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Simultaneous detection of three common potyviruses infecting cucurbits by multiplex reverse transcription polymerase chain reaction assay

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ABSTRACT

Watermelon mosaic virus (WMV), Zucchini yellow mosaic virus (ZYMV), and Papaya ring spot virus (PRSV-W) are the three most common potyviruses infecting cucurbits in the United States of America. In this study, a Multiplex reverse transcription polymerase chain reaction (RT-PCR) assay was developed for the simultaneous detection and differentiation of WMV, ZYMV, and PRSV-W. A mixture of specific primers set for each virus successfully amplified a distinct PCR product of 980 bp from the coat protein (CP) gene of WMV, 708 bp from the cylindrical inclusion (CI) gene of ZYMV and 496 bp from the helper component (Hc-Pro) gene of PRSV-W. Sanger sequencing confirmed that the amplified PCR products for each virus are specific. The sensitivity and specificity of these primers were tested by serial dilution assay of total RNA extracted from virus-infected samples for all three viruses. The detection limit of the Multiplex RT-PCR assay was between 10^{-5}–10^{-6}-fold dilution. The Multiplex RT-PCR assay was successfully applied for the detection of these viruses on 54 field samples, which were collected from two counties of Oklahoma during the 2018 growing season. Based on these results, this Multiplex RT-PCR assay is specific, rapid, and economical for the detection of three common potyviruses of cucurbits and has the potential to screen a large number of field samples of cucurbits against these viruses.

Cucurbits are major cash crops in the United States of America (USA) and are grown extensively in several southern states. The most common cucurbits cultivated in the USA are pumpkin (*Cucurbita maxima*), watermelon (*Citrullus lanatus*), squash (*Cucurbita pepo*), cantaloupe (*Cucumis melo*), and cucumber (*Cucumis sativus*). These cucurbits have a total production of about five million metric tons and a value of about 1.7 billion dollars in 2017 (NASS-USDA, 2018).

More than six dozen viruses have been reported worldwide that infect cucurbits and cause significant losses in quality and quantity of the product yield. Among these, the three most prevalent viruses infecting cucurbits in the USA are Watermelon mosaic virus (WMV), Zucchini yellow mosaic virus (ZYMV), and Papaya ring spot virus (PRSV-W) (Provvidenti et al., 1984; Grafton-Cardwell et al., 1996; Walters et al., 2003; Jossey and Babadoost, 2008; Ali et al., 2012a, b). Previous surveys of cucurbit viruses in the southern states showed that WMV, ZYMV, and PRSV-W are dominant viruses infecting various species of cucurbits (Ali et al., 2012a, b). All three viruses belong to the genus *Potyvirus* in the family *Potyviridae* and consist of a positive sense single-stranded RNA genome.

These three types of potyviruses produce similar symptoms on cucurbit plants such as mosaic and mottling, so it is difficult to distinguish them from one another in the field. In addition, all three viruses can occur as a mixed infection and are difficult to detect serologically using virus specific antibodies against each virus because they might cross react to each other. Similarly, reverse transcription polymerase chain reaction (RT-PCR) can detect these viruses, but individual RT-PCR (Uniplex PCR) is needed to detect one virus at a time. Hence, detection of multiple viruses using the above-mentioned techniques is time consuming, expensive, and laborious. Therefore, it is necessary to develop a Multiplex PCR that can detect all three viruses simultaneously in a single reaction.

Multiplex PCR has been reported to detect various plant viruses in mixed infections (Nie and Singh, 2000; Nassuth et al., 2000; Menzel et al., 2002; Ito et al., 2002). This technique is convenient, reliable, and sensitive. A Multiplex PCR assay has been reported by Kwon et al. (2014) for seven cucurbit viruses mostly prevalent in South Korea using the dual priming oligonucleotide primers. However, our objective in this study is to design a very simplistic Multiplex assay, which targets specifically three common potyviruses infecting cucurbits in the USA, and to make this assay reliable, and easily reproducible. The assay should also be cost effective while screening a large number of samples during epidemiological studies. Here, we report a Multiplex assay that

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Table 1
Primers used for the multiplex RT-PCR for the detection of WMV, ZYMV and PRSV-W.

Viruses	Primers	Sequence (5′–3′)	Primer length	Location (nt)[c]	Target gene	Product size (bp)	(Tm) °C
WMV	WMV-F[a]	5′-AAC ACA CAA CCA AGT GAA TT-3′	20	8862–8881	CP	980	50.5
	WMV-R[b]	5′-TAA CGA CCC GAA ATG CTA ACT-3′	21	9821–9841			54.0
ZYMV	ZYMV-F	5′-AGA ATG GAG TGA CGT TGG ATG T-3′	22	4559–4580	CI	708	56.2
	ZYMV-R	5′-TAG TTC TGG GTA ATG CTG CTG G-3′	22	5245–5266			56.8
PRSV-W	PRSV-F	5′-TGC ACG TCT GAT ATG GAT GTT A -3′	22	1802–1823	Hc-Pro	496	53.7
	PRSV-R	5′-TGA AATGAG CTT TTC CAG AAC G-3′	22	2276–2297			53.7

[a] F, sense primer.
[b] R, antisense primer.
[c] Location of the nucleotides on the target genomes is according to the complete genomes sequence of WMV-TX29 isolate (KU246036), ZYMV-BL-67 isolate (MK124612) and PRSV-W- TUL15 isolate (KY039583).

offers rapid, specific, and cost effective detection of three common potyviruses in the USA (WMV, ZYMV, PRSV-W).

The complete genome sequences of WMV-TX29 isolate (Accession no. KU246036) (Rajbanshi and Ali, 2016), ZYMV-BL-67 isolate (Accession no. MK124612) (Khanal and Ali, 2019), and PRSV-W-TUL15 isolate (Accession no. KY039583) (Ali, 2017) that were collected in Oklahoma and Texas and sequenced previously in our lab were used as a template for designing primers. Complete genome sequences of other WMV, ZYMV and PRSV-W isolates were also obtained for comparison from the GenBank database and aligned using Clustal X 1.81 software. Based on the alignment, highly conserved regions were selected among the respective viruses that covered the coat protein (CP) gene for WMV, the cylindrical inclusion (CI) gene for ZYMV and the helper component (Hc-Pro) gene for PRSV-W. Primers were designed for each virus in Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and were checked for primer dimer formation. All primers were synthesized by Integrated DNA Technology, Inc, Iowa, USA, and are listed in Table 1.

Pure virus cultures of all the three potyviruses (WMV, ZYMV and PRSV-W) mentioned above were maintained in squash seedlings (SEEDWAY, LLC, New York) in the growth chamber under controlled conditions. Total RNA was extracted from the leaves (about 0.1 g tissues) of virus-infected squash plants using the Tri-reagent method as reported previously (Ali et al., 2012b). Total RNA pellets were suspended in 50 µl of RNAse free water.

The UniPlex reverse transcription (RT) reaction was performed individually for all three potyviruses (WMV, ZYMV and PRSV-W) in a total volume of 20 µL. For each virus, 6 µL of total RNA obtained from virus-infected tissues was mixed with 6 µL of RNase free water and 1 µl (50 µM) of virus-specific reverse primer in a 1.5 ml tube, incubated at 85 °C for 2 min, and chilled on ice for 2 min. Immediately, 7 µL master mix containing 4 µL 5x RT buffer, 0.5 µL MMLV-RT reverse transcriptase enzyme (BioChain Institute Inc. USA), 2 µL of 10 µM dNTPs and 0.5 µL (1 U/µL) of RiboLock RNase inhibitor (ThermoFisher, Scientific), was added to the tube to make the final volume of 20 µL.

UniPlex PCR for WMV, ZYMV and PRSV-W was carried out in individual PCR tubes in 20 µL containing 1 µL each of fragment specific forward and reverse primers (10 µM) (Table 1) of each virus (WMV, ZYMV and PRSV-W), 10 µL of 2 x GoTaq® Green Master Mix (Promega, USA), 6.5 µL water and 1.5 µL cDNA from RT reaction. Negative PCR control contained 1.5 µL nuclease free water instead of cDNA from RT reaction. The PCR conditions were optimized and for optimum performance of the primers, the following parameters were used. Initial denaturation step at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing for 40 s at 48 °C and extension for 30 s at 72 °C with a final extension at 72 °C for 10 min.

The Uniplex RT-PCR successfully amplified the expected bands from their respective viruses at the same annealing temperature as confirmed by agarose gel electrophoresis. Individual DNA bands of 980 bp, 708 bp and 496 bp were obtained for WMV, ZYMV, and PRSV-W respectively (Fig. 1). A test for cross amplification of primers pairs confirmed that the primers were virus specific. The use of these three pairs of primers in the same reaction mixture did not show dimer formation or any cross amplification.

A Multiplex RT reaction was performed to detect simultaneously two or more viruses from virus-infected tissues in one reaction tube. Total RNA of each virus was mixed together in all possible combinations (WMV + PRSV-W, WMV + ZYMV, PRSV-W + ZYMV and WMV + PRSV-W + ZYMV). In double virus combinations, 3.5 µL total RNA of each virus while in triple virus combination, 2.5 µL of each virus was mixed with 0.8 µl of 50 µM reverse primer of each virus in all combinations. The rest of the RT reaction was the same as described above for Uniplex RT-PCR. The RT reaction was briefly centrifuged and incubated at 50 °C for 1 h. The RT reaction (cDNA) product was either used immediately or stored at −20 °C for further amplification by PCR.

A Multiplex PCR was performed in a 25.0 µL reaction mixture, which consists of 12.5 µL of 2 x Go Taq® Green Master Mix (Promega, USA), 1 µL (10 µM) each of forward and reverse primers for each virus, 1.5 µL of cDNA from RT-reaction and water as needed. Negative PCR control contained 1.5 µL nuclease free water instead of cDNA from RT reaction. The PCR cycles conditions were followed as mentioned above. The PCR products were run in 1% agarose gel and then visualized in UV illuminator after ethidium bromide staining.

All duplex PCR assays in three different combinations and Multiplex PCR assay for all three viruses together successfully amplified the respective virus bands (Fig. 1).

Another experiment was designed to validate the Uniplex, and Multiplex-PCR assays using spiked viral RNA in total RNA extracted from healthy squash seedling. Virus like particles (VLP) preparations of each virus was obtained from squash plants infected individually with the three viruses according to the procedure reported previously (Ali et al., 2012b). Viral RNA was then extracted from VLP of each virus using RNAeasy® Mini Plant Kit (QIAGEN, USA). The concentration of viral RNA was measured using NanoDrop 8000 UV spectrophotometer (ThermoFischer Scientific) and the final concentration for each virus was adjusted to 10 ng/µl in sterile water as a stock solution. In separate new tubes, three µl RNA of each virus from the stock solution was mixed with seven µl of total RNA extracted from healthy squash seedlings to make the final volume 10 µl of working solution. From the working solution, six µl RNA for each virus was used from the respective tubes in Uniplex RT reaction, while four µl for each virus was used in duplex and Multiplex RT reactions followed by PCR as mentioned above. PCR products from Uniplex, duplex and Multiplex RT-PCR assays showed successfully the expected amplification of the particular virus (s) in each combination (Fig. 2).

Another experiment was designed to determine the efficiency of the Multiplex RT-PCR to detect all three viruses directly from infected tissues together. Squash seedlings inoculated with individual viruses were maintained in a growth chamber. Leaf tissues were collected from infected plants showing distinct mosaic symptoms after 3 weeks of infection. Aliquots of virus-infected leaf tissues, each infected with a single type of virus, were mixed and crushed together in liquid

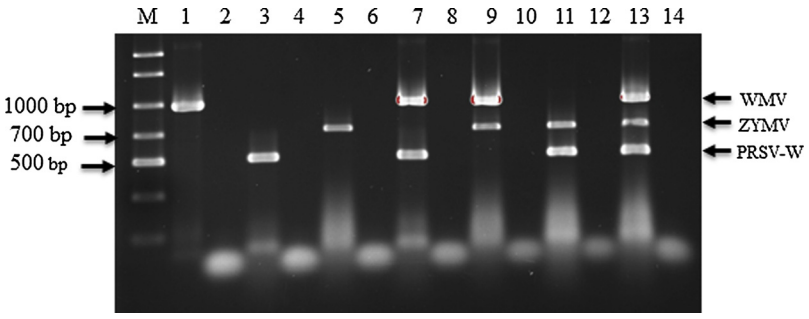

Fig. 1. Optimization of Multiplex RT-PCR for three potyviruses. Total RNAs of individual virus extracted separately was used either as a single or different combination in RT-PCR. PCR products (10 µl) were run on 1% agarose gel. M (Ladder), Lane 1 (WMV), Lane 3 (PRSV), Lane 5 (ZYMV), Lane 7 (WMV + PRSV-W), Lane 9 (WMV + ZYMV), Lane 11 (ZYMV + PRSV-W), Lane 13, (WMV + ZYMV + PRSV-W). Lanes 2, 4, 6, 8, 10, 12, and 14 are water with primers.

nitrogen. Total RNA (five µl) was used in RT reaction followed by Multiplex PCR assay as mentioned above. All the three potyviruses were successfully detected from infected tissues together by their respective primers in a single reaction. Analysis on agarose gel electrophoresis showed the presence of virus expected DNA bands in all three replicates indicating the consistent detection of three viruses in the RNA mixture (Fig. 3).

To determine the sensitivity, specificity and detection limit of the Multiplex RT-PCR assay, a 10-fold serial dilution was initially performed using the total RNA (˜10 ng each) obtained from infected tissues of WMV, ZYMV, and PRSV-W. All serially diluted total RNA mixtures was done in sterile water and used for Multiplex RT-PCR by the procedure described above. In addition, another experiment was performed using purified viral RNA obtained from VLP preparations above and added to total RNA obtained from healthy squash plants. One µl (10 ng/µl) of viral RNA of each virus was spiked with healthy squash RNA (seven µl) to make final volume of 10 µl and then it was used for 10-fold serial dilution (10^{-1} to 10^{-9}). Each dilution was used as a template for Multiplex RT-PCR as described above. All PCR products were run on 1% agarose gel and compared with each other.

Two different serial dilution experiments showed the ability of the Multiplex RT-PCR to detect the three viruses in low concentration. The primer mix successfully detected all three viruses from spiked viral RNA with total RNA from healthy squash (Fig. 5) and the results were comparable to that of viral RNA mixed in water (Fig. 4). All three viruses were detected in sample dilution up to 10^{-5} to 10^{-6}. Thus, this assay should be able to detect each virus in a mixed infection where one type of virus may be in significantly lower concentration than the other virus.

To test the specificity and validate the Multiplex RT-PCR assay for its practical application, 54 pumpkin samples collected from growers' fields in Tulsa and Blaine Counties of Oklahoma (Table 2) were tested by the Multiplex PCR assay as developed above. All the collected field samples showed typical mosaic symptoms and shoestring appearance.

The application of this Multiplex assay in 54 field samples showed that the assay could effectively detect single, double, and triple infection by the three potyviruses infecting cucurbits (Table 2). Analysis of PCR products on 1% agarose gel revealed that 11.11% (6/54) have

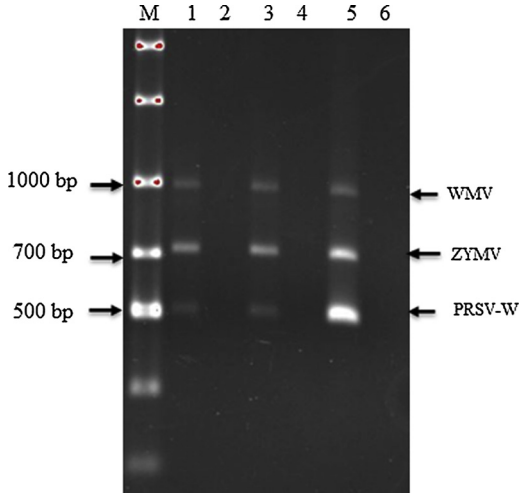

Fig. 3. Detection of three potyviruses by Multiplex RT-PCR in mixed virus-infected tissues. Leaves tissues infected by individual viruses were mixed and total RNA was extracted together. Experiment was repeated three times. PCR products were run in 1% agarose gel. M (Ladder), Lanes 1, 3 and 5 showed PCR bands of three viruses while lanes 2, 4 and 6 are water with primer mix.

triple infection, 42.5% (23/54) have double virus infection and 16.7% (9/54) of the samples were infected by a single virus. Out of 23 samples with double infection, 9.3% (5/54) were infected with WMV and ZYMV, while the remaining 33.3% (18/54) showed infection with both ZYMV and PRSV-W. None of the samples were infected alone with WMV or PRSV-W. Similarly, none of the samples were infected with double infection of WMV + PRSV-W (Table 2). Few representative field samples, which are infected with double and triple infection, are shown in Fig. 6.

To confirm that the amplified PCR products obtained in above experiments of each virus was specific and belonged to the corresponding virus, the expected PCR products from Uniplex, duplex and Multiplex were used for sequencing. Some of the PCR products for the positive field samples were also sequenced. PCR bands were excised from the gel

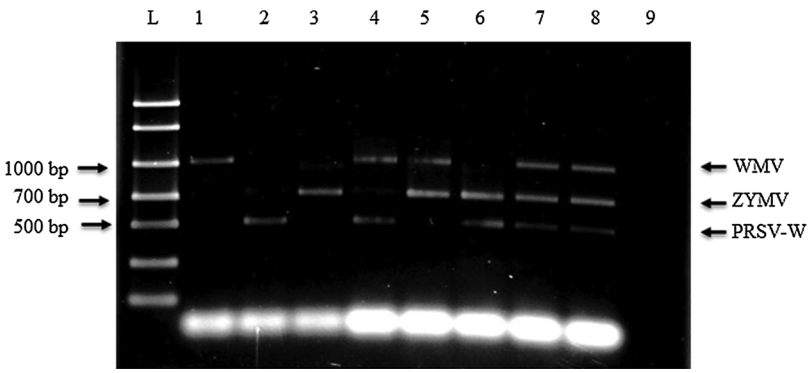

Fig. 2. Optimization of Multiplex RT-PCR for three potyviruses from purified viral RNA obtained from virus-like particle preparations (VLP). Viral RNA of each virus was mixed with total RNA from healthy squash seedlings. L- Ladder, Lane 1–3: Uniplex PCR for single viruses, Lane 4–6: duplex PCR for two viruses in different combination, Lane 7 and 8: replicates of Multiplex PCR for three viruses, Lane 9: virus-free squash plant RNA with all primers.

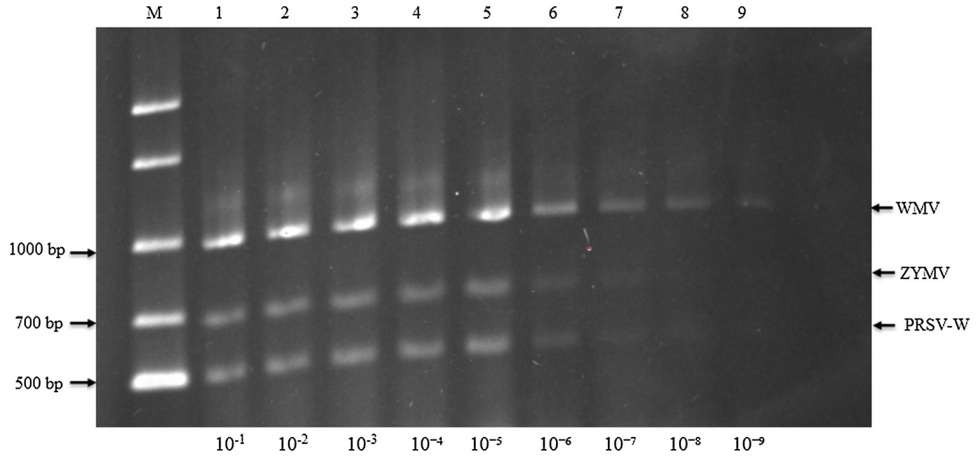

Fig. 4. Sensitivity of Multiplex RT-PCR assay. Total RNA from infected tissues of WMV, ZYMV and PRSV-W were mixed in equal concentration, serially diluted 10-fold and used in Multiplex RT-PCR assay. PCR products (10 µL) was run on 1% agarose gel. All three viruses were detected successfully by 10^{-5}–10^{-6} dilution.

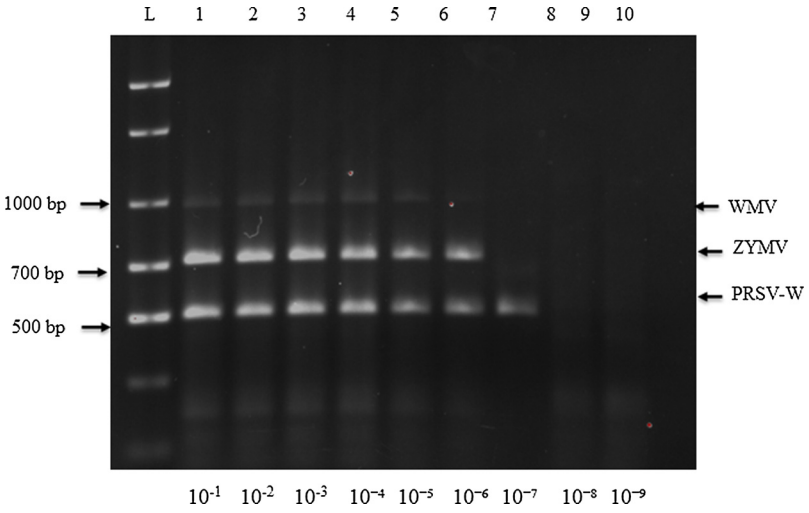

Fig. 5. Sensitivity of Multiplex RT-PCR assay using viral RNA spiked in total RNA from healthy squash seedling. Viral RNAs of known concentration (10 ng/µl) were serially diluted 10-fold (10^{-1}–10^{-9}) in total RNA from healthy squash seedlings. L = ladder, Lane 1–9, PCR products of a 10 fold serially diluted viral RNA mixtures of WMV, ZYMV and PRSV. Lane 10 shoed negative PCR control contained primers with water.

Table 2
Screening of 54 pumpkin samples by multiplex RT-PCR against the three potyviruses (WMV, PRSV-W, ZYMV) that were collected from grower's fields in two counties of Oklahoma.

Locations	Samples	Single virus infection			Double virus infection			Triple virus infection
		WMV	ZYMV	PRSV-W	WMV + ZYMV	PRSV-W + ZYMV	WMV + PRSV-W	PRSV-W + WMV + ZYMV
Blaine County	22	0/22	9/22	0/22	5/22	4/22	0/22	0/22
Tulsa County	32	0/32	0/32	0/32	0/32	14/32	0/32	6/32
	Total	0/54	9[a]/54	0/54	5[a]/54	18[a]/54	0/54	6[a]/54
	Percentage	0%	16.7%	0	9.3%	33.3%	0%	11.11%

[a] All the positive samples were confirmed by sequencing the PCR products.

and purified by gel-extraction purification kit (QIAGEN, USA). Gel purified PCR products were ligated into pGEM®- T Easy vector (Promega, USA) using the T4 DNA ligase cloning kit (Promega, USA) according to manufacturer's protocol. The ligation product was used for transformation in *Escherichia coli* strain DH5α competent cells (New England BioLabs, UK) using the heat shock method at 42 °C. The cells were grown on LBA plates overnight at 37 °C. Recombinant colonies (white) were confirmed by colony PCR and the products were used for dye terminator cycle sequencing. Sequencing was performed using the Applied Biosystems 3130 genetic analyzer, USA at the core facility of the Department of Biological Science, The University of Tulsa, Oklahoma.

Sequences acquired for each PCR products of the respective viruses obtained from virus-infected plants in the lab or field was run in BLAST program in www.ncbi.nlm.nih.gov. Blast results showed that each DNA fragment belonged to the respective virus (data not shown). These results further confirmed the specificity of the Uniplex and Multiplex RT-PCR assays and could be used in field samples for the detection of these three potyviruses.

These three potyviruses frequently occur as mixed infection in cucurbit fields and may be one of the factors in the severity of viral disease (Rochow and Ross, 1955; Vance, 1991). As shown in our previous study, most of the viral disease in cucurbit crops in Oklahoma as well as other states are primarily due to single and mostly mixed infection of WMV, ZYMV and PRSV-W (Ali et al., 2012a, b). Hence, a diagnostic RT-PCR assay which can simultaneously detect these specific Potyvirus is urgently required. Reliable and timely diagnosis of plant viruses is very important before formulating any disease management strategy.

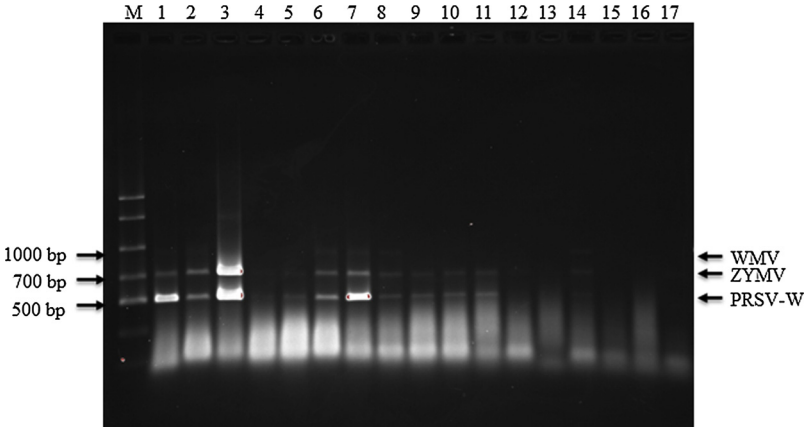

Fig. 6. Specificity of Multiplex PCR assay in 54 pumpkin samples collected from fields in two counties of Oklahoma during 2018 growing season. Sixteen representative field samples (Lanes 1–16) were run on 1% agarose gel. M (Ladder), Lanes 1–5, and 9–11 showed double infections while Lanes 6–8 and 14 showed triple infections. The remaining lanes without a band were not infected with the three potyviruses. Lane 17 = negative PCR control contained primers in water.

In conclusion, a specific, sensitive, rapid and economical Multiplex PCR was developed to simultaneously detect and differentiate three potyviruses (WMV, ZYMV and PRSV-W) in a single reaction. This Multiplex assay will be very useful in future epidemiological studies of these three potyviruses in cucurbits in the U.S. and screening for their resistance.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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