Simple Latex Agglutination Test for Detecting Flexuous Rod-Shaped Viruses in Forage Legumes

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ABSTRACT

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Flexuous rod-shaped viruses occurring in forage legumes were detected by a latex agglutination test (LAT). This simple, sensitive, reliable, and rapid serological procedure detected alfalfa latent (ALV), bean yellow mosaic (BYMV), clover yellow mosaic (CYMV), clover yellow vein (CYVV), red clover vein mosaic (RCVMV), white clover mosaic (WCMV), and peanut mottle (PMV) viruses directly from leaf sap extracts of greenhouse and field samples of various clovers and peas. The standard dilution procedure using purified virus preparations showed that LAT detected as little as 0.5, 0.5, 0.1, 0.2, 0.1, and 0.2 μg/ml of BYMV, CYVV, CYMV, RCVMV, WCMV, and ALV, respectively. In crude sap extracts (1:10, w/v) from pea and clover, the detectable dilution end point for these viruses varied from 4×10^{-2} to 4×10^{-4}. The capability of LAT to detect virus in composite samples of infected and uninfected leaflets was 1 in 20 for BYMV and CYVV, 1 in 40 for ALV and RCVMV, and 1 in 100 for WCMV and CYMV.

Several viruses are known to infect forage legumes in the southeastern United States. Some of these include bean yellow mosaic virus (BYMV), clover yellow vein virus (CYVV), clover yellow mosaic virus (CYMV), white clover mosaic virus (WCMV), red clover vein mosaic virus (RCVMV), alfalfa latent virus (ALV), and Wisconsin pea streak virus (WPSV) (2,3,5,6,9,10,12). Detection and identification of these viruses have been difficult because of similar symptomology, unreliable and cumbersome diagnostic tests, and the need for large sample numbers. A simple and reliable test is needed for detection of these viruses. The most obvious choice would be a serological test, but there are several disadvantages associated with the most commonly used procedures. Liquid and gel precipitin tests generally need large quantities of antisera and are adapted primarily for use with small sample sizes (1). Labeled antibody tests, such as enzyme-linked immunosorbent assay (ELISA), provide sensitivity but are time-consuming and need extra equipment and supplies (16). Therefore, a simple and rapid test that combines high specificity and sensitivity is needed.

A latex agglutination test (LAT) is a simple, sensitive, portable, and inexpensive test that has been used for detection of potato viruses (13). This study was undertaken to determine the sensitivity and potential application of LAT in forage legume virus research.

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MATERIALS AND METHODS

Virus isolates and antisera used in this study were BYMV-204-1 from S. Diachun and T. Pirone, University of Kentucky (10); CYVV, WCMV, and CYMV from O. W. Barnett, Clemson University (3); RCVMV-PV110 from D. P. Maxwell, University of Wisconsin (11,14); ALV-35 from M. K. Brakke, University of Nebraska (15); peanut mottle virus from C. Kuhn, University of Georgia (6); WPSV from L. Bos, Institute of Pathological Research, Netherlands (4,8); and peanut stunt virus (an isolate from white clover in Georgia). All but WPSV are known to be prevalent in the southeastern United States (2). Purity and identity of these viruses were confirmed by a host-range test (2) and sodium dodecyl sulfate-agar double-diffusion serology (7). Virus cultures were maintained in red clover (*Trifolium pratense* L.) clone L-12 or garden pea (*Pisum sativum* L.) cultivars Alaska and Perfected Wales.

All test plants were grown in 10-cm clay pots containing a soil-vermiculite mixture and maintained in a greenhouse at 25 C. Clovers and other small-seeded species were germinated in vermiculite before being transplanted to pots. Virus isolates were maintained in the greenhouse by biweekly mechanical transfers. Inoculum was prepared by triturating 1 g of leaf tissue in 1 ml of 0.025 M sodium phosphate buffer, pH 7.2. Conjugation of antiserum to latex beads and the test procedure were performed as described by Khan and Slack (13), except the latex beads (Sigma, 10% w/v) were diluted to about 0.6% (w/v). γ-Globulin fractions (IgG) from crude serum were precipitated with ammonium sulfate [equal volumes of saturated $(NH_4)_2SO_4$ with antiserum diluted with nine volumes of distilled water]. Mixtures were stirred in a cold room (4 C) overnight, then centrifuged at 10,000 rpm for 10 min. Resulting pellets were resuspended in 0.1 M tris-HCl buffer, pH 7.4 (to original volume of antiserum), and dialyzed against the same buffer to remove sulfate ions. Aliquots (5-ml) of several antiserum dilutions (e.g., 1:25, 1:50, 1:100, 1:200, 1:400, and 1:800) were mixed with equal volumes of latex beads and diluted 1:15 (v/v) in 0.85% NaCl. After incubating 1 hr at room temperature, the mixture was centrifuged for 30 min at 6,000 rpm (Sorvall SS-34 rotor). Sensitized latex particles were stabilized by washing the pellets twice in 0.02% polyvinylpyrrolidone in 0.85% NaCl. Final pellets were resuspended in 5 ml of 0.1 M Tris-HCl buffer, pH 7.4, containing 0.02% sodium azide. Preparations were filtered through Whatman No. 2 filter paper and stored at 4 C. Six dilutions in twofold increments of each antiserum latex conjugate were used to determine the optimum reaction range, with the last dilution giving a

Table 1. Minimum detectable concentration for detecting purified forage legume viruses in buffer and crude extract by the latex agglutination test

Virus	Purified virus (μg/ml) in Tris-buffered saline (pH 7.2)	Purified virus (μg/ml) in Crude sap extract
BYMV[a]	0.2[b]	0.5
CYVV	0.2	0.5
ALV	0.1	0.2
RCVMV	0.1	0.2
WPSV	0.1	0.2
CYMV	0.1	0.1
WCMV	0.1	0.1
PSV	0.5	0.5

[a] BYMV = bean yellow mosaic virus, CYVV = clover yellow vein virus, ALV = alfalfa latent virus, RCVMV = red clover vein mosaic virus, WPSV = Wisconsin pea streak virus, CYMV = clover yellow mosaic virus, WCMV = white clover mosaic virus, and PSV = peanut stunt virus.

[b] Each value is the mean of three replicates.

Table 2. Comparison of detection limits for legume viruses in *Pisum sativum* 'Alaska' and *Trifolium* spp. by latex agglutination test

Virus	Detectable dilution end point[a]						
	P. sativum 'Alaska pea'	*T. hybridum* 'Alsike'	*T. vesiculosum* 'Arrowleaf'	*T. incarnatum* 'Crimson'	*T. pratense* 'Red'	*T. subterraneum* 'Subterranean'	*T. repens* 'White'
ALV[b]	4×10^{-4}[c]	NH	NT	NT	NH	NT	NH
BYMV	1×10^{-3}	4×10^{-2}	8×10^{-2}	4×10^{-2}	4×10^{-2}	4×10^{-2}	NH
CYVV	1×10^{-3}	4×10^{-2}	8×10^{-2}	8×10^{-2}	4×10^{-2}	8×10^{-2}	4×10^{-2}
CYMV	8×10^{-3}	4×10^{-3}	2×10^{-3}	2×10^{-3}	2×10^{-3}	4×10^{-3}	2×10^{-3}
RCVMV	2×10^{-3}	NT	1×10^{-3}	NT	NT	NT	NH
WCMV	2×10^{-4}	2×10^{-4}	1×10^{-4}	2×10^{-3}	1×10^{-3}	2×10^{-4}	2×10^{-4}
WPSV	4×10^{-4}	NT	NT	NT	2×10^{-3}	NT	NT

[a] A mean agglutination value greater than 1.5 (scale of 1–5) was considered an end point. Each value is the mean of three replicates.
[b] ALV = alfalfa latent virus, BYMV = bean yellow mosaic virus, CYVV = clover yellow vein virus, CYMV = clover yellow mosaic virus, RCVMV = red clover vein mosaic virus, WCMV = white clover mosaic virus, and WPSV = Wisconsin pea streak virus.
[c] Dilutions are based on leaf tissue in TBS (g/ml); NH = nonhost, and NT = not tested.

positive reaction reported as the end point of fractioned serum.

Crude sap extract (CSE) antigen was prepared by triturating 0.1 g of leaf tissue in 1 ml of 0.1 M tris-buffered-saline, pH 7.2 (TBS). Leaf samples were taken from individual plants, or for composite samples, leaf disks were obtained by using a no. 8 corkborer.

To determine the sensitivity of LAT, the CSE for each virus was serially diluted in twofold and 10-fold increments. Each dilution was tested against the six sensitized latex dilutions. Agglutination values of 1 (weakest) to 5 (strongest) were used in assessing the reaction of each CSE antigen-antiserum latex conjugate combination (13). Values obtained from the highest positive dilution were averaged (usually three tests performed), and if the mean agglutination value was greater than 1.5, the dilution was considered a detectable dilution end point (DDEP). In some cases, the dilution end point was also tested by bioassay on local lesion hosts.

RESULTS AND DISCUSSION

Purified virus preparations and CSE reacted positively with homologous but not with heterologous serum-sensitized latex by agglutinating the latex beads of BYMV, CYMV, WCMV, RCVMV, ALV, or WPSV. Sap from uninoculated plants of garden pea cultivar Alaska, buffer, and tobacco leaves infected with potato viruses S, X, and Y, a carlavirus, potexvirus, and potyvirus, respectively, were used as controls. No agglutination of the sensitized latex preparations was observed against any of the controls.

The optimum antiserum dilution for each antiserum-sensitized latex preparation was determined. The antiserum dilution that detected the highest dilution of antigen was considered optimum. In each case, the optimum serum dilution was between 1:50 and 1:200. However, to ensure the detection of virus in composite samples, three serum dilutions near the optimum were used.

The minimum detectable virus concentration using LAT was determined using purified virus preparations (1 mg/ml). The minimum detectable concentration was 0.2 μg/ml for potyviruses BYMV and CYVV, 0.1 μg/ml for carlaviruses RCVMV and WPSV, and 0.1 μg/ml for potexviruses WCMV and CYMV (Table 1). Detection limits generally were higher when TBS was used as a diluent rather than CSE. This was probably due to the increased likelihood of nonspecific interference of the agglutination reaction when plant sap was added to the reaction.

The sensitivity of LAT for seven viruses from seven legume species is listed in Table 2. The detection limit depended on the host-virus combination used. BYMV and CYVV (potyviruses) could only be detected up to a dilution of 10^{-3}. RCVMV and ALV (carlaviruses) varied from 10^{-3} to 4×10^{-4}, whereas WCMV (potexvirus) was easily detected from various clovers up to a 10^{-4} dilution. The DDEP from pea sap was slightly higher than for clover sap for all viruses tested.

Limits for composite samples were determined using LAT (Table 3). CYMV and WCMV were easily detected from CSE prepared by combining one infected leaflet with 100 uninfected ones. However, the detection limit for various clovers infected with BYMV or CYVV was one in 40 to 80 leaflets, depending on the host plant used (Table 3). The technique was most efficient in testing field samples for multiple viruses when a ratio of one infected in 20 leaflets was used. This was an optimum level for all the viruses tested and made it possible to test large numbers of field plants.

In most cases, LAT was as sensitive as the infectivity test (Table 4). In a few

Table 3. Number of leaflets from which one infected leaflet could be detected for clover viruses by the latex agglutination test in composite samples

Host	No. of leaflets/composite sample			
	BYMV[a]	CYVV	CYMV	WCMV
Clovers				
Arrowleaf	80	80	100	100
Crimson	40	80	100	100
Red	40	40	100	100
White	NT[b]	40	100	100
Pea	100	100	100	100

[a] BYMV = bean yellow mosaic virus, CYVV = clover yellow vein virus, CYMV = clover yellow mosaic virus, and WCMV = white clover mosaic virus.
[b] NT = not tested.

Table 4. Comparison of the latex agglutination test with enzyme-linked immunosorbent assay and infectivity tests on indicator hosts

Virus	Detectable dilution end point by		
	LAT[a]	ELISA[a]	Indicator host
BYMV[b]	10^{-3}	10^{-3}	10^{-3}[c]
CYVV	10^{-3}	10^{-3}	10^{-3}[c]
WCMV	4×10^{-4}	10^{-4}	10^{-7}[d]
CYMV	2×10^{-3}	10^{-6}	10^{-5}[c]

[a] Data on ELISA obtained from Dr. M. R. McLaughlin (Mississippi State University). LAT and ELISA data taken from different plants. Virus titers may vary due to environmental conditions. These data are an average of three tests performed at different times of the year.
[b] BYMV = bean yellow mosaic virus, CYVV = clover yellow vein virus, WCMV = white clover mosaic virus, and CYMV = clover yellow mosaic virus.
[c] *Chenopodium amaranticolor.*
[d] *Vicia faba.*

instances, however, when clover field samples were tested for BYMV, CYVV, WCMV, CYMV, and RCVMV at a level of one infected in 100 samples, a discrepancy between LAT and the bioassay was observed. Some samples that tested negative by LAT showed lesions on bioassay; however, the number of lesions was very small, indicating an extremely low concentration. Therefore, a lower ratio (1:20) of infected to uninfected would have to be used to ensure detectability. LAT also compared favorably with ELISA in sensitivity of virus detection. The DDEPs were nearly equal in all instances, except for CYMV, which could be detected at a lower concentration with ELISA (Table 4).

The results of this study indicate that LAT is a sensitive, simple, rapid, and reliable procedure for diagnosis of several legume viruses. It allows testing of multiple viruses in composite samples. The test, which is sensitive enough for routine analysis of legume viruses, can identify one infected leaflet combined with 20 uninfected leaflets. This sensitivity can be as great as one in 100 leaflets for CYMV and WCMV. Individual plants can also be tested with a dilution limit of 1×10^{-3} to 4×10^{-4}, depending on the virus used. The test is inexpensive and uses minimal amounts of antiserum, time, and labor. LAT can be used for routine mass-indexing programs, such as certification, breeding, plant quarantine, and germ plasm screening. Therefore, LAT is an extremely efficient procedure that should help researchers working with legume viruses to meet their diagnostic needs. The LAT procedure may also have advantages in developing countries where sensitive tests such as ELISA are difficult because the necessary enyzmes are not available.

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