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ABSTRACT

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Wheat streak mosaic virus (WSMV) is transmitted by the wheat curl mite (WCM), *Aceria tosichella*. Immunofluorescent and dot-immunobinding assays were developed to detect the presence of WSMV in single WCM. Virus-specific immunofluorescent microscopy detected WSMV near the anterior end of viruliferous WCM. With dot-immunobinding assay, WSMV was detected in WCM fed on WSMV-infected wheat (*Triticum aestivum*) but not in WCM maintained on healthy plants. Both immunofluorescent and dot-immunobinding assays were sufficiently sensitive to detect WSMV in individual WCM, providing a means to determine the percentage of viruliferous WCM in field collections.

Wheat streak mosaic is an important disease of wheat (*Triticum aestivum* L.) in the Great Plains of the United States, Canada, and many other wheat-producing countries (3). Regional epiphytotics of the disease have occurred in Colorado, Idaho, Kansas, Nebraska, North and South Dakota, Montana, and Washington. The most serious losses (up to 100%) occur when winter wheat becomes infected in the fall (19). Plants may be severely stunted, with varying degrees of necrosis resulting in death of tillers, reduced seed set, and shriveled kernels (10,11). Wheat streak mosaic is caused by wheat streak mosaic virus (WSMV), a potyvirus with filamentous particles, about 700 nm long and 15 nm diameter (3).

WSMV is specifically transmitted by the wheat curl mite (WCM). Originally named *Aceria tulipae* Keifer (18), this WCM has recently been renamed *Aceria tosichella* Keifer (1). WSMV infections are most severe when volunteer wheat results from the shattering of seed heads by hail (19). Volunteer wheat plants are rapidly infested with WCM and serve as a green bridge allowing WCM and the virus to successfully survive the summer. WCM then in-

fest newly planted wheat in the fall. The role of late-emerging volunteer wheat and other alternate hosts in WSMV epidemiology is not well understood. Thus, it is important to predict the potential for WSMV transmission from such plants and to identify the proportion of WCM in a population carrying WSMV.

The importance of the WCM as a vector of WSMV stimulated a number of studies on the relationship between the WCM and the WSMV. Transmission assays have been used to identify viruliferous mites from field and greenhouse populations (12). Virus also was detected in the homogenates of WCM through manual inoculation of wheat leaves or by employing a serological precipitin-ring test (13). Electron microscopy has also been used to test for the presence of virus in WCM (20). However, all of these assay methods required several hundred WCM, and given the small size of the WCM (250 × 75 μm; 7), such tests can be cumbersome and time-consuming.

Recently, several adaptations and improvements were made for the detection of plant viruses (22). However, some of the more sensitive assays, such as enzyme-linked immunosorbent assay (ELISA), require a minimum amount of test sample to partially fill the microtiter plate wells. The volume of a single WCM is less than 1 nl. The 100- to 200-μl test sample volume used in ELISA represents a 100,000- to 200,000-fold dilution of the sample. Dot-immunobinding assay and immunofluorescent microscopy may avoid these problems. Immunofluorescent microscopy has been used to detect different plant viruses in individual insects, such as rice dwarf virus and wound-tumor virus in the leafhopper vector (8,9). A direct immunofluorescent antibody labeling technique was

used to detect WSMV in crude homogenates from 10 WCM reared on virus-infected wheat (17). Dot-immunobinding assays have been used to detect a variety of plant viruses, such as members of carlavirus, potexvirus, potyvirus (including WSMV), tobamovirus, and nepovirus groups (2,14,15,16), from plant tissue. These dot-immunobinding assays used plant tissue for virus detection. There is to date no report of detection of a plant virus in an insect vector by dot-immunobinding assay. In this paper, we report the results of two serological assays, immunofluorescent microscopy and dot-immunobinding assays, to detect WSMV in single WCM.

MATERIALS AND METHODS

Virus culture. The Sidney isolate of WSMV (5) was used in this research. The virus was maintained on greenhouse-grown wheat cv. Michigan Amber with frequent transfers. An extract of infected plants was prepared by grinding leaves with a mortar and pestle at a 1:10 dilution (wt/vol) in 0.02 M potassium phosphate buffer, pH 7.2. Seven to 10 days after planting, test plants were lightly dusted with Carborundum (600 grit), and the wheat (cv. Arapahoe) leaves were rub-inoculated with the extract. Inoculated plants were maintained in the greenhouse for 3 weeks under natural light conditions with a temperature range of 18 to 27°C.

Virus purification. WSMV was maintained in winter wheat cv. Michigan Amber and purified according to Brakke and Ball (4). Virus was extracted from five to six fully expanded leaves that were infected with WSMV for 2 to 3 weeks. The extract was prepared by grinding the leaves in a blender in 1 mg per ml of 0.01 M K_2HPO_4. The extract was subjected to one cycle of differential centrifugation (low speed 12,000 × g for 15 min and high speed 60,000 × g for 90 min). The pellets were resuspended in 0.01 M citrate buffer, pH 8.0. The resuspended pellets were clarified by centrifugation at 7,000 × g for 5 min at 4°C. The supernatant was further purified by sucrose density gradient centrifugation at 60,000 × g for 3 h. Gradients were fractionated at 254 nm with an ISCO (Instrumentation Specialties Co., Lincoln, NE) density gradient fractionator. Virus concentration was estimated by ultraviolet absorbance spectroscopy using an extinction coefficient of 3 cm²/mg at 254 nm (4).

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Wheat curl mites. A colony of aviruliferous WCM was started by transferring WCM eggs onto virus-free wheat seedlings with a single hair cemented to a small dowel. All WCM observations and manipulations were done with a stereoscopic microscope. The WCM colony was maintained on healthy wheat (cv. Arapahoe) plants in a growth chamber at 20°C for 13-h days and 14°C for 11-h nights. Care was taken to isolate the WCM colony from potential sources of WSMV contamination.

Viruliferous WCM were obtained by manually inoculating two- to three-leaf wheat plants with WSMV, Sidney isolate. Two weeks after inoculation, when symptoms were evident, aviruliferous WCM were transferred to the WSMV-infected plants. Transfer was accomplished by attaching excised WCM-infested leaves to the infected plants. WCM crawled from excised to attached leaves within 36 to 48 h, after which the excised leaves were removed. WCM were allowed to feed on WSMV-infected plants for a minimum of 1 week before being used in this study.

Field samples of WCM were collected from symptomatic wheat from commercial plantings at three different locations in western Nebraska. These WCM were tested for the presence of WSMV.

Indirect immunofluorescent microscopy. Samples were prepared by placing individual WCM in a grid pattern on glass slides coated with poly-L-lysine (Sigma Chemical Co., St. Louis, MO). Aviruliferous and viruliferous WCM were placed on separate glass slides. The WCM were gently pressed in place with a glass slide. This allowed the WCM to better adhere to the glass and forced internal fluids out. All of these procedures were done while examining WCM under a stereoscopic microscope. WCM were fixed with 2% paraformaldehyde by submerging slides for 2 h at 4°C, and then were washed three times with phosphate-buffered saline (PBS; 145 mM NaCl and 10 mM sodium phosphate, pH 7.2) with 0.1% Tween 20 (PBS-T). In some experiments, the WCM were fixed with 95% ethanol rather than paraformaldehyde to test the effectiveness of fixation. WCM were incubated for 2 h at room temperature with anti-WSMV rabbit immunoglobulin G, 1.3 mg/ml, diluted 1:1,000 in PBS-T buffer. The anti-WSMV rabbit immunoglobulin G was prepared against Sidney isolate of WSMV (supplied by R. C. Gergerich). The slides were washed three times for 10 min in PBS-T buffer, followed by incubation for 2 h at room temperature in fluorescein isothiocynate-conjugated anti-rabbit serum (Sigma), 1.1 mg/ml, diluted 1:100 in PBS buffer. After washing with PBS-T buffer, WCM were examined under an epifluorescent microscope (Nikon Optiphot, Melville, NY) using a fluorescein filter cube (illumination at 450 to 490 nm). Aviruliferous WCM were used as negative control. The experiment was performed three times.

Dot-immunobinding assay. A single WCM was crushed and ground with a glass rod in a porcelain spot plate with 10 to 15 µl of Tris-buffered saline (TBS; 50 mM Tris-HCl and 150 mM NaCl, pH 7.5) while being examined under a stereoscopic microscope. The extract from a single WCM was then serially diluted by five fivefold dilutions to determine the sensitivity of the assay. This dot-immunobinding assay was similar to the procedure of Towbin et al. (21). All steps were performed at room temperature. A nitrocellulose membrane (15 × 10 cm, pore size 0.45 µm; Bio-Rad, Richmond, CA) was marked with a grid of 1.0-cm squares with a no. 2 lead pencil. The membrane was cut to a size that would accommodate the number of samples in an individual test and was immersed in TBS buffer, pH 7.5, for 15 min by inserting one edge of the membrane into the buffer and allowing it to absorb the liquid by wick action. It then was dried on filter paper for 10 min. Three µl of the sample was placed in the center of each square of the nitrocellulose membrane and allowed to air-dry for 10 min. After blocking with 5% nonfat dry milk for 2 h in TBS-0.1% Tween buffer, pH 7.5 (TBS-T), it was incubated for 2 h in anti-WSMV antibodies, 1.3 mg/ml, diluted 1:1,000 in TBS-T buffer. The antiserum used was the same as that
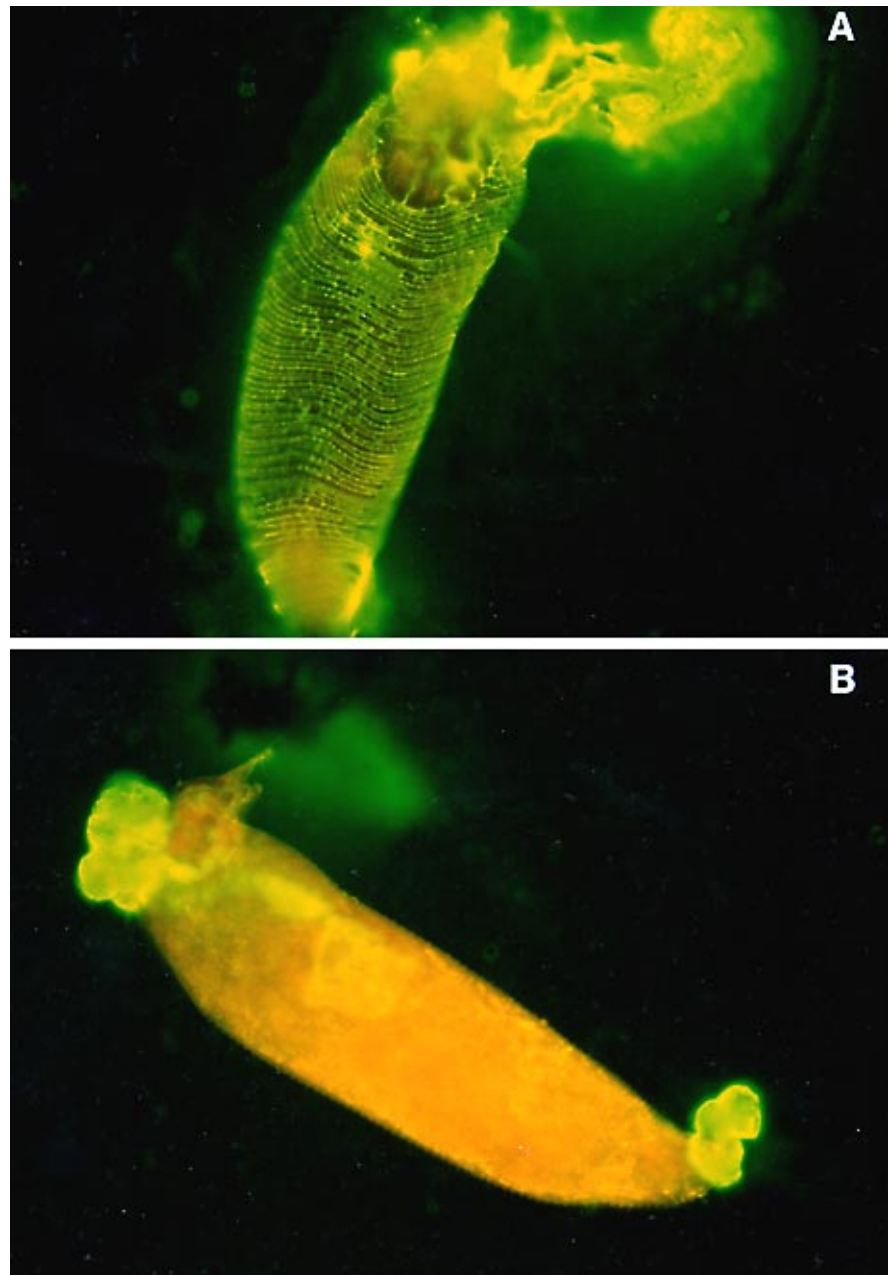

Fig. 1. Indirect fluorescent antibody detection of wheat streak mosaic virus (WSMV) antigen in wheat curl mites (WCM), *Aceria tosichella*. Detection of WSMV antigen in the body of a single WCM that **(A)** had fed on a WSMV-infected wheat plant, and **(B)** had fed on a healthy wheat plant.

used for the indirect immunofluorescent microscopy. The membrane then was washed three times for 10 min each in TBS-T buffer and incubated for 2 h in a 1:3,000 vol/vol (0.8 mg/ml) solution of anti-rabbit antibody conjugated to alkaline phosphatase (Sigma) in TBS-T buffer, pH 7.5. After washing, the presence of alkaline phosphatase was demonstrated by reacting the membranes with bromochloroindolyl phosphate/nitro blue tetrazolium substrate for 20 min. Aviruliferous WCM were used as a negative control, while purified WSMV was used as positive control. Three µl of a dilution series of a purified virus preparation was used to test the sensitivity of the assay. The experiment was performed three times.

RESULTS

Indirect immunofluorescent microscopy. Preliminary experiments using viruliferous and aviruliferous WCM established the optimum dilutions of first and second antibodies needed in subsequent immunofluorescence assays. Different dilutions of first (1:250, 500, 1,000, 2,000) and second (1:50, 100, 200, 400, 800) antibodies were tested. Satisfactory results were obtained with a 1:1,000 dilution of first and a 1:100 of second antibodies. In three experiments, 50 WCM from WSMV-infected wheat and 50 WCM from healthy wheat were subjected to indirect immunofluorescent labeling and then were examined microscopically using epiillumination. All WCM examined showed a bright yellow autofluorescence. However, all viruliferous WCM showed an additional brilliant apple-green fluorescence due to specifically bound fluorescein-labeled antibodies.

This fluorescence was most consistently seen along the body of the WCM (Fig. 1A). However, virus-specific fluorescence was also present at the anterior and posterior ends of some of the viruliferous WCM. This was probably caused by internal fluids squeezed out of the mouth and anus during slide preparation. Aviruliferous WCM never exhibited green fluorescence staining on the sides of the body (Fig. 1B), in contrast to viruliferous WCM (Fig. 1A). The extruded fluid at the anterior ends of some aviruliferous WCM showed a very weak nonspecific fluorescent staining (Fig. 1B), probably caused by the presence of low levels of antibodies to plant sap components in the WSMV antiserum. In contrast to aviruliferous WCM, the fluorescein staining of viruliferous WCM usually quenched or reduced the yellow autofluorescence, facilitating the differentiation of viruliferous and aviruliferous WCM. Samples could be stored at 4°C for 2 to 3 weeks without losing their antigenicity. Identical results were obtained with WCM fixed in either 2% paraformaldehyde or 95% ethanol.

Dot-immunobinding assay. Virus concentrations from 1 to 5 ng of WSMV per ml could be detected with the standard dot-immunobinding procedure described (Fig. 2). WSMV was also detected by spotting a 3-µl aliquot of a homogenate prepared by grinding a single viruliferous WCM in 10 µl of buffer (Fig. 2). WSMV could be detected in homogenate extract of a single WCM after a 1:125 dilution. No background reaction product was seen when extracts of aviruliferous mites were similarly assayed. The samples prepared after grinding WCM could be stored at 4°C for

2 to 3 weeks without losing their antigenicity.

This procedure was also used to detect WSMV in single WCM collected from symptomatic wheat from commercial plantings. In four different experiments, 21 out of 54 WCM tested positive for WSMV (Table 1). Aviruliferous WCM maintained on healthy wheat were used as a negative control. No aviruliferous WCM tested positive for WSMV.

DISCUSSION

Immunofluorescent microscopy and dot-immunobinding assays were effective in detecting viruliferous WCM obtained from WSMV-infected wheat and field-collected WCM. The most important criteria in evaluating diagnostic techniques are reliability, sensitivity, and cost. The immunofluorescence assay is more convenient for examining large numbers of mites, but it does require a microscope with fluorescence optics. Thus, the dot-immunobinding assay provides an alternative for laboratories without access to such a microscope. There was some nonspecific staining in immunofluorescent microscopy but very little background with dot-immunobinding assay. This is likely due to differences between the blocking steps used in the two procedures.

Dot-immunobinding assays are valuable because they are simple, sensitive, rapid, and economical. ELISA, especially the double antibody sandwich form (6), has been the method of choice for the detection and assay of plant viruses because it is sufficiently sensitive for most applications. Dot-immunobinding assays and fluorescent microscopy procedures have sensitivity similar to ELISA but produce results that are more difficult to quantify. Dot-immunobinding assays are potentially useful research and diagnostic tools in plant pathology, particularly in the study of pathogens transmitted by eriophyid mite vectors. The low volume of the sample required for dot-immunobinding assays is an important advantage over ELISA in analyzing samples without dilution.

Our results are similar to the work of Sinha and Paliwal (17), who could detect WSMV antigens in crude homogenates
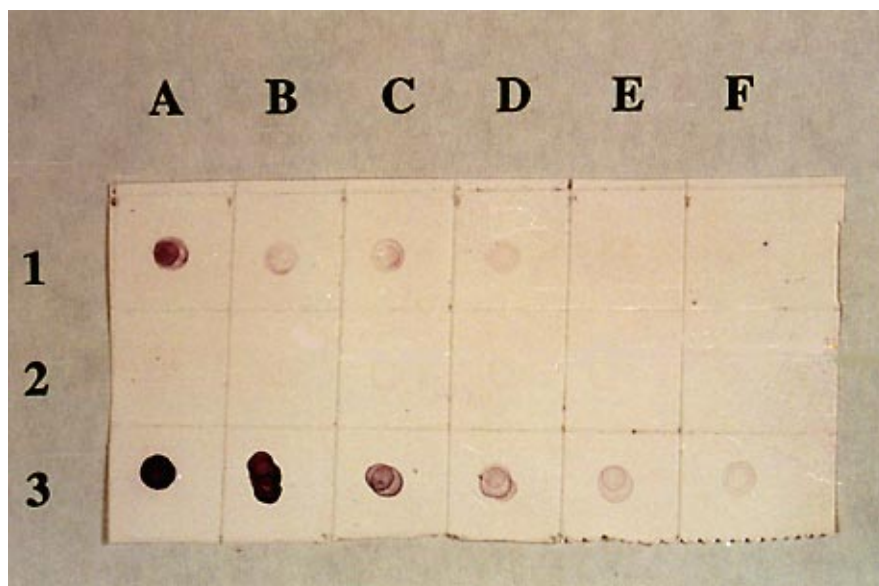

Fig. 2. Dot-immunobinding detection of wheat streak mosaic virus (WSMV). (Row 1) fivefold dilutions of an extract from a single viruliferous wheat curl mite (WCM); (row 2) dilutions of an extract from an aviruliferous WCM; and (row 3) purified WSMV. The concentrations of purified WSMV used were: (A) 5,000 ng/ml, (B) 1,000 ng/ml, (C) 200 ng/ml, (D) 40 ng/ml, (E) 8 ng/ml, and (F) 1 ng/ml.

Table 1. Dot-immunobinding assays for wheat streak mosaic virus (WSMV) in samples of wheat curl mites (WCM) collected from symptomatic wheat from commercial plantings

Experiment	WSMV positive	WSMV negative
1[a]	4	8
2[b]	5	7
3[c]	7	7
4[c]	5	9

[a] WCM collected from Kimball County, NE.
[b] WCM collected from Cheyenne County, NE.
[c] Two collections of WCM from Scotts Bluff County, NE.

prepared from WCM from virus-infected wheat plants by using direct immunofluorescent microscopy assays. In their work, WSMV was detected only in the body fluid released through the anus of mites. Our results showed that WSMV was detected in the body fluid released through the anus and the mouth of the WCM. Moreover, WSMV was also detected from both sides of the bodies of mites. This suggests that indirect immunofluorescent microscopy is more sensitive in detecting the presence of WSMV in single WCM.

These assays provide useful tools for virus detection in virus vectors, especially very small vectors, and we have successfully applied these techniques to field-collected WCM. Future investigations need to determine the correlation between the presence of WSMV in WCM populations and the ability of that WCM population to transmit WSMV. It should now be feasible to develop appropriate field sampling methods for testing large numbers of WCM. The proportion of viruliferous WCM in WCM populations on various hosts has hitherto not been examined closely because of difficulties with virus assays on single WCM. The ability to readily identify proportions of viruliferous individuals in WCM populations may allow more effective epidemiological disease transmission models to be developed.

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