An Enzyme-Linked Immunosorbent Blocking Assay for Comparing Closely Related Virus Isolates

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ABSTRACT

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Ouchterlony double diffusion comparison of tomato ringspot virus (TmRSV) isolated from apple (A), blueberry (B), geranium (G), and cymbidium orchid (O) showed that all four isolates had at least one antigenic determinant in common, and A- and B-TmRSV had at least one additional antigenic determinant in common, which was not shared by TmRSV-G or -O. An enzyme-linked immunosorbent blocking assay (EIBA), which compares the relative abilities of excess unlabeled heterologous and homologous antigen to block the binding of enzyme-labeled antigen to its immobilized homologous IgG, was developed to more closely scrutinize the serological relationships. EIBA revealed additional antigenic differences among the four TmRSV isolates not detected by double diffusion tests.

Tomato ringspot virus (TmRSV) is a member of the nepovirus group. As such, TmRSV is polyhedral in shape, 27 nm in diameter (13), and is transmitted by the nematodes *Xiphinema americanum* (15) and *X. rivesi* (L. B. Forer and C. A. Powell, *unpublished*). TmRSV is a multiple component virus consisting of three particles with sedimentation coefficients of 53S, containing no RNA, and 119 and 127S, containing single-stranded RNAs of molecular weights 2.2 and 2.3×10^6 daltons, respectively (1,10).

TmRSV has a wide host range including both woody and herbaceous species and causes several economically important diseases including stem-pitting (11) and yellow bud mosaic (2) of peach, decline (17) and yellow vein (6) of grape, ringspot of raspberry (12), and has been associated with a decrease in the production of geranium cuttings (3). In addition, TmRSV has been isolated from symptomatic apple (14), blueberry (8; R. F. Stouffer and C. A. Powell, *unpublished*), and cymbidium orchid (5).

Because of the wide range of hosts and diseases associated with TmRSV, there have been several serological studies comparing different isolates (2,6,15–17). These studies have shown little antigenic variation, although spurs were formed in double diffusion tests between a grape isolate and a peach isolate (6) and between a grape isolate and a tobacco isolate (15).

The purpose of our research was to assess the antigenic variability of isolates of TmRSV obtained from woody (apple and blueberry) and herbaceous (geranium and cymbidium orchid) hosts and to develop improved methodology for comparing closely related antigens. Our results show that although each of these isolates can be immunologically differentiated, they are remarkably similar considering their presumed divergent histories. Our results also demonstrate the feasibility of using an enzyme-linked immunosorbent blocking assay (EIBA) for comparing the serological relatedness of viruses.

MATERIALS AND METHODS

Virus isolates. Four isolates of TmRSV were used in this study: TmRSV-A, isolated from the rootstock suckers of a cultivar MM. 106 rootstock, red delicious-scion apple tree with the apple union necrosis disease by R. F. Stouffer; TmRSV-B, isolated from declining blueberry by R. F. Stouffer; TmRSV-G, isolated from *Pelargonium hortorum* 'Sincerity' with ringspot symptoms by M. A. Derr; and TmRSV-O, isolated from cymbidium orchid with chlorotic leaf streak by Goff and Corbett (5). Each isolate was transmitted from cucumber to *Chenopodium quinoa* Willd. via the nematode vector, *X. rivesi*. Sap from these infected *C. quinoa* was diluted so that it produced an average of one local lesion per plant when mechanically tranferred to other *C. quinoa*. One of these local lesions for each isolate was selected for transfer and subsequent virus increase.

Virus purification. Each isolate of TmRSV was transferred mechanically to *Petunia hybrida* L. 'Rose of Heaven' plants. In 7–10 days, plants showed ringspot symptoms characteristic of TmRSV infection. After an additional week, the infected leaves were harvested and stored frozen.

TmRSV was purified from 100 g of infected *P. hybrida* L. leaves homogenized in a Waring Blendor with 200 ml of purification buffer (0.05 M potassium citrate, pH 6.5, containing 0.1% thioglycollic acid). The sap was expressed through cheesecloth and centrifuged at 6,000 rpm for 20 min at 5 C in a Beckman Ti 45 rotor. The supernatant was centrifuged at 43,000 rpm for 1.5 hr at 5 C in a Beckman Ti 45 rotor. The virus was resuspended in purification buffer containing 1% Triton X-100 and placed at 4 C overnight. The virus was pelleted through a 10-ml pad containing purification buffer, 20% sucrose, and 1% Triton X-100 by centrifuging at 43,000 rpm for 2 hr. The virus was resuspended in purification buffer and centrifuged in 75–300 mg sucrose per milliliter 0.01 M potassium phosphate (pH 7.0) linear density gradients (24,000 rpm for 4 hr in a Beckman SW 27 rotor). The gradients were fractionated with an ISCO density-gradient fractionator and the virus was collected, concentrated by ultracentrifugation at 43,000 rpm for 2 hr, and stored frozen in 0.01 M potassium phosphate buffer, pH 7.0. The average yields for the isolates were approximately 1 mg, 1 mg, 2 mg, and 1 mg/100 g leaves for isolates A, B, G, and O, respectively. The four purified isolates were highly infectious, were precipitated by TmRSV antiserum (American Type Culture PVAS 15), but not by tobacco ringspot virus antiserum, and appeared to be free of contamination when examined in an electron microscope.

Antiserum production. Antiserum to each isolate was produced by the Department of Comparative Medicine, Pennsylvania State University, at the Hershey Medical Center Animal Research Farm. Two New Zealand white rabbits per virus isolate were injected intramuscularly for three consecutive weeks with a mixture of 0.5 ml of 2× phosphate buffered saline (PBS, single-strength consists of 0.02 M sodium phosphate, 0.15 M sodium chloride, pH 7.4) containing 0.175 mg TmRSV plus 0.5 ml of Freund's complete adjuvant. One week after the third injection, the rabbits were anesthetized by an intravenous injection of sodium pentobarbitol (40 mg/kg) and exsanguinated. All the antiserum used in these

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studies had a titer of 1:512 against 1 mg purified TmRSV per milliliter in Ouchterlony double diffusion tests, and did not react with healthy *P. hybrida* sap or purified tobacco ringspot virus.

Prior to use in enzyme-linked assays, 1 ml of crude antiserum was precipitated with 10 ml of saturated ammonium sulfate. The precipitate was collected by centrifugation (4), dissolved in 2 ml of 0.0175 M sodium phosphate buffer (pH 6.3) and dialyzed against several changes of buffer. The amount of protein in the serum was determined spectrophotometrically ($E_{278\ nm} = 1.4$) and mixed with 10 times this weight of Cellex D (Bio-Rad Laboratories). This thick suspension was stirred for 10 min, and the serum was separated from the cellulose by vacuum filtration. The procedure yielded serum enriched in gamma globulin.

Ouchterlony double diffusion tests. Disposable petri plates, 5 cm in diameter, were filled with 8 ml of 0.8% Ionagar No. 25 in 0.01 M potassium phosphate, 0.8% sodium chloride, pH 7.0. Wells 4 mm in diameter were cut at the corners and center of a regular hexagon 42 mm in circumference. The center wells were filled (0.02 ml) with a 1:2, 1:5, or 1:10 dilution of unfractionated antiserum. The peripheral wells were filled with 1 mg of virus in 1 ml of buffer. Antisera and virus dilutions were made in 0.01 M potassium phosphate, 0.85% sodium chloride, pH 7.0. After 24 hr at room temperature, the plates were observed for precipitation lines and spur formation.

Antigen conjugation. Antigen (TMV, TbRSV, or TmRSV) was linked to alkaline phosphatase in a manner similar to that used for antibody conjugation in enzyme-linked immunosorbent assay (ELISA) (4). One-tenth milligram of purified virus was mixed with 1.0 mg of alkaline phosphatase (Type VII; Sigma Chemical Co., St. Louis, MO 63178) in 0.5 ml PBS and dialyzed 17 hr against 1 L of PBS at 4 C. The next day glutaraldehyde was added to a final concentration of 0.05%, and this solution was incubated for 4 hr at room temperature. The glutaraldehyde was removed by dialysis for 3 days against three changes of 1 L of PBS at room temperature. The antigen conjugate was stored at 4 C and has retained the ability to combine with homologous antibody for at least 1 yr.

Enzyme-linked immunosorbent blocking assay. The enzyme-linked immunosorbent blocking assay (EIBA) is diagramatically represented in Fig. 1. First, 0.25 ml of 0.01 mg/ml of coating IgG in 0.05 M sodium carbonate (pH 9.6) is added to the wells of microtiter plates (Dynatech). In experiments comparing unrelated viruses, the coating IgG was specific for tobacco mosaic virus (TMV), TbRSV, or TmRSV-A. In experiments comparing TmRSV isolates the coating IgG was formed in response to TmRSV-A, -B, -G, or -O. Second, 0.25 ml of 0.01 mg/ml of blocking antigen in 0.02 M potassium phosphate, 0.15 M sodium chloride, 0.05% Tween-20, 2.0% polyvinylpyrrolidone 40 (pH 7.4) (PBS-Tween-PVP), or PBS-PVP-Tween alone is added. In experiments comparing unrelated viruses, the blocking antigens were TMV, TbRSV, and TmRSV. In experiments comparing TmRSV isolates, the blocking antigens were TmRSV-A, -B, -G, and -O. The plates are incubated at 4 C for 17 hr followed by washing as in standard ELISA. Third, 0.25 ml of a 1:800 dilution in PBS-Tween-PVP of alkaline phosphatase-conjugated antigen is added to wells previously coated with homologous IgG in the first step. In experiments comparing unrelated viruses, the conjugated antigens were TMV, TbRSV, and TmRSV. In experiments comparing isolates of TmRSV, the conjugated antigens were TmRSV-A, -B, -G, and -O. After incubation for 4 hr at 37 C and washing, 0.25 ml of the enzyme substrate *p*-nitrophenyl phosphate is added to each well. The enzyme activity was terminated after 1 hr by adding 0.05 ml of 6.0 M NaOH with the $A_{405\ nm}$ was determined spectrophotometrically.

In all experiments, a treatment would show the ability of homologous antigen, a heterologous antigen, or buffer (PBS-PVP-Tween) to block a conjugated antigen from combining with its homologous IgG. In experiments comparing unrelated viruses, there were 12 treatments that showed the ability of TMV, TbRSV, TmRSV, and buffer to block TMV, TbRSV, and TmRSV from combining with their homologous IgG. In experiments comparing TmRSV isolates, there were 20 treatments that showed the ability of TmRSV-A, TmRSV-B, TmRSV-G, TmRSV-O, and buffer to block TmRSV-A, -B, -G, and -O from combining with their homologous IgG. Each treatment was replicated 10 times per experiment in a completely randomized design. Each experiment was repeated at least three times with serum from at least two bleedings from at least two rabbits. Unused wells in microtiter plates were filled with water.

RESULTS

Ouchterlony double-diffusion tests. Antiserum (As) produced against each of the four TmRSV isolates (A, B, G, and O) was analyzed for subsets of antibody molecules, which could be precipitated by homologous, but not heterologous antigen. When As against TmRSV-A, -G, or -O was placed in the center well, no spurs were formed between the homologous antigen and any of the heterologous antigens. This indicated that all the antibody molecules (detectable by Ouchterlony double-diffusion) produced in response to TmRSV-A, G, or O were precipitated by the three heterologous TmRSV isolates (Fig. 2A, C, and D). However, when As against TmRSV-B was placed in the center well, no spur was formed between TmRSV-B and -A, but spurs were produced between TmRSV-B and -G and between TmRSV-B and -O, indicating that all the antibody molecules (detectable by Ouchterlony double-diffusion) produced in response to TmRSV-B were precipitated by TmRSV-A, but some of these antibody molecules were not precipitated by TmRSV-G or -O (Fig. 2B).

The Ouchterlony double-diffusion results are diagrammatically summarized in Fig. 3. Based on Ouchterlony results, each of the four TmRSV isolates have at least one antigenic determinant in common, and the apple and blueberry isolates have at least one additional antigenic determinant in common that is not shared by TmRSV-G or -O.

Analysis of unrelated viruses with EIBA. The results of a representative experiment are shown in Table 1. If the unlabeled blocking antigen is identical to the labeled antigen, the unlabeled antigen will completely block the labeled antigen from combining with its homologous IgG, and there will be no color change. This identity reaction has been observed for several viruses including the blocking of tobacco mosaic virus (TMV) by TMV, TmRSV by TmRSV, and tobacco ringspot virus (TbRSV) by TbRSV (Table 1). If the unlabeled antigen is unrelated to the labeled antigen, the unlabeled antigen will not block the labeled antigen from combining with its homologous IgG, and there will be a fast color change. This unrelated reaction has been shown by the inability of TMV, TmRSV, and TbRSV to block each other (Table 1). Data from duplicate experiments using serum from duplicate rabbits and serum from different bleedings were not quantitatively different from that in Table 1.

Analysis of TmRSV isolates with EIBA. The relative ability of each of the four isolates of TmRSV (A, B, G, and O) to block each isolate from combining with its homologous IgG was determined.

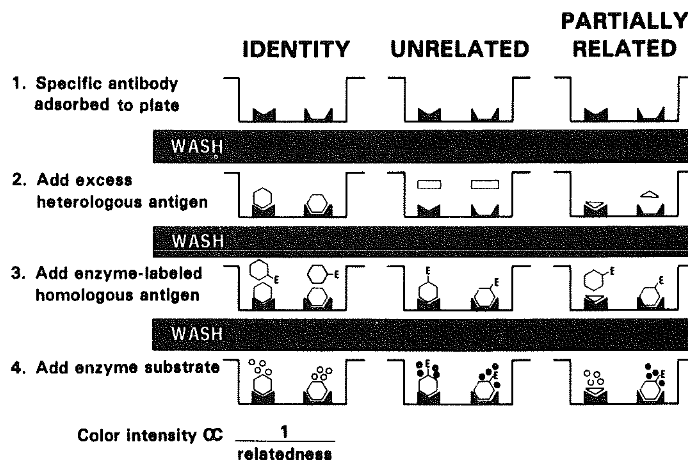

Fig. 1. Diagrammatic representation of the enzyme-linked immunosorbent blocking assay.

Buffer (PBS-Tween-PVP) served as a negative control (no blocking). Table 2 shows the results from a representative experiment. TmRSV-A, and -B, but not TmRSV-G or -O, completely blocked TmRSV-A from combining with its homologous IgG. Thus, TmRSV-A elicited some antibody molecules that had a greater affinity for TmRSV-A and -B, than TmRSV-G or -O. Each of the four TmRSV isolates completely blocked TmRSV-G from combining with its homologous IgG. Thus, all the detectable antibody molecules elicited by TmRSV-G had equal affinity for the four TmRSV isolates. None of the three heterologous TmRSV isolates completely blocked TmRSV-B from combining with its homologous IgG. Thus, TmRSV-B elicited some antibody molecules that only had a high affinity for TmRSV-B. TmRSV-O and -B, but not TmRSV-A or -G, completely blocked TmRSV-O from combining with its homologous IgG. Thus, TmRSV-O elicited some antibody molecules with equal affinity for TmRSV-O and -B, but reduced affinity for TmRSV-A or -G. Every isolate was capable of partially blocking every other isolate, indicating that all four of the TmRSV isolates elicited some common antibodies. Data from duplicate experiments with serum from duplicate rabbits and serum from different bleedings were not quantitatively different from that in Table 2. Alkaline phosphatase conjugated healthy petunia sap did not react with any of the TmRSV sera ($A_{405\ nm}$ = <0.1 after 1 hr). EIBA analysis of the four isolates of TmRSV is summarized diagrammatically in Fig. 4. Each of the four isolates have at least one antigenic determinant in common represented by the square-like symbol. The apple and blueberry isolates share at least one additional antigenic determinant represented by the triangle-like symbol. The blueberry and orchid isolates also share at least one additional antigenic determinant represented by the trapezoid-like symbol. The blueberry isolate had at least one unique antigenic determinant represented by the rectangle-like symbol.

A comparison of Figs. 3 and 4 shows the relative sensitivities of the Ouchterlony double diffusion and EIBA techniques for detecting subsets of antibody molecules. EIBA detected several additional antibody subsets and allowed more critical differentiation of the TmRSV isolates. TmRSV-G and -O, which were identical in double diffusion, were different in EIBA.

Direct comparisons between EIBA and standard ELISA (4) in detecting differences among related viruses were also made. In our tests, standard ELISA was not satisfactory for segregating TmRSV isolates. Each of the sera reacted equally well with each isolate over a range of antigen and antibody-conjugate concentrations (P = 0.05) (*unpublished*). However, it would be inappropriate to conclude that EIBA is better than standard ELISA for differentiating related viruses based on results from one laboratory with one virus system.

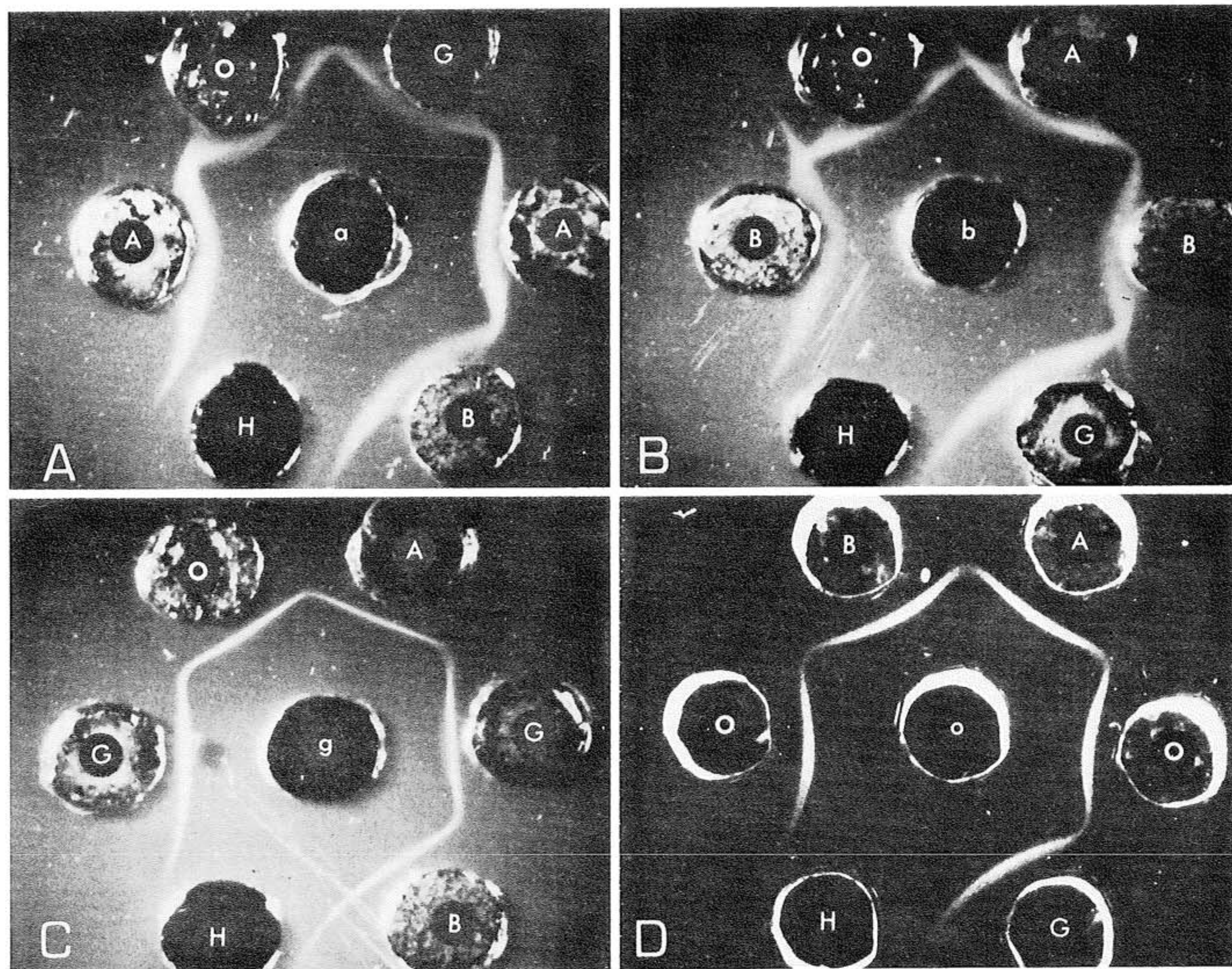

Fig. 2. Ouchterlony double diffusion tests showing cross-reactivity among isolates of tomato ringspot virus (TmRSV) from apple, A-TmRSV; from blueberry, B-TmRSV; from geranium, G-TmRSV; and from cymbidium orchid, O-TmRSV. Lowercase letters represent the antiserum against the corresponding virus isolate. The antiserum dilution was 1:10 and the virus concentration was 1 mg/ml in buffer. **A**, Reactivity of TmRSV isolates with anti-TmRSV-A serum. **B**, Reactivity of TmRSV isolates with anti-TmRSV-B serum. **C**, Reactivity of TmRSV isolates with anti-TmRSV-G serum. **D**, Reactivity of TmRSV isolates with anti-TmRSV-O serum.

OUCHTERLONY RESULTS

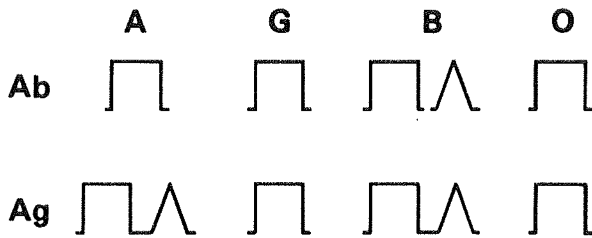

Fig. 3. Diagrammatic summary of the serological relationship among TmRSV isolates A, B, G, and O based on Ouchterlony double-diffusion. Ag, minimum antigenic determinants for each isolate; and Ab, detectable subsets of antibody molecules produced in response to each isolate.

EIBA RESULTS

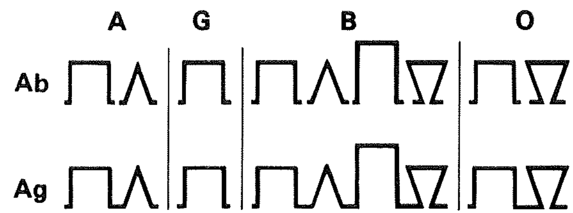

Fig. 4. Diagrammatic summary of the serological relationships among TmRSV isolates A, B, G, and O based on EIBA. Ag, minimum antigenic determinants for each isolate; and Ab, detectable subsets of antibody molecules produced in response to each isolate.

TABLE 1. Analysis of unrelated viruses with the enzyme-linked immunosorbent blocking assay

Blocking agent[b]	Virus-antibody system[a]		
	TMV	TbRSV	TmRSV
Buffer	1.416 a	1.317 a	1.614 a
TMV	0.185 b	1.016 a	1.314 a
TbRSV	1.010 a	0.138 b	1.317 a
TmRSV	1.117 a	1.618 a	0.143 b

[a]Refers to the homologous virus-antibody complex that results in a color change. The numbers are the average $A_{405\,nm}$ of 10 replications. The lower the number, the greater the blocking. Each letter indicates that the corresponding mean differed significantly from means in the same column, which are followed by other letters with a 5% chance of a Type I error.
[b]Nonconjugated antigens that were used to block the formation of conjugated antigen-homologous antibody complexes.

TABLE 2. Analysis of TmRSV isolates with the enzyme-linked immunosorbent blocking assay

Blocking agent[b]	Virus-antibody system[a]			
	TmRSV-A	TmRSV-B	TmRSV-G	TmRSV-O
Buffer	1.380 a	0.748 a	0.626 a	0.724 a
TmRSV-A	0.319 b	0.344 b	0.187 b	0.244 b
TmRSV-B	0.277 b	0.162 c	0.177 b	0.159 c
TmRSV-G	0.500 c	0.305 b	0.141 b	0.237 b
TmRSV-O	0.796 d	0.339 b	0.156 b	0.177 c

[a]Refers to the homologous virus-antibody complex that results in a color change. The numbers are the average $A_{405\,nm}$ of 10 replications. The lower the number the greater the blocking. Each letter indicates that the corresponding mean was significantly different from means in the same column, which are followed by other letters with a 5% change of Type I error.
[b]Nonconjugated antigens that were used to block the formation of conjugated antigen-homologous antibody complexes.

DISCUSSION

Our results show that four isolates of TmRSV from different hosts are antigenically related, but can be differentiated. It is unlikely that an isolate would be transmitted between the woody and herbaceous hosts. Apparently the sites on TmRSV that are most immunogenic are rarely subject to change via mutation. Many of the sites on the TmRSV coat protein that are responsible for the antigenicity are probably also necessary for its function(s).

Relative antigenic stability appears to be a general characteristic of the nepovirus group. Although there are several subgroups of tobacco ringspot and tomato black ring viruses (7,9), the serological variation is not great, considering the wide host range and extensive distribution of these viruses. In general, and in contrast to many other virus groups, individual members of the nepovirus group do not cross-react serologically with other members in their group.

The four isolates of TmRSV in this study are also similar in their biological properties. In limited experiments, they have identical host ranges. For example, both the apple and geranium isolates can induce the yellow bud mosiac disease in peach (L. B. Forer and C. A. Powell, *unpublished*). Some symptom differences among the isolates may occur on tobacco and petunia, but variables such as virus concentration, relative infectivity of the preparation, day length, and temperature influence the symptoms more than the particular isolate. Each of the four isolates of TmRSV is also transmitted by *X. rivesi*, but transmission efficiency comparisons have not yet been made. General biophysical properties, including sedimentation and particle size, are the same for TmRSV isolates. Comparative studies on the nucleic acids and protein have not yet been performed.

The EIBA was a simple technique for rapidly differentiating the four TmRSV isolates. EIBA has many advantages over Ouchterlony double-diffusion as well as many other commonly used techniques. First, it is highly sensitive. EIBA detected several subsets of antibody molecules not detected by double diffusion. EIBA owes its sensitivity to the catalytic nature of the detection mechanism, although in EIBA there is no amplification due to "sandwiching." A second advantage of EIBA is that the results can be quantitated as in standard ELISA by measuring the color spectrophotometrically. A third advantage of EIBA is that the technique easily lends itself to statistical analysis. Treatments can easily be replicated to provide a statistical basis for claiming the identity or differences among antigens within a predetermined confidence level. A fourth advantage of EIBA is that it measures the primary binding of antigen to antibody rather than precipitation, which is a much more complex chemical reaction involving additional variables.

There are many factors that contribute to serological differences among viruses. These differences are legitimate when based upon differences in the amino acid sequence(s) of the viral protein(s), which are a reflection of differences in the viral genome. However, antisera with different specificities are also obtained from different bleedings, individuals, and antigen preparations, although immunization conditions are identical. A sensitive technique such as EIBA has the drawback that it has the potential to lead to a conclusion that two antigens are different based on these nonreproducible factors. This does not appear to be the case with our analysis of the four isolates of TmRSV. However, whether nonreproducibility with EIBA proves to be a problem in other systems awaits further experimentation.

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