Serological Detection of Corn Stunt Spiroplasma and Maize Rayado Fino Virus in Field-Collected *Dalbulus* spp. from Mexico

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ABSTRACT

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Corn stunt spiroplasma (CSS) experimentally acquired from maize (*Zea mays*) was detected by enzyme-linked immunosorbent assay (ELISA) in extracts from individual as well as groups of five *Dalbulus maidis* and in extracts from individual *D. elimatus, D. gelbus, D. guevarai,* and *D. quinquenotatus*. CSS was detected by ELISA in all exposed but none of the unexposed individuals of all species. Infectivity assays gave the following transmission rates: *D. maidis* 100%, *D. elimatus* 83%, *D. gelbus* 0%, *D. guevarai* 25%, and *D. quinquenotatus* 50%. CSS infection of these species was detected more reliably by ELISA than by infectivity assay. Purified CSS antigen was detected by ELISA at 25 but not at 10 ng/ml. CSS was detected by ELISA in about 10% of 436 individual *Dalbulus* leafhoppers field-collected in Mexico in 1982, and maize rayado fino virus (MRFV) was detected in 5.5% of 828 leafhoppers collected in 1981 and 1982. Incidence of MRFV in maize plants in the field where the leafhoppers were collected in 1981 was 35–40%. As demonstrated by Wilk's statistic (W), the absorbance ($A_{405\ nm}$) values of the unexposed leafhoppers in ELISA were generally normally distributed. On the basis of this distribution, when the mean of $A_{405\ nm}$ values plus three times the population standard deviation for the unexposed controls was used to calculate the lower limit of acceptance for positive $A_{405\ nm}$ values, the type I error rate had an upper bound of 7% for a sample size (N) of 8 and of 1.3% for $N = 30$.

Enzyme-linked immunosorbent assay (ELISA) has been used to detect maize rayado fino virus (MRFV) in *Dalbulus maidis* (DeLong & Wolcott) (4,10–12) and the corn stunt spiroplasma (CSS) in both *D. maidis* (2) and *Euscelidius variegatus* (Kirsch) (9). To date, no reports of ELISA-detected CSS in field-collected vectors have been noted, but Saavedra (12) reported detection of MRFV by ELISA in field-collected *D. maidis*. In 1981 and 1982, we detected CSS and MRFV by ELISA in extracts from leafhoppers collected in a survey of leafhoppers on maize (*Zea mays* L.) and on wild *Zea* and *Tripsacum* in Mexico (6). We also related the percentage of viruliferous leafhoppers to incidence of MRFV in maize in the field and report our findings in this paper.

MATERIALS AND METHODS

Sources of leafhopper and maize samples. All *Dalbulus* leafhoppers for laboratory tests were taken from colonies maintained on healthy maize in the laboratory at Wooster, OH. CSS-exposed leafhoppers were given a 7-day acquisition access period (AAP) and a 14-day inoculation access period (IAP) before assays. All field samples of *Dalbulus* leafhoppers were collected from maize plots at the Centro Internacional de Mejoramiento de Maiz y Trigo (CIMMYT) near Texcoco, Mexico, in early October. Leafhoppers were collected at night with an ultraviolet light (BioQuip 2800 Series, 15W blacklight collecting light, BioQuip Products, Santa Monica, CA) and a white sheet on which leafhoppers alighted after disturbance of adjacent maize plants. Leafhoppers, collected with aspirators, were placed on maize leaves in plastic refrigerator boxes lined with moistened charcoal and plaster of paris bottoms to prevent desiccation of leafhoppers and host leaves during transport. Boxes were refrigerated until transported with ice in an insulated container. Leafhoppers were identified to species and sex. All leafhoppers from laboratory tests and those from the 1981 field collections were frozen until assayed by ELISA. For the 1982 field collection, only leafhoppers that survived transport to Wooster were assayed.

In 1981, maize leaf samples collected from the same field as the *Dalbulus* leafhoppers were taken from 33–34 plants in each of three rows about 6 m apart and at 2.7-m intervals within each row for a total of 100 samples. Leaves were collected without regard to disease symptoms. Control leaf tissues were healthy and MRFV-infected maize maintained in the greenhouse at Wooster.

Preparation of extracts. Individual or groups of five leafhoppers were ground in glass tissue homogenizers (13 × 2.5 cm) with Teflon pestles in 0.2 or 0.3 ml of PBS-Tween (PBS = 0.15 M sodium chloride + 0.02 M sodium phosphate + 0.02% sodium azide, pH 7.4; Tween 20 = 0.05% polyethylene sorbitan monolaurate) and 0.1 ml of each homogenate was transferred to a single well of an ELISA microtiter plate (Dynatech Laboratories Inc., Alexandria, VA).

Extracts were prepared from leaves (0.2 g) ground in PBS-Tween (0.8 ml) with a pestle in a mortar, and 0.1 ml of each extract was added without further treatment to each of two wells of a microtiter plate.

ELISA procedures. Antisera to CSS strain 1747 was from R. E. Davis (USDA, Beltsville, MD) and antisera to MRFV was produced as described previously (8). CSS and MRFV gamma globulins were purified and conjugated with alkaline phosphatase and ELISA was performed as described previously (8). The coating gamma globulin concentration was 1 μg/ml for antibodies to both CSS and MRFV, and the gamma globulin-enzyme conjugate dilutions were 1:400 for MRFV and 1:400 (leafhoppers) and 1:1,000 (purified CSS protein) for CSS. Sodium hydroxide (3 N) was added to wells after a 2-hr incubation to terminate reactions, and absorbances ($A_{405\ nm}$) were recorded. Measurements were made with either a Gilford Stasar II spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, OH) equipped with a rapid sampling system or with a Bio-Tek EIA Reader (Model EL307) (Bio-Tek Instruments Inc., Burlington, VT).

Tests for ELISA sensitivity. To determine the sensitivity of ELISA for detection of CSS antigen, a dilution series of purified CSS protein (provided by R. E. Davis, USDA, Beltsville) was prepared with PBS-Tween as the diluent to give 15 twofold or 2.5-fold dilutions ranging from 200 μg/ml to 5 ng/ml. These were tested in six replicates per

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dilution by ELISA.

To compare detection of CSS by ELISA vs. infectivity assay, early-instar to mid-instar *D. maidis, D. elimatus, D. gelbus* DeLong, *D. guevarai* DeLong, and *D. quinquenotatus* DeLong & Nault were assayed immediately after an AAP and a 14-day incubation period by ELISA or tested for infectivity by caging on test maize plants (cultivar Aristogold Bantam Evergreen) (five per plant) for a 7-day IAP. After the IAP, plants were sprayed with an insecticide, placed in an insect-containment greenhouse, and observed for symptoms.

Analysis of ELISA data. Reactions were judged positive for the pathogen when $A_{405\ nm}$ values were greater than the mean plus three times the population standard deviation for the unexposed leafhopper controls within the same microtiter plate. A set of the latter controls was included within each microtiter plate, because previously, when such controls were included in several plates tested at the same time, the controls for different plates were significantly different (D. T. Gordon, *unpublished*). When no assumptions were made about the underlying distribution of the absorbances for the unexposed leafhopper population and variations in the estimate of the population variance were ignored, the type I error rate (ie, the probability of classifying a pathogen-free sample as positive for the pathogen) in this classification had an upper bound of 11%. This rate was determined with the Chebychev inequality. If an underlying normal distribution was assumed, the estimated (95% probability) type I error rate had an upper bound of 7% for sample size (N) of 8 and of 1.3% for $N = 30$.

To determine the lower limit for detection of CSS antigen, mean $A_{405\ nm}$ values for each dilution of purified antigen were compared statistically with means for the adjacent more- and less-diluted preparations. A one-tailed t test at a level of $\alpha = 0.05$ was used to determine significant differences between the paired means.

RESULTS

Detection of laboratory-acquired CSS. In the test of whether ELISA could detect CSS in *D. maidis*, wells that contained extracts from five leafhoppers had visual ratings that ranged between 5 and 6 on a scale of 0–7 (0 = no color and 7 = maximum yellow coloration) and the mean $A_{405\ nm}$ was 0.993 (standard deviation [SD] = 0.196 and $N = 4$) with a minimum $A_{405\ nm}$ of 0.766 and a maximum of 1.240. For the unexposed controls with five leafhoppers per well, the mean $A_{405\ nm} = 0.072$, SD = 0.0029, and $N = 4$. For the single leafhopper extracts per well, visual ratings averaged 3.8, the mean $A_{405\ nm} = 0.538$, SD = 0.373, and $N = 10$. The corresponding unexposed controls had values of mean $A_{405\ nm} = 0.067$, SD = 0.004, and $N = 10$; no color was observed. All exposed leafhoppers were judged positive for CSS whether detected in extracts prepared from groups of five or from single leafhoppers. Because only about one-half of the extract was added to a well, CSS was detected from about one-half of a leafhopper. There was no apparent interference from materials in leafhopper extracts, because $A_{405\ nm}$ values for extracts from groups of five unexposed leafhoppers were similar to those for extracts from one unexposed leafhopper.

In further tests of the sensitivity of CSS detection for individual *D. maidis, D. elimatus, D. gelbus, D. guevarai*, and *D. quinquenotatus*, all $A_{405\ nm}$ values were positive for CSS (Table 1). Results confirmed the first test—that CSS could

Table 1. Detection of corn stunt spiroplasma (CSS) in five *Dalbulus* spp. by enzyme-linked immunosorbent assay (ELISA) and infectivity assay

	D. maidis		*D. elimatus*		*D. gelbus*		*D. guevarai*		*D. quinquenotatus*	
Assay	E[a]	NE[b]	E	NE	E	NE	E	NE	E	NE
ELISA										
Mean ($A_{405\ nm}$)[c]	0.36	−0.05	0.39	−0.05	0.51	0.06	0.39	0.06	0.23	0.06
SE[d]	0.020	0.003	0.063	0.002	0.037	0.004	0.048	0.003	0.037	0.005
No. positive[e]	19	0	25	0	20	0	12	0	10	0
No. total[f]	19	8	25	16	20	9	12	8	10	8
Infectivity assay	6/6[g]	NT[h]	5/6	NT	0/6	NT	1/4	NT	4/8	NT

[a] E = leafhoppers given a 7-day acquisition access period on CSS-infected maize followed by a 14-day incubation period before assays.
[b] NE = leafhoppers not exposed to infected plants.
[c] Mean absorbance at 405 nm for extracts of leafhoppers within each column.
[d] SE = standard error.
[e] Number of leafhoppers positive for CSS in ELISA. A partial summary of these results was published by Madden and Nault (5).
[f] Total number of leafhoppers assayed by ELISA.
[g] Numerator = number of plants showing symptoms of corn stunt after an inoculation access period (IAP) of 7 days with groups of five leafhoppers per plant. Denominator = total number of plants in test. All *D. gelbus* leafhoppers were dead by the end of the IAP and this probably explains the failure to transmit (5).
[h] NT = not tested.

Table 2. Numbers of individual *Dalbulus* spp. field-collected in Mexico that were positive and negative for corn stunt spiroplasma and the mean absorbance ($A_{405\ nm}$) values for these leafhoppers and for the unexposed, laboratory-reared *D. elimatus* as determined in individual microtiter plates of enzyme-linked immunosorbent assay (ELISA) for 1982 collection

	ELISA for CSS in field-collected *Dalbulus* spp. (no. of leafhoppers)		Mean $A_{405\ nm}$ for CSS detection by ELISA		
			Field-collected leafhoppers		Unexposed laboratory-reared
Plate no.	Positive	Negative	Positive	Negative	*D. elimatus*
1	15	62	0.103[a] (0.006)[b]	0.063 (0.001)[b]	0.068 (0.002)[b] (12)[c]
2	9	69	0.162 (0.007)	0.100 (0.003)	0.081 (0.005) (12)
3	2	76	0.222 (0.038)	0.105[d] (0.003)	0.092 (0.007) (12)
4	4	74	0.192 (0.028)	0.087 (0.002)	0.079 (0.006) (12)
5	11	67	0.127 (0.014)	0.068 (0.001)	0.066 (0.001) (12)
6	3	44	0.161 (0.013)	0.071 (0.002)	0.087 (0.003) (12)

[a] Threshold value for positives of plate 1 was 0.074, based on three times the standard deviation plus the mean $A_{405\ nm}$ of the unexposed, laboratory-reared *D. elimatus*.
[b] Number in parentheses is the standard error.
[c] Number of individual leafhoppers assayed.
[d] Threshold for positives of plate 3 was 0.113, calculated as described in footnote a.

be detected in extracts from individual *Dalbulus* leafhoppers experimentally exposed to a CSS source.

ELISA proved to be a more reliable detector of CSS infection of these species than the infectivity assay (Table 1), particularly with *D. gelbus*, which probably succumbed to the pathogen before transmission could occur (5).

Sensitivity of ELISA for detection of CSS antigen. Significant differences were obtained for antigen concentrations of adjacent pairs from 100,000 and 50,000 to 50 and 25 ng/ml. Thus, the lowest detectable CSS antigen concentration was 25 ng/ml and the saturation point was 100,000 ng/ml for our conditions. Concentrations between 25 and 100,000 ng/ml were used to demonstrate the relationship of $A_{405\ nm}$ to antigen concentration. The model $A_{405\ nm} = \alpha(C)^{\beta}\epsilon$ (ie, $\log A_{405\ nm}$ was linearly related to $\log C$) was fit to the data yielding $R^2 = 0.971$, where C = the concentration (ng/ml), α and β = the regression parameters, and ϵ = the random error variable.

Detection of CSS and MRFV in field-collected leafhoppers. Mean $A_{405\ nm}$ values for CSS-positive, field-collected *D. maidis* and *D. elimatus* (Table 2) were considerably lower (average of means = 0.161) than those for the experimentally acquired CSS (average of means = 0.375). In all, six microtiter plates were used and there were appreciable differences in $A_{405\ nm}$ values among plates, as judged by the mean $A_{405\ nm}$ values for the unexposed leafhoppers of the six plates. Of special note, the mean $A_{405\ nm}$ for the CSS-negative leafhoppers of plate 3 was slightly higher than that for the CSS positives of plate 1 because of the considerable difference in threshold values for the two plates. Mean $A_{405\ nm}$ values for field-collected *Dalbulus* spp. judged positive for CSS ranged from 0.101 to 0.222, with the average 2.04 times that for the unexposed leafhoppers, which had mean values from 0.066 to 0.092. Similarly, the average for the former was 1.95 times that of the CSS-negative, field-collected leafhoppers. Only about 10% of all the field-collected leafhoppers assayed were positive for CSS (Table 3). Slightly more *D. elimatus* were positive for CSS than *D. maidis*, but the difference was not significant.

For 1981 collections, MRFV was detected in 31 of 464 (6.7%) field-collected *Dalbulus* spp. by ELISA (Table 4). For 1982, these figures were 16 of 364 (4.4%). The difference for the 2 yr was not significant (Table 3). Absorbance means for the MRFV-positive, field-collected *Dalbulus* spp. were 1.7–3.8 times those for the unexposed, laboratory-reared *D. maidis* (negative controls) in 1981 and 2.0–2.75 times those for negative controls in 1982. Means ($A_{405\ nm}$) of the MRFV-positive, field-collected *Dalbulus* spp. were about 2.9 times the means for MRFV-negative, field-collected *Dalbulus* spp. in 1981 and about 2.1 for comparable leafhoppers in 1982.

In 1981, MRFV was detected in a significantly higher percentage of *D. maidis* than in *D. elimatus* (Table 3). In 1982, *D. elimatus* was infected with a significantly higher percentage of CSS than MRFV. Four of the 1982 leafhoppers (one female *D. elimatus*, one female *D. maidis*, and two male *D. maidis*) were positive for both MRFV and CSS.

Detection of MRFV in field-collected maize. Assays of leaf samples from the Mexican maize field in 1981 gave 35 of 100 positive for MRFV by ELISA. For the positive samples, mean $A_{405\ nm} = 0.275$ and SD = 0.163. For the negative, field-collected samples, mean $A_{405\ nm} = 0.072$, SD = 0.017, and $N = 60$. Five additional samples were scored positive but had mean $A_{405\ nm}$ values that were only slightly higher than the value for statistical significance. For these five samples, mean $A_{405\ nm} = 0.116$ and SD = 0.010.

Table 3. Estimated percentage ($\hat{p}$) of individual *Dalbulus maidis* and *D. elimatus* leafhoppers field-collected in Mexico that were positive for corn stunt spiroplasma (CSS) and maize rayado fino virus (MRFV) and number of each species tested by enzyme-linked immunosorbent assay (ELISA) for 1981 and 1982 collections

	CSS		MRFV			
	1982		1981		1982	
Species	$\hat{p}$	No. tested[a]	$\hat{p}$	No. tested[b]	$\hat{p}$	No. tested[c]
D. maidis	8.6 (±5)[d]	163	10.4 (±4)[d]	250	7.4 (±5)[d]	149
D. elimatus	11.0 (±4)	273	2.3 (±2)	214	2.3 (±2)	215
Totals	10.1 (±3)	436	6.7 (±2)	464	4.4 (±2)	364

[a] Numbers of *Dalbulus* leafhoppers according to species and sex: 126 male *D. maidis*, 37 female *D maidis*, 207 male *D. elimatus*, and 66 female *D. elimatus*.
[b] Numbers of *Dalbulus* leafhoppers according to species and sex: 223 male *D. maidis*, 27 female *D. maidis*, 167 male *D. elimatus*, and 47 female *D. elimatus*.
[c] Numbers of *Dalbulus* leafhoppers according to species and sex: 112 male *D. maidis*, 37 female *D. maidis*, 149 male *D. elimatus*, and 66 female *D. elimatus*.
[d] Number in parentheses when added to $\hat{p}$ yields the 95% confidence interval (CI) for the true percentage positive for the pathogen as calculated by $\hat{p} \pm [t\sqrt{\hat{p}(100-\hat{p})/(N-1)+1/2N}]$, where $\hat{p}$ = estimated percentage positive for pathogen, N = no. tested, and t = t value from table for $(N-1)$ degrees of freedom for 95% CI, as described by Cochran (1).

Table 4. Numbers of individual *Dalbulus* spp. field-collected in Mexico that were positive and negative for maize rayado fino virus (MRFV) and the mean absorbance ($A_{405\ nm}$) values for these leafhoppers and for the unexposed, laboratory-reared *D. maidis* as determined in individual microtiter plates of enzyme-linked immunosorbent assay (ELISA) for 1981 and 1982 collections

		ELISA for MRFV in field-collected *Dalbulus* spp. (no. of leafhoppers)		Mean $A_{405\ nm}$ for MRFV detection by ELISA		
				Field-collected leafhoppers		Unexposed laboratory-reared
Year	Plate no.	Positive	Negative	Positive	Negative	*D. maidis*
1981	1	14	66	0.500 (0.130)[a]	0.172 (0.004)[a]	0.167 (0.009)[a] (10)[b]
	2	2	78	0.786 (0.320)	0.190 (0.004)	0.206 (0.015) (10)
	3	1	79	0.426[c]	0.165 (0.005)	0.248 (0.065) (10)
	4	8	72	0.177 (0.017)	0.092 (0.002)	0.090 (0.005) (8)
	5	4	76	0.139 (0.008)	0.068 (0.002)	0.065 (0.005) (8)
	6	2	62	0.121 (0.001)	0.060 (0.001)	0.056 (0.004) (14)
1982	1	2	70[d]	0.374 (0.074)	0.132 (0.006)	0.149 (0.011) (18)
	2	1	71	0.458[c]	0.261 (0.010)	0.224 (0.017) (18)
	3	9	63	0.559 (0.051)	0.273 (0.008)	0.221 (0.017) (18)
	4	2	70	0.401 (0.008)	0.190 (0.006)	0.146 (0.019) (18)
	5	2	75	0.334 (0.019)	0.148 (0.007)	0.168 (0.014) (12)

[a] Number in parentheses is the standard error.
[b] Number of individual leafhoppers assayed.
[c] No standard error with only one positive sample.
[d] Number includes one *D. gelbus* leafhopper; the remainder were either *D. elimatus* or *D. maidis*.

Statistical evaluation of ELISA absorbance data. To characterize the underlying distribution of the $A_{405\ nm}$ values for the unexposed leafhopper controls in tests for CSS, values obtained from an unexposed leafhopper sample of 30 individuals were tested by Wilk's (W) statistic for normal distribution (13). The W statistic was greater than the 98th percentile and we concluded that there was not sufficient evidence to reject the null hypothesis that the distribution of the $A_{405\ nm}$ values was normal.

The W statistic (13) was also used to determine whether there was sufficient evidence to reject the hypothesis that the distributions of $A_{405\ nm}$ values for the 22 sets of unexposed leafhoppers listed in Tables 1, 2, and 4 plus the values for eight sets of unexposed *D. maidis* tested for MRFV by Gingery et al (4) were normal. For these samples, 22 of 30 samples did not show sufficient evidence to reject the null hypothesis of an underlying normal distribution. The remaining eight samples, for which the W statistic justified rejection of the null hypothesis, were right (positive) skewed. This implies that the mean was greater than the 50th percentile (median). Thus, in each of these eight samples, the mean plus three population standard deviations would be at a percentile less than that for a symmetric distribution, such as the normal distribution. This percentile decrease implies that for these samples, the error rate in this classification was probably larger than that assumed for the normal distribution.

DISCUSSION

Our lower limit of detection of purified CSS antigen was 25 ng/ml. Eden-Green (2) reported a minimum detection level of less than 10^5 CSS (about 1 ng of protein) per milliliter by ELISA from in vitro culture, and Raju and Nyland (9) were able to detect 0.01 μg (10^4–10^5 cells per milliliter) of CSS protein per milliliter of pure culture.

The fact that ELISA would not discriminate between intact CSS cells and cell fragments or proteins suggests that it may overestimate CSS titer, but this lack of discrimination should not affect detection of infected leafhoppers by ELISA.

We failed to confirm Eden-Green's (2) report of interference by *D. maidis* extracts when the equivalent of four leafhoppers per sample extract was assayed for CSS by ELISA. However, like Eden-Green, we were able to detect laboratory-acquired CSS in the equivalent of individual *D. maidis* leafhoppers without leafhopper extract interference. In our tests, the amount of leafhopper extract per assay was about two to three times that used by Eden-Green.

The relatively low $A_{405\ nm}$ values for field-collected compared with laboratory-exposed leafhoppers posed a problem in establishing CSS-positive values. The causes for these low values are unknown. The delay between collection and assay or the conditions for transport may have reduced CSS titer. Serological differences in the Mexican CSS may have decreased values, or simply a lower CSS titer for field-infected compared with laboratory-infected *Dalbulus* spp. may have been responsible. Also, the pathogenicity of CSS to *D. elimatus* (5) may have resulted in early death of severely infected individuals; these may have had higher CSS titers and would not have been assayed because of early death. However, CSS is not pathogenic to *D. maidis* and thus reduced longevity of infected individuals would not have been a factor in reduced values.

For MRFV-positive, field-collected leafhoppers, mean $A_{405\ nm}$ values were about two to four times those for unexposed controls compared with 2.5–3.33 times for MRFV-positive, laboratory-exposed *D. maidis* relative to unexposed controls (D. T. Gordon, *unpublished*). Data for the latter comparison were from a previous study (4) wherein leafhoppers also were assayed individually. Thus, unlike the results for CSS, the field-collected, MRFV-positive *Dalbulus* spp. had values comparable to those for the laboratory-exposed, positive leafhoppers.

In our study, we detected MRFV and CSS coinfecting four of 364 (1%) leafhoppers tested. Gamez (3) obtained a higher rate (8%) of dual infection of *D. maidis* by infectivity assay.

Because 100% of laboratory-exposed *D. maidis* and 80–100% of *D. elimatus* were positive for CSS by ELISA and infectivity assay, a high percentage of field-collected individuals of the two species was expected to be positive in ELISA. However, our data indicated that the CSS infection potential for these two *Dalbulus* spp. was not realized in the field.

Average MRFV infection rates for laboratory-exposed *D. maidis* were 12% for single leafhoppers ($N = 1,753$) assayed for infectivity (7), whereas leafhoppers assayed by ELISA in a previous study had a rate of 30% (4; D. T. Gordon, *unpublished*). Average MRFV infection rates determined by ELISA for field-collected *D. maidis* of 8.9% for the 2 yr of our study were considerably less than the laboratory rates. Similarly, ELISA detected 11 and 25% MRFV-infected *D. maidis* collected from early- and late-planted maize, respectively, in Costa Rica (12).

For Costa Rica, incidence of MRFV-infected maize was 22.6 and 41.7%, respectively, for the two plantings (12). For the Mexican maize in our study, incidence was 35–40%. Thus, the data for MRFV incidence in maize and *D. maidis* from Mexico and Costa Rica were similar.

Statistical analyses of ELISA values for detection of pathogens in vectors have assumed a normal distribution of the unexposed control $A_{405\ nm}$ values (4,10). Our demonstration of the reasonableness of the assumption of a normal distribution for most of our sets of negative control values and the positive skew of the distributions for these established an error rate that was probably less than that for the normal distribution. As pointed out in Materials and Methods, when the threshold value is established at three times the population standard deviation above the negative control means, the distribution of these negative control values determines the error rate that can range at most from 1.3% ($N = 30$) to 7% ($N = 8$) assuming a normal distribution or at most 11% with no distributional assumptions.

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