

# Serological Characterization of Wheat Streak Mosaic Virus Isolates

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## ABSTRACT

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Wheat streak mosaic (WSM) is a destructive disease of wheat (*Triticum aestivum* L.) caused by wheat streak mosaic rymovirus (WSMV). Serological assays are commonly used to detect WSMV using polyclonal antibodies (PABs) or monoclonal antibodies (MABs). Wheat suspected to be infected with WSMV did not react with MABs made to the Oklahoma State University (OSU) isolate of WSMV, but did react to PABs made to the OSU isolate. The differences among six additional isolates of WSMV were determined using enzyme-linked immunosorbent assay (ELISA), Western blot, protein fingerprinting, and serological specific electron microscopy. Results indicate the existence of serologically distinct strains of WSMV. This finding is important in detecting WSMV, in breeding for resistance to WSM, and in investigating the epidemiology of WSM.

Wheat streak mosaic (WSM) is a destructive disease of wheat (*Triticum aestivum* L.) caused by wheat streak mosaic rymovirus (WSMV) (5,6,42). This virus is transmitted by the wheat curl mite, *Aceria tosichella* Keifer (33,38,39). Symptoms of WSM on wheat vary widely and are affected by temperature, cultivar, environment, and strain of WSMV (10,36).

Although the characterization of WSMV isolates has been limited (5,6), Carroll et al. (10) used symptom expression on the wheat cv. Michigan Amber to compare eight isolates of WSMV collected in Montana. They reported that seven isolates caused mild symptoms similar to American Type Culture Collection (ATCC) isolate PV-91, and one isolate caused severe symptoms similar to ATCC isolate PV-57.

WSMV may occur in mixed infections with soilborne wheat mosaic virus, barley yellow dwarf virus (BYDV), and Agropyron mosaic virus (AgMV) (27,30). Therefore, identifying the virus in symptomatic plants is critical for accurate diagnosis and for formulating appropriate control and cultivar recommendations. Serological assays are commonly used for detection of WSMV using polyclonal antibodies (PABs) or monoclonal antibodies (MABs) (31,32,40). Preliminary reports have indi-

cated serological differences among isolates of WSMV (26) and nucleotide sequence variability in the coat protein region of WSMV isolates (11,14). Identification of WSMV serotypes could have important implications in breeding for resistance to WSM and investigating the epidemiology of WSM, because differences in antigenicity are associated with vector transmissibility, host range, and virulence (2,4,25). The objective of this study was to determine protein and serological heterogeneity of isolates of WSMV.

## MATERIALS AND METHODS

**Virus isolates, maintenance and purification.** Seven isolates of WSMV from different wheat-producing areas of the United States were used (Table 1). In addition, an isolate of WSMV collected in the field in Oklahoma (designated 994) was used in Western blots with MABs. All isolates were increased and maintained in the hard red winter wheat cv. Blue Jacket and isolated from infected wheat as previously reported (17,31,32).

**Antibodies.** Production and reactivity of the PABs and MABs used in this study have been reported (31,32). Based on results in competitive enzyme-linked immunosorbent assay (ELISA), as previously described (3,15), the three MABs used in this study (32C-1, 32C-6, and 33A-1) reacted to the same or spatially close epitopes.

**ELISA.** Two ELISA methods were used in analysis of the isolates. Indirect double antibody sandwich ELISA (DAS-ELISA) was used with PABs to WSMV as the primary antibody. Then one of the three MABs to WSMV was used as the secondary antibody, followed by a goat-anti-mouse alkaline phosphatase conjugate (32). The direct antigen plating ELISA (DAP-ELISA) was used with PABs. For

this assay, virus-infected leaf tissue was ground in carbonate coating buffer (0.05 M carbonate buffer, pH 9.6; 0.5 g/5 ml) and added directly to ELISA plate wells (100  $\mu$ l per well). Samples were probed with IgG from PABs (1  $\mu$ g/ml) followed by goat-antirabbit antibody conjugated to alkaline phosphatase (Sigma Chemical Co., Sigma A-3687) and then substrate (31).

When either ELISA was conducted, three wells were used for each sample. Additionally, three wells of noninfected plant sap were included for each plate as a control and to provide a zero baseline, and three wells of wheat infected with the Oklahoma State University (OSU) isolate (detected by both MABs and PABs) were included as the positive control. Positive ( $\geq 0.100$ ) and negative ( $< 0.100$ ) absorbance ( $A_{405}$ ) thresholds were set based on previous evaluation of material for WSMV (17,32).

**Protein fingerprinting.** For proteolysis, 10  $\mu$ l of the chemical reagents or 1  $\mu$ l of protease was added to 50  $\mu$ l of isolated virus (1 mg/ml) suspended in 0.01 M citric acid (pH 8.0). The cleavage agents used were CNBr, *N*-chlorosuccinimide (24), formic acid, trypsin (32), chymotrypsin, papain, V8 protease, and pepsin. Except for CNBr and acetonitrile, all treatments were diluted in 0.125 M Tris-HCl buffer (pH 6.8) to obtain a 1 mg/ml concentration of the cleavage agent. For the CNBr treatment, 225 mg of CNBr was first dissolved in the acetonitrile solution (100  $\mu$ l) and adjusted to a final volume of 150  $\mu$ l. Then, 20  $\mu$ l of this reagent was added to 200  $\mu$ l of 0.6 N HCl plus 200  $\mu$ l of 0.125 M Tris-HCl, (pH 6.8). Acetonitrile was not used for proteolysis but as a carrier solution for the CNBr and to prevent confounding of the resultant peptide digestion profile with the formic acid digestion. An acetonitrile control was included (28). The concentrations of virus and proteolytic agent used were determined in preliminary experiments that resulted in discernible banding patterns. This approach was necessary, as treatment of WSMV with proteolytic agents results in significant loss of protein and thus loss of bands detectable by protein staining or Western blotting (9,32). Experiments were then conducted a minimum of three times to assure reproducibility of treatment.

All proteolytic-virus solutions were incubated for 1 h at 20 to 25°C, or for the formic acid solution at 37°C. Enzymatic proteolysis was terminated by adding an

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equal volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and heating for 5 min at 95°C. For the chemical digestion treatments, 50 µl of acetone was added to each sample prior to the addition of sample buffer. Each sample was placed at -20°C for 30 min. Samples were allowed to warm to 25°C, then centrifuged at 10,000 rpm for 10 min in a microfuge. The aqueous phase was removed, and samples were air-dried in a fume hood for 5 min. Fifty µl of sample buffer was added, and the samples were heated as above prior to SDS-PAGE.

**SDS-PAGE and Western blot.** SDS-PAGE and Western blot analyses were conducted with either PABs or MABs, as previously described (31,32). The major capsid protein band of the type member of WSMV has a molecular weight ( $M_r$ ) in an SDS-PAGE gel at 46 kDa, with usually three bands from 31 kDa to this 46 kDa band (7,9,30,32). However, as the ratio of effective size to charge of the SDS-protein complex of WSMV coat protein differs from that of standard proteins (9), precise interpretation of molecular weights of proteins in SDS-PAGE and Western blots should be guarded. In the gel stained with Fast Stain (ZION Biotech Corp., Newton, MA) many low  $M_r$  bands were apparent at the bottom of the discontinuous 12% resolving gel after digestion. Consequently, 15% resolving gels and 4 to 20% precast gradient gels (BioRad Corp., Hercules, CA) were used in analysis of these peptides.

Gels were dried in a slab gel drying unit (Ann Arbor Plastics, Ann Arbor, MI) using a 10% glycerol solution to prevent rapid drying and cracking of gels. Dried gels and most blots were then scanned using the Quantity One software by PDI, and the digitized information was downloaded to a Power Mac computer, using the Macintosh SuperPaint software, for production of photographic prints.

**Serological specific electron microscopy (SSEM).** Labeling of each of the seven WSMV isolates was attempted with

PABs and the three MABs. AgMV and PABs produced to AgMV (PABs courtesy of D. Seifers, Kansas State University, Agricultural Experiment Station, Hays) were included as controls. Procedures used in preparation for SSEM were as previously reported (41), with the following differences. Solutions, including the Abs, were kept on ice until needed. The Abs were diluted 1:10 in Fetal Bovine Serum-Glycine-PBS (FBG-PBS), and the grids were allowed to remain for 30 to 40 min on the surface of the droplet, carrying over as little excess fluid as possible from the previous droplet. The protein-A 15 nm gold-labeled conjugate was prepared by diluting 1:15 in 5% FBG-PBS and placing 6-µl droplets for each grid on the Parafilm. Grids were transferred to a 5% uranyl-acetate for 10 min and then dipped 15 times in a beaker of water before removing excess water with filter paper. The grids were air-dried in a laminar flow hood prior to viewing on a JEOL-100 CXII scanning transmission electron microscope at 80 kV.

## RESULTS

**ELISA and Western blots.** Differential reaction of isolates to MABs was observed in both ELISA and Western blots. All isolates were detected with PABs in ELISA or Western blot (Table 1). In ELISA and Western blots with MAB 33A-1 and MAB 32C-6, only isolates OSU, PV-57, and PV-91 were detected (Table 1, Fig. 1). MAB 32C-1 did not react to any WSMV isolate in Western blot (Table 1). Although results from the competitive binding assay with the three MABs indicated that they reacted to the same or a spatially close epitope, results from the Western blots indicated that MAB 32C-1 reacted to a neotope that was destroyed when the virus was denatured for SDS-PAGE.

**Protein fingerprinting. Fast Stain.** Nondigested capsid proteins of the majority of WSMV isolates were approximately 45 kDa (Fig. 2A), as previously reported (7,9,30,32). Several bands below and sometimes above 45 kDa were observed

from several of the isolates. The use of chemical cleavage agents followed by Fast Stain did not yield significant information about the isolates. Migration of capsid protein in SDS-PAGE appeared inhibited by treatment with formic acid, *N*-chlorosuccinimide or CNBr. Acidity (pH < 1) of the solutions may have inhibited migration. Treated capsid protein was precipitated with acetone, but no capsid cleavage was evident for treatment with acetonitrile or formic acid (not shown). The CNBr digestion provided little information (Fig. 2B). Cleavage of OSU, PV-57, and PV-91 resulted in a number of bands of various molecular weights. The lack of bands for COLO, PV-106, and OK964 was likely due to the inhibition of migration of the proteins as indicated above, since protein was known to have been loaded to the gel. The TAMU isolate was also cleaved by CNBr, as indicated by the faintness of the bands, but partial digest products were not detected. Capsid digested with *N*-chlorosuccinimide gave similar digestion products for PV-57 and PV-91, with the loss of the 31- to 46-kDa doublets and occurrence of bands of 14 to 25 kDa (not shown). Similarly treated TAMU had bands from 6 to 31 kDa and doublets from 31 to 46 kDa, and COLO had three bands ≤21 kDa.

Of the proteases used, distinctive banding patterns were produced by chymotrypsin, which resulted in loss of the 31- to 46-kDa capsid proteins and the appearance of products <21 kDa for many of the isolates (Fig. 3A). The OSU isolate appeared least affected by chymotrypsin. Papain cleavage resulted in banding patterns that more readily differentiated the isolates (Fig. 3B). PV-91 was readily degraded, with multiple bands at <14 kDa resulting, followed by COLO and then the other isolates. Cleavage of the COLO isolate by chymotrypsin or papain resulted in the most similar banding pattern compared to the banding pattern obtained with the two enzymes with the seven isolates.

Digestion with V8 protease did not greatly differentiate the isolates (Fig. 3C).

**Table 1.** Source of wheat streak mosaic virus isolates and reaction in enzyme-linked immunosorbent assay (ELISA), Western blot (WB), and serologically specific electron microscopy (SSEM)<sup>a</sup>

Isolate	Source	Reaction to polyclonal antiserum			Reaction to monoclonal antibodies								
		ELISA	WB	SSEM	MAB 33A-1			MAB 32C-6			MAB 32C-1		
		ELISA	WB	SSEM	ELISA	WB	SSEM	ELISA	WB	SSEM	ELISA	WB	SSEM
OSU	E. Sebesta, Stillwater, OK	+	+	+	+	+	+	+	+	+	+	-	+
COLO	B. Hammond, Fruita, CO	+	+	+	-	-	-	-	-	-	-	-	-
OK964	Texas County, OK	+	+	+	-	-	-	-	-	-	-	-	-
TAMU	C. Rush, Bushland, TX	+	+	+	-	-	-	-	-	-	-	-	-
PV-57	ATCC	+	+	+	+	+	+	+	+	+	+	-	+
PV-91	ATCC	+	+	+	+	+	+	+	+	+	+	-	+
PV-106	ATCC	+	+	+	-	-	-	-	-	-	-	-	-

<sup>a</sup> Positive assay (+) in ELISA (OD<sub>405</sub> ≥ 0.100), in Western blot a visibly discernible band and in SSEM visibly discernible binding of protein-A gold.

COLO appeared the most readily digested. Trypsin digestion could not be used to separate the isolates because the banding pattern was similar among all seven isolates (data not shown) and as observed in earlier reports (9,32).

**Western blot analysis of proteolyzed capsid with PAbs.** Some distinct differences were observed among some isolates for those chemical agents that produced protein bands that reacted with the PABs. The banding pattern seen with chymotrypsin and PABs was most similar between TAMU and PV-106 (Fig. 4A). There was some similarity in banding patterns obtained in Fast Stain gels (which stain all the protein bands of sufficient concentration) (Fig. 3A) and Western blots (in which proteins that are immunologically reactive are detected) (Fig. 4A), but there were also many bands that were observed in one treatment that were not evident in another treatment. Similar observations were made with Western analysis of V8 protease-treated virions (Fig. 4B) when compared to Fast Stain gels (Fig. 3C). With *N*-chlorosuccinimide, PV-106 and TAMU produced two doublets between 14 and 21 kDa, COLO had no bands <31 kDa, PV-57 and PV-91 were similar in banding pattern, and migration was limited for the OSU isolate, most likely due to pH (data not shown). There were no distinct banding differences seen in Western blots using PABs of trypsin-treated virions; the patterns were the same, as reported earlier for the OSU isolate (32) and by others using the type strain (9).

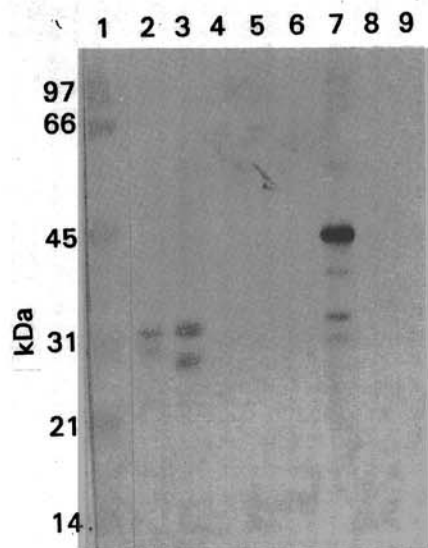


Fig. 1. Migration pattern of eight wheat streak mosaic virus (WSMV) isolates in a 4 to 20% precast gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel probed with monoclonal antibody 33A-1. Two  $\mu$ l of BioRad's Kaleidoscope prestained standards in lane 1, followed by OSU, PV-91, COLO, OK994, OK964, PV57, TAMU, and PV-106.

**Western blot analysis of proteolyzed capsid with MABs.** The isolates detected with MABs (OSU, PV-57, and PV-91) and cleaved by CNBr, chymotrypsin, or protease V8 produced similar banding patterns (not shown). Trypsin-digested proteins probed with MABs resulted in the patterns previously reported (32). No reaction occurred with the papain or *N*-chlorosuccinimide digestion products (data not shown).

**SSEM.** PABs bound along the length of the virion of each isolate (Fig. 5), as did MABs for isolates that reacted to MABs in ELISA (Fig. 6). MAb 32C-1, which did not react to any isolate in Western blot, did react to disrupted OSU, PV-57, and PV-91 isolates in SSEM (Fig. 7). No binding was seen on control grids.

## DISCUSSION

The results indicate that WSMV isolates can be distinguished by serological reactivity. The reactions of OSU, PV-57, and PV-91 to MABs distinguished these isolates from the others. The lack of reactivity by MAb 32C-1 under denaturing conditions, but reactivity in nondenaturing conditions, suggests MAB 32C-1 was produced by a discontinuous antigenic determinant (neotope) in the intact virion

coat protein. Based on previous competitive ELISA, the neotope was determined to be near the epitope to which the other MABs react. Reaction of MABs 32C-6 and 33A-1 to both native and denatured OSU, PV-57, and PV-91 indicates these MABs react to continuous antigenic determinants (metatopes).

The SSEM was conducted to attempt to determine the location of antigenic determinants and serotype relatedness of these isolates. Others (13,23) have shown that a particular set of Abs bind only at one end of a virus particle, at both ends, or all along the virion. In this study, PABs and MABs bound similarly along the length of the virus and to disrupted virions. Langenberg (21) also found that PABs to WSMV bound along the virion. Binding of Abs throughout the formvar grid initially was thought to have been nonspecific binding; however, such an array of gold particles was never seen in the various control grids (e.g., those lacking Abs, protein-A gold, or virus). The binding of Abs throughout the grid is believed to be due to Ab binding to disrupted virions produced by repeated freeze-thaw cycles. Disrupted virions were not observed in preparations only frozen once. Antibody binding to disrupted virion subunits has previously been reported

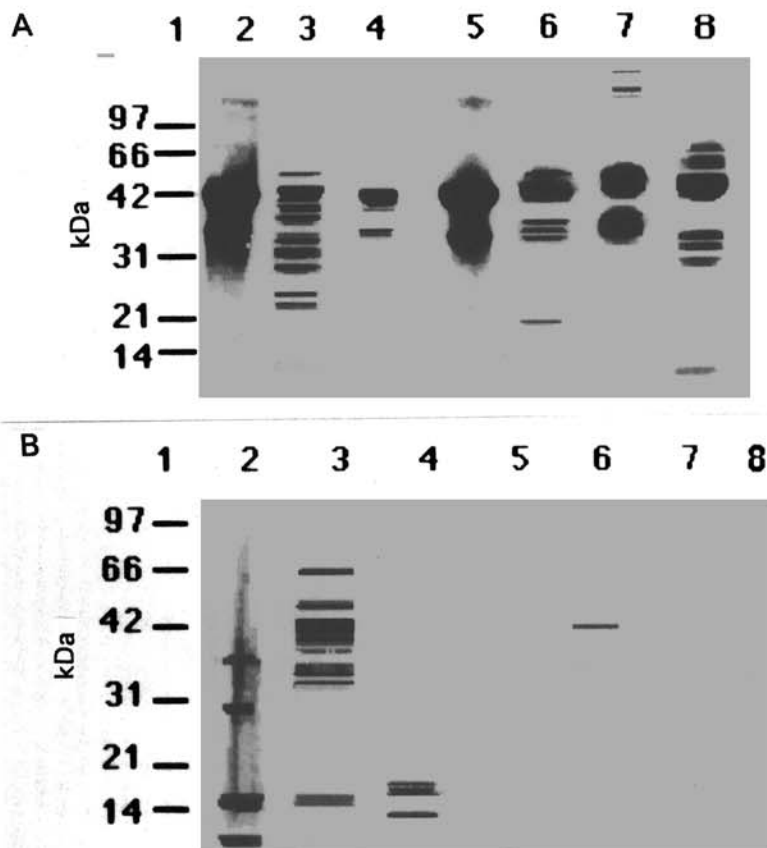


Fig. 2. Migration pattern of seven wheat streak mosaic virus (WSMV) isolates in a 4 to 20% precast gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Two  $\mu$ l of BioRad's Kaleidoscope prestained standards in lane 1, followed by OSU, PV-57, PV-91, COLO, TAMU, PV-106, and OK964: (A) untreated and (B) CNBr treated.

(12,13). Because all MABs reacted in SSEM with the three isolates that were detected in DAS-ELISA, SSEM did not help explain why MAB clone 32C-1 did not react under the denaturing Western blot conditions. The capsid was most likely not disrupted by freeze-thaw cycles to the extent that occurs in sample preparation for SDS-PAGE. Thus, the neotope was conserved and detected in SSEM.

The use of chemical and proteolytic cleavage agents on the capsid protein of the isolates for the protein fingerprinting

also indicated differences among isolates. The multiple bands seen below the typical 31- to 46-kDa range for the coat protein of the untreated purified viruses were probably due to degradation by host plant proteases during the purification procedure. We (32), as others (8,9,30), have observed capsid degradation and reaction of PABs with the degradation products. The protein bands observed at 66 kDa with the Fast Stain may be aggregations of intact and partially degraded protein (9,32). This is particularly true for this study, as a high

amount of protein was loaded to the gels to facilitate detection of small amounts of degradation or cleavage products. The 66-kDa proteins reacting with PABs, and to the MABs as well in the case of the OSU, PV-57, and PV-91 isolates, probably resulted from protein aggregation and thus poor gel migration.

Conducting protein fingerprint assays with seven digestion agents and seven WSMV isolates resulted in a large number of combinations for determining treatment conditions to compare isolates. However, the use of only one or two digestion agents such as trypsin or formic acid would have led to a spurious conclusion that no differences existed among the isolates. This work resulted in identification of cleavage agents to distinguish the isolates that could be useful for additional protein fingerprinting by capillary electrophoresis or HPLC (35).

The isolates could be divided into several groups, but from a more practical standpoint, the groups can be subdivided into reactive and nonreactive to MABs. The N- and C-termini of the coat protein of potyviruses are located on the protein surface (1,34,35). The N-terminus is the most immunodominant region in potyvirus particles and is variable among viruses and strains (18-20). Therefore, epitopes located in this area should elicit virus-specific antibodies. The PABs and MABs used in this study were produced to freshly purified virions of the OSU isolate (32). Thus, it was initially an enigma when WSMV isolates nonreactive to the MABs were found. The OSU isolate is believed to have been obtained originally from H. H. McKinney, who collected the type strain of WSMV (ATCC PV-57). Results presented in this paper suggest that the OSU isolate and PV-57 are very similar, if not the same viral strain. Furthermore, both PV-57 and OSU have been observed to have similar virulence in preliminary field tests at Stillwater, OK. The other isolate collected by H. H. McKinney, ATCC PV-91, also reacted to the MABs. However, in preliminary field tests at Stillwater, PV-91 causes mild symptoms in the hard red winter wheat cultivars being evaluated. PV-57 and PV-91 have been used in many scientific studies since first collected. The mite vector was used to transfer PV-57 from infected to healthy wheat before the virus isolate was donated to ATCC. Perhaps continual mechanical transfer of PV-91 is associated with the mild symptoms produced by this isolate.

Records from the Oklahoma State University Diagnostic Lab indicate that ELISA using MABs was once a useful diagnostic procedure for WSMV. Only recently has diagnostic evaluation (by MAB-ELISA) of wheat samples suspected to be infected with WSMV ceased to be consistently positive. Isolates obtained from the adjacent states of Texas and Colorado, and

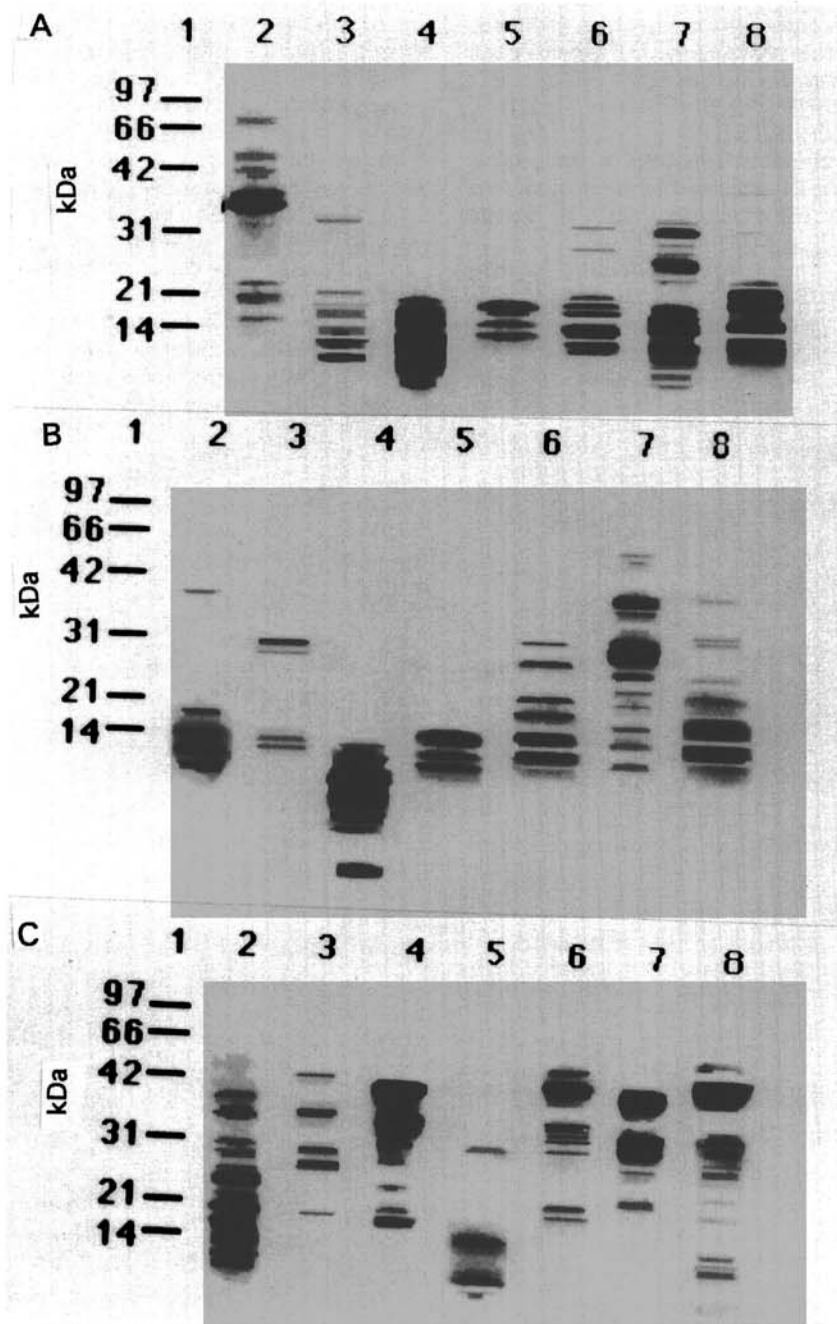


Fig. 3. Migration pattern of seven wheat streak mosaic virus (WSMV) isolates in a 4 to 20% precast gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Two  $\mu$ l of BioRad's Kaleidoscope prestained standards in lane 1, followed by OSU, PV-57, PV-91, COLO, TAMU, PV-106, and OK964: (A) chymotrypsin treated, (B) papain treated, and (C) V8 protease treated.

from the panhandle of Oklahoma, reacted to PABs but not to MABs. These isolates include not only those used in this paper but others subsequently collected. Geographically distant WSMV samples (Oregon) also gave a positive reaction to WSMV with PABs but not MABs (data not shown). Recently, an isolate of WSMV reactive to MABs was collected near the Oklahoma border in south central Kansas (D. Seifers, *personal communication*). This suggests that the lack of reactivity to MABs cannot be attributed simply to the maintenance of the OSU isolate by mechanical inoculation.

The existence of WSMV serotypes has potential significance in breeding for resistance or tolerance to WSM and in studying the epidemiology of this disease because studies of other vector-transmitted viruses have shown that differences in serological reactivity are associated with altered virulence, vector transmissibility, and host range (2,4,16,25). For example, studies conducted by Skaria et al. (37)

demonstrated that reaction to BYDV was both cultivar and virus-isolate specific. Ranieri et al. (29) examined this further and demonstrated differences in resistance and tolerance to isolates of the PAV, MAV, RPV, and RMV serotypes of BYDV, and concluded it was important to identify the specific virus strains used in testing for tolerance and resistance to BYDV. More recently, Lei et al. (22) reported that two SGV serotype isolates of BYDV exhibited differences in aphid transmissibility that could influence their relative occurrence and epidemiology; i.e., the TX-SGV isolate of BYDV was readily transmitted by a range of vector aphids, whereas the NY-SGV isolate showed a much greater vector specificity. Although these findings are in a different virus group (luteoviruses [BYDV] versus rymoviruses [WSMV]), the potential significance of serologically distinct isolates having differing biological properties associated with virulence or vector transmissibility cannot be ignored

and should be investigated. Current studies are underway with strains used in this study to determine if there are strain-cultivar interactions and to identify the sequence variability in the coat protein region of these isolates.

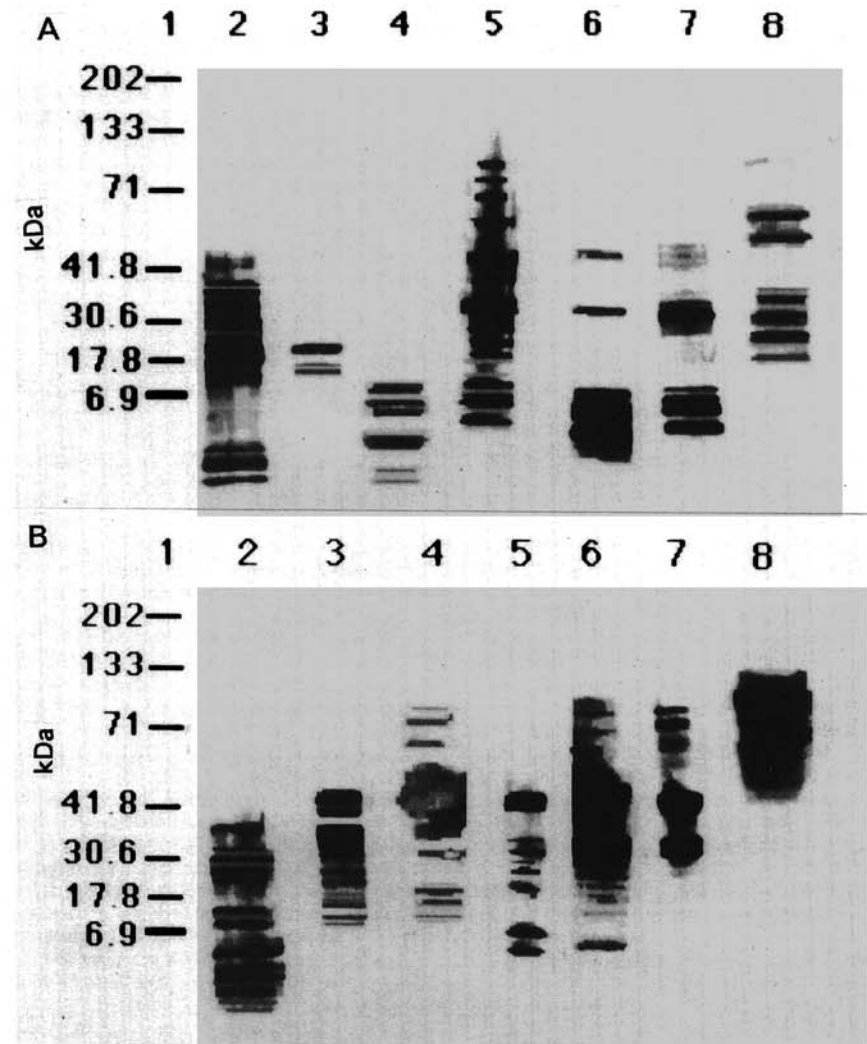


Fig. 4. Migration pattern of seven treated wheat streak mosaic virus (WSMV) isolates in a 4 to 20% precast gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel probed with polyclonal antibodies. Two  $\mu$ l of BioRad's Kaleidoscope prestained standards in lane 1, followed by OSU, PV-57, PV-91, COLO, TAMU, PV-106, and OK964: (A) chymotrypsin treated and (B) V8 protease treated.

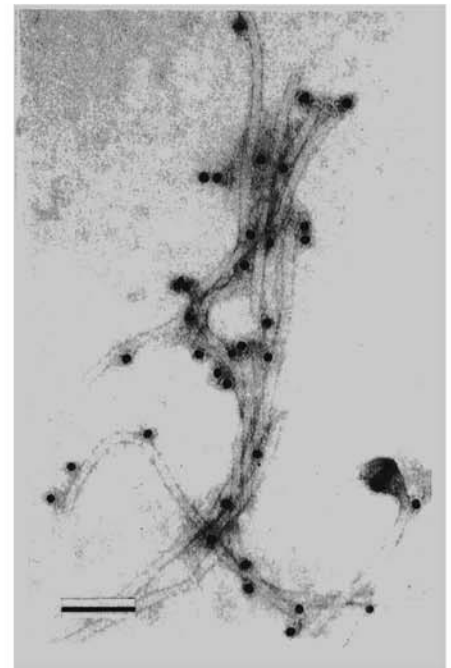


Fig. 5. Electron micrograph of COLO isolate labeled with polyclonal antibodies to wheat streak mosaic virus (WSMV) followed by 15 nm of gold-labeled antirabbit antibody. Bar = 150 nm.

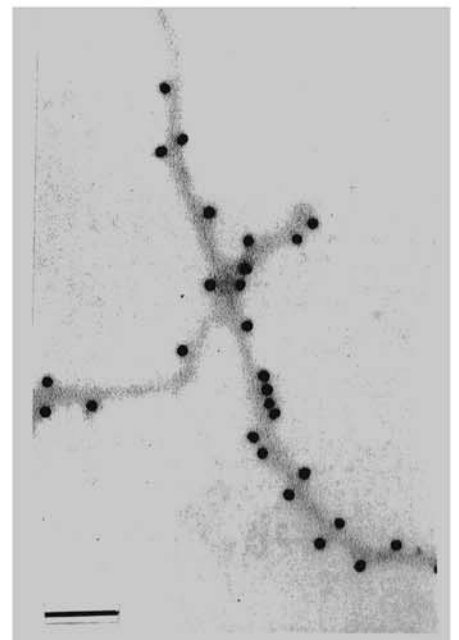


Fig. 6. Electron micrograph of PV-57 isolate labeled with monoclonal antibody 32C-1 to wheat streak mosaic virus (WSMV) followed by 15 nm of gold-labeled antimouse antibody. Bar = 112 nm.

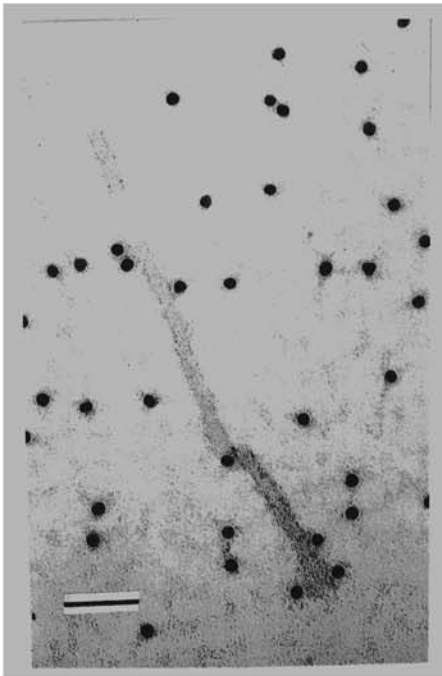


Fig. 7. Electron micrograph of disrupted OSU isolate labeled with monoclonal antibody 32C-1 to wheat streak mosaic virus (WSMV) followed by 15 nm of gold-labeled antimouse antibody. Bar = 100 nm.

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