed with an appropriate amount of plant homogenate in each well to obtain the final concentration of the conjugate as those used in the standard ELISA procedure. Two hundred microliters of mixtures containing 100–175 μl of samples and 25–100 μl of conjugates were added in each well. For the detection of viruses in insects, individual insects were placed in each well of a coated plate. The insect was smashed with a vinyl chloride rod (ϕ 6 mm), which was then wiped with a paper towel and used for smashing the other ones. Conjugate of 200 μl at the same dilution as the one used in standard ELISA was added to each well. After incubation with the sample-conjugate mixture for 1–2 hr at 37 C, plates were subjected to reaction assessment.

Latex suspension was sensitized with each antiserum following the procedure described by Omura et al (16). For PHA, formalinized sheep erythrocytes (4) were sensitized with antiserum (2,23) of RDV, RTBV, RTYV, RSV, or RGSV.

For extraction of RSV and RGSV in rice leaves or individual insects for LF, 0.05 M Tris-HCl, pH 7.2, containing 0.02% PVP was used. This buffer caused little nonspecific aggregation when used for extracting healthy control plants (16). For RDV, RGDV, RRSV, RTBV, RTSV, and RWV, 0.01 M PB, pH 7.0, containing 0.01 M $MgCl_2$ and 0.1% Tween 20, was used because of pronounced specific aggregation in this buffer (16). For detection of RBSDV and RTYV in viruliferous insects (27), 0.01 M citrate buffer containing 0.85% NaCl, pH 6.0, was used. Rice leaves and individual insects were homogenized with 0.85% NaCl solution (saline) for PHA. Extract of 100 μl was mixed in a small test tube with 100 μl of sensitized latex for LF or with 300 μl of sensitized sheep erythrocytes for PHA. When U-bottom-well microtiter plates were used in PHA, the amount of sensitized erythrocytes could be reduced to 100 μl, and the time required for sample preparation was reduced. Scoring of the results could be done 20 min after shaking in LF and 3 hr after incubation at room temperature in PHA.

For LF and PHA, small pieces of plant specimens were crushed directly by glass rods in test tubes with 0.1 ml of buffer and then two drops of sensitized latex or 300 μl of sensitized sheep erythrocytes was added. All serological assays were conducted with similar samples at the same time, and the assays were repeated at least three times at different time.

## RESULTS

**Detection of virus antigens in plants.** Reciprocals of dilution end points for the positive reactions of viruses in rice plants in the four methods are shown in Table 1. In general, detection of the viruses with systemic infection (RDV, RSV, and RGSV) was easier than the detection of phloem-restricted viruses (RGDV, RBSDV, RRSV, RTBV, RTSV, RWV, and RTYV). Most viruses tested were detected by ELISA and simplified ELISA. RDV, RGSV, and RSV were also detected by LF and PHA. The other viruses were hardly or not detected by LF and PHA. RBSDV and RTYV were not detected in LF because of the nonspecific reactions previously reported (27). Also, RTYV was not detected by PHA because of the inhibition of erythrocytes agglutination (27). Viruses in infected plants other than rice were also detected by the methods described earlier. Both RBSDV and RSV were detected easily from infected maize plants, and RSV was also detected from infected wheat.

The best ratios of sample and conjugate for the detection of each virus in simplified ELISA were as follows: 175 μl of sample to 25 μl of conjugate for RSV; 150 μl to 50 μl for RGDV, RBSDV, RRSV, RTSV, RWV, RTYV, and RGSV; and 100 μl to 100 μl for RDV and RTBV.

Two RDV strains have been reported (13)—the ordinary (O) strain and the severe (S) strain, which causes severe stunting. SDS-polyacrylamide gel electrophoresis of the structural proteins showed differences in the mobility of their major outer capsid proteins, suggesting structural changes in the protein (13). The antiserum against RDV-O detected both the RDV-O and the RDV-S strains.

The efficiency of detection was compared for fresh and frozen leaf samples. There was no difference in $A_{410}$ values in ELISA and simplified ELISA and no difference in the results by LF and PHA. The use of roots is recommended for practical diagnosis of three plant reoviruses (RGDV, RBSDV, and RRSV) because antigen concentrations appeared to be higher in roots than in leaves (*data not shown*), as reported earlier for RGDV and RRSV (16). The concentration of another plant reovirus, RDV, in rice leaves was greater than that in roots.

Highly diluted samples are generally considered to have less nonspecific reactions in serological assays (25). In LF and PHA, RDV, RSV, and RGSV were detected in saps diluted 1:100, whereas RGDV, RRSV, RTBV, RTSV, and RWV were detected only in saps diluted less than 1:10. For practical detection of the latter group of viruses in plants, healthy controls are indispensable and skills are required for the judgment of the results. In ELISA and simplified ELISA, the dilution end point (i.e., sensitivity) generally increased when the incubation time for substrate reaction

**Table 1.** Reciprocal dilution end points of positive reactions of four serological methods for detection of rice viruses

| Virus[a] | Serological method[b] | | | |
|---|---|---|---|---|
| | ELISA | Simplified ELISA | LF | PHA |
| Rice dwarf virus | 128,000 | 32,000 | 8,000 | 16,000 |
| Rice gall dwarf virus | 5,120 | 1,280 | 40 | NT |
| Rice black-streaked dwarf virus | 1,280 | 80 | – | NT |
| Rice ragged stunt virus | 640 | 80 | 10 | NT |
| Rice tungro bacilliform virus | 2,560 | 160 | 40 | 40 |
| Rice tungro spherical virus | 2,560 | 160 | 80 | NT |
| Rice waika virus | 3,200 | 640 | 40 | NT |
| Rice transitory yellowing virus | 25,600 | 25,600 | – | – |
| Rice stripe virus | 512,000 | 64,000 | 16,000 | 16,000 |
| Rice grassy stunt virus | 64,000 | 32,000 | 8,000 | 16,000 |

[a] Rice plant infected with each virus was used as the antigen.
[b] – = Negative reaction, NT = not tested.

was prolonged until it reached the plateau. Based on the results, $A_{410}$ was read 0.5 hr after the addition of the substrate for RSV and RGSV detection; 1 hr after for RDV, RTBV, or RTYV; and 2 hr for RGDV, RBSDV, RRSV, RTSV, or RWV.

In LF, in which samples were smashed directly in test tubes, positive reactions were easily visibly distinguishable in crude saps from 1 to 32 $mm^2$ of leaf tissues infected with RDV, RSV, or RGSV. To detect RTBV and RTSV, leaf pieces larger than 8 $mm^2$ were needed and it was necessary to read the results under a microscope. When leaf pieces larger than 32 $mm^2$ were used, the homogenate obtained was viscid and it was diffcult to read the results even under a microscope (16). RGDV and RRSV were not detected in leaves or roots (16) by LF. RDV, RGSV, and RSV were easily detected from small leaf pieces after the simple extraction method by PHA.

**Detection of virus antigens in insects.** Vectors were stored at −70 C. All of the viruses that are transmitted in a persistent fashion were easily detected in individual insect vectors by ELISA and simplified ELISA. RDV, RGDV, RSV, and RGSV were detected by LF in individual vectors, and RDV, RSV, and RGSV could be detected by PHA in individual insects. Viruses that are transmitted transovarially (RDV, RGDV, and RSV) were detected not only in individual adult insects but also in nymphs right after hatching. None of the methods used detected RWV, RTSV, and RTBV antigens in their respective insect vectors. RBSDV and RRSV were not detected by LF in vectors because of nonspecific reactions (*data not shown*) or inhibition of flocculation (16), probably by host components. RTYV was not detected by LF or PHA in viruliferous insects (27).

The assays varied in their detection efficiency of RDV, RGDV, and RTYV in the common vectors. In simplified ELISA, individual insects exposed to each virus resulted in a range of $A_{410}$ values (Table 2). Insects exposed to RDV gave $A_{410}$ readings higher than those of insects exposed to RGDV, suggesting efficient multiplication of RDV in the vectors. In the case of RTYV, individual insects that have been exposed to the virus gave $A_{410}$ values ranging from 0 to >1.5, whereas unexposed insects gave values <0.08. The insects that gave $A_{410}$ values of ≥0.1 can be considered infected. In serological testing, setting thresholds for positive reaction is essential for reliability of the tests (24,25). The antisera that we used gave very low background levels, i.e., $A_{410}$ values <0.1 in ELISA and simplified ELISA. Therefore, we set the threshold as a positive reaction at an $A_{410}$ value ≥0.1 after appropriate incubation with the substrate. For LF, we read the results by observing aggregations of latex particles with the naked eye. The stereo microscope (e.g., ×10–20) was also used for confirming the results if necessary (25). When half numbers of red blood cells were agglutinated, we counted them as positive ones in PHA. These results suggest that the use of a healthy control is indispensable in setting the threshold level for positive detection of virus antigens in their vectors.

**Evaluation of serological methods.** Of the serological methods used, ELISA showed the highest sensitivity for all of the viruses (Table 1). However, ELISA took 1 or 2 days to obtain results after several steps and complicated procedures (26). On the other hand, simplified ELISA tests, which also could be used for all of the viruses, can be accomplished in 2–4 hr after somewhat simplified procedures. LF and PHA took 1–4 hr and also were simple procedures. The sensitivity of PHA was almost the same as that of LF. PHA was not used for RGDV, RBSDV, RRSV, RTSV, or RWV detections, because one PHA test required more than four times the γ-globulin than LF did in sensitizing sheep erythrocytes.

In mass inspection of viruliferous insects, considerable time for sample preparation is required for testing in LF, PHA, or ELISA. Therefore, the smashing method (one push of individual insects with a vinyl chloride rod directly in a well) provides sufficient extraction of the virus antigen. The time requirement for simplified ELISA can be greatly shortened this way. By adopting the direct smashing method, simplified ELISA has a comparative advantage over LF and PHA.

**Table 2.** Detection of three viruses in individual leafhoppers (*Nephotettix nigropictus*) by simplified ELISA

| $A_{410}$ values | Virus[a] | | | |
|---|---|---|---|---|
| | RDV | RGDV | RTYV | Unexposed |
| 0.00–0.05 | 4[b] | 7 | 17 | 90 |
| 0.05–0.1 | 3 | 1 | 7 | 7 |
| 0.1–0.2 | 0 | 0 | 14 | 0 |
| 0.2–0.4 | 0 | 0 | 11 | 0 |
| 0.4–0.6 | 0 | 16 | 6 | 0 |
| 0.6–0.8 | 0 | 35 | 12 | 0 |
| 0.8–1.0 | 3 | 25 | 2 | 0 |
| 1.0–1.5 | 8 | 11 | 17 | 0 |
| 1.5+ | 38 | 0 | 7 | 0 |

[a]RDV = Rice dwarf virus, RGDV = rice gall dwarf virus, RTYV = rice transitory yellowing virus.
[b]Number of *Nephotettix nigropictus*.

## DISCUSSION

The four serological methods were applicable for the detection of virus antigens in infected rice plants or viruliferous insect vectors. Generally, the order of virus detection sensitivity was: ELISA ≥ simplified ELISA ≥ LF ≥ PHA. On the other hand, the order of simplicity of the tests was: LF ≥ PHA ≥ simplified ELISA ≥ ELISA.

In Japan, serological methods have been applied on a large scale in an RSV disease forecasting program by monitoring the percentage of RSV carriers in overwintering vector small brown plant hopper populations. RSV causes one of the most serious diseases of rice in East Asia, is persistently transmitted by the vector, and, furthermore, is transovarially transmitted at a high rate (29). RSV disease incidence is known to be correlated with the percentage of viruliferous insects in overwintering vector populations. Therefore, serological methods such as LF and PHA have been employed every spring to determine the percentage of RSV carriers in many locations. Based on the results obtained in these experiments, the use of simplified ELISA is recommended instead of LF and PHA for the forecasting program. The reuse of the microplates (28) is expected to reduce testing costs further. In addition to the improvements in testing methods, the monoclonal antibody technique was applied for establishing antibody supply systems to supply enough antibody for annual consumption in large amounts in Japan (20). The detection of rice viruses in Japan was made more practical by the development of antibody supply systems and simple and cost-effective serological methods. Simplified ELISA has been proven to be useful in screening rice varieties for resistance to RSV (11), because the scoring of screening materials for resistance was based on symptoms because of the lack of simple diagnosis and difficulty in handling many samples at once.

Now, a user can choose one of several serological methods depending on their objective, sample number, laboratory facilities available, and availability of skilled personnel. If coating γ-globulin and conjugate for ELISA or sensitized latex or sheep erythrocytes are supplied, the assays will be used more widely in the countries. The establishment of practical serodiagnosis will undoubtedly strengthen integrated control of the virus diseases of rice.

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