Purification of the NY-RMV and NY-SGV Isolates of Barley Yellow Dwarf Virus and the Production and Properties of Their Antibodies

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ABSTRACT

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Methods were developed to purify the NY-RMV and NY-SGV isolates of barley yellow dwarf virus (BYDV) in amounts adequate for antiserum production and serological studies. Yields of NY-RMV (from leaves of Clintland 64 oats) and NY-SGV (from roots of Moore barley) were 0.26–0.39 and 0.7–2.7 mg/kg, respectively. Polyclonal antisera to each virus were developed by injecting rabbits and mice. Murine monoclonal antibodies to each virus were also prepared. The specificity of the polyclonal antisera in enzyme-linked immunosorbent assays (ELISA) depended on the format of the test used. Immunoassays in which virions were captured on antibody-coated microtiter plates were more specific than immunoassays in which virions were coated onto microtiter plates. The results confirmed serological relationships between NY-SGV and other serotypes grouped as group 1 BYDVs and indicated a more distant relationship between NY-RMV and RPV serotypes, usually grouped as group 2 BYDVs. Cross-reactivity exhibited by the monoclonal antibodies may reflect immunological similarities in certain epitopes among various isolates of BYDV.

The term barley yellow dwarf virus (BYDV) defines a range of serotypes or strains of cereal-infecting luteoviruses (27,35). North American isolates fall into five serotypes—MAV, PAV, SGV, RPV, and RMV—represented by five isolates so named, with the prefix NY (New York) (28), and maintained in a collection at Cornell University established by W. F. Rochow. These serotypes were originally distinguished by their aphid vector specificities (10,11,15,26) and later by serology (1,29). Serological and other properties indicate closer relationships between the MAV, PAV, and SGV serotypes grouped as group 1 than between these and the RPV and RMV serotypes, group 2 (27,35).

All isolates of BYDV are difficult to purify. MAV-, PAV-, and RPV-like isolates have been purified with yields ranging from 0.5 to 6.0 mg per kilogram of tissue extracted (4,9,12,21,24), which are meager relative to some viruses but have proved adequate for the production of polyclonal (1,18,29) and monoclonal antibodies (MAbs) (5,7,13,25,32). Greater difficulties have been encountered in the purification of RMV- and SGV-like isolates (23,29). For the NY-RMV isolate, Rochow and Carmichael (29) reported very poor yields from infected leaves, although they were able to produce specific antisera in rabbits with their virus preparations. For NY-SGV, Hu et al (14) reported a yield of 94 μg from over 16 kg of infected leaves, whereas Rochow et al (30) reported 540 μg from 13 kg. Hu et al (14) immunized chickens and obtained antibodies from egg yolks. However, these antibodies were difficult to use for diagnosis, especially in double-antibody sandwich (DAS) enzyme-linked immunosorbent assays (ELISA). MAbs suitable for routine specific diagnosis of RMV- and SGV-like isolates were not available when the present work was initiated.

Difficulties in purifying RMV- and SGV-like isolates and the absence or shortage of polyclonal and monoclonal antibodies to them have been a constraint in their study and in the development of a range of diagnostic reagents for BYDV. We report here on improved purification of the NY-SGV and NY-RMV isolates, their use in the preparation of polyclonal antisera and MAbs, and on the characteristics of the antibodies produced. A preliminary note on purification was published previously (37).

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MATERIALS AND METHODS

Virus isolates. For virion purification and antiserum production we used subcultures of the authentic NY-RMV and NY-SGV isolates (15,26), supplied from the Cornell University collection by W. F. Rochow and by S. Gray. Subcultures of the authentic NY-MAV and NY-RPV isolates (originally supplied from the same collection by W. F. Rochow), a PAV-like isolate (P-PAV) from Indiana (12), and other isolates supplied by colleagues (detailed in table footnotes) were used for comparative studies.

Propagation. Initially, batches of infected leaves containing NY-RMV and NY-SGV were obtained from S. Gray (37). Young *Avena byzantina* (K. Koch) 'Coast Black' seedlings were inoculated with appropriate aphids (*Rhopalosiphum maidis* (Fitch) for NY-RMV and *Schizaphis graminum* (Rondani) for NY-SGV), grown for 3 wk in a greenhouse at Cornell University, and then shipped frozen to the Purdue laboratory for purification. Subsequently, for studies reported here, the viruses were propagated at Purdue University in growth chambers at 20 ± 1 C with a 14-hr photoperiod (200 $\mu E \cdot s^{-1} \cdot m^{-2}$).

NY-RMV was efficiently propagated by scattering viruliferous aphids from infected barley (*Hordeum vulgare* L. 'Hudson') culture plants on to 7- to 10-day-old oat (*Avena sativa* L. 'Clintland 64') seedlings grown in 8-cm-diameter polystyrene cups (eight to 10 plants per cup). For propagating NY-SGV, because of the injurious effect of *S. graminum* feeding on oats and barley and the need to use nymphal stages for optimal transmission (38), seedlings (two per polystyrene cup) were infested individually. Clintland 64 oat was used as the source plant for NY-SGV because obvious reddening symptoms developed in this host. Symptoms in barley cv. Moore, the purification host, were not obvious. Aphids were allowed to acquire NY-SGV by feeding on infected Clintland 64 plants for 2–3 days. Nymphs were then transferred individually (about five to 10 per plant) to 8-day-old seedlings, left for 3 days, and then killed with a pyrethrin insecticide. For both viruses, shoots (all aerial parts) or roots were harvested 14 days after the beginning of the inoculation access period. Shoot tissue was cut into 2- to 5-cm lengths and frozen at −80 C for subsequent use. Soil was washed from roots, which were then blotted dry and frozen at −80 C.

Cultivar and culture effects. Several oat and barley cultivars were tested as propagation hosts for NY-RMV and NY-SGV. In experiments with NY-RMV, seedlings were germinated and grown in rolls of seed germination paper (12 seeds/roll) to save space. Rolls were placed upright in a tray with 1 cm of Knop's nutrient solution (31), which was replenished on alternate days with nutrient solution or tap water. Seedlings

were thinned to four per roll, inoculated at the one-leaf stage with viruliferous *R. maidis* (10 aphids per plant, 3-day inoculation access period), and grown in a growth chamber. With NY-SGV, procedures were the same except that plants were grown in polystyrene cups (two per cup). With both isolates, uninfested plants of each host were also grown for negative control tissue. Virus production in both the roots and shoots was assessed by DAS-ELISA (3) at intervals after inoculation access.

Purification. The purification procedure developed for both viruses was based on that of Hammond et al (12) for purifying the P-PAV isolate from oat shoots. Its main features were as follows. Infected frozen or cold, fresh tissue (ideally Clintland 64 oat leaves for NY-RMV and Moore barley roots for NY-SGV) was extracted in 0.5 M phosphate buffer, pH 6.0, at 1:3 (w/v) by repeated blending (30 sec every 15 min during 3 hr) in a 4-L Waring blender. The homogenate was squeezed through cheesecloth, clarified by a brief blending with 0.2 volume of chloroform and *n*-amyl alcohol (2:1, v/v), and centrifuged at 7,000 rpm for 10 min in a Sorvall GSA rotor. Virus was then precipitated from the supernatant by stirring 60–90 min with 10% polyethylene glycol (PEG) M_r 8,000, and sedimented by centrifugation at 8,000 rpm for 25 min. Pellets were resuspended overnight in buffer (1 ml per 25 ml of original volume). After clarification by a further low-speed centrifugation, virus was pelleted from this suspension by centrifugation at 50,000 rpm for 2 hr through a 30% sucrose pad occupying one-fourth of Beckman 60 Ti rotor tubes. Pellets were resuspended for at least 1 hr in 1.0 ml of buffer. Virus was then separated by rate-zonal ultracentrifugation by layering 1- to 1.5-ml samples on sucrose density gradients formed by freezing and thawing 10.5–11 ml of 20% sucrose solution in Beckman SW41 tubes, which were then centrifuged at 37,000 rpm for 2 hr. Gradients were analyzed for ultraviolet (UV) absorbance at 254 nm in an ISCO model D gradient fractionator equipped with a type 6 optical unit and a UA-5 absorbance monitor. Virions were detected on sucrose density gradients as UV absorbance peaks at the same level as virions in similar gradient preparations of the P-PAV isolate and containing particles indistinguishable in electron micrographs from P-PAV virions. Virus yields were compared on the basis of the relative sizes of peaks in the appropriate regions of UV absorption profiles of density gradient analyses. Virus preparations were resuspended after PEG precipitation and during subsequent steps in 0.1 M phosphate, pH 7.0. All purification steps were performed the cold (4 C). The concentration of virus preparations from sucrose gradients was estimated assuming $E^{0.1\%}_{260nm} = 8.0$ (4), and preparations were stored as collected, frozen at −80 C. For purifying NY-SGV from Moore barley roots, tissue was initially ground in liquid nitrogen, then blended in 0.5 M phosphate buffer, and the extract was then incubated with 2% Extractase P20X (Finnsugar Biochemicals Inc., Schaumberg, Illinois) for 3 hr at room temperature. The remaining procedure was as for NY-RMV.

Variations of these procedures investigated were the tissue used; variations in extraction, purification, and concentration procedures; and the use of enzymes (Cellulase Onozuka R-10, Yakult Biochemicals, Nishinomiya, Japan; Pectinase, INC Nutritional Biochemicals, Cleveland, Ohio; and Extractase P20X) in extraction. The effects of modifying various procedures were examined by processing split samples of tissue for analysis by rate-zonal density gradient centrifugation (12) and occasionally by ELISA. Results of both kinds of analysis were consistent. Most work was done with samples infected with NY-RMV, because this isolate was easier to culture reliably, but experiments with NY-SGV indicated that the effects of the various treatments were similar for both viruses. In fact, both behaved essentially like the P-PAV isolate previously investigated in this laboratory (12).

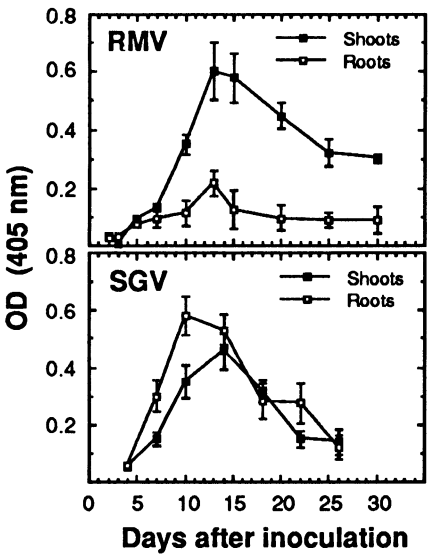

Fig. 1. Variations in NY-RMV (top) and NY-SGV (bottom) concentration over time in Clintland 64 oats. Seedlings were inoculated at 5 days old and maintained in a growth chamber at 20 ± 1 C with a 14-hr photoperiod at 200 $\mu E \cdot s^{-1} \cdot m^{-2}$. Each point represents the mean of enzyme-linked immunosorbent assay values for three to six plants (NY-RMV) or six plants (NY-SGV) harvested at the times indicated. Bars indicate standard deviations. Mean values for noninfected control samples did not exceed 0.04.

Polyclonal antisera. Three New Zealand white rabbits (RMV1, 2, and 3) were immunized with NY-RMV and one (SGV1) with NY-SGV. Initial immunizations were with 50–70 μg of untreated purified virion preparations emulsified with Freund's complete adjuvant and injected intradermally (18) at 20 sites (RMV1, SGV1), intramuscularly at 4 sites (RMV3), or both intradermally and intramuscularly (RMV2). Booster injections were with 30–60 μg preparations emulsified with Freund's incomplete adjuvant and were given 10–50 days after the initial immunization or, if more than one booster was given, at intervals of 7–21 days. Apart from the initial immunizations with RMV1 and SGV1, where virions were pelleted by ultracentrifugation to concentrate them sufficiently for injection, all other injections, both initial and booster, were with virion fractions from sucrose gradient separations without further concentration.

Immunoglobulins (Igs) were concentrated from crude sera by ammonium sulfate precipitation (3). Sometimes, to improve specificity, crude sera were first cross-absorbed against healthy plant proteins (3,19). For this, antisera were mixed 1:1 with a preparation of healthy plant proteins prepared as for infected tissue (12) but without fractionation on sucrose density gradients. After incubation for 1–2 hr at 37 C, the mixture was centrifuged for 10 min at 10,000 rpm in a Sorvall SS-34 rotor to remove antibody-healthy antigen aggregates. The supernatant fluid was then diluted 1:1 with phosphate-buffered saline (PBS) (3), loaded onto a column equilibrated with PBS (protein A-Sepharose CL-4B, Sigma P-3391). Unbound proteins were washed through with 50 ml of PBS. The bound immunoglobulin was eluted with 0.1 M citrate, pH 3.0. Fractions containing immunoglobulin were neutralized, combined, and dialyzed against three 1-L changes of half-strength PBS. Alkaline phosphatase conjugates were prepared as described by Clark et al (3).

Monoclonal antibody production. Monoclonal antibodies (MAbs) were given suffixes indicating the virus isolate used for immunization (i.e., RMV or SGV) followed by the number of the fusion, the abbreviation P (for Purdue University, where all MAbs were screened and selected in our laboratory), and the antibody preparation number; for example, MAb RMV1-P1. The antibody subclass and light-chain component of each MAb were determined by ELISA using the class- and subclass-specific anti-mouse immunoglobulins IgA, IgG1, IgG2a, IgG2b, IgG3, IgM, kappa light chain, and lambda light chain (Zymed Laboratories, Inc., San Francisco, California).

Cell lines secreting MAbs to the NY-SGV or NY-RMV isolates were derived from three separate fusions: one (RMV1) with NY-RMV, carried out by the Cell Science Laboratory, University of Illinois, Urbana; and two (RMV2 and SGV1) with NY-RMV and NY-SGV,

respectively, by the Cell Culture Laboratory, Purdue Cancer Center, Purdue University. Fusions, hybridoma cell culture, and ascitic fluid production were performed by the two laboratories, but mouse immunization and hybridoma screening and selection were done by the authors.

Fusions at the Illinois laboratory employed the non-immunoglobulin-secreting mouse myeloma cell line SP/0-Ag14, and fusions at the Purdue laboratory employed the mouse myeloma cell line M5, derived from SP/0-Ag14 (D. Asai, *personal communication*). Cell culture and fusions were as described by D'Arcy et al (5) with minor variations for the work done by the Purdue laboratory.

ELISA procedures. DAS-ELISA procedures for polyclonal antisera were as described previously (3,8), with Immulon 2 (Dynatech Laboratories, Inc., Chantilly, Virginia) or Corning 25801 (Corning Inc., Corning, New York) microtiter plates, but with 100-μl sample volumes. Extracts were prepared for DAS-ELISA by grinding tissue 1:10 or 1:20 (w/v), in 0.1 M phosphate buffer, pH 7.0, with a homogenizer (Ultra-turrax, Tekmar Co., Cincinnati, Ohio) or by pulverizing in liquid nitrogen with a mortar and pestle followed by grinding in buffer. They were usually incubated overnight at 6 C on ELISA plates previously coated with homologous polyclonal immunoglobulin for 3 hr at 37 C in 0.05 M sodium carbonate, pH 9.6. Following this, conjugate preparations were incubated in the plates for 3 hr at 37 C. Polyclonal immunoglobulin for coating was usually 1 μg/ml, and conjugates were diluted 1:1,000 in an extract of healthy Clintland 64 oats in PBS-Tween to reduce background reactions (3,19). Plates were read 30–60 min after substrate (*p*-nitrophenol phosphate) in diethanolamine buffer, pH 9.8, was added. Twofold dilutions of freshly thawed purified virion preparations (initial concentration, 1.0 μg/ml) in healthy shoot or root extracts were used to confirm reaction linearity. For the critical estimation of virus concentrations, freshly purified virion preparations were used.

For triple-antibody sandwich (TAS)-ELISA (3) with MAbs in cell culture supernatants or ascitic fluid, antigen was trapped by the homologous polyclonal rabbit immunoglobulin (2 μg/ml) from sap extracts or purified preparations incubated overnight at 6 C. Blocking was with 5% defatted milk powder in PBS-Tween (1–2 hr at room temperature) after the coating step, but this was found to be unnecessary with sap extracts. Culture supernatants, diluted 1:2 with PBS-Tween-polyvinylpyrrolidone (PVP), were incubated for 2 hr at 37 C. Ascitic fluid diluted in PBS-Tween-PVP-ovalbumin was usually used at 1:5,000 and incubated 2 hr at 37 C. Reacting MAbs were initially detected with an alkaline phosphatase-labeled goat anti-mouse conjugate detecting IgG, IgM, and IgA (Sigma). Subsequently, for IgM MAbs, an anti-mouse IgM-specific alkaline phosphatase conjugate (Sigma) (1:1,000 in PBS-Tween-PVP) was used; and for IgG MAbs, an anti-mouse IgG (H+L chain) alkaline phosphatase conjugate (Bethesda Research Laboratories, Gaithersburg, Maryland) (1:2,000) was used. Incubations were for 2 hr at 37 C. Plates were read 30–60 min after substrate was added.

In some instances, for example where crude sera were being titrated, a reverse-TAS-ELISA was used (i.e., a MAb was used for trapping, a rabbit polyclonal antibody was used as the second antibody, and an anti-rabbit alkaline phosphatase conjugate was used for the detecting antibody). Where MAbs did not adhere efficiently to plates, they were trapped using goat anti-mouse immunoglobulin (1 μg/ml) previously coated onto the plate, resulting in an ELISA assay with four layers of antibodies.

RESULTS

Cultivar and culture effects. Results from ELISA for several experiments indicated that under the growth chamber conditions used peak virion production of both NY-RMV and NY-SGV in roots and shoots of Clintland 64 oats and other hosts occurred about 14 days after inoculation (Fig. 1). Therefore, this culture time was adopted for routine use. Results from ELISA also indicated that, among oat and barley cultivars examined as culture hosts, Clintland 64 and Coast Black oat shoots were the best sources of NY-RMV virions, and Moore barley roots were the best source of NY-SGV virions (Table 1).

Yields of NY-RMV virions purified from shoot tissue of Clintland 64 oats grown in the growth chamber were slightly better than from Coast Black oats (Fig. 2A) and ranged from 0.26 to 0.39 mg/kg (11 experiments), with a mean A_{260}/A_{280} ratio of 1.67. In five separate purifications of NY-SGV virions, yields of Moore barley roots were 0.7–2.7 mg/kg of tissue, which was far more than the corresponding shoots (Fig. 2C). The wide range in yields reflected inefficient infection of the plants, a problem with NY-SGV (38). Preparations had an average A_{260}/A_{280} ratio of 1.52. NY-SGV virion preparations from the roots of Clintland 64 and Coast Black oats contained impurities that interfered with their resolution on rate-zonal density gradients. However, NY-SGV virions were purified successfully from the shoots of these hosts (Fig. 2E) and from Moore barley shoots (Fig. 2C), but yields were only 0.2–0.3 mg/kg of tissue.

Results of both ELISA and purification experiments (Fig. 2D) indicated that NY-SGV production was about twofold greater in roots of Moore barley plants grown in the growth chamber than in roots of plants grown in a greenhouse, but virus concentrations in shoots remained similar. Thus, mean ELISA values for shoot extracts made at 1:10 (w/v) were 0.44 (greenhouse) and 0.48 (growth chamber) (standard error of difference between means [SED] = 0.03),

Table 1. Production of NY-RMV and NY-SGV isolates of barley yellow dwarf virus in roots and shoots of various cereal cultivars sampled 14 days after inoculation

Isolate Host and cultivar	Plants infected[x]	Mean ELISA values[y]	
		Shoots	Roots
Expt. 1 NY-RMV			
Oat	8	1.19 a[z]	0.42 d
Clintland 64	8	1.23 a	0.40 d
Coast Black	8	0.93 b	0.37 d
California Red	7	0.87 bc	0.50 cd
Algeribee			
Barley	8	0.76 bc	0.74 b
Hudson	8	0.89 bc	0.97 a
Moore	8	0.72 c	0.68 bc
Luther	...	0.19	0.21
SD			
Expt. 2 NY-SGV			
Oat			
Clintland 64	8	0.15 b	0.20 c
Coast Black	5	0.21 a	0.27 c
California Red	6	0.23 a	0.12 c
Barley			
Hudson	3	0.08 c	0.35 bc
Moore	8	0.19 ab	1.11 a
Luther	8	0.20 ab	0.67 b
SD	...	0.05	0.31

[x] Number of infections out of eight plants inoculated.
[y] Two wells per sample; samples randomized on plates. DAS-ELISA = Double-antibody sandwich enzyme-linked immunosorbent assay. ELISA values for healthy controls ranged from 0.08 to 0.12.
[z] In each experiment, means in each column followed by the same letter are not significantly different from others in the same column at $P = 0.05$, by Fisher's (protected) LSD test.

and mean ELISA values for root extracts were 1.29 (greenhouse) and 1.72 (growth chamber) (SED = 0.07, $n = 10$).

Effects of variations in purification procedure—extraction and clarification. For NY-RMV purified from Clintland 64 oat shoots, extraction at pH 5.5–6.0 and buffer concentrations of 0.5 M gave best results. Freezing tissue improved separation of virions from other constituents slightly but reduced yields by about 25–30%. Repeated blending of leaf tissue in buffer (12) rather than blending once gave somewhat improved yields, and it improved clarification of extracts. Pulverizing tissue in liquid nitrogen before buffer extraction did not improve yields compared with those from extracts that were repeatedly blended. Treating shoot extracts (blended once) for 3 hr at room temperature with 2% Extractase P20X (21) to macerate fiber and cell walls gave slightly lower yields of NY-RMV than repeated blending, whereas incubation with Extractase P20X overnight at room temperature reduced yields to about one-half (Fig. 2B). In contrast to this, although yields of NY-SGV or NY-RMV from Coast Black oat shoots were not improved by incubating extracts with 2% cellulase plus 4% pectinase (Fig. 2E), incubating Moore barley root extracts with Extractase P20X did improve yields of NY-SGV by about one-third to one-half (Fig. 2F). Stirring extracts with 2% Triton X-100 for 0.5–1 hr before emulsification with chloroform and *n*-amyl alcohol improved extract clarification and aided in separating and removing the aqueous phase following centrifugation, but it had no effect on yields of either NY-RMV or NY-SGV.

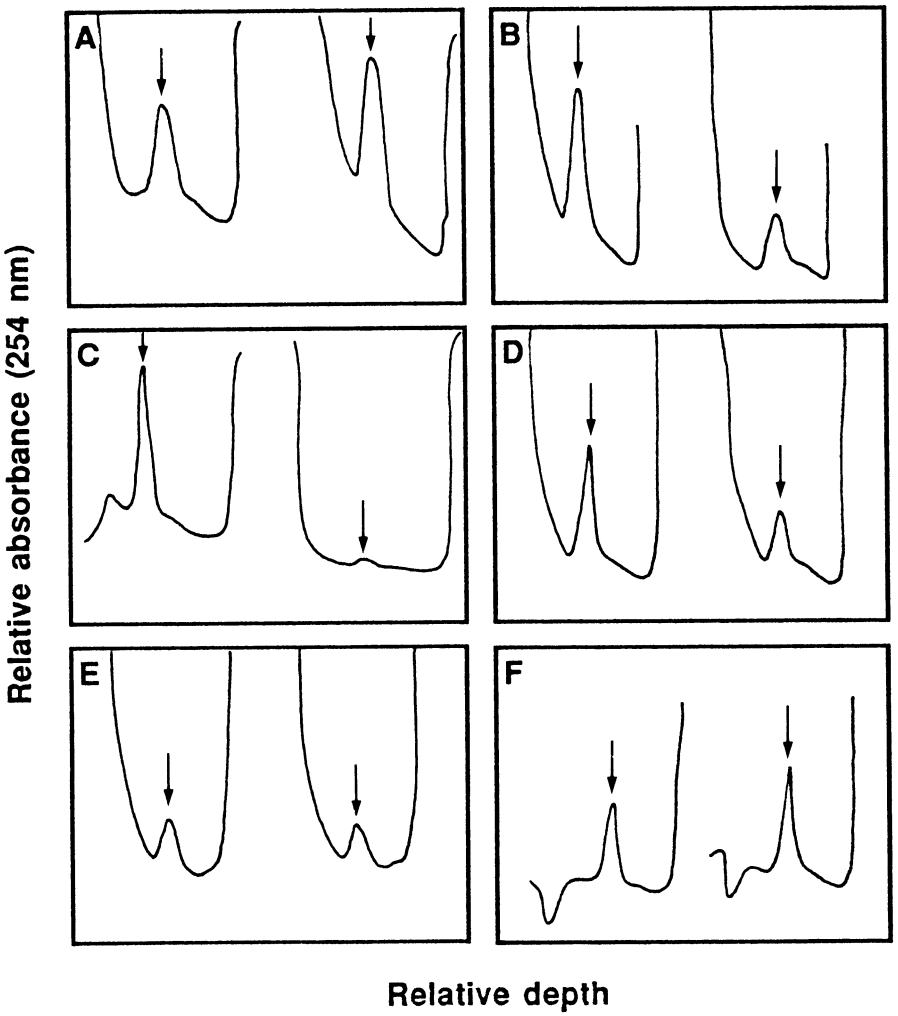

Fig. 2. Ultraviolet absorbance profiles (A_{254}) for paired rate-zonal density gradient centrifugations, in six typical experiments comparing the relative yields of virions purified from samples containing the same weights of tissue, as described in Methods: **(A)** NY-RMV purified from (left) Coast Black oat and (right) Clintland 64 oat shoots (210 g each, absorbance monitor set at 0.5 range); **(B)** NY-RMV purified from 250-g lots of Clintland 64 oat shoots by (left) repeated blending of extracts, 30 sec every 15 min for 3 hr, or (right) incubation of extracts (blended once) with 2% Extractase P20X overnight at room temperature (0.5 range); **(C)** NY-SGV purified from (left) Moore barley roots, or (right) shoots (90 g each, 1.0 range); **(D)** NY-SGV purified from Moore barley roots grown in (left) a growth chamber and (right) a greenhouse (May) (11 g each, 0.2 range); **(E)** NY-SGV purified from Coast Black oat shoots by (left) repeated blending of extracts, 30 sec every 15 min for 3 hr, or (right) incubation of such extracts with 2% cellulase/4% pectinase for 4 hr at room temperature (250 g each, 0.5 range); **(F)** NY-SGV purified from Moore barley roots by (left) repeated blending of extracts, 30 sec every 15 min for 3 hr, or (right) incubation of extracts (blended once) with 2% Extractase P20X 2.5 hr at room temperature (1.0 range). Virion peaks are indicated by arrows.

Concentration and final purification steps. When NaCl concentrations were varied between 0 and 0.5 M at the PEG-precipitation stage, yields of NY-RMV were not affected, although yields of NY-SGV were slightly improved with 0.1–0.3 M NaCl. Both NY-SGV and NY-RMV seemed stable overnight at 4 C; no loss of virus was noted in preparations stored overnight at 4 C before analysis on sucrose gradients compared with the same preparations analyzed immediately. However, losses of 50–70% were frequently encountered in concentrating virion preparations collected from sucrose gradients by ultracentrifugation. In attempts to find an alternative to ultracentrifugation, dialysis against 20% polyethylene glycol M_r 20,000 and ultrafiltration (Millipore immersible molecular separation kit, 10,000 nominal M_r limit) were found to be unreliable, although these methods were used to concentrate some virion preparations for monoclonal antibody production. NY-RMV virions seemed especially unstable to these treatments.

Final virion yields were generally much lower than predicted by ELISA values of starting extracts. For example, in one experiment where NY-RMV virions were purified from Clintland 64 shoots, ELISA using MAb RMV1-P1 (which apparently reacts only with intact virions; see Discussion) indicated that the initial extract contained about 2.2 mg of virions, but the final yield was only 0.36 mg, a loss of 84%.

Stability of NY-RMV and NY-SGV in dry leaf and frozen tissue. NY-RMV ELISA activity was stable in air-dried leaves and was similar to that of samples stored frozen at either −80 C or −20 C (Table 2). In contrast, ELISA activity of NY-SGV-infected dry leaf samples was very low relative to that of frozen samples, suggesting instability or insolubilization of virions during drying (Table 2). Other tests in this laboratory confirmed a substantial loss of ELISA activity for NY-SGV in dry leaf samples, whereas NY-RMV, like other BYDV isolates, can be stored for many months in dry leaf form with little loss.

Properties of rabbit polyclonal antisera. The reactions of NY-RMV and NY-SGV and other polyclonal rabbit antisera in a typical set of DAS-ELISA experiments with isolates representative of the five BYDV serotypes and two isolates of beet western yellows virus (BWYV) are summarized in Table 3. Antisera to NY-RMV (RMV3 bleed 2) and NY-SGV (SGV1 bleed 3) were both very specific, cross-reacting only negligibly or not at all with heterologous serotypes. The

MAV-PS1 antiserum cross-reacted with P-PAV and with NY-SGV serotypes, whereas the NY-RPV antiserum cross-reacted with the two isolates of BWYV.

The specificity of the NY-RMV antisera was confirmed in tests of crude sera (Table 4), where immunoglobulin activity was not impaired by conjugation with alkaline phosphatase (7). None of 28 bleeds from the three rabbits immunized with NY-RMV, taken over a period of 260 days after primary injections, reacted with NY-RPV in crude leaf extracts. However, an antiserum from a rabbit immunized with NY-RPV did react weakly with NY-RMV (Table 4), indicating that the two isolates were related serologically. This relationship was more distant than that between group 1 serotypes (NY-MAV, P-PAV, and NY-SGV). In similar tests with 12 bleeds taken over a period of 230 days after primary injection from a rabbit immunized with NY-SGV, where antigens were trapped from crude leaf extracts with a MAb specific for group 1 serotypes, one typical bleed gave ELISA values of 1.61 with NY-SGV, 0.59 with NY-MAV, 0.19 with P-PAV, and 0.00 for healthy extract.

Table 2. Influence of storage for two months as dry leaf or frozen tissue on DAS-ELISA values for extracts of 0.5-g samples (fresh weight) of Clintland 64 or Moore barley infected with the NY-RMV or NY-SGV isolates of barley yellow dwarf virus, respectively[v]

Storage conditions	ELISA value[w] Experiment 1 (NY-RMV)	Experiment 2 (NY-SGV)
Tissue stored at −80°	0.74 a[x]	1.28 a
Tissue stored at −20°	0.72 a	0.71 b
Air-dried tissue[y]	1.04 a	0.14 c
Tissue dried over $CaCl_2$[y]	0.93 a	0.18 c
LSD 0.05	0.28	0.27
Virion preparations[z]		
undiluted	1.53	1.75
diluted ×2	0.89	1.01
diluted ×4	0.53	0.63
diluted ×8	0.31	0.36
Healthy tissue control	0.11	0.04

[v] Extracts were 1:10 (fresh weight/v) of tissue in 0.1 M phosphate buffer, pH 7.0. DAS-ELISA = double-antibody sandwich enzyme-linked immunosorbent assay.
[w] Mean of 10 samples, two wells per mean randomized on the plate.
[x] Means in each column followed by the same letter are not significantly different from others in the same column at $P = 0.05$, by Fisher's (protected) LSD test.
[y] NY-RMV tissue was air-dried 5 days, then stored dry or over $CaCl_2$ at room temperature (25 C). NY-SGV tissue was dried over $CaCl_2$ for 1 mo, then either stored dry or over $CaCl_2$ at room temperature.
[z] Purified virion preparations at 250 ng/ml.

Properties of murine polyclonal antibodies. Serological relationships among group 1 and group 2 serotypes of BYDV were also investigated using polyclonal antisera to NY-RPV and NY-RMV from the mice used for the RPV1 and RMV1 fusions, respectively. This also enabled comparison and evaluation of two ELISA procedures: trapping virions with homologous rabbit polyclonal immunoglobulins, and coating virions onto microtiter plates in 0.1 M phosphate buffer, pH 7.0. With both mouse antisera, when virions were trapped with rabbit antibodies, little or no reaction was observed with heterologous isolates (Fig. 3A and C). However, when virions were coated directly onto microtiter plates, strong reactions occurred with all heterologous isolates, including NY-MAV and NY-PAV (Fig.

Table 3. Reactions of polyclonal antisera and monoclonal antibodies used in DAS- and TAS-ELISA, respectively, with extracts of various isolates of five serotypes of barley yellow dwarf virus (BYDV) and two beet western yellows virus (BWYV) isolates from different locations[x]

Isolate[y]	Polyclonal antisera[z] MAV-PS1	P-PAV	NY-SGV	NY-RPV	NY-RMV	Monoclonal antibody[z] RMV1-P1	RMV2-P2	RMV2-P3	RPV1-P1	SGV1-P1	BWYV
NY-MAV	0.57	0.03	0.04	0.01	0.02	0.01	0.10	0.01	0.03	0.08	0.08
MAV-PS1	1.60	0.04	0.05	0.01	0.02	0.01	0.18	0.01	0.04	0.06	0.07
MEX-MAV	1.23	0.04	0.06	0.01	0.02	0.01	0.71	0.02	0.06	0.08	0.08
P-PAV	0.33	1.08	0.05	0.01	0.02	0.00	0.12	0.01	0.04	0.05	0.05
NY-SGV	0.30	0.05	1.34	0.01	0.02	0.00	0.31	0.02	0.03	0.29	0.05
ID-SGV	0.31	0.06	1.70	0.02	0.02	0.00	0.57	0.01	0.04	0.67	0.04
NY-RPV	0.02	0.02	0.02	1.32	0.02	0.01	0.08	1.95	0.67	0.19	1.91
UK-RPV	0.02	0.01	0.01	0.44	0.01	0.00	0.12	0.66	0.01	0.27	1.43
MEX-RPV	0.03	0.02	0.02	1.03	0.02	1.57	0.15	0.01	0.03	0.71	1.70
NY-RMV	0.02	0.02	0.02	0.01	0.65	1.03	0.67	1.30	0.05	0.03	0.24
MEX-RMV (maize)	0.02	0.02	0.01	0.04	0.11	0.97	0.07	1.26	0.02	0.10	0.22
BWYV-NZ3	0.02	0.01	0.01	0.24	0.00	0.00	0.25	0.74	0.01	0.13	1.47
BWYV-NZ4	0.02	0.01	0.01	0.15	0.00	0.01	0.24	0.30	0.02	0.09	1.07
Healthy controls											
Clintland 64	0.02	0.02	0.02	0.01	0.02	0.00	0.01	0.00	0.01	0.04	0.05
Crambe	0.02	0.01	0.01	0.01	0.00	0.00	0.03	0.00	0.00	0.02	0.01

[x] Antigens were crude extracts of fresh or dry leaf in 0.1 M phosphate buffer, pH 7.0, at 1:10 (w/v) for fresh leaf and 1:30 (w/v) for dry leaf. DAS = double-antibody sandwich, TAS = triple-antibody sandwich, ELISA = enzyme-linked immunosorbent assay.
[y] MAV-PS1 is a subculture of NY-MAV (20). ID-SGV is an isolate from wheat collected by S. Halbert, University of Idaho; MEX-MAV, MEX-RPV, and MEX-RMV (maize) are isolates from small grain cereals or maize collected in Mexico. MEX-RPV was typical of two of six Mexican RPV serotype isolates tested. BWYV-NZ3 and BWYV-NZ4 are beet western yellows virus isolates from oilseed rape and shepherd's purse, respectively (J. Fletcher, Plant Diseases Division, DSIR, Canterbury Agriculture and Science Centre, Lincoln, Canterbury, New Zealand); UK-RPV is isolate R568 (2). Further details on isolates are given in the text.
[z] In DAS-ELISA tests with polyclonal antisera to the isolates indicated, the homologous immunoglobulin was used for trapping antigen. In TAS-ELISA tests with monoclonal antibodies, BYDV antigens were trapped with a mixture of polyclonal antisera reacting with PAV, MAV, SGV, RPV, and RMV serotypes, whereas BWYV was trapped with a specific polyclonal antiserum supplied by R. Stace-Smith, Agriculture Canada, Vancouver, British Columbia. The BWYV MAb was supplied by P. Ellis and R. Stace-Smith.

3B and D). Similar results were also obtained with rabbit polyclonal antisera when virions were coated directly onto microtiter plates (36).

Properties of murine monoclonal antibodies. Only small numbers of BYDV-specific, MAb-secreting hybridoma cell lines were obtained (1, 11, and 1, respectively, for the RMV1, RMV2, and SGV1 fusions). Possible technical reasons for this include the low amounts of immunogen used for immunization and that fusion could not always be done at the optimum time (3 days) after immunizations, because myeloma cell lines were insufficiently expanded or mice were sick and had to be sacrificed earlier than desirable. Four cloned, virus-specific, MAb-secreting cell lines were selected for further study, and ascitic fluid was produced to these. The MAbs RMV1-P1, RMV2-P2, and RMV2-P3, were IgM, and MAb SGV1-P1 was IgG2b; all had kappa light chains. Although these MAbs did bind to microtiter plates, none was able to subsequently trap virions in reverse TAS-ELISA. Also, none reacted with virions coated directly to microtiter plates, only to virions trapped by the homologous antivirus immunoglobulin.

Reactions of these MAbs in ELISA tests with a range of luteovirus isolates are summarized in Table 3, together with those of a MAb (RPV1-P1) that was derived from a fusion made with NY-RPV by the Cell Culture Science Laboratory, University of Illinois, following the protocol for the RMV1 fusion. MAb RMV1-P1 reacted with both RMV-like isolates and with an RPV-like isolate from Mexico, but it did not react with NY-RPV or an RPV-like isolate from Great Britain (2). MAbs from the RMV2 and SGV1 fusions exhibited a number of cross-reactions with other BYDV and luteovirus isolates. MAb RMV2-P3, typical of 60% of the virus-specific MAbs derived from this fusion, reacted with both RMV-like isolates and two of three RPV-like isolates as well as with the two BWYV isolates from New Zealand. MAb RMV2-P2, less typical of the virus-specific MAbs obtained from the RMV2 fusion, reacted with NY-RMV but not with a Mexican RMV-like isolate from maize. It also reacted with both SGV-like isolates, one MAV-like isolate (but not two others), and both BWYV isolates (Table 3). MAb SGV1-P1 reacted with both SGV-like isolates but also with all three RPV-like isolates. In contrast to these results, MAb RPV1-P1 was very specific, reacting only with NY-RPV but not with other RPV or RMV-like isolates.

Table 4. Reactions of selected rabbit polyclonal antisera prepared against NY-RMV and NY-RPV with healthy, NY-RMV, and NY-RPV antigens[w]

Rabbit Bleed[x]	Days after initial immunization	Mean ELISA[y]		
		Healthy	NY-RMV	NY-RPV
RMV1				
3	51	0.00	1.25	0.00
14	254	0.00	1.04	0.03
RMV2				
4	73	0.01	1.18	0.01
7	87	0.01	0.78	0.01
RMV3				
2	56	0.02	1.61	0.04
7	77	0.01	1.22	0.03
RPV				
4,8,11[z]	84,260,383	0.01	0.15	1.67
Preimmune RMV1	...	0.00	0.00	0.00

[w] All bleeds from each rabbit were tested and reacted similarly. Results for the bleeds containing the highest titer of antiviral antibodies and one later bleed for each rabbit are presented here. Antisera were used at a dilution of 10^{-4} (crude serum). Antigens were in crude sap extracts and were trapped using the RMV2-P3 monoclonal antibody, which was initially trapped by goat anti-mouse immunoglobulin (1 μg/ml) previously coated to the microtiter plates. The detecting antibody was goat anti-rabbit conjugated with alkaline phosphatase (1:1,000 dilution).

[x] Rabbits are identified according to the antigen used to immunize them, followed by a number to differentiate them.

[y] Enzyme-linked immunosorbent assay.

[z] A mixture of 1 ml of each bleed.

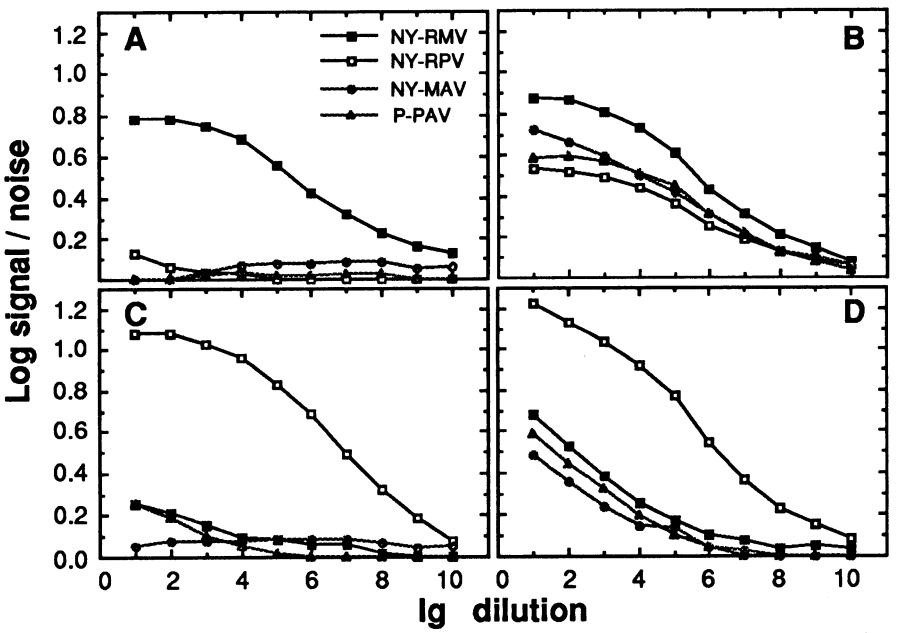

Fig. 3. Reactions in enzyme-linked immusorbent assays (ELISA) of twofold dilutions (starting from 1:1,000) of murine polyclonal antisera to NY-RMV **(A and B)** or NY-RPV **(C and D)**, with purified NY-RPV, NY-RMV, NY-MAV, or P-PAV (at 1 μg/ml). Antigens were either trapped with homologous polyclonal rabbit immunoglobulin at 10 μg/ml **(A and C)** or were coated onto the ELISA plate surface **(B and D)**. The detecting antibody, used at 1:1,000, was goat anti-mouse immunoglobulin conjugated with alkaline phosphatase. For the calculation of signal/noise ratios (i.e., values for antigen reactions vs. background values) ELISA values were adjusted by blanking against air so that the readings for healthy control extracts were 0.1–0.2.

DISCUSSION

As with other luteoviruses, critical factors in optimizing yields of NY-RMV and NY-SGV were choice of host, tissue type, culture time, and environmental conditions. Thus, with NY-SGV, our success was predicated on discovering that roots of Moore barley could provide exceptional yields (2–3 mg/kg), comparable with the best reported for other serotypes (4,12), and a large improvement on previous yields reported for this isolate (14,30). Typical yields of NY-RMV, obtained from shoots of Clintland 64 oats, were about one-tenth of those for NY-SGV. For both viruses, losses during purification presented problems, and final yields were much lower than ELISA values for the starting extracts

indicated should be possible. NY-SGV appeared to be more stable during purification, but its ELISA activity did not survive air-drying in leaves as well as that of NY-RMV or other BYDV serotypes. This observation has significance in relation to the use of dried leaves in diagnosing BYDV in survey samples (17).

As found in tests with other luteoviruses (22), the serological relationships indicated between NY-RMV and NY-SGV and other BYDV serotypes by ELISA tests apparently depended on the procedures used and their probable influence on virion integrity. Thus, experiments in which virions were trapped by using polyclonal antibodies from either rabbits or mice gave results consistent with those indicated by previous tests (1,35). Group 1 isolates showed interrelationships, but the group 2 isolates showed only slight relationship to each other and none to group 1 isolates. However, when virions were directly coated onto microtiter plates, obvious cross-reactions occurred between group 1 and group 2 isolates. Luteovirus virions can degrade in 0.05 M carbonate, pH 9.6, the buffer used for coating (5,7). Diaco et al (7) and Murphy and D'Arcy (22) attributed cross-reactions between distantly related luteoviruses in their experiments to this effect exposing internal epitopes which are hidden on relatively undegraded virions trapped with polyclonal antibodies. However, in our study, virions coated on ELISA plates were diluted in 0.1 M phosphate, pH 7.0, in which they are stable (5,7). In this case, a possible reason for the exposure of hidden epitopes could be that in binding to the plate surface, virions are broken up or distorted, so exposing hidden epitopes. Similar considerations have been applied in interpreting the results of dot immunoassay tests of crude extracts from infected plants applied directly to nitrocellulose membranes (16) and have been discussed by Vincent et al (34) in relation to recently discovered genome homologies among the luteoviruses. Deduced amino acid sequences for the coat protein encoding portions of the genomes of MAV-PS1, P-PAV, NY-RPV, and other luteoviruses have revealed several regions of homology, which may therefore encode similar epitopes.

None of the MAbs selected reacted with virions coated directly onto microtiter plates, only to antigens trapped by the homologous antivirus immunoglobulin. If, as discussed above, virions degrade on binding to plate surfaces, this suggests that the MAbs react with conformation-dependent epitopes or neotopes, i.e., epitopes that disappear on dissociation of virions (33), and that they therefore react only with intact virions.

MAbs from the RMV2 and SGV1 fusions cross-reacted with various other luteovirus isolates in ELISA tests in which antigen was captured by polyclonal antibodies (Table 3). The occurrence of MAbs reacting broadly with a range of BYDV isolates and other luteoviruses has also been noted in other work (6,7).

MAb RMV1-P1 may be useful as a specific MAb for RMV serotypes. However, it did react with two of six Mexican RPV serotype isolates tested, although not with NY-RPV or an RPV-like isolate from Great Britain (Table 3). In contrast, MAb RMV2-P3 had a wider spectrum of reactivity and might find use as a broadly reacting probe for group 2 serotypes (RPV and RMV), complementing another MAb developed in this laboratory that detects group 1 serotypes (MAV, PAV, and SGV) (C.-H. Lei, Purdue University, *unpublished*). It reacted with all RMV and RPV serotype isolates tested except for the two RPV serotype isolates from Mexico that reacted with MAb RMV1-P1. Both these MAbs need to be tested against a greater range of BYDV and luteovirus isolates to verify their range of reactivity.

Cross-reactions of MAb SGV1-P1 with RPV serotypes were avoided by use of the NY-SGV polyclonal immunoglobulin instead of a mixture of antisera (Table 3) for trapping antigen. In our experience, this immunoglobulin did not trap RPV serotypes. Hence, MAb SGV1-P1 could still be used in TAS-ELISA formats as a specific probe for SGV serotypes. However, this MAb also requires testing against a greater range of BYDV isolates and other luteoviruses to verify its specificity. The other MAbs appear to be less useful. MAb RPV1-P1 was extremely specific, reacting only with the homologous NY-RPV and not with the U.K. and Mexican RPV-like isolates tested. Its range of reactivity with other U.S. RPV-like isolates has yet to be established. Reactions of MAb RMV2-P2 were inconsistent among various serotypes, precluding its use as a reliable probe for either specific or broad spectrum detection.

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