RT-PCR Method for Detecting Cowpea Mottle Carmovirus in *Vigna* Germ Plasm

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ABSTRACT

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A highly sensitive reverse transcription-polymerase chain reaction (RT-PCR) method was developed to detect cowpea mottle carmovirus (CPMoV) in newly acquired germ plasm of *Vigna* spp. It detected virus in tissues diluted up to 10^{-9} . The preferred primers were designed from the RNA replicase cDNA sequence of CPMoV. These primers were able to detect CPMoV in plants infected with 10 different isolates of the virus. There were no cross-reactions with either bean mild mosaic or melon necrotic spot carmoviruses or any of the common cowpea viral pathogens tested. The RT-PCR method was up to 10^5 times more sensitive than direct antigen coating enzyme-linked immunosorbent assay (DAC-ELISA) in detecting CPMoV. The RT-PCR method gave no false positive reaction as is sometimes seen with ELISA.

Cowpea mottle carmovirus (CPMoV) was first described from Nigeria (9) as a causal agent of a serious disease of cowpea (*Vigna unguiculata* (L.) Walp. subsp. *unguiculata*) and bambarra groundnut (*V. subterranea* (L.) Verdc.). Cowpea yield reductions of more than 75% were reported from Nigeria (9). The virus continues to occur throughout Nigeria, where it was found in 8 to 10% of cowpea samples surveyed (11). It is readily transmitted by sap inoculations and by several chrysomelid beetle vectors (4,12). The virus is transmitted in the seed of cowpeas at a rate up to 10% depending on the genotype of the line and the time between infection and flowering (4,10), but many lines produced no more than 0.4% infected seeds (1). Because it is seed transmitted and has potentially serious effects once established in a growing area, CPMoV is of great importance in international exchange of *Vigna* germ plasm and in germ plasm collections.

Gillaspie et al. (5) described a procedure for detecting and eliminating exotic seed-borne viruses from cowpea seed lots. It involves growing seedlings from each seed lot, testing for viruses by enzyme-linked immunosorbent assay (ELISA), and then growing the healthy mother plants to produce virus-free seed that can be increased for storage and distribution. A shortcoming of this method for CPMoV is that with

some cowpea genotypes, there are occasional borderline ELISA reactions. The virus could not be isolated from many of these plants by bioassay on cowpea indicator plants.

The genome of CPMoV is a positive sense ssRNA of 4,029 nucleotides with six major open reading frames (ORFs) (14). ORF2 encodes the putative RNA replicase (REP) and ORF5 encodes the capsid protein (CP). To establish a more specific and sensitive test for CPMoV, primers were designed corresponding to both the REP and the CP coding regions of the nucleic acid and were evaluated for use in a reverse transcription-polymerase chain reaction (RT-PCR) detection method.

MATERIALS AND METHODS

Virus isolates and propagation. A number of isolates of CPMoV were used to develop the method and to test the efficacy for different isolates. Virus isolates ISU, AT, Mokwa, Ibadan, Moor Plantation, and Bambarra groundnut came from Nigeria, and isolates 23-48/96, 23-49/96, 23-50/96, and 23-83/96 came from Togo. We had five isolates of Mokwa and six each of Ibadan, Moor Plantation, and Bambarra groundnut. Isolate ISU has been described and sequenced (6,10,14) and is the one from which the primers were designed. Two additional carmoviruses, bean mild mosaic virus (BMMV) and melon necrotic spot virus (MNSV), were supplied by V. Hari and D. M. Rochon, respectively, and were used to evaluate the specificity of the primers.

Crude extracts of CPMoV isolates were inoculated to cowpea cv. Early Ramshorn grown at 27°C for 12 h light and 23°C for 12 h dark in pots of MetroMix 300. For production of seeds from infected plants, two pots of five seedlings each of PI

582567, 582568, 582575, 582658, 582659, 582660, 582661, 583521, and Early Ramshorn were inoculated with isolate ISU at the primary leaf stage and grown until mature seeds were harvested. California Blackeye no. 5 seedlings were inoculated as long as 4 weeks after the primary leaf stage and became infected. After drying for 7 to 10 days, seeds were sown in flats of 50 each, and the seedlings were grown until the third-trifoliate stage. When testing the cross-reactivity/specificity of the primers among CPMoV isolates, cowpea accession PI 471475 was used for propagating the virus. BMMV and MNSV were used as purified virus and were not increased for the cross-reactivity tests with other carmoviruses.

Serological testing of plants to verify infection was done by direct antigen coating enzyme-linked immunosorbent assay (DAC-ELISA), as described by Gillaspie et al. (5). G. Thottappilly provided the polyclonal antiserum against CPMoV-AT. DAC-ELISA was used to test tissue extracts with all isolates and to test seedlings in seed transmission studies.

Nucleic acid extraction. Initial experiments were conducted using CPMoV-RNA from purified virus. Isolates ISU and AT were multiplied in cowpea cv. Early Ramshorn for 2 weeks and purified either by differential ultracentrifugation and sucrose density gradient centrifugation as described (4), or by a modified method. The latter involved extraction of the virus in 0.1 M potassium phosphate, pH 7.0, followed by treatment with 2% Triton X-100 and precipitation of the virus with 10% polyethylene glycol (8,000 MW) plus 0.1 M NaCl (13). The virus was further purified on a 10 to 40% sucrose density gradient at 100,000 × g ultracentrifugation for 2 h. Virus bands were collected by needle puncture, and the virus was sedimented at 110,000 × g for 2.5 h. Virus pellets were resuspended in 0.1 M phosphate, pH 7.0. RNA extraction from the purified virus preparations was performed by incubation at 37°C with 1% sodium dodecyl sulfate (SDS) and 1 mM EDTA, followed by phenol:chloroform (1:1) extraction. The RNA was precipitated by addition of 2.5 volumes of cold ethanol and 0.1 volume of 3 M sodium acetate, pH 5.2, and incubation at -20°C for 1 h. Nucleic acid was pelleted at 14,000 × g for 10 min in a microfuge. The pellet was washed with 70% cold ethanol, vacuum dried, and suspended in nuclease-free water. The RNA was extracted from purified BMMV and MNSV virions in the same manner.

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Total RNA was extracted from fresh tissue as described by MacKenzie et al. (7). This method utilized a modified RNeasy Miniprep technique (Qiagen Inc., Chatsworth, CA). RNA was eluted from columns with RNase-free water, and RNA preparations were stored at -20°C . All RNAs were quantified based on A_{260} readings.

Primer design. Primers (Table 1) were designed from sequences available in GenBank accession number NID g885949 using a computer program (DesignerPCR 1.03 program, Research Genetics, Huntsville, AL) (14). Two strategies were used for selecting primer sequences. The first strategy utilized three sets of nonredundant primers. One set targets the region between nt 2,674 and nt 3,777, which includes the 3' end of the CP gene and its flanking 3' untranslated region (UTR) (CP3381F and UTR3969R). A second set of primers targets the REP gene between nt 776 and nt 2,263 (REP1667F and REP1990R). The third set targets a region that includes both the replicase gene and the movement protein gene, sharing one primer with the previous set (REP1667F and MOV2666R).

A second approach to primer design was based upon the report of Morozov et al. (8), in which highly conserved areas within the RNA polymerase region were used to design degenerate primers for detection of several carmoviruses. Two degenerate forward primers corresponding to regions II and IV and two degenerate reverse primers corresponding to regions V and VI of the polymerase region were designed so that there were four possible combinations of these primers. All primers were synthesized by Genosys Biotechnologies, Inc., Woodlands, Texas.

RT-PCR. For first-strand c-DNA synthesis, 25 pM of the reverse primer was added to either 0.3 μg of CPMoV RNA extracted from purified virus or 3 μg of total RNA from infected leaf tissue in a total volume of 11 μl . The reaction was incubated at 94°C for 2 min and immediately chilled to 4°C . After primer annealing, 4 μl of $5\times$ first-strand buffer, 2 μl each of 0.1 M DTT and 10 mM dNTPs, 0.25 μl of SUPERScript RT RNase H⁻ Reverse Transcriptase (Gibco BRL), and 0.75 μl of RNase-free water were added. The reaction mix (20 μl) was then incubated at 37°C for 1 h followed by a treatment at 94°C for 2 min to inactivate the enzyme.

Reverse transcription products (1 to 3

μl) were used in a PCR reaction consisting of $1\times$ PCR buffer, 1.5 to 3 mM MgCl_2 , 0.2 mM dNTPs, 5 pM each of upstream and downstream primers, and 1 U *Taq* DNA polymerase (Amplitaq, Perkin Elmer or Promega, Madison, WI). The nondegenerate primer pairs from the REP region gave good amplification at 1.5 mM MgCl_2 . The primers from the CP region and the degenerate primers did not perform as well as these two pairs, so the specifics of these will not be mentioned. Cycling times and temperatures were as follows: REP1667F/REP1990R and REP1667F/MOV2666R = one cycle of 94°C for 2 min, 25 cycles of 94°C for 1 min, 52.5°C for 2 min, 72°C for 3 min, one cycle of 72°C for 10 min. Products were analyzed by electrophoresis in a 1.5% agarose gel in $1\times$ TBE buffer.

The PCR products from the nondegenerate primer reactions were directly sequenced to determine whether the DNA sequences matched the predicted amplified sequence, and the results were compared with known sequences by BLAST search of GenBank (2,3).

DNA sequencing and data analysis. Amplified fragments were visualized on 1.2% agarose gels, and DNA concentrations were determined by comparison with a quantitation standard (*Hind*III fragments of bacteriophage lambda DNA). Single-stranded DNAs and unincorporated dNTPs were degraded by treating the PCR reaction mixtures with a mixture of enzymes. A portion (5 to 10 μl) of each reaction was incubated at 37°C for 15 min with exonuclease I (10 U) and shrimp alkaline phosphatase (1 U) (Amersham Life Science, Inc., Arlington Heights, IL). The enzymes were inactivated by heating at 80°C for 15 min, and an aliquot of the treated PCR products (5 to 10 ng of DNA per 100 bp of product) was used directly in sequencing reactions. Sequencing primers were the same as those used in the initial PCR reactions.

PCR products were sequenced using *Taq* cycle sequencing with fluorescence-based chain termination chemistry (Perkin-Elmer/Applied Biosystems) and automatic DNA sequencers (Perkin-Elmer/Applied Biosystems, models 373A or 377). Sequences of both DNA strands were determined. DNA sequence traces (electropherograms) were edited, and DNA contigs were assembled with the aid of a computer program (Sequencer, v 3.0; Gene Codes Corp., Ann Arbor, MI).

Primer sensitivity and specificity. Two approaches were used to test the sensitivity of the primers. First, RT-PCRs were performed using 10-fold dilutions (10^0 to 10^{-9}) of the purified viral RNA as template. Second, leaf disks were removed from healthy and diseased leaves and diluted as follows: 1/10 (one virus-infected leaf disk: nine healthy disks), 1/50, 1/100, 1/500, and 1/1,000. The disks were removed with a cork borer of a size such that the total weight of leaf disks was about 0.5 g. The disks were then extracted as described above, and the total RNA was used as template for RT-PCR.

Specificity of the primers was tested against BBMV-RNA and MNSV-RNA from purified virus. In addition, tissue samples of alfalfa mosaic virus, blackeye cowpea mosaic potyvirus, cowpea chlorotic mottle bromovirus, cowpea severe mosaic comovirus, cucumber mosaic cucumovirus, peanut stunt cucumovirus, southern bean mosaic sobemovirus, sunn hemp mosaic tobamovirus, and white clover mosaic potexvirus were extracted and tested to determine if any common cowpea pathogens would react with the primers.

Comparison of RT-PCR and DAC-ELISA. Several approaches were used to compare ELISA and RT-PCR. The first approach utilized leaf disks as described above for testing sensitivity in RT-PCR. Each leaf disk dilution (1/10 to 1/1,000) was ground in an extraction buffer, pH 7.1, used for DAC-ELISA (5), and the serology test was conducted on these samples using the polyclonal CPMoV antiserum. These data were then compared with those obtained by RT-PCR on the leaf disk dilution. The second approach involved diluting purified CPMoV (starting concentration of about 5 mg/ml based on A_{260}) and also crude leaf extracts in the DAC-ELISA extraction buffer mentioned above. A portion of each dilution was used in an ELISA (200 μl in each of two wells in the microtiter plate). A 400- μl sample of each was extracted by the phenol:chloroform method described above and used in RT-PCR.

RESULTS

Sensitivity and specificity of primers. Both pairs of primers from the REP region (Table 1) were found to be highly sensitive and produced a single product upon amplification. The CP primers also produced a single amplification product and had a low sensitivity compared with the REP primers. All of the degenerate primer pairs tested gave inconsistent results and sometimes produced erroneous bands of the incorrect size (data not shown). The primers from the REP region were judged to be most reliable and were used almost exclusively in the remaining tests.

The RT-PCR results for the replicase primer pairs assayed against extracts from both CPMoV-infected and healthy tissues indicated a single product of the expected

Table 1. Primers designed for detecting cowpea mottle virus

Primer designation	Primer sequence (5'-3', base pairs)	Predicted PCR product size
Nondegenerate		
REP ^a forward-1667F	AAC AAC GGA GAT GAC TGT GT	
REP reverse-1990R	TAC GCT TGC TGT ATG GGT AT	1667F/1990R = 323 bp
MOV ^b reverse-2666R	TTG AAG AGA GGG AGG AGA AG	1667F/2666R = 999 bp

^a RNA replicase coding region.

^b Movement protein coding region.

size for each pair with the infected tissue (Fig. 1). DNA sequences obtained for the 323-bp fragment amplified by REP1667F/REP1990R matched the expected CPMoV sequences published for the ISU product. The 323-bp fragment amplified by REP1667F/REP1990R for CPMoV-AT matched the published sequence with 13 base substitutions (Fig. 2). Primers REP-1667F/REP1990R detected CPMoV-ISU in one infected plant mixed with 99 healthy plants, and REP1667F/MOV-2666R produced a product at a dilution of one infected plant per 49 healthy plants, but no bands were visible at further dilutions of infected with healthy plants (Fig. 3A). Results using serial dilutions of purified viral RNA were similar. Primer pair REP1667F/REP1990R produced a specific PCR product at a 10^{-6} dilution ($\sim 2.6 \times 10^{-1}$ pg/ μ l) of CPMoV-AT-RNA, but primer pair 1667F/2666R produced a faint band at 10^{-5} dilution of the RNA (Fig. 3B). The lesser sensitivity for the 1667F/2666R primer pair is probably an artifact of PCR in which the longer fragment cannot be replicated as efficiently as the shorter one.

The comparisons of DAC-ELISA and RT-PCR for detection of CPMoV indicated that PCR is much more sensitive (Table 2). ELISA detected virus in one infected plant in 75, compared with the REP1667F/REP1990R primer pair, which detected virus in one plant in 1,000, as discussed in the previous paragraph. The ELISA results with the diluted purified virus indicated an end point at 10^{-7} dilution, and the PCR (Fig. 3C) detected virus at 10^{-12} dilution or $\sim 5 \times 10^{-3}$ pg/ml (no end point was obtained in the PCR). ELISA of the crude tissue extract dilution series yielded a 10^{-6} end point, but the PCR (Fig. 3D) detected virus at 10^{-9} dilution (no end point obtained).

Fig. 1. Reverse transcription-polymerase chain reaction (RT-PCR) bands in agarose gel with cowpea mottle virus-ISU. Lanes 2 and 3 = RNA from virus-infected and healthy cowpea, respectively, with primer pair REP1667F/REP1990R; lanes 4 and 5 = RNA from infected and healthy cowpea, respectively, with REP1667F/MOV2666R. Lanes 1 and 6 = markers (100-bp ladder).

	1		50
AT	AACAACGGAG	ATGACTGTGT	GGTTATTACC GAGTCCAAGA ACGTTGATTT
Togo	AACAACGGAG	ATGACTGTGT	GGTTATTACC GAGTCCAAGA ACGTTGATTT
ISU	<u>AACAACGGAG</u>	<u>ATGACTGTGT</u>	GGTTATTACC GAGTCCAAGA ACGTTGATTT
	51		100
AT	CGTATGCAAG	CAGTTGGAAC	GATTCCGCGA TTACGGGTTT ACGTGCATAG
Togo	CGTATGCAAG	CAGTTGGAAC	GATTCCGCGA TTACGGGTTT ACGTGCATAG
ISU	CGTATGCAAG	CA ATTGGAAC	GATTCCGCGA CT ATGGGTTT ACGTGCATAG
	101		150
AT	CTGAGGAACC	AGTGTATGAG	TTGGAAAAGC TGGAGTTCTG CAATCTACAC
Togo	CTGAGGAACC	AGTGTATGAG	TTGGAAAAGC TGGAGTTCTG CAATCTACAC
ISU	CTGAGGAACC	AGTGTATGAG	TTGGAAAAGC TGGAGTTCTG TA ATCTACAC
	151		200
AT	CCACTGTTTG	ATGGCTCCTC	CTGGACGGTG ATGCGGAAAC CAAGTGTTC
Togo	CCACTGTTTG	ATGGCTCCTC	CTGGACGGTG ATGCGGAAAC CAAGTGTTC
ISU	CCACTGT AT G	ATGGCT CT TTC	CTGGACGGTG ATGCGGAAAC CAAG CG TTC
	201		250
AT	ATTAGCCAAG	GACACCTATT	GTGTGGCTGG ATGGAACAAC ACCAAGGATG
Togo	ATTAGCCAAG	GACACCTATT	GTGTGGCTGG ATGGAACAAC ACCAAGGATG
ISU	ATT GGC TA AG	GACACCTATT	GTGTGG CA AGG ATGGAACAAC ACCAAGGATG
	251		300
AT	CAGCTAGTTG	GCTCAATGCC	ATCGGTCAAT GTGGTGCAGC CATCACAGGC
Togo	CAGCTAGTTG	GCTCAATGCC	ATCGGTCAAT GTGGTGCAGC CATCACAGGC
ISU	CAG CC AGTTG	GCTCAATGCC	AT T GGTCAAT GTGGTGCAGC CATCACAGGC
	301		323
AT	GGTATACCCA	TACAGCAAGC	TTA
Togo	GGTATACCCA	TACAGCAAGC	GTA
ISU	GGT <u>ATACCCA</u>	<u>TACAGCAAGC</u>	<u>GTA</u>

Fig. 2. DNA sequence alignment for 323 bases from the replicase gene of three cowpea mottle virus isolates. Variant nucleotides are in bold type, and primer sequences (REP1667F and REP 1990R) are underlined.

Specificity of the primers was tested against other carmoviruses, BMMV-RNA and MNSV-RNA from purified virus, as well as against tissue extracts of cowpeas infected with other common cowpea pathogens. BMMV-RNA, MNSV-RNA, and the other cowpea viral-pathogen extracts produced no 323-bp fragment in PCR with REP1667F/REP1990R or a 999-bp fragment with REP1667F/MOV2666R. Interestingly, the degenerate primer pairs mentioned earlier were generally observed to amplify fragments of the same size using CPMoV, BMMV, and MNSV templates, indicating an unacceptable level of cross-reactivity in this assay (data not shown).

Specificity of primers. To determine the range of reaction of the primers to different CPMoV isolates, 29 samples representing 11 separate isolates were increased on cowpea PI 471475 in growth chambers, and total RNA was extracted from each. RT-PCR was performed using the REP1667F/REP1990R primer pair, and all 29 extracts produced the expected band (data not shown). Sequencing was performed on PCR products obtained from ISU, AT, and several Togo isolates. DNA sequences from the 323-bp fragment amplified by primer pair REP1667F/

REP1990R were identical for all isolates from Togo. Furthermore, the Togo sequences were almost identical to those derived from the AT isolate (there was a single base difference at position 321, Fig. 2). The AT sequence was more divergent, with 13 base substitutions, compared with ISU.

Primers for seedborne virus detection.

The results obtained from tests of seedlings from a total of 1,000 seeds from eight different infected cowpea genotypes indicated CPMoV was not transmitted, as determined by both RT-PCR and ELISA. Samples were bulked to five plants per sample for serology and to 50 plants per sample

Table 2. Comparison of enzyme-linked immunosorbent assay (ELISA) and reverse transcription-polymerase chain reaction (RT-PCR) sensitivity for crude extract and pure virus of cowpea mottle carmovirus-ISU

Dilution ^a	Crude extract		Pure virus	
	ELISA(A ₄₀₅)	RT-PCR ^b	ELISA(A ₄₀₅)	RT-PCR ^b
10 ⁻³	0.937	+	nt	nt
10 ⁻⁴	0.938	+	0.943	+
10 ⁻⁵	0.928	+	0.937	+
10 ⁻⁶	0.738	+	0.917	+
10 ⁻⁷	0.036	+	0.224	+
10 ⁻⁸	—	+	—	+
10 ⁻⁹	—	+	—	+
10 ⁻¹⁰	nt	nt	—	+
10 ⁻¹¹	nt	nt	—	+
10 ⁻¹²	nt	nt	—	+

^a Samples for direct antigen coating-ELISA diluted in 0.15 M NaCl, 0.036 M Na₂HPO₄, 0.016 M NaH₂PO₄, 0.013 M Na DIECA, 0.003 M NaN₃, pH 7.1.

^b Samples for RT-PCR were extracted from the various dilutions for ELISA by incubation at 37°C for 1 h in sodium dodecyl sulfate (SDS) and EDTA followed by treatment with chloroform/phenol (1:1 vol/vol) and precipitation in ethanol. Primer set REP1667F/REP1990R was used, and results can be seen in Figure 3C and D.

Fig. 3. (A) Reverse transcription-polymerase chain reaction (RT-PCR) bands in agarose gel with tissue dilutions of cowpea mottle virus (CPMoV)-ISU-infected cowpea. Lanes 2 to 8 = 1 infected plant/10 plants, 1/50, 1/100, 1/500, 1/1,000, RNA from purified virus, and healthy cowpea, respectively, with primer pair REP1667F/REP1990R; lanes 9 to 15 = 1 infected plant/10 plants, 1/50, 1/100, 1/500, 1/1,000, RNA from purified virus, and healthy cowpea, respectively, with REP1667F/MOV2666R. Lanes 1 and 16 = markers (100-bp ladder). (B) RT-PCR bands in agarose gel with dilutions of CPMoV RNA. Lanes 2 to 6 = 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷ CPMoV-AT RNA, respectively, with primer pair REP1667F/REP1990R; lanes 7 to 11 = 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷ CPMoV-AT RNA, respectively, with REP1667F/MOV2666R. Lanes 1 and 12 = markers (100-bp ladder). (C) RT-PCR with a dilution series of purified CPMoV-ISU (starting virus concentration of ~5 mg/ml). Lanes 2 to 10 = 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹, 10⁻¹⁰, 10⁻¹¹, 10⁻¹² dilutions, respectively, tested with REP1667F/REP1990R. Lane 11 = RNA (~250 ng/μl) from purified CPMoV. Lanes 1 and 12 = markers (50-bp ladder). (D) RT-PCR of CPMoV-ISU-infected tissue. Lanes 2 to 8 = 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹ dilutions, respectively, tested with REP1667F/REP1990R. Lanes 1 and 9 = markers (50-bp ladder).

for RT-PCR. There were 10 instances where ELISA gave marginal results (false positives) with some samples. When these plants were then tested by RT-PCR, they were found to be negative for CPMoV. Further attempts to achieve seed transmission with CPMoV inoculated into California Blackeye no. 5 from 2 to 4 weeks after the primary leaf stage also failed to yield any virus in the 120 seeds tested.

DISCUSSION

This RT-PCR procedure, based on the sensitivity and specificity of at least one set of primers, is the most sensitive method developed to date to detect CPMoV. The fact that seed transmission did not occur in our tests does not eliminate seedborne CPMoV from consideration as a problem in germ plasm exchange, but rather indicates that seed transmission occurs at low rates as reported by others (1,4,12). In a laboratory/greenhouse study of seed transmission of CPMoV (10), plants inoculated at the primary leaf stage produced 1% infected seeds; whereas plants inoculated when the second trifoliate leaves were expanding, produced 6% infected seeds. This tendency for greater seed transmission when plants are infected later in the season could explain some of the differences in seed transmission rates that have been observed. This virus is a very serious threat in the movement of genetic resources of *Vigna* because of its potential for severe damage to crops. In the tests comparing RT-PCR and ELISA (Fig. 3C and D), twice the volume was extracted for RT-PCR as was used for each well in the ELISA, so these end comparative dilutions may be misleading. A similar problem exists with the tissue dilution test comparisons mentioned previous to these tests. However, this twofold volume difference is insig-

nificant when compared to the PCR assay, which is three to five orders of magnitude more sensitive.

Nondegenerate primers designed from the replicase gene are clearly a better choice for sensitive, accurate detection of CPMoV than the degenerate primers used in these tests. The use of these primers in RT-PCR offers a definite increase in sensitivity over the ELISA detection methods and will solve many problems arising with marginal ELISA results (false positives). False positive results occurred with cowpea plants of several different genotypes, but not all plants arising from a particular seed lot showed this response. Furthermore, dilutions of 1/100 did not eliminate the response in earlier studies, nor did it work here (5). Many seed lots in the collection are not genetically homogeneous. Apparently there are proteins among plants of certain seed lots that give a nonspecific serological reaction.

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