Original Article

Evaluation of a non-radioactive dot-blot nucleic acid hybridization as a detection tool for citrus leaf blotch virus in Cuba

Evaluación de la hibridación de ácidos nucleicos no radiactivo *dot-blot* como herramienta de detección para citrus leaf blotch virus en Cuba

http://opn.to/a/AzuqG

**Lester Hernández-Rodríguez [1] [2] [*], Gabriel García-García [1] [3], Juana María Pérez-Castro [1],
Lochy Batista-Le Riverend [1], Victoria Zamora-Rodríguez [1], Inés Peña-Barzaga [1],
Pedro Luis Ramos-González [1] [4]**

[1]Instituto de Investigaciones en Fruticultura Tropical, P.O. Box 11 300, 7th Ave. # 3005, Playa, La Habana, Cuba.
[2]Instituto Nacional de Investigación Agropecuaria (INIA), Estación Experimental INIA Salto Grande. Camino al Terrible, CP 50000, Salto, Uruguay.
[3]Analytical Food Laboratories, 860 Greenview Drive, Grand Prairie, TX 75050, USA.
[4]Instituto Biológico de São Paulo. Av. Conselheiro Rodrigues Alves 1252, São Paulo 04014-002, Brazil.

ABSTRACT: A non-radioactive dot-blot nucleic acid hybridization method was evaluated for detecting citrus leaf blotch virus (CLBV, *Flexiviridae*: *Citrivirus*) in total RNA extracts from kumquat trees. DNA fragments partially encompassing the open reading frames encoding the polymerase (RdRp) and the movement (MP coat (CP) proteins of CLBV were used to generate digoxigenin (DIG-11-dUTP) probes by PCR. Probes were individually generated using cloned DNA fragments of the Cuban CLBV isolate as templates. In the assays, several working conditions, including the use of different membranes, hybridization and washing buffers were tested. The method detected up to 40 pg of a plasmid containing the complete CLBV genome, as well as viral genomic sequences in RNA extracts of naturally infected trees of 'Nagami' kumquat (*Fortunella margarita*) and 'Round' kumquat (*F. japonica*). Results support considering the method developed an appropriated tool to be used after its validation by the programs of sanitation, quarantine, and certification of citrus propagation materials in Cuba.

Keywords: budwood, certification, *Citrivirus*, CLBV, diagnosis, *Flexiviridae*, *Fortunella*.

RESUMEN: Se evaluó un método de hibridación de ácidos nucleicos no radioactivo en formato *dot-blot* para la detección precisa de citrus leaf blotch virus (CLBV: *Citrivirus*) en extractos de ARN totales de árboles de cítricos. Los fragmentos de ADN que conforman parcialmente los marcos de lectura abiertos que codifican la polimerasa (RdRp), la proteína de movimiento (MP) y la proteína de la cubierta (CP) de CLBV se utilizaron para generar sondas marcadas con digoxigenina (DIG-11-dUTP) mediante PCR. Para generar las sondas se utilizaron fragmentos de ADN clonados a partir del aislado de CLBV de Cuba. Se evaluaron varias condiciones de trabajo que incluyeron el uso de diferentes membranas, soluciones de hibridación y tampones de lavado. Con el método evaluado fue posible detectar hasta 40 pg de un plasmidio que contiene el genoma completo de CLBV, así como material genómico del virus en extractos de ARN de plantas de kumquat 'Nagami' (*Fortunella margarita*) y kumquat 'Round' (*F. japonica*) infectadas de forma natural con el virus. Los resultados evidenciaron que el método desarrollado es una herramienta útil y altamente recomendable, una vez sea validado, para su uso en los programas de saneamiento, cuarentena y certificación del material de propagación de cítricos en Cuba.

Palabras clave: certificación, CLBV, *Citrivirus*, *Flexiviridae*, diagnóstico, *Fortunella*, material de propagación.

INTRODUCTION

Citrus leaf blotch virus (CLBV) has been detected in *Citrus* sp. and its relatives' genera around the world, including California (USA), Italy, Spain, Japan, Australia, New Zealand, Cuba, and China (1-8). In nature, besides plants of the family *Rutaceae*, CLBV infects plants of sweet cherry [*Prunus avium* (L.) L.], kiwifruit [*Actinidia deliciosa* (A. Chev.) C. F. Liang & A. R. Ferguson], and peony [*Paeonia lactiflora* Pall.] (9-12). Viral transmission mainly occurs through vegetative propagation of infected materials and, at a lower rate, by infected seeds (13). CLBV can also be mechanically transmitted to the herbaceous plants *Nicotiana cavicola* N.T.Burb., *Nicotiana benthamiana* Domin and *Nicotiana occidentalis* H.-M. Wheeler (2,14-16), but its vector-mediated transmission has not been demonstrated.

Citrus leaf blotch virus is the type-species, and so far, the only assigned to the genus *Citrivirus*, family *Betaflexiviridae* (17). CLBV virions are filamentous, with 960 nm length and 14 nm width, and shelter a positive-sense, single-stranded RNA molecule (gRNA) of 8,747 nt as genome (2,18,19). CLBV gRNA comprises three open reading frames (ORFs) and two untranslated regions (UTRs) at its 5′ and 3′ ends. ORF1 encodes a ~227 kDa polyprotein that harbors the methyl-transferase (MET), AlkB-like, Out-like peptidase, papain-like protease (PRO), helicase and RNA-dependent RNA polymerase (RdRp) domains (19). ORF2 and ORF3 encode the ~40 kDa cell-to-cell movement protein (MP) and the 41 kDa coat protein (CP), respectively (19). Both MP and CP are translated from independent subgenomic RNAs (sgRNAs), which are synthesized at different rates during the infectious cycle (20,21).

Traditionally, CLBV detection is conducted by biological indexing assays in which the graft-inoculated seedlings of 'Dweet' tangor (*Citrus tangerina* Hort. Ex Tan. × *C. sinensis* [L.] Osb.) develop stem pitting and those of 'Etrog' citron (*C. medica* L.) show chlorotic blotching on the young leaves (2,14). Moreover, procedures based on nucleic acid hybridization and reverse transcription followed by end-point or quantitative polymerase chain reaction (RT-PCR or RT-qPCR) have also been developed (22-24).

In 2016, two CLBV-infected plants of *Fortunella* sp. were detected in the western region of Cuba (6). After the detection of the virus, a wide survey was conducted by the Cuban System for Production of Citrus Certified Budwood to restrain the virus dissemination. However, that survey was mostly conducted using an RT-PCR method as a diagnostic tool. It represented a limitation due to the high cost per analysis, which even at present restricts the number of samples to be certified. As an attempt to overcome this limitation, this work describes the development and evaluation of a non-radioactive dot-blot nucleic acid hybridization method for the detection of CLBV. This methodology is considered cheaper and appropriate when large quantities of samples are required to be processed (25,26).

MATERIAL AND METHODS

Viral isolates and total RNA extraction

CLBV sub-isolates used in this study were graft-propagations of plants containing the Cuban CLBV isolates FM (sub-isolates: FM-1 and FM-2) and FJ (sub-isolates: FJ-1 and FJ-2), previously biologically and molecularly characterized (6). All plants were maintained in a temperature-controlled glasshouse (22-26 °C) following technical instructions for citrus cultivation (27). Total RNA extracts were obtained from 100 mg of tender leaves (24) using the TRIzol LS Reagent kit, according to the manufacturer's instructions (Invitrogen, Scotland, UK). Additionally, RNA extracts from a healthy 'Etrog' citron tree obtained from certified seeds and maintained under temperature-controlled glasshouse (22-26 °C) were used as negative controls.

DIG DNA labeling

Non-radioactive probes were generated using the *PCR DIG-labeling and Detection kit* (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Each probe was obtained in an independent reaction adding the primer pairs KU-54/KU-55 for the RdRp amplicon [468 bp] (28); KU-8/KU-34 for MP

[674 bp] ([19](#)) and KU-18/KU-19 for the CP [437 bp] ([28](#)). DNA templates in PCR reactions were 10 ng of each plasmid containing the RdRp, MP and CP DNA fragments from the Cuban CLBV FM isolate ([6](#)). DNA labeling process was verified simultaneously by comparing the amplicon sizes of DIG PCR products, those of the unlabeled ones (amplicon of the same PCR reactions without DIG) ([29](#)), and the bands of the DNA molecular weight marker (100 bp ladder, Promega, Madison, USA) in 1.5 % agarose gels electrophoresis. Electrophoresis was carried out in 1X TAE buffer (40 mM Tris-acetate, pH 8.3, 1 mM EDTA) at 100 V for 30 min, and the products were visualized on a UV-transilluminator after ethidium bromide staining ([30](#)). Finally, DNA labeled probes were purified using a High Pure PCR Product Purification Kit (Roche Diagnostic) after electrophoresis on a low-melting-point 0.8 % agarose gel with the same conditions described above. DNA and RNA concentrations and sizes (only for DNA fragments) were determined by visual comparison with the DNA molecular weight marker bands (100 bp ladder, Promega, Madison, USA) on the agarose gel.

Nucleic acid hybridization experiments
General hybridization conditions

Nucleic acid hybridization was carried out as recommended by the Dig Luminescent Detection Kit manual (Roche Diagnostics, Mannheim, Germany). Twenty µL of sample solution was dripped on nylon membranes (different types of membranes were tested, further described for each experiment). Plant RNA (~10 µg) and DNA (mass varied in each sample, indicated below in each experiment) samples were denatured in 50 % formamide at 65 °C for 15 min. and at 95 °C for 10 min, respectively, and immediately chilled on ice. The samples were spotted onto the membranes using a commercial device (Bio-Dot, Bio-Rad) and fixed during 2 h at 80°C in a Grant BOEKEL HIR12 hybridization oven (Grant/ Boekel, Cambridge, UK). Membranes were prehybridized for 1 h at 50 °C in Church buffer ([30](#)). After a change of the Church buffer, the membranes were hybridized at 60°C for 16 h with the DIG-probe mixture (100 ng x cm^2), previously denatured at 95°C for 10 min. The membranes were washed twice, the first with 1X SSC (3 M NaCl, 0.3 M sodium citrate, pH7) at 60°C for 15 min and the second with 0.5X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7) also at 60 °C. Subsequently, the membranes were incubated at room temperature in Blocking Solution (Roche Diagnostics, Mannheim, Germany). Afterward, the membranes were incubated with the anti-DIG antibody (1:1000) for 30 min at room temperature, according to the standard procedure (Roche Diagnostics, Mannheim, Germany). Chemiluminescence was achieved using CPD-star substrate (Roche Diagnostics, Mannheim, Germany), as recommended in manufacturer instructions, and detected in Kodak S-Omat films at 37°C for 30 min. In all the assays, an intra-assay replicate of each sample was included, and all the experiments were replicated at least once.

Evaluation of nucleic acid hybridization conditions

To evaluate the best nucleic acid hybridization conditions, a first experiment with two treatments was conducted to evaluate the performance of three types of membranes and blocking solutions. In this experiment, the RdRp probe was used as recommended ([22](#)). Treatment 1 included three membrane types that differed in surface charge and manufacturer: (i) Hybond-N (Amersham, England), (ii) Hybond-N (Roche Diagnostics, Mannheim, Germany) and (iii) Hybond-N + (Roche Diagnostics, Mannheim, Germany). Treatment 2 evaluated four variants of blocking solutions before the anti-DIG antibody incubation: (i) 1 X PBS, 1 % PVP K25, 0.05 % Tween 20 and 1 % BSA; (ii) 1 X PBS, 1 % PVP K25, and 1 % BSA; (iii) 1 X PBS, 0.05 % Tween 20, and 1 % BSA, and (iv) Blocking Solution (Roche Diagnostics, Mannheim, Germany). Each membrane included spots that contained a calibration curve with decreasing quantities of 25 ng, 5 ng, 1 ng, and 200 pg of a plasmid containing the complete genome cloned of the Spanish CLBV isolate, kindly provided by Dr. José Guerri, Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain. The molecular characterization of the two Cuban CLBV isolates (FM and FJ) showed nucleotide

identities over 95 % with the Spanish CLBV isolate ([6](#)).

Selection of the probe

After selection of the best nucleic acid hybridization conditions, a new experiment was conducted to test different combinations of the probes: (i) one probe (RdRp, MP, or CP) per trial, (ii) a mixture of the probes MP and CP, or (iii) all probes together (RdRp + MP + CP). Each membrane included spots that contained a calibration curve with decreasing quantities of the plasmid with the complete genome of the Spanish CLBV isolate cloned (100 ng, 50 ng, 25 ng, 5 ng, 1 ng, 200 pg, and 40 pg).

Evaluation of the non-radioactive dot-blot nucleic acid hybridization for the detection of CLBV in total RNA extracts of infected plants

To evaluate the capability of the method for detecting CLBV in total RNA extracts of infected plants, a final test was conducted using the best nucleic acid hybridization conditions and probe combination that were determined in the experiments described above. Total RNA extracts of the plants containing CLBV sub-isolates FM-1, FM-2, FJ-1, and FJ-2 were used to evaluate the detection of the virus by the assayed method in nucleic acid preparations. Two replicates from plants infected with each CLBV sub-isolate (20 µL with ~10 µg of total RNA) were transferred onto the membrane. Besides, the membrane included a calibration curve comprised of 100 ng, 50 ng, 25 ng, 5 ng, 1 ng, 200 pg, and 40 pg of the plasmid with the complete genome of the Spanish CLBV isolate cloned. An 'Etrog' citron seedling RNA solution (20 µL with ~10 µg of total RNA) was used as the negative control. Samples with spots showing hybridization signals three times more intense than those of the negative controls by visual observation were considered positive.

RESULTS AND DISCUSSION

CLBV non-radioactive probes labeling

DNA amplicons of the expected sizes, 468 bp, 675 bp, and 437 bp, were obtained in the non-DIG assays (non-labeled controls) with the RdRp, MP and CP primer pairs, respectively ([Figure 1](#), lanes 1, 3, and 5). When DIG-11-dUTP

were used, PCR products showed a markedly higher weight ([Figure 1](#), lanes 2, 4, and 6) indicating the incorporation of the modified nucleotide in the probes ([29](#)). Bands of the MP and CP probes were stronger than that of the RdRp probe, suggesting that optimization of this PCR reaction might be required.
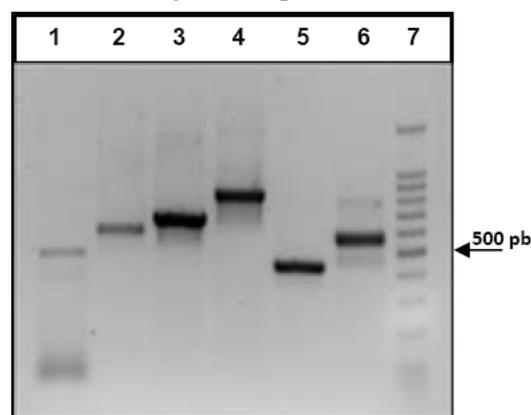

Figure 1. Amplified products in the PCR DIG-labeling of RdRp, MP and CP CLBV non-radioactive probes (lanes 2, 4, and 6) and same PCR products without DIG incorporation (lanes: 1, 3, and 5). Lane 7: Molecular weight marker 100 bp ladder (Promega). / Productos amplificados en el PCR de marcaje con DIG de las sondas no radiactivas RdRp, MP y CP CLBV (carriles 2, 4 y 6) y los mismos productos de PCR sin incorporación de DIG (carriles: 1, 3 y 5). Carril 7: marcador de peso molecular escalera de 100 pb (Promega).

Optimization of non-radioactive hybridization conditions

Positive hybridization signals were obtained in all the trials using the RdRp probe ([Fig. 2](#)). However, the combination of the lowest background and highest signal intensity was verified using the Hybond-N+ membrane (Roche Diagnostics, Mannheim, Germany) and the commercial Blocking Solution (Roche Diagnostics, Mannheim, Germany) ([Fig. 2](#), position D-III). In the experiment, intra- and inter- assay replicates showed similar intensities, showing the reproducibility of the results. Thus, this membrane and blocking solutions were chosen for the following assays.

Detection sensibility of the CLBV non-radioactive probes

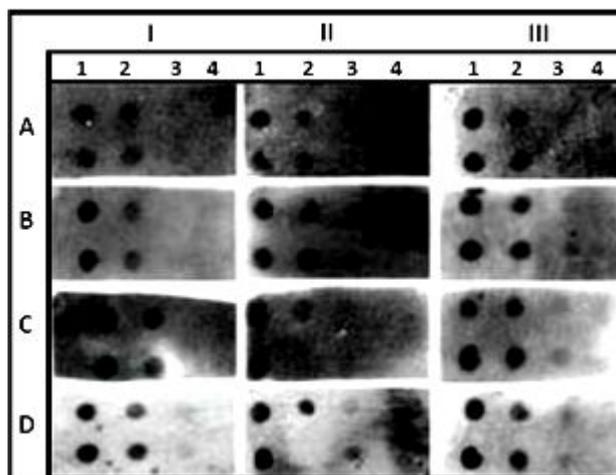All the probes combinations reacted with the CLBV genome, although with different intensity

Figure 2. Hybridization of the CLBV non-radioactive RdRp probe with the plasmid containing the CLBV complete genome transferred onto three different membranes: columns I- Hybond-N (Amersham, England); II- Hybond-N (Roche Diagnostics, Mannheim, Germany), and III- Hybond-N+ (Roche Diagnostics, Mannheim, Germany), using four different blocking solutions: rows A: 1X PBS, 1 % PVP K25, 0.05 % Tween 20, 1 % BSA; B: 1X PBS, 1 % PVP K25, 1 % BSA; C: 1X PBS, 0.05 % Tween 20, 1 % BSA and D: Roche Blocking Solution (Roche Diagnostics, Mannheim, Germany). Lanes 1, 2, 3, and 4 contained serial dilutions of the plasmid (1- 25 ng, 2- 5 ng, 3- 1 ng, and 4- 200 pg). All the samples transferred to membranes had two replicas. / Hibridación de la sonda RdRp no radioactiva de CLBV con el plásmido que contiene el genoma completo de CLBV transferido a tres membranas diferentes: columnas I-Hybond-N (Amersham, Inglaterra); II- Hybond-N (Roche Diagnostics, Mannheim, Alemania) y III- Hybond-N + (Roche Diagnostics, Mannheim, Alemania), utilizando cuatro soluciones de bloqueo diferentes: filas A: 1X PBS, 1 % PVP K25, 0.05 % Tween 20, 1 % de BSA; B: 1X PBS, 1 % PVP K25, 1 % BSA; C: 1X PBS, 0.05 % Tween 20, 1 % BSA y D: Solución de bloqueo Roche (Roche Diagnostics, Mannheim, Alemania). Los carriles 1, 2, 3 y 4 conttuvieron diluciones en serie del plásmido (1-25 ng; 2-5 ng; 3-1 ng y 4-200 pg). Todas las muestras se transfirieron a las membranas por duplicado.

(Fig. 3). In single-probe trials, the CLBV-containing plasmid was detected with the same analytical sensitivity (~1 ng) (Fig. 3, signals in lane 5). Interestingly, the use of probes MP and CP led to weaker spots, almost imperceptible at the highest dilutions (200 pg and 40 pg) (Fig. 3, lane 6). It should be noticed, however, that positive signals could not be ruled out for an intermediate value of the plasmid dilution between 1 ng and 200 pg. Analogous to the previous experiment, intra- and inter- assay replicates showed similar intensities.

Moreover, since the CP template is only 30 nt shorter than RdRp, the lengths of the DNA fragments of the probes may not account for the observed difference in the signal intensity. Conversely, DIG-11-dUTP might be more frequently incorporated in the MP and CP probes than in the RdRp probe. DIG-11-dUTP is incorporated in the *de novo* synthesized DNA fragments during polymerization in the substitution of thymine (T) (33). Thus, CLBV CP amplicon shows 60 % of A + T [263 (A+T) / 437 (A+T+C+G)]; whereas this value in the RdRp amplicon is slightly lower [55 %: 257 (A+T) / 468 (A+T+C+G)], representing a highest probability of DIG-11-dUTP incorporation in the DNA fragments when the probe is synthesized (30,31). In addition, RdRp and CP probes are smaller (< 207 bp) than the derived from the MP, which shows an approximately similar A+T composition [382 (A+T) / 675 (A+T+C+G)]. Other factors might bias the DIG-11-dUTP assimilation into *de novo* DNA fragments and their causes have been discussed elsewhere (31).

Four different sgRNAs have been described in CLBV-infected plants, two of which are 3' co-terminal and the other two are 5' co-terminal with gRNA (20). The two 3' co-terminal sgRNAs are 3 and 2 kb in size, encoding the MP and CP
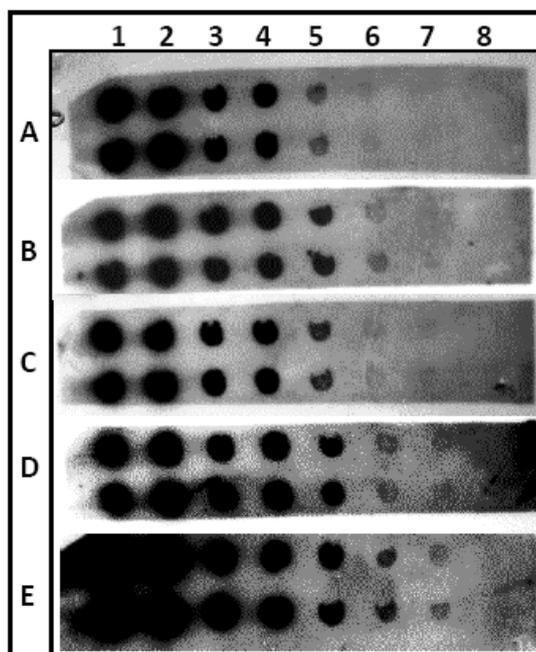
Figure 3. Hybridization of the CLBV non-radioactive probes with a plasmid containing the complete genome of the Spanish CLBV isolate to determine the detection sensibility of different probe combinations. Probes in rows: A- RdRp, B- MP, C- CP, D- CP+MP, and E- CP+MP+RdRp. Columns: 1 to 7, serial dilutions of the plasmid with CLBV complete genome containing: 1- 100 ng, 2- 50 ng, 3- 25 ng, 4- 5 ng, 5- 1 ng; 6- 200 pg, 7- 40 pg, and 8- 20 µL (~10 µg) of RNA of a healthy 'Etrog' citron (negative control). All the samples were applied with two replicates. / Hibridación de las sondas no radiactivas de CLBV con un plásmido que contiene el genoma completo del aislado de CLBV español para determinar la sensibilidad de detección de diferentes combinaciones de sondas. Sondas en filas: A- RdRp, B- MP, C- CP, D- CP + MP y E- CP + MP + RdRp. Columnas: 1 a 7, diluciones en serie del plásmido con genoma completo CLBV que contuvieron: 1-100 ng; 2- 50 ng; 3- 25 ng; 4- 5 ng; 5- 1 ng; 6- 200 pg; 7-40 pg y 8-20 µL (~ 10 µg) de ARN de un cidro 'Etrog' sano (control negativo). Todas las muestras se transfirieron a las membranas por duplicado.

genes, respectively ([20]). This suggests a higher accumulation of the CP probe target in the infected tissues. Regarding viral expression particularities and our own results, the mixture of probes RdRp+MP+CP will be used in the subsequent assays for CLBV detection.

Detection of CLBV in total RNA preparations of infected plants by non-radioactive hybridization of nucleic acids

Autoradiography of the membranes revealed the presence of CLBV in the total RNA extracts of four plants tested FJ-1 and FJ-2 isolates from *F. japonica* and FM-1 and FM-2 from *F. margarita* ([Figure 4], spots from C1 to C8). The intensity of the obtained signals was equal or stronger than the verified using 5 ng of the plasmid containing the CLBV genome cloned ([Figure 4], spots A4 and B4), suggesting a high quantity of CLBV sequences in the evaluated samples. No signals corresponding to the negative control were observed ([Figure 4], spots A8 and B8). Intra- and inter- assay replicates of points corresponding to the curve of serial dilutions showed similar intensities, whereas RNA extracts sample signals displayed some differences.

In this study, it was not possible to test a citrus host other than *Fortunella* sp. The species of the host, and sometimes the organ and tissue used as sources of the RNA extracts, are factors influencing the success of a given detection methodology for a particular pathogen ([25]). CLBV has been detected by non-radioactive nucleic acid hybridization in infected 'Eureka' lemon [*C. limon* (L.) Burm. f.], 'Marsh' grapefruit [*C. paradisi* Macf.], 'Nules' clementine (*C. reticulata* Blanco), 'Navelina' sweet orange (*C. sinensis* L. Osbeck), and 'Nagami' kumquat in the
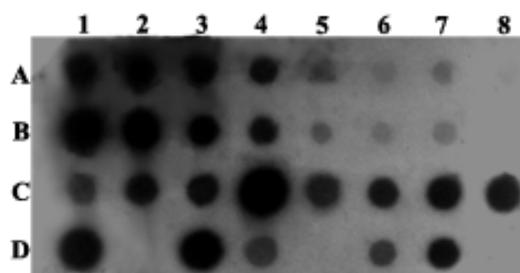
Figure 4. Detection of CLBV in the RNA extracts of *F. margarita* (FM) and *F. japonica* (FJ) sub-isolates [originated from field trees infected with CLBV] by nucleic acid hybridization using the CP +MP+RdRp probe mixture. Lanes A and B: Curve of serial dilutions containing decreasing quantities of the plasmid with CLBV complete genome, A1 and B1: corresponded to 100 ng, A2 and B2: 50 ng, A3 and B3: 25 ng, A4 and B4: 5 ng, A5, and B5: 1 ng, A6 and B6: 200 pg, A7 and B7: 40 pg. A8 and B8 contained 20 μL (~10 μg) of RNA extracts from healthy Etrog citron tissues. Lane C contained FM and FJ sub-isolates samples (20 μL with ~10 μg of RNA extracts): C1, C2: FM-1; C3, C4: FM-2; C5, C6: FJ-1 and C7, C8: FJ-2. / Detección de CLBV en los extractos de ARN de subaislados de *F. mar*garita (FM) y *F. japonica* (FJ) [originados de árboles de campo infectados con CLBV] por hibridación de ácido nucleico usando la mezcla de sonda CP + MP + RdRp. Carriles A y B: curva de diluciones en serie que contienen cantidades decrecientes del plásmido con genoma completo de CLBV, A1 y B1: correspondieron a 100 ng, A2 y B2: 50 ng, A3 y B3: 25 ng, A4 y B4: 5 ng, A5 y B5: 1 ng, A6 y B6: 200 pg, A7 y B7: 40 pg. A8 y B8 contenían 20 μL (~ 10 μg) de extractos de ARN de tejidos sanos de citron Etrog. El carril C contuvo muestras de subaislamientos FM y FJ (20 μL con ~ 10 μg de extractos de ARN): C1, C2: FM-1; C3, C4: FM-2; C5, C6: FJ-1 y C7, C8: FJ-2.

greenhouse, but its detection in infected leaves of 'Pineapple' sweet orange (*C. sinensis*) failed (22). In this regard, finding the best tissue to be used for CLBV detection and of the particularities of the virus infection in different hosts under Cuban climatic conditions support the need for novel studies.

CLBV detection using the TaqMan real-time RT-PCR technique has evidenced that the number of copies of the viral genome depends on both the infected citrus species and its cultivar (24). Moreover, the use of a one-step RT-PCR based method, which is 10-fold more sensitive than a non-radioactive nucleic acid hybridization assay (25), allowed accurate detection of CLBV in all kinds of infected tissues (22). For these reasons, using up to ten independent samples per tree in hybridization tests to detect CLBV has been recommended to reduce the risk of false negatives (22-24). However, due to the high number of samples to be processed in the certification of propagation materials, the use of PCR-based techniques may still result expensive for some laboratories worldwide (25,26). Alternatively, the nucleic acid hybridization technique, which is considered a high throughput

process, can be followed by PCR based methods to confirm negative results (25,26).

Diseases caused by bacteria, phytoplasmas, viruses, and viroids cause a great economic impact on agriculture worldwide (32). The two main effects are: (i) a decrease in crop productivity, and, indirectly, (ii) an increase in production costs due to the implementation of disease management programs (25,26). For this reason, for early identification of the pathogen and prevention of its spread, there is a constant demand for new methods with higher sensitivity, specificity, and reliability (25,26).

The hybridization method developed allowed detecting CLBV in infected citrus trees. After a required validation process, according to the international regulation of the OIE (World Organization for Animal Health) (33,34), the non-radioactive CLBV nucleic acid hybridization could be incorporated into the Cuban program for the production of citrus certified budwood and the quarantine and surveillance programs. This methodology revealed itself as a potentially useful tool to support the citrus agroindustry in Cuba.

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