Detection of Diverse Maize Chlorotic Mottle Virus Isolates in Maize Seed

e-Xtra*

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Abstract

Maize chlorotic mottle virus (MCMV) has driven the emergence of maize lethal necrosis worldwide, where it threatens maize production in areas of East Africa, South America, and Asia. It is thought that MCMV transmission through seed may be important for introduction of the virus in new regions. Identification of infested seed lots is critical for preventing the spread of MCMV through seed. Although methods for detecting MCMV in leaf tissue are available, diagnostic methods for its detection in seed lots are lacking. In this study, ELISA, RT-PCR, and RT-qPCR were adapted for detection of MCMV in maize seed. Purified virions of MCMV isolates from Kansas, Mexico, and Kenya were then used to determine the virus detection thresholds for each diagnostic assay. No substantial differences in response were detected among the isolates in any of the three assays. The RT-PCR and a SYBR Green-based RT-qPCR assays were >3,000 times more sensitive than commercial ELISA for MCMV detection. For ELISA using seed extracts, selection of positive and negative controls was critical, most likely because of relatively high backgrounds. Use of seed soak solutions in ELISA detected MCMV with similar sensitivity to seed extracts, produced minimal background, and required substantially less labor. ELISA and RT-PCR were both effective for detecting MCMV in seed lots from Hawaii and Kenya, with ELISA providing a reliable and inexpensive diagnostic assay that could be implemented routinely in seed testing facilities.

Keywords: maize chlorotic mottle virus, maize lethal necrosis, diagnostics, seed health

Maize chlorotic mottle virus (MCMV; species *Maize chlorotic mottle virus*, genus *Machlomovirus*, family *Tombusviridae*) is a positive-sense single-stranded RNA virus first discovered in maize (*Zea mays* L.) from Peru (Castillo and Hebert 1974). MCMV is now endemic in a number of areas worldwide including the United States, Argentina, China, Democratic Republic of Congo, Ecuador, Ethiopia, Kenya, Mexico, Rwanda, Spain, Sudan, Tanzania, Uganda, Thailand, and Taiwan (reviewed in Redinbaugh and Stewart 2018). Although MCMV alone can cause production losses in maize, coinfection with any of several maize-infecting potyvirids results in the synergistic disease maize lethal necrosis (MLN), which has much greater economic impact (Redinbaugh and Stewart 2018). Annual losses caused by MLN in Kenya were estimated at 22% of production, with a value of about US$180 million per year (De Groote et al. 2016).

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MCMV can be transmitted to plants mechanically, by thrips (Cabanas et al. 2013; Jiang et al. 1992) and beetles (Nault et al. 1978), through soil (Mahuku et al. 2015; Phillips et al. 1982), and via seed (reviewed in Redinbaugh and Stewart 2018). Although MCMV is suspected of moving into MLN-free regions via seed, results demonstrating this are lacking. Because maize seed is frequently moved between regions, countries, and continents as food, feed, crop seed, and research material, the potential for MCMV movement via seed has increased demand for robust approaches for detecting the virus in seed lots.

Although a number of laboratory and commercial assays are available for detection of MCMV in leaf tissue, few have been specifically developed for detecting MCMV in seed (Redinbaugh and Stewart 2018), including a seed-adapted RT-qPCR assay (Zhang et al. 2011). Our objective was to develop or adapt sensitive and reliable ELISA, RT-PCR, and SYBR Green-based RT-qPCR (Stewart et al. 2017) assays for detecting the presence of MCMV in maize seed lots (as virus infectivity is not demonstrated here, we will use the term "presence" to refer to MCMV detected in and/or on seed). We compare the responses of three MCMV isolates, representative of the virus's genomic variability (Braidwood et al. 2018), in each of the assays to ensure they are broadly applicable.

Materials and Methods

Virus isolates and plant materials. Freeze-dried leaves from maize inoculated with the MCMV-Kenya (MCMV-KE) and MCMV-Mexico (MCMV-MX) isolates were delivered to Wooster, OH, and stored at –80°C prior to use. The isolates were developed from infected maize collected from the Marula farm at Naivasha, Kenya, and the International Maize and Wheat Improvement Center's research station in Tlaltizapan, Morelos, Mexico, respectively, and were maintained by serial passage to susceptible maize at each location. MCMV-Kansas (MCMV-KS; Niblett and Claflin 1978) was maintained in Wooster by serial passage on susceptible maize.

Seed of maize inbred line Oh28 was produced at the Ohio Agricultural Research and Development Center, Wooster, OH, where MCMV has not been reported. Commercially produced seed of sweet corn hybrids Spirit (Syngenta, Wilmington, DE) and Early Sunglow (Park Seed Company, Greenwood, SC) was assumed to be MCMV-free. Seed lots of maize grown in Kenya were purchased in local markets. Seed from Hawaii was provided by Agdia (Elkhart, IN).

Purification of MCMV isolates from Kansas, Kenya, and Mexico. To generate MCMV-infected leaf tissue, 100 seeds of maize inbred line Oh28 were planted as previously described (Louie 1986) and transferred to a growth chamber with 12-h/12-h light/dark (600 μmol/m^2/s) and 25°C/21°C temperature cycles. At 7 days postplanting, the first two leaves of each plant were inoculated with one of the three MCMV isolates as described (Louie 1986). Symptomatic leaf tissue (70 g) was collected at 30 days postinoculation and used for virus purification by a minor modification of the method developed for panicum mosaic virus (PMV; Niblett and Paulsen 1975) in which differential centrifugation of the virus suspension was replaced with centrifugation at 158,420 × *g* for 260 min. Viral protein concentration was estimated with the Bio-Rad Protein Assay Kit using bovine serum albumin as a standard (Hercules, CA) according to the manufacturer's instructions. Virion purity was assessed by SDS-PAGE and Western blot analysis as previously described (Redinbaugh et al. 2002). Purified virus samples were aliquoted, frozen in liquid nitrogen, and stored at –80°C until use. RNA was extracted from purified virions using the Direct-zol RNA MiniPrep Plus kit (Zymo Research Corporation, Irvine, CA) following the manufacturer's instructions. RNA concentrations were estimated using a NanoDrop (Thermo Scientific, Wilmington, MA).

ELISA. A commercial DAS-ELISA kit (Agdia) was used for MCMV detection, with some modification of the manufacturer's protocol. Briefly, 96-well plates were incubated overnight at 4°C with capture antibody (1:200 v/v with coating buffer) and then washed three times with phosphate-buffered saline containing Tween 20 (PBST, Agdia). Samples (100 μl per well), prepared as outlined below, were applied to two replicate wells, and the plates were incubated overnight at 4°C in a closed plastic box containing a moist paper towel. Plates were then washed seven times with PBST, incubated with the alkaline phosphatase enzyme conjugate for 1 h at 37°C, and washed six times before adding alkaline phosphatase substrate. The absorbance of samples at 405 nm (A_{405}) was determined at room temperature at 20 and/or 60 min after addition of substrate using a FilterMax F5 Multi-Mode Microplate reader (Molecular Devices, San Jose, CA). Samples were considered positive if the average sample absorbance was greater than twice the mean absorbance of healthy controls. General extraction buffer (GEB, Agdia) and extracts from leaves of MCMV-KS infected plants (Early Sunglow or Spirit) served as negative and positive controls for ELISA performance, respectively.

Limit of detection (LoD) for MCMV in ELISA. Serial dilution of purified virions was used to determine the LoD. Twenty seeds of an MCMV-free commercial sweet corn hybrid (Early Sunglow or Spirit) were incubated overnight at room temperature with 20 ml of GEB. The seed plus GEB was vortexed for 20 s, and 200 μl of liquid was taken directly as the MCMV-free "seed soak solution" (SSS). MCMV-free seed extracts (SExt) were then prepared by grinding the remaining liquid and seeds with a Conair 710-ml food processor (Cuisinart, Stamford, CT) for 30 s. Purified virions were added to SSS and SExt to produce virion protein concentrations of 10^{-3} to 10^{-6} μg of virion protein per 100 μl. MCMV-free SSS and SExt served as healthy controls.

LoD for MCMV in one-step RT-PCR. Serial dilution was used to determine the detection limit for MCMV by RT-PCR. As a control for potential background amplification or amplification inhibition from seed components, 20 Early Sunglow or Spirit seeds were incubated overnight at room temperature in 10 ml of distilled water and then vortexed for 20 s, and an aliquot (2 μl) was taken directly as the seed soak water (SSW). The seed soak RNA (SSR) was isolated from 250 μl of SSW with the Direct-zol RNA Miniprep Plus kit (Zymo Research Corporation) following the manufacturer's protocol. RNA (1 μl) isolated from MCMV-KS, -MX, or -KE virion preparations was mixed with 2 μl of either SSR or SSW for RT-PCR to give 1 fg to 1 ng of RNA isolated from virions. One-step RT-PCR was carried out with primers MCMV-2452F (5′-AGTGGAGGTAGGCAGAGTCA-3′) and MCMV-3111R (5′-TCCAACAGCAATGTTTTCCA-3′) designed to amplify a 660-bp region of the MCMV replicase gene. Reactions (25 μl) contained 3 μl of sample, 1× GoTaq Buffer, 1.25 U of GoTaq DNA polymerase (Promega, Madison, WI), 5 mM DTT, 800 nM of each primer, 200 μM dNTPs, 4 U of RNAseOUT (Invitrogen, Carlsbad, CA), and 7 U of Superscript III (Invitrogen). Thermal cycling was carried out at 55°C for 40 min, 94°C for 2 min, followed by 33 cycles at 94°C for 15 s, 55°C for 20 s, and 72°C for 40 s. A final elongation at 72°C for 7 min was done. Amplicons were analyzed in a 1% agarose gel containing ethidium bromide. Three independent experiments were performed, and these produced similar results for each virus isolate.

To test the specificity of the primers, maize leaf tissue was collected from individual maize plants infected with wheat mosaic virus (WMoV, Stewart et al. 2013b), Johnsongrass mosaic virus (JGMV, Stewart et al. 2013a), maize chlorotic dwarf virus (MCDV, Chaouch-Hamada et al. 2004), maize dwarf mosaic virus (MDMV, Stewart et al. 2012), maize fine streak virus (MFSV, Tsai et al. 2005), maize necrotic streak virus (MNeSV, Louie et al. 2000), maize rough dwarf virus (MRDV, Louie and Abt 2004), maize rayado fino virus (MRFV, Zambrano et al. 2013), sugarcane mosaic virus (SCMV, Jones et al. 2011), and wheat streak mosaic virus (WSMV, Jones et al. 2011). Total RNA was isolated from leaf tissue with the Direct-zol RNA MiniPrep Plus kit, and 5 ng of RNA for each sample was used for RT-PCR as outlined above. Three independent experiments produced similar results.

LoD for MCMV in RT-qPCR. The LoD for MCMV by RT-qPCR was determined using serial dilution of MCMV RNA (1 fg to 10 pg) added to 1 μl of total RNA isolated from MCMV-free seed (Spirit) as outlined above. RT-qPCR was carried out as described (Stewart et al. 2017), with duplicate or triplicate technical replications for each qPCR reaction run on a CFX96 Touch (Bio-Rad). Each isolate was evaluated in three independent experiments. Cq values, amplification curves, and melt curves were determined using CFX Manager 3.1 (Bio-Rad). The standard curve and its equation and the amplification efficiency were calculated considering only data in the linear dynamic range (Kralik and Ricchi 2017; Papic et al. 2017). The LoD was the lowest amount of RNA for which MCMV was detected in all replicates across experiments. Assay variability was assessed through the intra- and interassay coefficients of variation and the limit of quantification, defined as the lowest concentration above or equal to the LoD with a coefficient of variation less than 25% (Kralik and Ricchi 2017).

MCMV detection in seed lots. Twenty seeds from each of 10 lots from Kenya and Hawaii were soaked overnight at room temperature in 20 ml of GEB, and the SSS and SExt were produced and evaluated for the presence of MCMV by ELISA as outlined above. For RT-PCR, 20 seed from 10 Kenyan seed lots and 10 seed from eight Hawaiian seed lots (the lower number of seed reflects limited seed availability) were soaked overnight in 0.5 ml of sterile water per seed. After vortexing for 20 s, the SSW (3 μl) was directly tested for the presence of MCMV by RT-PCR as outlined above.

To test detection of a single MCMV-infested seed in sublots by ELISA, 100 g of MCMV-free seed (Early Sunglow) was weighed into 0.95-liter Mason jars (Ball, Broomfield, CO), and one seed from lot KE-G was added to each jar. The jar was then shaken by hand for 20 s. GEB (150 ml) was added, and the samples were incubated on an orbital shaker at 200 rpm overnight at room temperature (20 to 23°C). After removing aliquots for the SSS, the SExt was prepared in a 3.3-liter food processor as outlined above. Samples were stored at 4°C prior to ELISA as outlined above. Between samples, processor parts contacting the samples were washed in 1% D-256 One-Step Disinfectant (VEDCO, St. Joseph, MO) and then rinsed with water. In separate experiments, a single MCMV-positive seed was mixed with 100 g of MCMV-free seed as above and then ground to a powder in a coffee grinder. Ten grams of dry ground seed was added to 15 ml of 1× GEB and mixed by vortexing. The extract was used directly for ELISA as outlined above.

Statistical analyses. A linear mixed-model analysis of variance using the GLIMMIX procedure in SAS 9.4 (SAS, Cary, NC) was used to examine the effects of seed background and the time of absorbance measurement on ELISA responses, with experiment considered as a random effect. A_{405} determined at 20 and 60 min after substrate addition was log-transformed prior to analysis. Differences

between treatments were assessed using the *lsmeans* statement in Proc GLIMMIX.

Results

MCMV purification. MCMV isolates from Kansas (KS; Niblett and Claflin 1978), Mexico (MX), and Kenya (KE; Wangai et al. 2012) were purified using a procedure previously developed for PMV, the tombusvirid most closely related to MCMV. Protein concentration in purified virion preparations was estimated at 2.6 to 7.0 μg/μl. SDS-PAGE indicated a single protein band at 25 kDa in all three virion preparations. A band of this size reacted strongly with polyclonal antisera raised against MCMV-KS on Western blots (data not shown). RNA isolated from the purified virions yielded from 0.2 to 0.5 μg of RNA per μg of virion protein. Infectivity of the purified virion preparations was verified by leaf-rub inoculation of an aliquot (0.5 ng of virion protein) to susceptible maize prior to use (data not shown). The virion preparations and isolated viral RNA were used to evaluate the sensitivity and specificity of ELISA, RT-PCR, and RT-qPCR for the detection of MCMV.

Specificity and sensitivity of ELISA for detection of MCMV isolates. Preliminary experiments indicated that MCMV could be detected in both the buffer used to soak seed (SSS) and in seed extracts (SExt) from MCMV-infested seeds (data not shown). Because seed components may interfere with virus detection in ELISA (Albrechtsen 2006), the effects of SSS and SExt produced from MCMV-free seed relative to GEB and healthy maize leaf samples on ELISA absorbance were tested. No difference in ELISA absorbance for GEB and SSS for maize hybrids (Early Sunglow and Spirit) was detected at 20 or 60 min after addition of alkaline phosphatase substrate ($P > 0.05$, Fig. 1). However, ELISA absorbances at 405 nm of SExt from this MCMV-free seed were greater than the buffer control ($P < 0.001$) at both 20 min and 60 min, with mean SExt absorbance being more than twice that of GEB after 60 min ($P < 0.001$). Differences in ELISA background absorbance were detected among maize hybrids and inbred lines from the United States and East Africa, but these were not related to seed origin or hybrid versus inbred character (Supplementary Table S1). Leaves from healthy control plants, freeze-dried samples of which are frequently used as negative controls for commercial ELISA, were not included in these experiments. However, in separate experiments, no differences in absorbance were detected between GEB and extracts of healthy leaf tissue 20 min after addition of color reagent (Supplementary Table S2).

Serial dilution of purified virions was used to determine the LoD for MCMV isolates using ELISA. In these experiments, SSS and SExt were produced using MCMV-free seed from two maize hybrids, and samples were considered positive if the ELISA absorbance

Table 1. Limit of detection (LoD) for three maize chlorotic mottle virus (MCMV) isolates using ELISA[a]

		μg of protein detected			
		20 min[c]		60 min	
Isolate	**Background[b]**	**SSS**	**SExt**	**SSS**	**SExt**
Kansas	Early Sunglow	10^{-5}	10^{-5}	10^{-6}	10^{-5}
	Spirit	10^{-4}	10^{-4}	10^{-6}	10^{-5}
Mexico	Early Sunglow	10^{-5}	10^{-4}	10^{-5}	10^{-5}
	Spirit	10^{-4}	10^{-4}	10^{-5}	10^{-5}
Kenya	Early Sunglow	10^{-6}	10^{-6}	10^{-6}	10^{-6}
	Spirit	10^{-6}	10^{-6}	10^{-6}	10^{-6}

[a] The LoD was the lowest amount of viral protein for which all replicates in three independent experiments had an absorbance at 405 nm greater than twice the mean absorbance of the negative controls.

[b] Hybrid seed used to generate the background seed soak solution (SSS) and seed extract (SExt) for ELISA.

[c] Time after addition of alkaline phosphatase substrate at which absorbance value was taken.

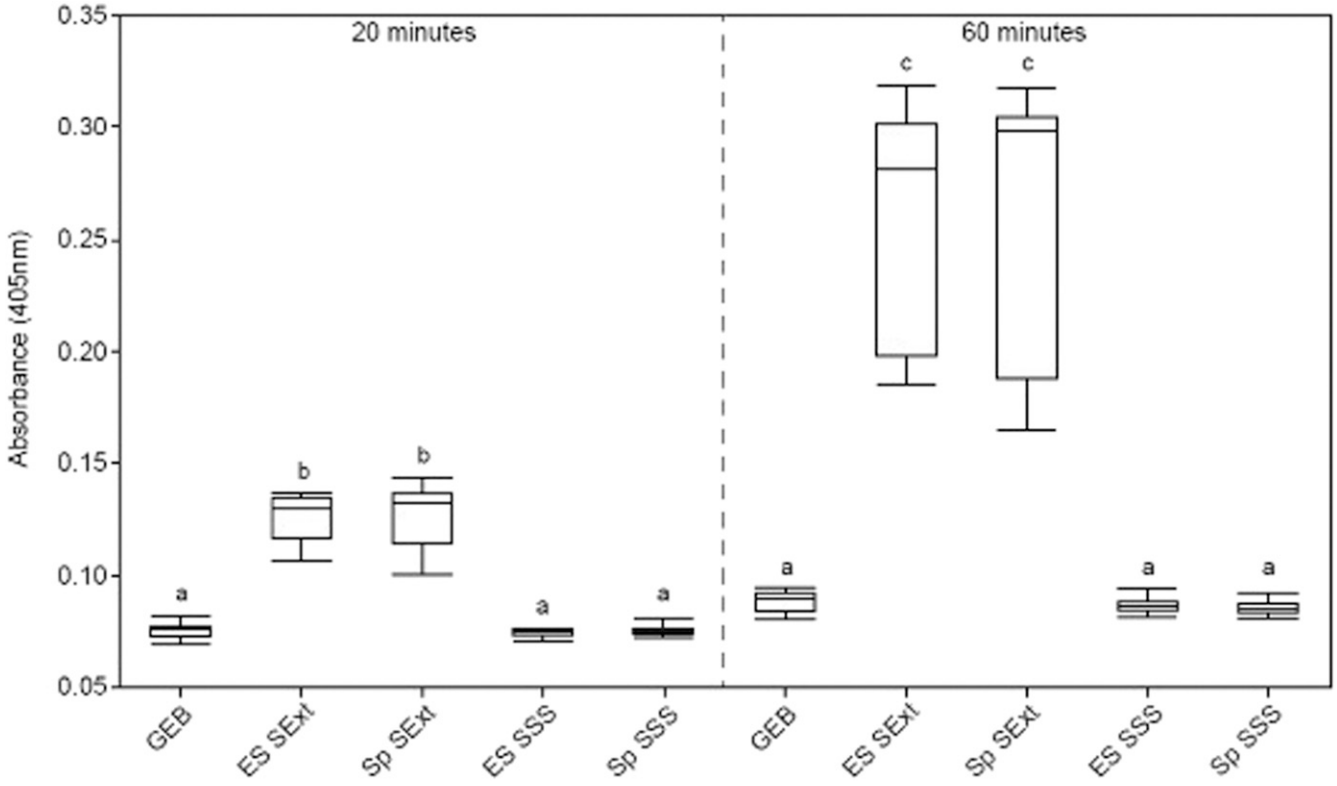

Fig. 1. The effect of maize seed soak solution (SSS) and extract (SExt) on ELISA background. SSS and SExt were generated from maize chlorotic mottle virus-free seed for two sweet corn hybrids and used in ELISA as outlined in the Materials and Methods. Sample absorbances were determined at 20 and 60 min after addition of substrate. GEB = general extraction buffer; ES = Early Sunglow; and Sp = Spirit. Box plots represent the absorbance at 405 nm for 24 samples from three independent experiments. Boxes with the same letter are not different ($P > 0.05$ in ANOVA of log-transformed data).

was more than twice that of control samples with no added MCMV. For MCMV-KE, ELISA detected 1 pg of virion protein in both the SSS and SExt at both 20 and 60 min after adding color reagent (Table 1). For MCMV-MX and MCMV-KS, the LoD was 10 to 100 pg after 20 min and 1 to 10 pg after 60 min.

Specificity and sensitivity of one-step RT-PCR for detection of MCMV isolates. To alleviate problems observed with background amplification of maize sequences after RT-PCR with previously designed primers (Wangai et al. 2012) (data not shown), primers MCMV-2452F and MCMV-3111R were designed to amplify a 660-bp conserved portion of the MCMV replicase open reading frame. The specificity of these primers in RT-PCR was tested against RNA from the three MCMV isolates, another maize-infecting tombusvirid (MNeSV), and nine phylogenetically unrelated maize-infecting viruses (WMoV, JGMV, MCDV, MDMV, MFSV, MRDV, MRFV, SCMV, and WSMV). RT-PCR produced the expected 660-bp amplicon for all three MCMV isolates but not for any of the other 10 viruses tested (Fig. 2).

Serial dilution of RNA isolated from purified virions was used to estimate the LoD for the three MCMV isolates using one-step RT-PCR. For MCMV-KS diluted into SSW or SSR, amplicons were clearly detected using 10 fg of viral RNA, with faint amplification observed using 1 fg of viral RNA (Fig. 3). Similar results were obtained in each of three independent experiments for each of the three virus isolates (data not shown).

LoD for MCMV by RT-qPCR. Serial dilution of viral RNA was used to determine the LoD for MCMV for the SYBR Green-based RT-qPCR assay developed by Stewart and coauthors (2017). Final LoD were 1 fg, 1 fg, and 10 fg for MCMV-KS, MCMV-MX, and MCMV-KE, respectively (Table 2). Amplification was detected at lower concentrations for some samples of each isolate, with coefficients of variation for the lowest levels of RNA detected of less than 25%. These data indicate that the limit of quantification for each isolate corresponded to the LoD (Supplementary Table S3).

MCMV detection in seed lots. Seed harvested from MCMV-infected maize from Hawaii and seed purchased from local markets in MLN-endemic areas of Kenya were tested for the presence of MCMV using ELISA and RT-PCR. For ELISA, the SSS and SExt were both tested, and the SSW was used for RT-PCR. Seed from maize raised in Ohio (Oh28), used as a control, was negative for MCMV by both RT-PCR and ELISA (Table 3). Two seed lots from Hawaii (HI-A and HI-B) were consistently negative for MCMV by ELISA and RT-PCR (Table 3). With one exception (one of three SSS samples of lot HI-C), the remaining seed lots from Hawaii were uniformly positive for MCMV by both ELISA and RT-PCR. With one exception (KE-E), seed lots purchased in Kenya tested either negative (KE-A, KE-B, and KE-C) or positive (KE-D, KE-F, KE-G, KE-H, KE-I, and KE-J) for MCMV in all three experiments by ELISA. RT-PCR tests varied among experiments for two seed lots from Kenyan markets (KE-D and KE-I) and produced negative results in all experiments for two seed lots (KE-H and KE-J) that were

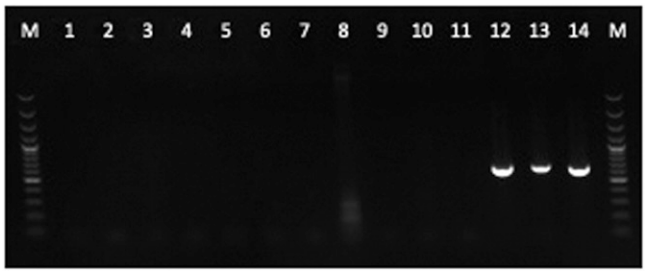

Fig. 2. Specificity of RT-PCR primers for detection of maize chlorotic mottle virus (MCMV). RT-PCR using primers MCMV-2452F and MCMV-3111R was carried out with 5 ng of total RNA isolated from leaves of maize plants infected with the following: lane 1 = wheat mosaic virus; lane 2 = Johnsongrass mosaic virus; lane 3 = maize chlorotic dwarf virus; lane 4 = maize dwarf mosaic virus; lane 5 = maize fine streak virus; lane 6 = maize necrotic streak virus; lane 7 = maize rough dwarf virus; lane 8 = maize rayado fino virus; lane 9 = sugarcane mosaic virus; lane 10 = wheat streak mosaic virus; lane 11 = water control; lane 12 = MCMV-KE; lane 13 = MCMV-KS; lane 14 = MCMV-MX; and M = 100-bp DNA ladder.

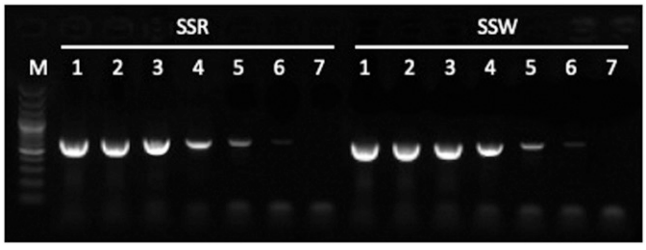

Fig. 3. Sensitivity of RT-PCR for detection of maize chlorotic mottle virus (MCMV). RT-PCR was carried out using serial dilution of RNA purified from MCMV-KS virions into seed soak RNA (SSR) and seed soak water (SSW) prepared as outlined in the Materials and Methods. M = 100-bp DNA ladder; lane 1 = 100 pg of MCMV RNA; lane 2 = 10 pg; lane 3 = 1 pg; lane 4 = 100 fg; lane 5 = 10 fg; lane 6 = 1 fg; and lane 7 = water control.

Table 2. Limit of detection (LoD) for three maize chlorotic mottle virus (MCMV) isolates using RT-qPCR

	LoD for MCMV RNA (ng)[a]			
MCMV isolate	**Exp 1**	**Exp 2**	**Exp 3**	**Final[b]**
Kansas	10^{-6}	10^{-7}	10^{-8}	**10^{-6}**
Mexico	10^{-6}	10^{-7}	10^{-6}	**10^{-6}**
Kenya	10^{-5}	10^{-6}	10^{-5}	**10^{-5}**

[a] For each experiment (Exp), the LoD is the lowest amount of RNA for which MCMV was detected in all replicates.
[b] The final LoD is the lowest amount of RNA for which MCMV was detected in all replicates across experiments and is indicated in bold.

Table 3. Detection of maize chlorotic mottle virus (MCMV) in maize seed lots with ELISA and RT-PCR

		ELISA		
Lot	**Origin**	**SSS[a]**	**SExt**	**RT-PCR, SSW**
Oh28	Ohio (USA)	0/3[b]	0/3	0/3
KE-A	Kenya	0/3	0/3	0/3
KE-B	Kenya	0/3	0/3	0/3
KE-C	Kenya	0/3	0/3	0/3
KE-D[c]	**Kenya**	**3/3**	**3/3**	**2/3**
KE-E	**Kenya**	**1/3**	**1/3**	**3/3**
KE-F	Kenya	3/3	3/3	3/3
KE-G	Kenya	3/3	3/3	3/3
KE-H	**Kenya**	**3/3**	**3/3**	**0/3**
KE-I	**Kenya**	**3/3**	**3/3**	**2/3**
KE-J	**Kenya**	**3/3**	**3/3**	**0/3**
HI-A	Hawaii	0/3	0/3	0/3
HI-B	Hawaii	0/3	0/3	0/3
HI-C	**Hawaii**	**2/3**	**3/3**	**NT[d]**
HI-D	Hawaii	3/3	3/3	NT
HI-E	Hawaii	3/3	3/3	3/3
HI-F	Hawaii	3/3	3/3	2/2
HI-G	Hawaii	3/3	3/3	3/3
HI-H	Hawaii	3/3	3/3	3/3
HI-I	Hawaii	3/3	3/3	3/3
HI-J	Hawaii	3/3	3/3	3/3

[a] SSS = seed soak solution; SExt = seed extract; and SSW = seed soak water.
[b] Number of MCMV-positive samples/the number of samples tested in three independent experiments. For ELISA, samples were considered positive if the absorbance at 405 nm 20 min after addition of color reagent was greater than twice the healthy controls. For RT-PCR, samples were considered positive if an amplicon was detected after agarose gel electrophoresis and visualization under UV light.
[c] Bold indicates seed lots for which inconsistencies in detection were found among detection methods.
[d] NT = not tested due to limited seed availability.

positive for MCMV by ELISA in all experiments. The sequences of amplicons from samples HI-H, HI-F, and KE-G were those expected for isolates from Hawaii and Kenya, respectively (data not shown).

A lack of remnant seed prevented assessing whether inconsistencies in ELISA results for KE-E and HI-C were due to lower rates of MCMV presence or assay insensitivity. Therefore, the sensitivity of ELISA for detection of a single infested seed harvested from MCMV-inoculated plants in a 100-g sample (approximately 700 seeds) of MCMV-free seed was assessed. For each of 46 samples tested in three independent experiments, both the SSS and SExt were positive for MCMV. None of 10 SSS or SExt samples consisting of MCMV-free seed alone were positive. The ratios of mean absorbance for samples containing an infested seed to samples containing MCMV-free seed alone were greater than 10 for both the SSS and SExt (Supplementary Table S4), suggesting ELISA was sufficiently sensitive to detect a single positive seed in the tested samples (Table 3). Because seed quality testing labs may grind maize seed samples for use in multiple assays, the sensitivity of ELISA for detecting one MCMV-infected seed in 100-g dry-ground seed samples was also tested. In these experiments, 23 of 24 samples from three independent trials were positive for MCMV, with none of 12 control samples being positive.

Discussion

Although MCMV isolates from around the world have a high degree of genome sequence identity (Braidwood et al. 2018; Redinbaugh and Stewart 2018), differences in virus detection among laboratories (Adams et al. 2013; Mahuku et al. 2015), among samples collected from different regions, or using different antibodies or primer pairs have led to speculation that ELISA and RT-PCR might not be useful for routine detection of MCMV (Adams et al. 2013; Fentahun et al. 2017). Therefore, we used isolates from Kenya, Mexico, and the United States to assess differences in the utility of ELISA, RT-PCR, and RT-qPCR detection of MCMV. These three isolates have 96 to 98% sequence identity and are representative of genomic diversity in the virus species (Braidwood et al. 2018; Redinbaugh and Stewart 2018). MCMV-HI (Table 3) shares 96% sequence identity with the other three isolates. Coat protein amino acid sequences for the four isolates are 99 to 100% identical, similar to the 99 ± 1% (mean ± standard deviation) sequence identity found for isolates from Asia, Africa, North America, and South America. The high degree of similarity among the isolates and the largely similar responses found for the three isolates in ELISA, RT-PCR, and RT-qPCR indicate that all three diagnostic approaches are useful for detecting the presence of MCMV on seed.

Selection of biologically similar negative controls (healthy tissue) can be important for avoiding false positive and negative interpretations of ELISA (Sutula et al. 1986). In this study, mean ELISA absorbance for extracts (SExt) of MCMV-free seed was 1.7 and 3 times greater than that for extraction buffer (GEB) at 20 and 60 min after adding color reagent, respectively (Fig. 1). Maize seed genotype may also influence background development for SExt (Supplementary Table S1). These results indicate the need for including appropriate MCMV-free controls if seed extracts are used for ELISA, especially if longer color reagent incubation times are used. Although genetically similar negative controls may not be readily available in areas where the virus is endemic, commercially produced negative controls derived from MCMV-free seed are becoming available. In contrast, mean absorbances for SSS samples were similar to those for MCMV-free leaf tissue and GEB (Supplementary Table S2), and ELISA was similarly sensitive for SSS and SExt samples. The SSS is recommended for sample preparation for detecting MCMV on seed by ELISA because of its low background, similar sensitivity to extracts, and reduced labor requirements.

The new primer pair for MCMV detection by RT-PCR, designed to alleviate problems with background amplification of maize sequences, has 100% identity with the corresponding regions of all 49 MCMV genome sequences evaluated including isolates from the Americas (including Hawaii), Asia, and Africa. RT-PCR using the primer pair specifically amplified an approximately 660-bp fragment of the viral replicase from all three MCMV isolates and did not amplify similar fragments from any of 10 other maize-infecting viruses, including another tombusvirid. RT-PCR produced clearly detected amplicons using 10 fg of input RNA for each of the three MCMV isolates (Fig. 3). The RT-PCR assay here used either the buffer or water used to soak seed samples, avoiding the labor and materials required for RNA isolation. MCMV-free seed and RNA-free controls are important for detecting any inadvertent contamination. Inclusion of a control for the detection threshold and confirmation of isolate identity by sequence analysis may also be warranted.

The LoD, or the lowest amount of RNA for which MCMV was detected in all replicates across experiments, was similar for the SYBR Green-based RT-qPCR and RT-PCR assays (Tables 2 and 4). Like RT-PCR, RT-qPCR is susceptible to laboratory contamination and requires MCMV-free seed and RNA-free controls. Highly specific TaqMan-based RT-qPCR assays for MCMV are also available, and these have sensitivities similar to or greater than that of the SYBR Green assay used here (Adams et al. 2013; Liu et al. 2016; Zhang et al. 2011). The RT-qPCR assays use RNA purified from seed, which may increase cost and time per sample required. However, these assays can also be more amenable to automation than RT-PCR assays that require gel analysis.

ELISA and RT-PCR were used to examine remnant seed lots from Hawaiian research plots and Kenyan markets for the presence of MCMV. With two exceptions (HI-C and KE-E), ELISA detected virus presence or absence of MCMV identically in three experiments using SSS and SExt from seed lots from Hawaii and Kenya (Table 3). It is possible that the inconsistency of MCMV detection in seed lots HI-C or KE-E reflected an uneven distribution of the virus in these lots or a lack of assay sensitivity. Insufficient seed was available to test for virus distribution in the small seed lot; however, MCMV was detected by ELISA in 46 of 46 samples in which a single seed from MCMV-positive lot KE-G was mixed into 100 g of MCMV-free seed, suggesting the inconsistency was not the result of assay insensitivity.

RT-PCR produced similar results to ELISA for lots of seed from MCMV-infected maize plants from Hawaii. However, differences were observed between ELISA and RT-PCR for detecting the presence of MCMV for two seed lots purchased in Kenyan markets (KE-H and KE-J). In addition, RT-PCR did not detect the virus in all three experiments for two other seed lots (KE-D and KE-I). These inconsistencies could reflect the smaller number of seeds used for the RT-PCR analysis (10 versus 20 seeds for ELISA), a lack of homogeneity in samples purchased in markets, lower stability of viral RNA relative to viral coat protein, and/or variability in the viral RNA sequence. Although the limited supply of seed from these lots prevented retesting for the cause of the observed variability, it is less likely that poor primer binding was the cause of RT-PCR variability, because the primers were 100% complementary to the target sites in

Table 4. Assay detection thresholds and costs for maize chlorotic mottle virus (MCMV) detection

Assay	Material detected	Cost/sample[a]	Detection limit	
			μg[b]	Virions[c]
ELISA	Coat protein	$0.94	1.00E-04	13,300,000
RT-PCR	RNA	$4.97	1.00E-08	4,100
RT-qPCR	RNA	$10.72	1.00E-08	4,100

[a] Approximate cost of reagents per sample. Costs were calculated based on U.S. market costs as of February 2020. Reagents included in calculation (at list price): ELISA, reagent set MCMV (5,000 test wells, Agdia) and buffer pack DAS/TAS direct ELISA (5,000 test wells, Agdia); RT-PCR, OneTaq one-step RT-PCR kit (30 reactions, NEB), PCR tubes; RT-qPCR, Sso Fast EvaGreen Supermix (5,000 reactions, Biorad), iScript cDNA synthesis 500 reactions (Biorad), Directzol RNA miniprep (200, (Biorad), low profile PCR plates (50, Bio-Rad), plate sealers (100, Bio-Rad).

[b] Lowest amount of coat protein or RNA always detected across isolates in each assay.

[c] Theoretical number of virions detected based on virion RNA and coat protein contents of 2.4E-12 and 7.5E-12 μg/virion, respectively.

49 genome sequences representing the diversity of known isolates. Given the similar results for detection of MCMV for ELISA and RT-PCR in seed from plants known to be infected with MCMV, non-homogeneous distribution of contaminated seed in lots purchased in a market is a likely explanation for the variable RT-PCR results. This points to the need for development of robust subsampling strategies to test for the presence of MCMV in large seed lots for which the infection status of the mother plants is not known.

The ELISA, RT-PCR, and RT-qPCR assays used here detect the presence of MCMV in seed with sensitivity and specificity. Rough calculations for a virion consisting of a viral RNA of 4,438 bp and 180 coat protein subunits (25,158 kDa) gives an estimate of 2.4E-12 and 7.5E-12 μg/virion, respectively, consistent with previous estimations of a virion with 25% RNA and 75% protein (Goldberg and Brakke 1987). These calculations indicated that RT-PCR and RT-qPCR were several orders of magnitude more sensitive than ELISA on a "per virion" basis (Table 4). However, all three diagnostic assays detect viral components, and do not distinguish infectious from non-infectious virus. The ability to detect a single MCMV-infested seed in a 100-g (700 seeds) sample indicates ELISA is sufficiently sensitive for most seed diagnostic applications. ELISA also has the advantages of having the lowest materials cost per sample (Table 4), using reagents that are stable for shipping and storage at 2 to 8°C, requiring less specialized equipment than the molecular assays and not requiring specialized training. However, the assay takes longer to run than the molecular assays. Although RT-PCR and RT-qPCR are significantly more sensitive and take less time than ELISA, these have a higher cost per sample, require technically skilled personnel, use reagents with specific shipping and storage requirements, and need specialized equipment. All three approaches are amenable to laboratory automation. The excellent sensitivity, low cost, and lower resource requirements of the ELISA make it a great choice for many seed testing applications. However, RT-PCR or RT-qPCR might better fit some laboratories' needs if, for example, simultaneous detection of multiple pathogens, very high sensitivity, maximum throughput, or high labor costs are major considerations. Other diagnostic platforms for MCMV detection are available, including reverse transcription loop-mediated isothermal amplification (Chen et al. 2017) and ImmunoStrips (Agdia), but these diagnostics are lower throughput and would require adaptation for use with seed. The three diagnostic approaches for detecting the presence of MCMV in seed samples presented here provide the basis for future research on MLN epidemiology and for the potential development of accredited standardized diagnostic protocols for MCMV detection by seed testing laboratories.

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