Establishment of a non-radioactive nucleic acid hybridization technique for begomovirus detection

✉ Raidel Rodríguez[1], Pedro Luis Ramos[1], Vivian Doreste[1], Karelia Velázquez[2], Rudy Peral[1], Alejandro Fuentes[1] and Merardo Pujol[1]

[1]Departamento de Plantas. Centro de Ingeniería Genética y Biotecnología. Ave 31 e/ 158 y 190. AP 6162, CP 10600, Playa, Ciudad de la Habana, Cuba. Tel.: (53-7)- 271-8164; Fax: (53-7)- 331779; E-mail: raidel.rodriguez@cigb.edu.cu

[2]Instituto de Investigaciones de Cítricos y otros Frutales, Ciudad de la Habana, Cuba.

ABSTRACT

In this work, we established a non-radioactive nucleic acid hybridization technique (Dot blot) for begomovirus detection. Two probes were constructed: a generic probe (TYLCV *coat protein* gene) and a specific probe (intergenic region and the 5′ end of the TYLCV *coat protein* gene). The efficacy, analytical and diagnostic sensitivity, analytical and diagnostic specificity, positive and negative prediction values and repeatability were determined using the designed probes. Assays using the generic probe showed values of efficacy, sensitivity, specificity and repeatability between 85-100 percent, whereas the assays employing the TYLCV specific probe showed percentages higher than 98% for all parameters analyzed. The generic probe can be used to detect different begomoviruses, while the specific one can only be used to detect *Tomato yellow leaf curl virus*.

Keywords: begomovirus, TYLCV, validation, non-radioactive hybridization

Biotecnología Aplicada 2003;20:164-169

RESUMEN

Establecimiento de una técnica de hibridación no radioactiva de ácidos nucleicos para la detección de begomovirus. En este trabajo se describe el establecimiento de una técnica de hibridación de ácidos nucleicos no radioactiva (Dot blot) para la detección de begomovirus. Dos sondas fueron construidas: una sonda genérica a partir del gen de la *proteína de la cápsida* de TYLCV y una sonda específica que se corresponde con la región intergénica y el extremo 5′ del gen de la *proteína de la cápsida* de TYLCV. La eficacia, la sensibilidad analítica y diagnóstica, la especificidad analítica y diagnóstica, los valores de predicción negativos y positivos y la repetibilidad fueron determinados para las dos sondas estudiadas. Los ensayos que usaron la sonda genérica mostraron valores de eficacia, sensibilidad, especificidad y repetibilidad entre un 85-100%, mientras los ensayos que emplearon la sonda específica mostraron porcentajes por encima de un 98% en todos los parámetros analizados. La sonda genérica puede ser usada para detectar diferentes begomovirus, mientras la sonda específica solamente puede ser empleada para detectar *Tomato yellow leaf curl virus*.

Palabras claves: begomovirus, TYLCV, validación, hibridación no radioactiva

*I*ntroduction

Geminiviridae is a plant virus family whose members produce serious damage to several crops worldwide, especially in tropical and subtropical regions (e.g. Middle East, Africa, Europe, Central America, Japan, United States, Caribbean basin) [1-3]. Viruses of this family are grouped in four genera (*Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus*), which differ in genome organization, host range and vectors [4].

Increases of begomovirus associated epidemics in Cuba since mid 1989 are due to a rise in whitefly infestation in a variety of crops. Thus, three different begomoviruses have been detected in tomato plantations: *Tomato yellow leaf curl virus* (TYLCV), *Tomato mottle Taino virus* (ToMoTV) and *Tomato mosaic Havana virus* (ToMHV) (the last two viruses were renamed according to Fauquet *et al.*)[4-10]. Other begomoviruses have also been detected infecting other crops in Cuba. A strain of *Bean golden yellow mosaic virus* (BGYMV) a Mexican isolate infecting beans has been molecularly characterized [11]. During 1989-1992, BGYMV caused yield depressions in a number of bean plantations, and yield losses of 100% in certain bean cultivars were reported at Holguín province. Also, the presence of a new begomovirus (Tobacco leaf rugose virus, TbLRV) affecting tobacco has been recently reported [12].

Tomato yellow leaf curl virus (TYLCV), which is the most widely spread virus in Cuba, produces up to 100% yield losses [5, 8]. Affected tomato plants are stunted, the shoots having short internodes and small leaves, that are curled, leathery and chlorotic. The most significant effect of TYLCV infection is flower abscission. Usually, less than one in ten flowers bear fruits, thus severely reducing yields. The TYLCV genome consists of a single strand (ssDNA) circular molecule of 2787 nucleotides [13], which encodes six open reading frames (ORF). ORFs V1 and V2 are on the virion-sense strand, while ORFs, C1, C2, C3, and C4 are located on the complementary-sense strand. Complementary and sense ORFs are separated by an intergenic region (IR) of approximately 300 nucleotides. V1 encodes the coat protein (CP), which is responsible for the encapsidation of the genome and is involved in the movement of the virus in the plant and its transmission by the vector insect [1].

The widespread occurrence of epidemics associated with begomoviruses and their potential threat to crop production, make it essential to develop

1. Moriones E, Navas-Castillo J. Tomato yellow leaf curl virus, an emerging virus complex causing epidemics worldwide. Virus Res 2000; 71(1-2):123-134.

2. Anderson PK, Morales FJ. The emergence of new plant diseases. The case of insect-transmitted plant viruses. Ann N Y Acad Sci 1994; 740:181-194.

3. Polston JE, Anderson PK. The emergence of whitefly-transmitted geminiviruses in tomato in the western hemisphere. Plant Dis 1997; 81:1358-1369.

4. Fauquet CM, Maxwell DP, Gronenborn B, Stanley J. Revised proposal for naming geminiviruses. Arch Virol 2000; 145(8): 1743-1761.

5. Herrera L, Guerra O, Ramos PL, Peral R, Echemendia AL, Ramírez N *et al*. Techniques for detection of Tomato Yellow Leaf Curl Geminivirus (TYLCV) in infected plants and viruliferous whiteflies. Biotecnología Aplicada 1999; 16(4):237-242.

6. Martinez ZY, de Blas C, Quinones M, Castellanos C, Peralta EL, Romero J. Havana tomato virus, a new bipartite geminivirus infecting tomatoes in Cuba. Arch Virol 1998; 143(9):1757-1772.

✉ Corresponding author

procedures for geminivirus detection in plants for disease management. Several attempts have been made including both serological, polymerase chain reaction (PCR) and DNA hybridization methods. Serological assays have met limited success with the whitefly-transmitted geminiviruses [14]. This is due to several deficiencies related with the low and moderate antigenicity of the geminivirus coat proteins, together with the low amount of CP recovered from infected plants [15]. Both factors make it difficult to obtain antibodies having high quality and specificity. In contrast, PCR and nucleic acid hybridization methods have provided sensitive techniques for the detection and identification of geminiviruses in infected plants and viruliferous whiteflies [5, 16, 17]. DNA probes are commonly labeled with radioisotopes as ^{32}P and ^{35}S. Alternatively, several non-radioactive agents (i.e. fluorescein, biotin, digoxigenin, metals (colloidal gold), enzymes (alkaline phosphatase and peroxidase) and systems conjugated with avidin biotin) have been used to avoid radioisotopes [18, 19]. Non radioactive-labeled probes can be safely handled and used after prolonged storage [20].

Here, we describe the establishment and validation of a diagnostic method for begomoviruses affecting several crops in Cuba, based on the non-radioactive hybridization of viral nucleic acids.

Materials and Methods

Plant material and DNA extracts

Samples of geminivirus-infected and healthy plants, collected in different regions of Havana, were used as the sick and healthy controls. One gram of plant tissue was collected for each sample. DNA extractions were carried out according to the procedure described by Dellaporta *et al.* [21].

PCR evaluation of DNA extracts

Samples from putative geminivirus-infected plants were screened by PCR using different primer sets (Table 1). The amplification reaction was carried out with the degenerate oligonucleotide pair C-D for begomovirus detection. The conditions were basically similar to those described by Rojas *et al*. [22], but the annealing temperature was set to 42 ºC. For TYLCV, PCR was carried out using the oligonucleotide pair A-B and the annealing temperature was 50 ºC. Healthy samples were always included in PCR evaluations.

Table 1. List of primers used in PCR.

Primer	Sequence (5′-3′)	Position in the viral genome
A	aatccatggtatgtcgaagcg accagg	5′ end of TYLCV CP, Complementary chain.
B	taaccatggttaatttgatatt gaatcatag	3′ end of TYLCV CP, Viral chain.
C*	gcatctgcaggcccacatygt cttyccngt	1978, Viral chain.
D*	aatactgcagggcttyctrtac atrgg	496, Complementary chain.
E	aaggatccaagcttacgagg catgttgaaatgaatc	5′ end of TYLCV IR, Complementary chain.

* Codes for degenerate positions: y,c/t;n,a/c/g/t;r,a/g. (Rojas *et al*. [22])

Sample preparation for dot blot assays

DNA extracts were diluted in TE 1X (Tris-HCl 10 mM, pH 8; EDTA 10 mM), heated for 5 min at 100 ºC and incubated at 4 ºC before using. Twenty µL of each sample were applied through on the commercial device (Hybri-Dot manifold, Gibco, USA) into nitrocelulose membranes (Amersham Pharmacia Biotech, USA). DNA was cross-linked to the membrane by exposure to ultraviolet light (in a crosslinker device Amersham Pharmacia Biotech, USA).

Non-radioactive labeling of DNA probes

Two different probes were obtained to discriminate between TYLCV-infection and the infection caused by other geminiviruses. The specific probe was a 500 nt fragment, which included the intergenic region and the 5' end of the TYLCV *cp* gene. On the other hand, the generic probe was the TYLCV *cp* gene (800 nt). The TYLCV *cp* gene was obtained by PCR using the TYLCV-specific oligonucleotides A and B (Table 1). PCR was carried out as mentioned above. The DNA fragment used as a template for the synthesis of the specific probe was obtained by PCR (oligonucleotides E and D, Table 1). The annealing temperature was 55 ºC.

Labeling reactions were carried out with 50 ng of DNA as a template, using the components and methodologies described by Gene Imagen Random Prime Labeling Kit (Amersham Pharmacia Biotech, USA). The probes obtained were stored at –20 ºC for later use.

Non-radioactive nucleic acid hybridization

The hybridization and detection conditions were performed according to the Gene Imagen Random Prime Labeling Kit (Amersham Pharmacia Biotech, USA). Hybridizations were done for 16-18 h at 60 ºC. Two washings, the first with 1X SSC (NaCl 3 M, sodium citrate 0.3 M, pH 7) at 60 ºC for 15 min and the second with 0.5X SSC (NaCl 3 M, sodium citrate 0.3 M, pH 7) were also carried out at 60 ºC.

Detection limits of the assays

Dot blot assay detection limits were determined using both the generic and specific probes. Dilutions of 5 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg of the TYLCV genome, cloned in the pZ200 vector and supplied with the DNA extract of a healthy plant, were evaluated. Signal intensity of the hybridization spots was determined using the Molecular Analyst software 1.4.1 (Biorad, USA). The calibration curves were determined from the regression analysis (Microsoft Excel, Microsoft, USA). The detection limit was defined as the lowest concentration to which the intensity value of the virus-containing sample spot was two fold higher than the intensity value of the healthy sample spots. This limit was also considered the cut-off value of the assay. The behavior of the signal intensity in three intra-assay and two inter-assay replicates were studied.

Determination of evaluation indicators

One-hundred and thirty-two samples, previously evaluated by PCR (see above), were assayed in the hybridization tests using the generic and specific probes. Samples infected with TYLCV, BGYMV, *Macroptilium yellow mosaic virus* (MaYMV) [23],

7. Ramos PL, Guerra O, Peral R, Rivera-Bustamante R, Oramas P. *Taino Tomato Mottle Virus*, a new bipartite geminivirus from Cuba. Plant Dis 1997; 81:1095.

8. Ramos PL, Guerra O, Dorestes V, Ramírez N, Rivera-Bustamante R, Oramas P. Detection of TYLCV in Cuba. Plant Dis 1996; 80(10):1208.

9. Cordero M, Ramos PL, Hernandez L, Fernandez AI, Echemedia AL, Peral R *et al*. Identification of *Tomato mottle Taino begomovirus* strains in Cuban potato fields. Phytoparasitica. in press 2003.

10. Ramos PL, Guevara-Gonzalez RG, Peral R, Ascencio-Ibanez JT, Polston JE, Arguello-Astorga G *et al*. *Tomato mottle Taino virus* pseudorecombines with PYMV but not with ToMoV: Implications for delimitation of *cis*- and *trans*-acting replication specificity determinants. Arch Virol. 2003; 148(9):1697-1712.

11. Echemendia AL, Ramos PL, Peral R, Fuentes A, Gonzalez G, Sanpedro J *et al*. Cuban isolate of Bean golden yellow mosaic virus is a member of the Mesomaerican BGYMV group. Plant Dis 2001; 85(9):1030.

12. Dominguez M, Ramos PL, Echemedia AL, Peral R, Crespo J, Andino V *et al*. Molecular Characterization of Tobacco leaf rugose virus, a New Begomovirus Infecting Tobacco in Cuba. Plant Dis 2002; 86(9):1050.

13. Palanichelvam K, Kunik T, Citovsky V, Gafni Y. The capsid protein of tomato yellow leaf curl virus binds cooperatively to single-stranded DNA. J Gen Virol 1998; 79 (Pt 11):2829-2833.

14. Harrison BD. Recognition and differentiation of seven whitefly-transmitted geminiviruses from India and their relationship to Africa cassava mosaic and Thailand mung bean yellow mosaic viruses. Ann Applied Biology 1991;118:299-308.

15. Harrison BD. Advances in geminivirus research. Annu Rev Phytopathol 1985; 23:55-82.

16. Navot N, Pichersky E, Zeidan M, Zamir D, Czosnek H. Tomato yellow leaf curl virus: a whitefly-transmitted geminivirus with a single genomic component. Virology 1991; 185(1):151-161.

17. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning. A laboratory manual. Second edition ed. New York.: Cold Spring Harbor Laboratory Press, 1989.

18. Mathews JA, Kricka LJ. Analytical strategies for the use of DNA probes. Anal Biochem 1988; 169:1-25.

19. Wilcheck M, Bayer EA. The avidin-biotin complex in bioanalytical applications. Anal Biochem 1988; 171:1-32.

20. Nakahara KHT, Hataya T, Uyeda I. A simple, rapid method of nucleic acid extraction without tissue homogenization for detecting virods by hybridization and RT-PCR. J Virol Methods 1999; 77:47-58.

21. Dellaporta S, Wood J, Hicks JB. A plant DNA minipreparation: version II. Plant Mol Biol 1983; 1:19-21.

22. Rojas MR, Gilbertson RL, Russell DR, Maxwell DP. Use of degenerate primers in the polimerase chain reaction to detect whitefly-transmitted geminiviruses. Plant Dis 1993; 77(4):340-347.

TbLRV and ToMoTV were employed as positive reference controls when the generic probe was assayed (all of them were kindly supplied by Ramos-González, CIGB, Havana). DNA samples from healthy plants were used as negative reference controls.

When the TYLCV-specific probe was tested, only TYLCV-infected samples were employed as positive reference controls. DNA samples from healthy plants and DNA samples from plants infected with other geminiviruses above mentioned, were included as negative reference controls. Statistical analyses of results were carried out by the Student test ($p<=0.05$).

Two intra-assay replicates were used for the samples analyzed with both probes. The evaluation indicators such as efficacy, diagnostic and analytical sensitivity, diagnostic and analytical specificity, negative and positive prediction values were determined [24-27].

Results and Discussion

Confirmation of the presence/absence of begomovirus in the reference controls

PCR was selected as the reference technique to evaluate the performance of the dot blot test developed in this study. All DNA extracts obtained from symptomatic and healthy plants were evaluated to verify if the begomoviruses were present. To accomplish this, PCRs were carried out using a set of degenerate primers (C and D, Table 1), which were designed by Rojas *et al*. [22] to detect a wide range of begomoviruses. The presence of begomoviruses was determined through the visualization of amplified fragments on 0.8% agarose gel (Figure 1, Lanes 2, 3, 5, 6 and 7). In contrast, healthy plants were corroborated when any product was amplified from their nucleic acid extracts (Figure 1, Lane 4).

Usually, after the PCR using primers C-D, begomoviruses can be separated into two different groups because they yield different size bands (i.e. Old and New world groups with 1.4 and 1.2 kb amplicons, respectively). However, this assay did not allow us to reveal the identity of a specific begomovirus. Furthermore, this PCR gives amplicons that are smaller than the predicted size for some New World geminivirus groups and results could be confusing. Hence, specific primers for this virus were designed (primers A and B, Table 1) and used to discriminate the presence of TYLCV among other begomoviruses occurring in Cuba. Amplicons of around 800 bp were observed on the 0.8% agarose gel, which corresponded to the *cp* gene of this virus (Figure 2, Lanes 2, 3, 4, and 5). Amplification signals were not present either in healthy samples or in those infected with geminiviruses other than TYLCV (Figure 2, Lanes 6 and 7). From the 132 samples analyzed, 81 showed geminivirus characteristic amplicons (65: TYLCV infected and 16: other begomovirus), and the rest were confirmed to be healthy samples.

Validation of the non-radioactive dot hybridization using the generic and specific probes

After labeling the probes, the reference controls previously tested as described above, were used to evaluate the analytical specificity of the dot blot hybridization assays in preparation here. Also, detection limits using both types of probes were assessed. In this case, they were determined using decreasing serial dilution of the viral DNA.

The use of the generic probe (TYLCV *cp*) showed intensity values in a range of 4.3 to 18.5 for the TYLCV-containing samples. These values were as average, 3.4 fold higher than the values shown by the healthy ones (intensity value range: 2.2 to 3.9) (Figure 3A). Dot blot using the generic probe was able to detect as little as 10 pg of TYLCV (Figures 4A and 5A). Additionally, the generic probe was assayed to detect the presence of other begomoviruses found in Cuba. Although it reacted with all the viruses used here, detection limits for each one were different. In a first attempt, the hybridizations were made at 50 ºC. The presence of ToMoTVwas detected in plant DNA extracts containing as little as 9.1 ng of this virus. Meanwhile, detection limits for MaYMV, TbLRV and BGYMV were set at 14 ng, 286 ng and 541 ng, respectively. When hybridizations were carried out at 60°C, detections were highly impaired and spots were only observed when high amounts of viral DNA were assayed (i.e. 909 ng of ToMoTV, 455 ng of MaYMV and 541 ng of BGYMV). The generic probe was unable to detect TbLRV in this condition even when 286 ng of the viral DNA were assayed.

23. Ramos PL, Fernandez AI, Castrillo G, Diaz L, Echemedia AL, Fuentes A *et al*. Macroptilium yellow mosaic virus, a New Begomovirus Infecting *Macroptilium lathyroides* in Cuba. Plant Dis 2002; 86(9):1049.

24. Burkhardt U, Mertens T, Eggers J. Comparison of two Commercially Available Ant-HIV ELISAs: Abbott HTLV III EIA and Du Pont III ELISA. J Virol Methods 1987; 23(3):217.

25. Kantor I, Berrera L, Ritacco V, Miceli I. Utilidad del enzimoinmunoensayo en el diagnóstico de la tuberculosis. Bol Ofic San Pan 1991; 6(110):461-470.

26. van Kerckhoven IK, Vercauteren KG, Piot P, van der Groen P. Comparative evaluation of 36 Commercial assays for detecting antibodies to HIV. Bull Wold Health Organization 1991;69(6):753-760.

27. Argote E, López G. Pautas para evaluar la calidad de los juegos diagnósticos basados en la técnica ELISA. Rev Cub Cient Vet 1995; 24(2):16-19.

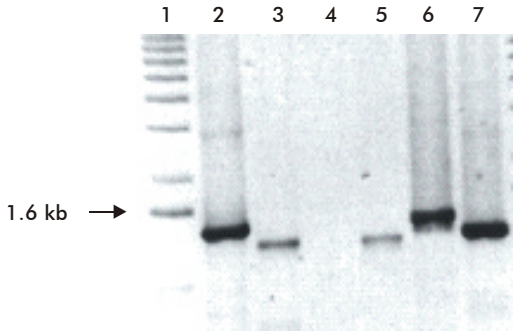

Figure 1. Polymerase chain reaction (PCR) amplification of 1.4 and 1.2 kb DNA fragments using the degenerate oligonucleotides C and D. Lane 1- MW-1Kb ladder size marker (Gibco, USA). Lanes 2-3 and 5-7-Extracts of DNA from infected plants. Lane 4-Extract of DNA from a healthy plant.

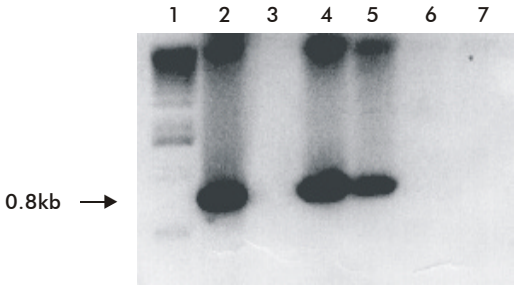

Figure 2. Polymerase chain reaction (PCR) amplification of 800 bp DNA fragments using the oligonucleotides A and B. Lane 1- MW-1Kb ladder size marker (Gibco, USA). Lanes 2, 3, 4 and 5-Extracts of DNA from TYLCV infected plants. Lane 6- Extract of DNA from infected plant with other begomovirus. Lane 7- Extract of DNA from healthy plant.

The high identity percentage between the begomovirus *cp* genes is well known and it is the reason why these DNA fragments are usually selected for geminivirus detection assays by DNA hybridization. In our case the TYLCV *cp* gene was useful to detect the presence of several begomovirus as predicted according to its identity values with the cognate gene from the other viruses analyzed (ToMoTV 67.1%, TbLRV 66.4%, BGYMV 67.9% and MaYMV 69.2%). However, we also demonstrate here, a significant reduction in detection limits when the TYLCV *cp* probe is used in heterologous hybridizations, especially for TbLRV and BGYMV.

The use of the TYLCV-specific probe (common region, CR) showed better resolution between infected and healthy samples than the generic probe. Intensity values for the positive samples ranged between 6 and 31.7 (5 fold higher than the negative values), while the values of by the negative ones ranged between 2.7 and 3.7 (Figure 3B). Dot blots using the specific probe allow us to detect 100 pg of TYLCV (Figures 4B and 5B). Samples infected with other begomoviruses found in Cuba were not revealed as expected, given the low identity percentage between their common regions (ToMoTV 43.2%, TbLRV 41.1%, BGYMV 44.3% and MaYMV 43.9%).

In general, the intensity values of the samples tested with the generic probe were significantly superior to the intensity values of the samples analyzed using the specific probe ($p<=0.05$). Probably, different lengths of both probes (800 bp for *cp* gene and 500 bp for CR) may account for the different intensity values. Larger fragments are more able to incorporate fluorescein-labeled dUTP nucleotides to the probe and this increases the intensity of the signal obtained.

When a pathogen diagnosis test is performed, the assessment of several parameters that warrant its quality is a prerequisite. To address this, indicators such as efficacy, diagnostic sensitivity, diagnostic specificity, and negative and positive prediction values were evaluated. Reference controls, previously verified by PCR, were used for a population assay. Basic values needed to estimate the validation indicators of the non-radioactive dot blot hybridization technique were determined for both generic and specific probes (Table 2). After data processing, the two variants showed evaluation indicators of over 85%, which were considered satisfactory (Table 3). In the case of the use of the generic probe, the efficacy was 93%, while the other variables remained between a 85% and 100%. In contrast, an efficacy of 100% was obtained when the specific probe was used. Other evaluation indicators were also 100%, which showed the effectiveness of this probe. The assays that were carried out to determine the repeatability of the methods analyzed showed 100% correspondence.

The results described above demonstrated that the use of the TYLCV-specific probe allowed a better detection precision than the generic probe, although both probes can be used for the detection of geminiviral pathogens. Selection of a particular probe must be determined according to the level of specificity required in a given diagnosis.

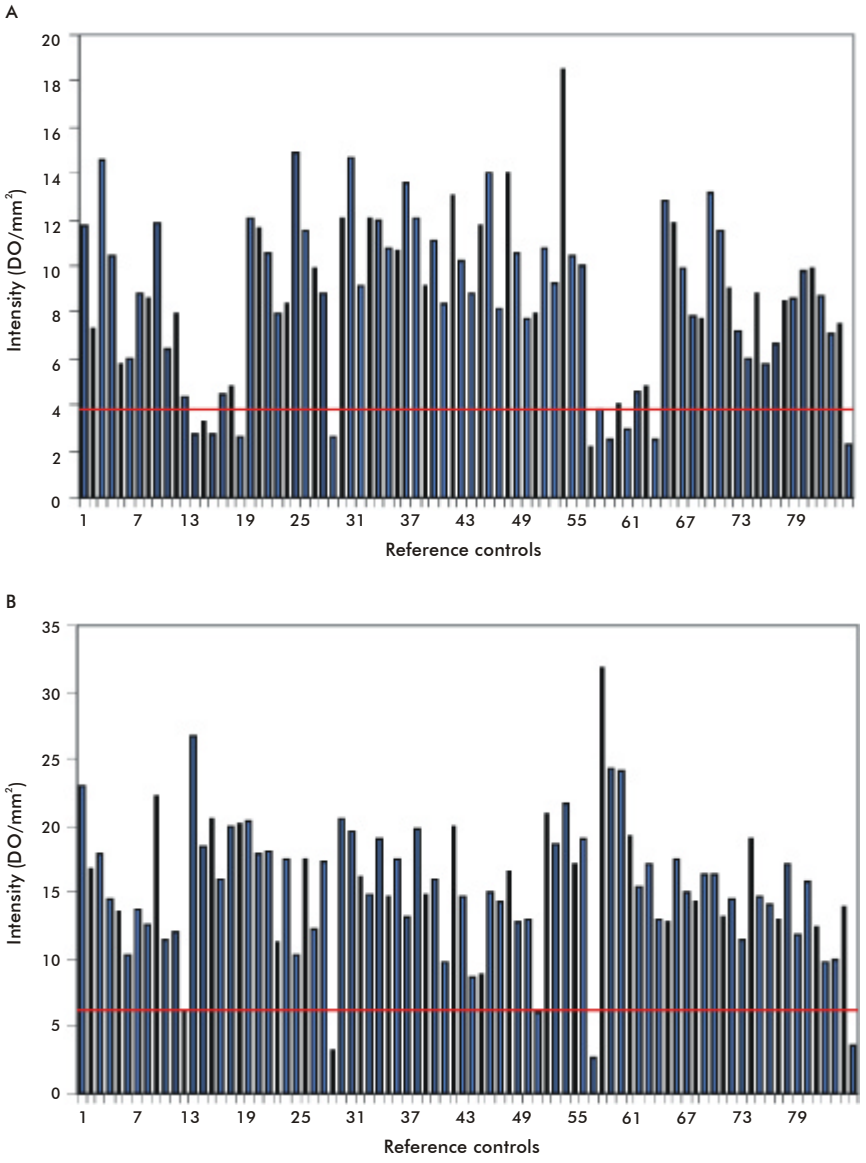

Figure 3. Signal intensity values from reference controls in the non-radioactive nucleic acid detection using the generic (A) and specific (B) probes. (Molecular Analyst Software, 1992-1995, version 1.4.1; Biorad, USA). Samples 1-27, 29-55, 57-83: Positive reference controls; 28, 56 and 84: Negative reference controls. Red lines represent the cut-off values.

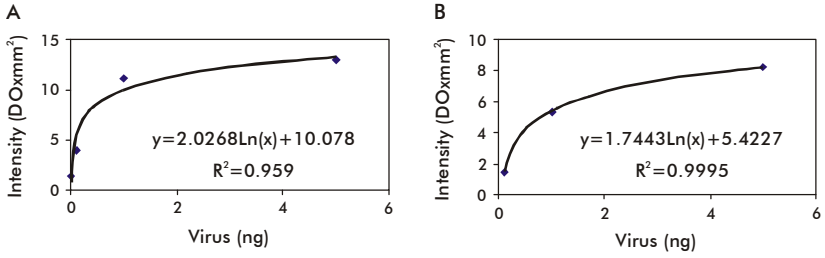

Figure 4. The calibration curve of the non-radioactive nucleic acid detection using the generic (A) and specific (B) probes (Molecular Analyst software, 1992-1995, version 1.4.1; Biorad, USA).

In general, indicators obtained here resemble the parameters shown by similar molecular techniques

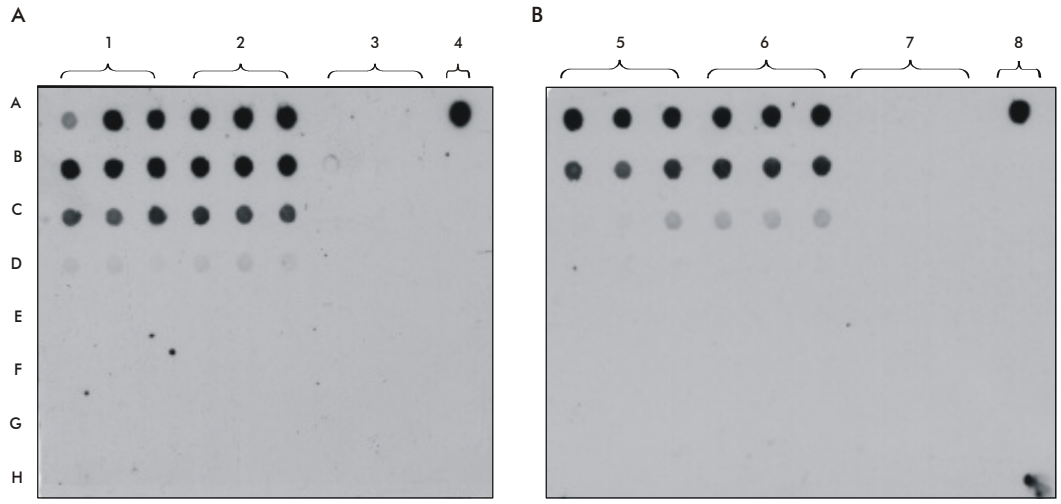

Figure 5. Comparison of analytical sensitivity from the full-length TYLCV genome cloned in a PZ200 vector by Dot blot hybridization using fluorescein-labeled probes. A- Generic probe. B- Specific probe. Columns 1-2 and 5-6- Inter-assay replicates that contains eight dilutions (from top to bottom) of pZTYLCV: 5 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg. Columns 3 and 7-Negative controls (pZ200). Spot 4 and 8- Positive controls (pZTYLCV).

for previously developed virus and viroid diagnosis [28, 29]. In our case analytical sensitivity obtained using both the generic or the specific probe was higher or similar to that reported by some authors that describing the diagnosis of viral pathogens. Caciagli et al. [30], showed the TYLCV diagnosis by Dot blot hybridization based on chemioluminiscent detection of the virus or cloned viral genome using a digoxigenine labeled probe. In that report, detection limits varied from film to film in a range between 38 and 320 pg. Also, González *et al*. [28], used a chemioluminiscent system for viroid diagnosis and they were allowed to obtain hybridization signals of up to 1 ng of the target.

Although radioactive methods have been widely used for several purposes, including geminivirus detection [5, 31], the introduction of non-radioactive techniques have been necessary due to the environmental and technological disadvantages of the radioactive procedure. Several authors have reported the use of the non-radioactive technique using markers such as biotin, fotobiotin and digoxigenine, which are able to detect viral concentrations as low as the radioactive techniques [32-35]. Our results also support this and consolidate the use of the non-radioactive technique for phytopatogen diagnosis in our country. Specifically, they show the feasibility of this method for the detection of geminiviruses affecting economically important crops in Cuba.

Table 2. Basic values to determine the validation parameters from a non-radioactive nucleic acid hybridization technique using the generic and specific probes. tp: true positives, tn: true negatives, fp: false positives, fn: false negatives.

		CONTROLS*			
		infected		healthy	
Generic probe	Positive	72	tp	9	fp
	Negative	0	fn	51	tn
Specific probe	Positive	81	tp	0	fp
	Negative	0	fn	51	tn

*Tested by PCR as described in Material and methods.

Table 3. Validation parameters determined from non-radioactive nucleic acid hybridization technique using the generic and specific probes.

PARAMETERS	GENERIC PROBE	SPECIFIC PROBE
Diagnostic sensitivity	100%	100%
Diagnostic specificity	85%	100%
Efficacy	93%	100%
Positive prediction value	89%	100%
Negative prediction value	100%	100%
Analytical sensitivity	10 pg	100 pg
Repeatability	100%	100%

Acknowledgements

The authors wish to thank Drs. Alberto Salazar Rodríguez and Jorge Luis Pérez (Centro de Ingeniería Genética y Biotecnología, La Habana, Cuba) for their technical support and critical review of this manuscript.

28. Gonzalez L, Soto M, Ortíz MI, Peralta EL. Determinación de parámetros analíticos de las técnicas de hibridación de ácidos nucleicos para la detección del viroide del tubérculo ahusado de la papa (PSTVd). Fitopatología 2001; 36(1):24-33.

29. Martinez ZY. Contribucion al conocimiento de geminivirus que afectan el cultivo del tomate (*Lycopersicon esculentum*) en Cuba. [dissertation]. San Jose de las Lajas: Universidad Agraria de la Habana; 1998.

30. Caciagli P, Bosco D. Quantitative determination of tomato yellow leaf curl geminivirus DNA by chemiluminescent assay using digoxigenin-labeled probes. J Virol Methods 1996; 57(1):19-29.

31. Waterhouse PM, Gerlach WL, Miller WA. Serotype-specific and general luteovirus probes from cloned cDNA sequences of barley yellow dwarf virus. J Gen Virol 1986; 67:1273-1284.

32. Li SF, Onodera.S, Sano T, Yoshida K, Wang GP, Shikata E. Gene diagnosis of viroid: Comparisons of return-PAGE and hybridization using DIG-labelled DNA and RNA probes for practical diagnosis of Hop Stunt, Citrus Exocortis and Apple Scar Skin viroids in their natural host plants. Ann Phytopatol Soc Jpn 1995; 61:381-390.

33. Nakahara KHT, Hataya T, Sugimoto T, Kimura I, Shigata E. A mixture of synthetic probes labelled with biotin for the sensitive detection of potato spindle tuber viroid. J Virol Methods 1998; 71:219-227.

34. Romero-Durban JC, Cambra M, Duran-Vila N. A simple imprint-hybridization method for detection of viroids. J Virol Methods 1995; 55:37-47.

35. Singh RP, Boucher A, Laksman DK, Tavantzis SM. Multimeric non-radioactive cRNA probes improve detection of potato spindle tuber viroid (PSTVd). J Virol Methods 1994; 49:221-234.

Received in May, 2003. Accepted for publication in September, 2003.