Detection of 41 Isolates of Necrotic Ringspot, Apple Mosaic, and Prune Dwarf Viruses in *Prunus* and *Malus* by Enzyme-Linked Immunosorbent Assay

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ABSTRACT

McMorran, J. P., and Cameron, H. R. 1983. Detection of 41 isolates of necrotic ringspot, apple mosaic, and prune dwarf viruses in *Prunus* and *Malus* by enzyme-linked immunosorbent assay. Plant Disease 67:536-538.

Enzyme-linked immunosorbent assay (ELISA) detected 41 isolates of ilarvirus in *Prunus* and *Malus* representing the entire symptomatic and serological range of necrotic ringspot virus (NRSV), apple mosaic virus (ApMV), and prune dwarf virus (PDV). Three isolates reported to be NRSV were not detected. Of 11 antisera used, only NRSV-G, RMV, PDV-B were required to detect all 41 isolates. These three antisera, which were commercially available, were combined to detect any of the 41 isolates in singular assays. Results of a comparative bioassay using *Chenopodium quinoa* and *Cucumis sativus* are discussed.

The group of virus isolates originally referred to as the Prunus ringspot virus group (19), then as ILAR viruses (10), and currently as three members of the ilarvirus group in Tricornaviridae (27) are widespread within certain genera of the Rosaceae. Programs designed to ensure that nursery stocks are free of these viruses have been based primarily on the use of woody indicator species, especially Shirofugan flowering cherry (*Prunus serrulata* L.). Such indexes, though accurate and sensitive, require a great deal of time and labor. Detection of these viruses serologically via enzyme-linked immunosorbent assay (ELISA) has been very successful in both "native" woody and herbaceous hosts (1,4,6, 14,18,23–25). Before the ELISA technique could be applied to the large-scale virus indexing programs for detection of these viruses in nursery stock, more information was required on how commercially available antisera react in ELISA to the wide range of virus isolates of this group that is found in nature. Specifically, information was needed to determine the possible combinations of antisera that could facilitate detection of the naturally occurring spectrum of isolates in this virus group.

Using the Ouchterlony double-diffusion technique (ODD), Fulton (9,10) and others (2,3,20–22,26) distinguished four serogroups within the original ILAR virus group, a necrotic ringspot virus (NRSV) (11), an apple mosaic virus (ApMV) (13), a prune dwarf virus (PDV) (12,15), and a North American line pattern virus (NLPV) (21) serogroup. Although the PDV and NLPV serogroups were totally distinct, the NRSV and ApMV serogroups showed distant serological relatedness in the ODD reactions.

Because antigens and antisera that have distant or intermediate relationships in ODD tests often give negative readings in ELISA (16,25), it was suggested (1,16) that detailed evaluation of all antigen/antisera reactions within a particular group be carried out before any large-scale testing was done to ensure that all known strains could be detected. In work reported here, bioassays employing *Cucumis sativus* and *Chenopodium quinoa* are presented. An abstract of some of these results was published previously (17). This research was conducted to document strains of ilarvirus that can be detected by ELISA using the presently available antisera and thus facilitate the use of this diagnostic technique in virus certification programs.

MATERIALS AND METHODS

Virus isolates. Forty-four virus isolates originally from various *Prunus, Rosa, Malus, Humulus*, and *Corylus* spp. were examined (Table 1). Previous investigators in 12 states and three countries had made the original isolations (McMorran, *unpublished*), most of which are currently part of the *Prunus* and *Malus* spp. virus collection at the IR-2 repository at Prosser, WA (7). All but the following 11 isolates were received from P. Fridlund at IR-2; rose virus-4, rose mosaic virus (R. W. Fulton's isolate) (in cucumber and periwinkle), and Paradise isolate of ApMV (in apple) from R. H. Converse (Corvallis, OR); almond calico

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and cherry rugose isolates of NRSV (in cherry) from G. Nyland (Davis, CA); hop virus isolates "A" and "I" (in cucumber) from K. Skotland (Prosser, WA); a "mild" and a cherry rugose isolate of NRSV (in cherry) from G. Mink (Prosser, WA); the Danish line pattern isolate of NRSV (in cucumber) from ATCC (Rockville, MD); and an ApMV isolate (in filbert) from H. R. Cameron (Corvallis, OR). All isolates received from P. Fridlund had been indexed periodically on Shirofugen flowering cherry and other hosts (P. Fridlund, *personal communication*) to confirm virus identity. Isolates not received from P. Fridlund were mechanically indexed on a host range of seven commonly used herbaceous indicator hosts (8,19; McMorran, *unpublished*) to check conformity with symptoms reported on these hosts (detailed results of index available from J. P. McMorran).

Antisera. The 11 antisera used included six NRSV antisera prepared against isolates "G," "H," "RA," and "464A" by R. W. Fulton, cherry rugose-15B by G. Nyland, and Stecklenberg "PRSV" by R. Casper (Braunschweig, West Germany); three ApMV serogroup antisera prepared against an RMV isolate and Paridise ApMV by R. W. Fulton, and HopV-I by C. B. Skotland; a PDV antisera prepared against the "B" isolate by R. W. Fulton, and one against an English isolate by H. R. Cameron. NRSV-"Stecklenberg," NRSV-G, NRSV-H, RMV, ApMV-P, PDV-B antisera were available from the ATCC. All antisera were purified to obtain the immunoglobulin fraction (IgG) and conjugated to alkaline phosphatase using standard techniques (5).

ELISA. The ELISA technique used was that described by Clark and Adams (5) with coating IgG and conjugated IgG being used at 2 μg/ml for ELISA with a single antiserum and 1.25 μg/ml (each) when IgG from three antisera were combined for ELISA. Young leaf material from infected and healthy hosts (*Prunus, Malus, Rosa, Corylus, Humulus*, and *Cucumis*) was ground at 1:10 (w/v) with buffer (0.2 M phosphate buffered saline, pH 7.4, with 20 g/L polyvinyl-pyrrolidone-40, 2 g/L egg albumin, and 0.2 g/L NaN_3). Incubation periods were 4 hr at 37 C for the coating and conjugate fixation stages and overnight at 4 C for the sap-filled plates. All ELISA tests reported were conducted from March through May in Gilford EIA cuvettes (part #1413 × 79 Gilford Inst. Labs Inc., Oberlin, OH) and read at 405 nm λ in 15–30 min using a Gilford Inc. PR-50 plate reader. Each sample was tested in adjacent duplicate wells.

Each test plate was standardized by adjusting the A_{405} values for "buffer wells" (wells that had not been filled with plant sap but in all other aspects identical to "sample wells") to 0.00. In all cases, the A_{405} for sample (S) and healthy (H) wells was recorded at ≥0.01. A positive test was defined as one in which the absorbance at 405 nm (A_{405}) of the sample wells was >3× the A_{405} of the virus-free control wells (plant of the same species and growth stage).

Bioassay. Bioassays of 35 isolates on *C. quinoa* and *C. sativus* were compared with ELISA. Material in *Prunus* and *Malus* sp. was ground at 1:5 (w/v) with 0.06 M phosphate buffer, pH 7.5, which was 0.2 M 2 mercaptoethanol and sodium diethyldithiocarbamate. The homogenate

Table 1. Sources of necrotic ringspot (NRSV), apple mosaic (ApMV), hop (HopV), and prune dwarf (PDV) viruses[a]

Virus	Strain[b]	No. isolates	Original host(s)	Reference
NRSV	Not designated	17	*Prunus domestica, P. avium, P. cerasus, P. armoniaca, P. triloba, P. americana, P. persica*	7,11, J. P. McMorran, *unpublished*
	Necrotic (H,G)	2	*P. cerasus, P. pennsylvania*	8
	Recurrent (A,E)	2	*P. cerasus*	8
	Almond calico	1	*P. dulcis*	20
	Cherry rugose	2	*P. amygdulus, P. avium*	18,20
	Rose virus 4	1	*Rosa dilecta*	3
	Danish line pattern	1	*P. domestica*	10
ApMV	Paradise	1	*Malus sylvestris*	13
	Other	5	*M. sylvestris*	7
	Unknown	1	*Corylus alalla*	J. P. McMorran, *unpublished*
	Rose mosaic	1	*Rosa setigera*	9
HopV	"A," "I"	2	*Humulus lupulus*	2,24
PDV	Not designated[c]	8	*P. avium, P. cerasus, P. domestica*	7,12, J. P. McMorran, *unpublished*

[a] For information on original isolate designations, investigators involved in collection, and immediate virus sources used in tests presented here, see references 7 and 17.
[b] As denoted by original investigators.
[c] Includes isolates originally described as causing apricot gummosis, sour cherry yellows, and prune dwarf diseases.

Table 2. Results of enzyme-linked immunosorbent assay testing

Serogroup	No. isolates tested	No. virus isolates detected by antisera of serogroup listed			No. isolates not detected
		NRSV	ApMV	PDV	
Necrotic ringspot	26	23	0	(1)[a]	3
Apple mosaic	10	0	10	0	0
Prune dwarf	8	0	0	8	0

[a] ( ) = "Weak positive," ie, A_{405}, sample/A_{405}, "healthy" >3, but much less than other positives on the same plate.

Table 3. Results of single vs. combined antiserum enzyme-linked immunosorbent assay[a]

Virus serogroup[b]	Average positive S/H value[c]		No. positives[d]		No. CAT false[e]		% CAT/SAT agreement[f]
	SAT	CAT	SAT	CAT	Positives	Negatives	
NRSV	52	37	62	61	4	1	94
ApMV	65	53	19	18	1	1	92
PDV	107	21	51	47	0	4	93
Total	75	33	132	126	5	6	93

[a] SAT = single antiserum ELISA, CAT = combined antiserum ELISA (necrotic ringspot virus-G, rose mosaic virus-F, prune dwarf virus-B).
[b] NRSV = necrotic ringspot, ApMV = apple mosaic, and PDV = prune dwarf serogroups.
[c] S/H value = sample A_{405} value/"healthy control" A_{405} value (with buffer well at 0.00 A_{405}) where the S and H values are always recorded as ≥0.01. The maximum S/H value possible was 280, minimum was 1.
[d] ELISA positive = S/H value >3; 41 total virus isolates used, some in two or more hosts. The total number of ELISA tests involving each serogroup was 83, 24, and 59 for NRSV, ApMV, and PDV, respectively, with 135 total tests (some with ≥ two viruses).
[e] "False positive" = SAT indicates no virus, whereas CAT has S/H vlaue >3. "False negative" = SAT indicates virus present while CAT has S/H value <3.
[f] % Agreement = [(total no. samples) − (no. CAT false positives + no. CAT false negatives)] ÷ (total no. samples).

Table 4. Comparison of enzyme-linked immunosorbent assay and bioassay for ILAR-b virus detection

Virus serogroup	No. isolates tested	No. isolates detected by		No. isolates not detected
		ELISA	Bioassay[a]	
Necrotic ringspot	22	18	21	0
Apple mosaic	6	6	6	0
Prune dwarf	7	7	5	0
Total	35	31	32	0

[a] *Chenopodium quinoa* and *Cucumis sativus*.

was applied by pestle to leaves or cotyledons lightly dusted with 600-mesh Carborundum, then immediately rinsed with tap water.

RESULTS

As shown in Table 2, all but three of the 44 isolates reported to be ilarviruses were detected in ELISA by one or more of the 11 antisera. The three isolates not detected were PRSV-(MC-15), PRSV-Japanese Blood Dwarf (IR-2 #119-17 and 119-3) and Casper's RV-4, which had been transferred through cucumber for 7 yr. The three serogroups defined previously for these isolates by Ouchterlony double-diffusion tests, ie, NRSV, ApMV, and PDV, were identifiable in these studies; however, there was no evidence of any antigen/antisera reaction between the NRSV and ApMV serogroups by ELISA. Significantly, all isolates within each serogroup were detectable by a single antiserum, so only NRSV-G, RMV, and PDV-B antisera were required to detect 41 isolates. When these three antisera were combined for a single ELISA, all 41 isolates previously detected with single-antiserum ELISA were detected (Table 3). This technique, however, was less sensitive (gave reduced virus sample/healthy A_{405} ratios) and less accurate (increased number of false positive or false negative samples). In most cases, the reduction in reliability of the combined antiserum ELISA over single-antiserum ELISA was due to an increase in background A_{405} levels and not a reduction in the A_{405} value of the sample isolates. Average agreement of conclusions from the two techniques was 93%. All false positive or false negative samples were borderline cases (ie, gave sample/healthy A_{405} ratios of 3–5).

The bioassay was essentially equivalent to the ELISA in terms of number of samples detected, 32/35 versus 31/35 of the isolates tested, respectively (Table 4). Yet the ELISA was much easier to conduct and interpret and could be done in less time over a much greater portion of the year on samples of *Prunus* and *Malus*.

DISCUSSION

Before ELISA can be used by state and regional certification agencies in programs designed to ensure that *Prunus* and *Malus* nursery stock is free of NRSV, ApMV, and PDV, there must be confidence that all isolates present will be detected. In this study, all but three of 44 isolates were detected using three commercially available antisera. The lack of detection of three isolates could be due to inaccurately assigning these isolates to this group in the first place, although error in sample collection and/or experimental technique is possible. Although the three-antiserum ELISA was less accurate than the single antiserum tests, its usefulness as a diagnostic alternative could be greatly improved if all samples with "borderline" absorption values were rerun using a single antiserum ELISA (frozen or refrigerated homogenates from the initial test could be used).

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