# Leaf-Dip Serology for the Determination of Strain Relationships of Elongated Plant Viruses

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Cooperative investigation of the USDA Agricultural Research Service and the Nebraska Agricultural Experiment Station. Research conducted under Project No. 21-12. Published as Journal Series, Paper No. 3523, Nebraska Agricultural Experiment Station.

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I thank M. K. Brakke for help and the following donors for the viruses used in this study: hordeum mosaic virus, J. T. Slykhuis; barley stripe mosaic virus and strains, A. O. Jackson; poa semilatent virus, A. O. Jackson (virus originated from J. T. Slykhuis; antiserum to poa semilatent virus was a gift from R. I. Hamilton to A. O. Jackson); ryegrass mosaic virus, R. Goss and C. J. Gould.

Accepted for publication 6 July 1973.

## ABSTRACT

Electron microscopic identification of plant viruses by the leaf-dip serology method of Ball and Brakke was extended to determine a relationship of ryegrass mosaic virus (RMV) and wheat streak mosaic virus (WSMV) and the strains A and B of maize dwarf mosaic virus (MDMV). Antiserum to WSMV reacted with RMV in leaf-dip serology and in liquid drop microprecipitin tests but not in gel-diffusion plates. MDMV-B antiserum reacted with MDMV-A in leaf dips and in gel-diffusion plates. Antiserum to a related virus did not coat the related particles uniformly but could be seen to adhere in a spiral arrangement along the particles, indicating serological differences in viral protein subunits along the virus rods. No relationship was found between barley stripe mosaic virus (BSMV) and poa semilatent virus (PSLV) or between hordeum mosaic virus (HMV) and WSMV.

Phytopathology 64:128-131

*Additional key words:* electron microscopy, negative stain.

The leaf-dip procedure of Brandes (3) for electron microscopic identification of rod-shaped viruses has been extended by Ball and Brakke (1) to include serological identification of the virus. Leaf dips were made in a drop of diluted antiserum to the suspected virus instead of in a drop of distilled water. Leaf-dip serology has proved to be a practical, useful, convenient, rapid means of positive identification of elongated viruses in Nebraska. This report points out that leaf-dip serology can also be used to complement or extend information on the relationships of rod-shaped viruses not detectable by the plate gel-diffusion method.

MATERIALS AND METHODS.—*Viruses and hosts.*—Maize dwarf mosaic virus (MDMV), strains A and B, were isolated in Nebraska and maintained, respectively, in Johnsongrass [*Sorghum halepense* (L.) Pers.] and sweet corn (*Zea mays* L. 'Golden Cross Bantam'); wheat streak mosaic virus (WSMV), type strain, in 'Cheyenne' wheat (*Triticum aestivum* L.); ryegrass mosaic virus (RMV) in 'English' ryegrass (*Lolium perenne* L.); barley stripe mosaic virus (BSMV), type strain, North Dakota 18 strain, and Argentine mild strain in 'Moore' barley (*Hordeum vulgare* L.); poa semilatent virus (PSLV) in 'Larker' barley; and hordeum mosaic virus (HMV) in Cheyenne wheat.

Leaf dips were made as described by Ball and Brakke (1) in antiserum diluted 1:250 with 0.001 M ammonium acetate. However, the droplet was not allowed to dry. Instead, excess liquid was removed after varying reaction times, as described under Results, with a wedge of filter paper followed by negative staining according to the leaf-dip method (1).

A cut was made across a freshly picked leaf and the cut surface then was passed three to four times through the antiserum droplet on the grid. With high titered virus (BSMV) usually a single cut was sufficient. In all other cases, an additional 10-15 small cuts were made perpendicular to the first cut to increase the number of virus particles. For each antiserum dilution a control test against the homologous virus was performed to insure that antiserum was reactive.

RESULTS.—*Maize dwarf mosaic virus.*—Antiserum to MDMV-B strain covered the MDMV-B particles entirely (Fig. 1) during the 5-10 min the reaction was allowed to take place in the drops. Longer reaction times did not increase the amount of antibody attached to the virus. The same antiserum dilution only partly covered MDMV-A particles (Fig. 2) in 5-10 min and a longer reaction time (30 min) did not appreciably increase the amount of bound antibody. MDMV-A was unstable and frequently present as short, broken rods in dips made from infected Johnsongrass leaves. Virus in infected Johnsongrass juice, obtained by pressing leaves between the jaws of pliers, was more stable. Equal volumes of juice and ammonium acetate-diluted antiserum were mixed on the grid and allowed to react for various times. Results were the same as those shown in Fig. 2 in that virus particles were only partly covered.

Antiserum to MDMV-B reacted against both MDMV-B and MDMV-A in high-pH agar gel-diffusion plates (7). Precipitin lines were observed after overnight incubation of the plates at 37 C. Heavier lines were observed against the MDMV-B wells than against the MDMV-A wells. Antiserum to MDMV-A was not available and reciprocal tests were not made.

*Wheat streak mosaic, ryegrass mosaic, and hordeum mosaic viruses.*—Wheat streak mosaic virus was entirely covered by WSMV antiserum in 10 min (Fig. 3). The WSMV antiserum also partly coated RMV. Antibodies to

WSMV were interpreted as covering the RMV particles in a spiral fashion (Fig. 4). Reaction times of 10 min to 24 hr produced the same results. For long reaction times, antiserum, at the dilution used for dips, was mixed with an equal volume of partially purified RMV. Undiluted antiserum to WSMV did not react with RMV in high-pH gel-diffusion plates. Precipitin lines against WSMV were observed after overnight incubation of the plates at 37 C, but no lines against the RMV wells were observed after 1 wk of incubation. The concn of virus used in the wells was approximately 0.5 mg/ml. In liquid drop microprecipitin reactions RMV (initial concn ~ 0.4 mg/ml) reacted with a WSMV antiserum dilution of 1:1 to 1:8. However, RMV also reacted with a normal serum dilution of 1:1 and 1:2.

No antibodies were attached to HMV in sufficient numbers to be visible when HMV-infected wheat leaves were dipped in WSMV antiserum (Fig. 5). No reaction of HMV against WSMV antiserum was observed in gel-diffusion plates or in liquid drop microprecipitin reactions.

As a control on the positive reaction of RMV and WSMV antiserum, RMV-infected leaves were dipped in MDMV-B antiserum and allowed to react for 10-30 min. Results were negative (Fig. 6).

*Poa semilatent and barley stripe mosaic viruses.*— Antiserum to PSLV reacted in leaf dips with PSLV from barley leaves by coating the particles uniformly (Fig. 7). Results of dips of BSMV type strain, North Dakota 18 strain, and Argentine mild strain in PSLV antiserum were negative. Results for the reverse, dips of PSLV-infected barley leaves in sera to the three BSMV strains, also were negative. Figure 8 shows the result of a dip made in BSMV-type antiserum of PSLV (uncoated)- and BSMV (coated)-infected leaves. Agar gel-diffusion reactions between BSMV-type antiserum and PSLV were also negative. No differences among the three BSMV strains could be detected by leaf-dip serology.

*Storage of antiserum dilutions.*— Antiserum dilutions of 1:250 were made in 5-ml lots and stored frozen after each use. Repeated melting and refreezing did not alter the effectiveness of the serum dilutions. Because only a few drops were used in each test, the 5-ml lots lasted several mo, even under intensive use.

DISCUSSION.— Leaf-dip serology can indicate the degree of serological relation of viruses by partial (RMV in WSMV antiserum) or complete (BSMV type strain in Argentine mild strain antiserum) coverage of the virus particles with antibody. Antigenic differences among virus protein subunits along a virus particle probably occur in MDMV-A and RMV. Antibody can be seen attached in a spiral fashion for both viruses (Fig. 2 and 4). Whether the differences exist because of subunit arrangement or because of a difference of amino acid sequence remains to be seen.

Ryegrass mosaic virus failed to react with undiluted WSMV antiserum in gel-diffusion plates to form visible precipitin lines. It is possible that the WSMV antiserum titer to RMV was too low to produce visible precipitin bands in gel-diffusion plates since a positive reaction was obtained in the microprecipitin test with an antiserum dilution of 1:8. The antibody molecules attached to MDMV-A do not appear to be present in greater numbers than those attached to RMV, yet MDMV-A reacted in gel-diffusion plates with MDMV-B antiserum. However, the reaction of individual virus particles with antibody molecules and the reaction between antibodies and several virus particles are not analogous. At any rate, the leaf-dip method detected a relationship between RMV and WSMV at a final antiserum dilution of 1:500.

MDMV-A has been classified as distinct from isolates of MDMV which do not infect Johnsongrass, on the basis of serological and host range studies (15). The leaf-dip test also indicates that MDMV-A is quite distinct serologically at least from MDMV-B (Fig. 1 and 2).

Ryegrass mosaic virus has been reported not to be related serologically to WSMV (14) but the method of testing was not mentioned. Hordeum mosaic virus was distantly related serologically to WSMV (11) but no relationship was detected by leaf-dip serology (Fig. 5) in WSMV antiserum. It has been previously reported that WSMV antiserum did not react with HMV (13). The distant relationship of HMV and WSMV was based on a low titer of HMV antisera to Agropyron mosaic virus (AMV) and on AMV antiserum having a low titer against WSMV. Possibly antiserum titers higher than the one used here are required to definitely establish a relationship. Brandes and Bercks (4) have pointed out that very high titers are needed to detect distant serological relationships. However, Brandes and Bercks were concerned primarily with precipitin reactions.

Antisera to PSLV and BSMV had a low measure of cross reactivity with the heterologous virus (12) but it was indicated that further tests would be necessary before a relationship between the two viruses could be established. Results of leaf-dip serology performed by Polák and Slykhuis (9) with BSMV and PSLV are confirmed. Results obtained by experiments reported here are the same as shown in their report.

Van Regenmortel (16) has shown that contaminating host antigens from widely differing host plants may be serologically related and a serological relationship between unrelated viruses could be simulated. This inherent danger would be present particularly in rabbits immunized for a prolonged period [see Matthews (8)]. Leaf-dip serology would be more reliable for the detection of strain relationships than methods that depend on the formation of a visible precipitation reaction in gels or liquids. Moreover, and this has been previously stressed (1), with leaf-dip serology, one can be certain that it is the virus reacting and not a host contaminant. Because the flexuous rod-shaped viruses are difficult to purify, the problem of host contamination is serious, especially with trace reactions.

Identification of long rod-shaped plant viruses has been hampered by technical difficulties. Rod-shaped plant viruses diffuse poorly through agar and prolonged incubation periods are usually necessary. Several methods have been devised to overcome the slow diffusion or nondiffusion by prior alkaline degradation of the antigen (10), the addition of detergent to the agar diffusion plate (2, 5, 6), or by combining a high pH with detergent incorporation into the gels (7). Most of these methods require concentration of antigens to obtain sufficient amounts for visible precipitin lines of those viruses occurring in low titer in the host plant; e.g., WSMV. Antiserum to a strain of a particular virus may not be available, the virus may be susceptible to enzymatic attack during purification and thus obscure a relationship, or the virus may require a disproportionate

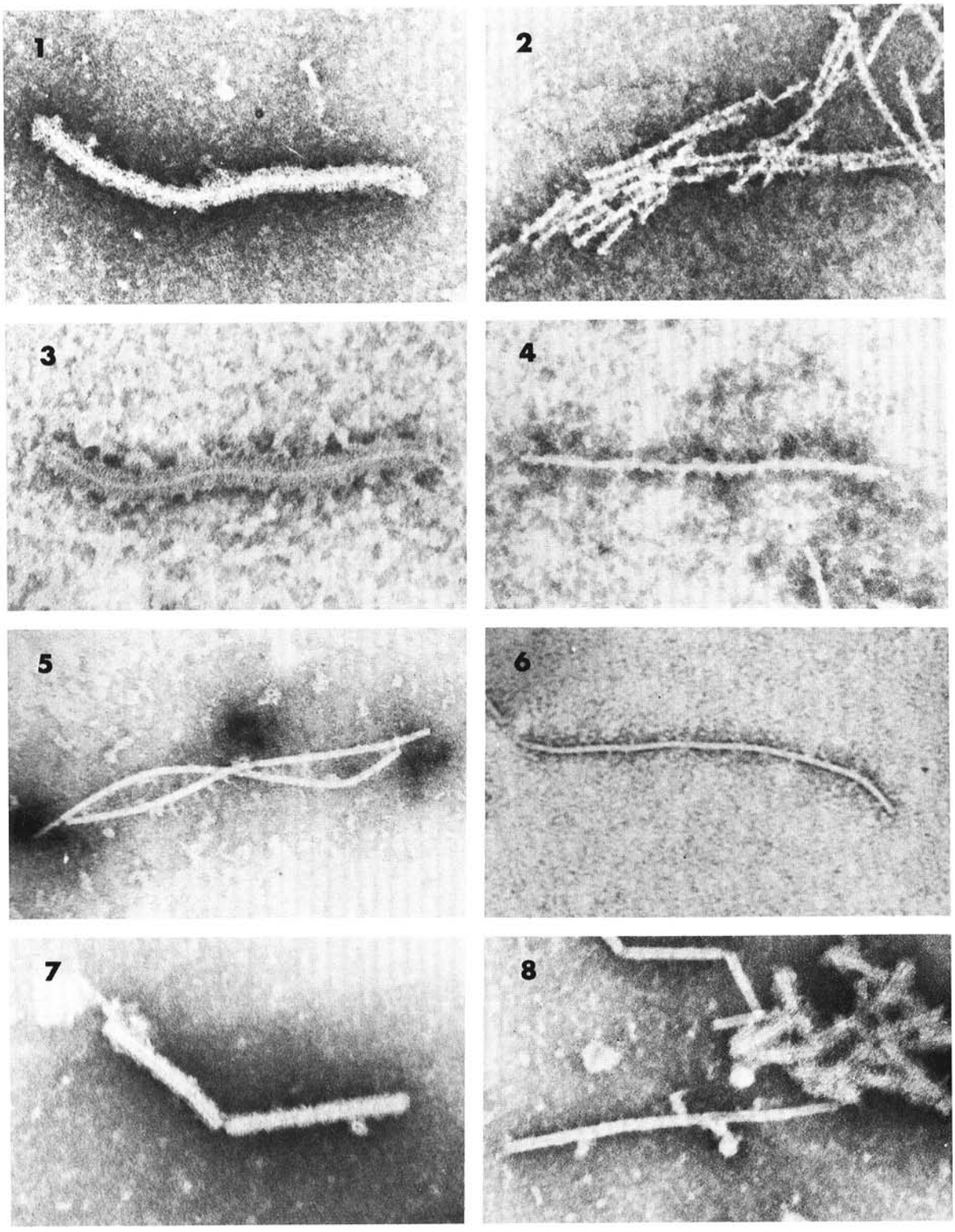


**Fig. 1-8.** Leaf-dip serology for identification of strain relationships of elongated plant viruses. All magnifications, ×90,000. **1)** Maize dwarf mosaic virus (MDMV-B) in MDMV-B antiserum showing complete coverage of virus particle by antibody. **2)** MDMV-A from Johnsongrass dipped in MDMV-B antiserum. Partial coverage only. **3)** Wheat streak mosaic virus (WSMV) dip in WSMV antiserum. **4)** Ryegrass mosaic virus (RMV) dip in WSMV antiserum. Virus partly covered. **5)** Hordeum mosaic virus (HMV) in WSMV antiserum; no reaction apparent. **6)** RMV in MDMV-B antiserum. No reaction apparent for this virus unrelated to MDMV. **7)** Poa semilatent virus (PSLV) in PSLV antiserum, showing a positive reaction. **8)** PSLV and BSMV-type dipped in BSMV antiserum, showing no reaction with PSLV rods and positive reaction with BSMV.

amount of time to produce for use in serological tests other than the leaf-dip method. Leaf-dip serology for the electron microscopic identification of plant viruses (1) overcomes many of the obstacles just mentioned. Reaction products of antibody and host contaminants, if present, do not interfere with the observed reaction of virus and antiserum. Provided antiserum is available, the method positively identifies morphologically similar viruses infecting a common host plant; e.g., WSMV and MDMV in corn. In addition, the method can show the existence of relationships not detected by gel diffusion and much faster and with less antiserum than the microprecipitin test, provided an electron microscope is available.

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