Citrus huanglongbing: validation of Real-Time PCR (qPCR) for the detection of *Candidatus* Liberibacter asiaticus and *Candidatus* Liberibacter americanus in Colombia

Citrus huanglongbing: validación de PCR en tiempo real para la detección de *Candidatus* Liberibacter asiaticus y *Candidatus* Liberibacter americanus en Colombia

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ABSTRACT

Citrus huanglongbing (HLB) is the most destructive citrus disease. Two of the three known HLB-associated *Candidatus* Liberibacter species were recently found to be present in the Americas. In this study, eggs, nymphs and adults of *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) and suspect citrus plant materials were collected in 25 municipalities in the departments of Cundinamarca, Santander, Valle del Cauca, Meta and Quindio (Colombia). The detection sensitivity, specificity and assay performance of the 16S rDNA-based real-time PCR (qPCR) were validated for the field survey of the disease in Colombia. The validation confirmed the reliability and robustness of the real-time PCR method for the detection of HLB bacteria in host citrus plant tissues and the vector *D. citri*. The diagnosis was performed for *Candidatus* Liberibacter asiaticus (*Ca*. L. asiaticus) and for *Candidatus* Liberibacter americanus (*Ca*. L. americanus) on 168 citrus plant material samples and 239 insect samples. Neither *Ca*. L. asiaticus nor *Ca*. L. americanus were detected in the host plants or insects vector, confirming the absence of the disease in the citrus-producing areas of Colombia.

Key words: *Diaphorina citri*, HLB, Psyllidae, qPCR, 16S rDNA.

RESUMEN

La enfermedad de los cítricos conocida como huanglongbing (HLB) es considerada como la más destructiva para este cultivo. De las tres especies de *Candidatus* Liberibacter asociadas a HLB, dos han sido recientemente reportadas en América. En el presente trabajo, huevos, ninfas y adultos de *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) y material de plantas de cítricos sospechosas fueron colectadas en 25 municipios de los departamentos de Cundinamarca, Santander, Valle del Cauca, Meta y Quindio (Colombia). La detección, sensibilidad, especificidad de los ensayos realizados a partir de la región 16s del ADN ribosomal, mediante la prueba de PCR en tiempo real para la detección de la bacteria causante de HLB, fue validada para el monitoreo de la enfermedad en Colombia. La validación confirmó la confiabilidad y robustez del método de PCR en tiempo real para la detección de la bacteria en tejido de plantas de cítricos y en el insecto vector *D. citri* y se realizó el diagnóstico para *Candidatus* Liberibacter asiaticus (*Ca*. L. asiaticus) y para *Candidatus* Liberibacter americanus (*Ca*. L. americanus) en 168 muestras de tejido vegetal y en 239 muestras de insectos. Ninguna de las dos variantes de la bacteria fue detectada en plantas e insectos, confirmando la ausencia de la enfermedad en las áreas citrícolas de Colombia.

Palabras clave: *Diaphoroina citri*, HLB, Psyllidae, qPCR, 16S rDNA.

Introduction

The disease known as citrus huanglongbing (HLB) is caused by the non-cultivated alpha subdivision of proteobacteria, with the *Candidatus* status "*Candidatus* Liberibacter", which lives in the phloem of citrus plants (Tsai and Liu, 2000; Tsai *et al*., 2002) and is disseminated through vegetative propagation and insect vectors, making it difficult to control (Hung *et al*., 2004; Manjunath *et al*., 2008). HLB was detected initially in countries on the Asian and African continents and, more recently, in countries in the Americas, such as Argentina (Senasa, 2013), Costa Rica (SFE, 2011), Belize (Manjunath *et al*., 2010), Cuba (Martínez *et al*., 2009), Mexico (NAPPO, 2009), Dominican Republic (Matos *et al*., 2009), the USA (Halbert, 2005; Manjunath *et al*., 2008) and Brazil (Teixeira *et al*., 2005), with large losses for citrus farmers (Bové, 2006).

Three *Candidatus* sp. of the pathogen have been described that affect citrus crops, of which *Ca*. Liberibacter asiaticus

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is the most widely distributed (Halbert and Manjunath, 2004, Teixeira *et al*., 2005). The American and Asian variants are transmitted by *Diaphorina citri*; additionally, the Asian variant is more tolerant to high temperatures, close to 30°C (Garnier *et al*., 2000). The African variant, caused by *Candidatus* Liberibacter africanus, develops between the temperatures of 22 and 25°C and is transmitted by *Trioza erytreae* (Del Guercio) (Bové, 2006; Lin *et al*., 2010). The American variant was detected for the first time in the state of Sao Paulo (Brazil), and the name proposed for this new HLB etiologic agent was *Candidatus* Liberibacter americanus (Teixeira *et al*. 2005).

The *Instituto Colombiano Agropecuario*, ICA, reported the *Diaphorina citri* psyllid vector for first time in 2007 in nursery plants and on citrus farms in the departments of Valle del Cauca and Tolima in Colombia (Ebratt *et al*., 2011a). It was later determined that the insect was present in all of the Andean regions in the departments of Risaralda, Caldas, Quindío, Antioquia, Norte de Santander, Santander, Huila, Cauca, Nariño and Cundinamarca, in the Caribbean regions in the departments of Córdoba, Cesar, Bolívar, Atlántico, and in the Orinoquia regions in the departments of Casanare, Meta and Vichada, with a potential infestation of 95% of the citrus production area in Colombia (Ebratt *et al*., 2011a). This fact was further worsened due to the closeness of countries where the vector insect has been detected and where the presence of the HLB disease has been confirmed, which is why Colombia is ranked as having a high phytosanitary risk of presenting this serious pathology on citrus plantations.

Although the visual symptoms favor the detection of the presence of HLB (Roistacher, 1991) on citrus plantations, it is only by using more sophisticated detection methods that are based on electronic microscopy, Enzyme-Linked Immuno-Sorbent Assays with monoclonal antibodies (ELISA), HLB specific fluorescent marking substances (Schwarz, 1968). Recently, the implementation of PCR and real-time PCR methods that have been used in many countries for the detection of the three causal agents of HLB based on the 16S ribosomal DNA region and other regions of the bacterial genome (Lin *et al*., 2010), that the presence of the disease can be truly diagnosed.

For all the reasons mentioned above, the present study aimed to determine the presence and geographic distribution of *D. citri* in the Andean and Orinoquia regions of the departments of Cundinamarca, Santander, Valle del Cauca, Meta and Quindio and determine the presence of HLB through the evaluation of citrus leaf tissue and *D. citri* insect vectors using conventional and real-time PCR with the use of specific primers based on some genomic sequences, such as the 16S ribosomal DNA region, as a contribution to the implementation and validation of a diagnostic method in the sampling and detection processes for this disease in Colombia.

Materials and methods

Capture and taxonomic identification of Psyllids

Nymph and adult psyllids were collected between December of 2012 and December of 2013 from plants of the Rutaceae family. In the Andean and Orinoquia regions, 262 farms were sampled. These farms were located in 25 producing municipalities in the departments of Cundinamarca, Santander, Valle del Cauca, Quindio and Meta (Colombia). Each of the collected samples were made up of psyllid nymphs or adults and were saved in vials with 95% ethanol that were labeled with the identification data and geographic location of the sites. Additionally, a form with data on the citrus planted area, the species used, age and observed natural enemies of *D. citri* and farm management was completed.

The taxonomic identification was done at the facilities of the National Phytosanitary Diagnostics Laboratory of the ICA. The content of each vial was transferred to Petri dishes with 75% ethanol and each of the specimens were observed using a stereoscope and microscope to detail the morphological diagnostic characteristics suggested in the keys for the immature stages proposed by Blackwell (2005) and Burckhardt (1987) (Figs. 1 and 2).

FIGURE 1. Stages of nymph development in *D. citri*.

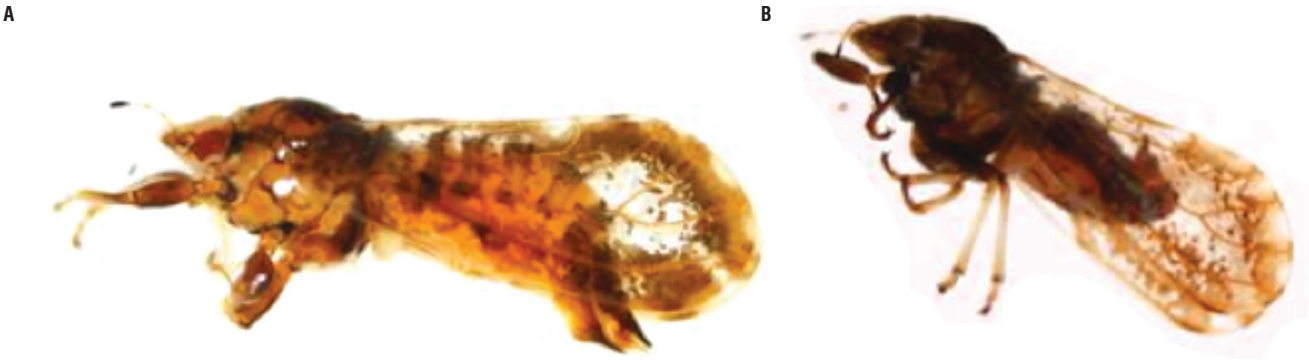

FIGURE 2. Adult stage of *D. citri*. A, female; B, male.

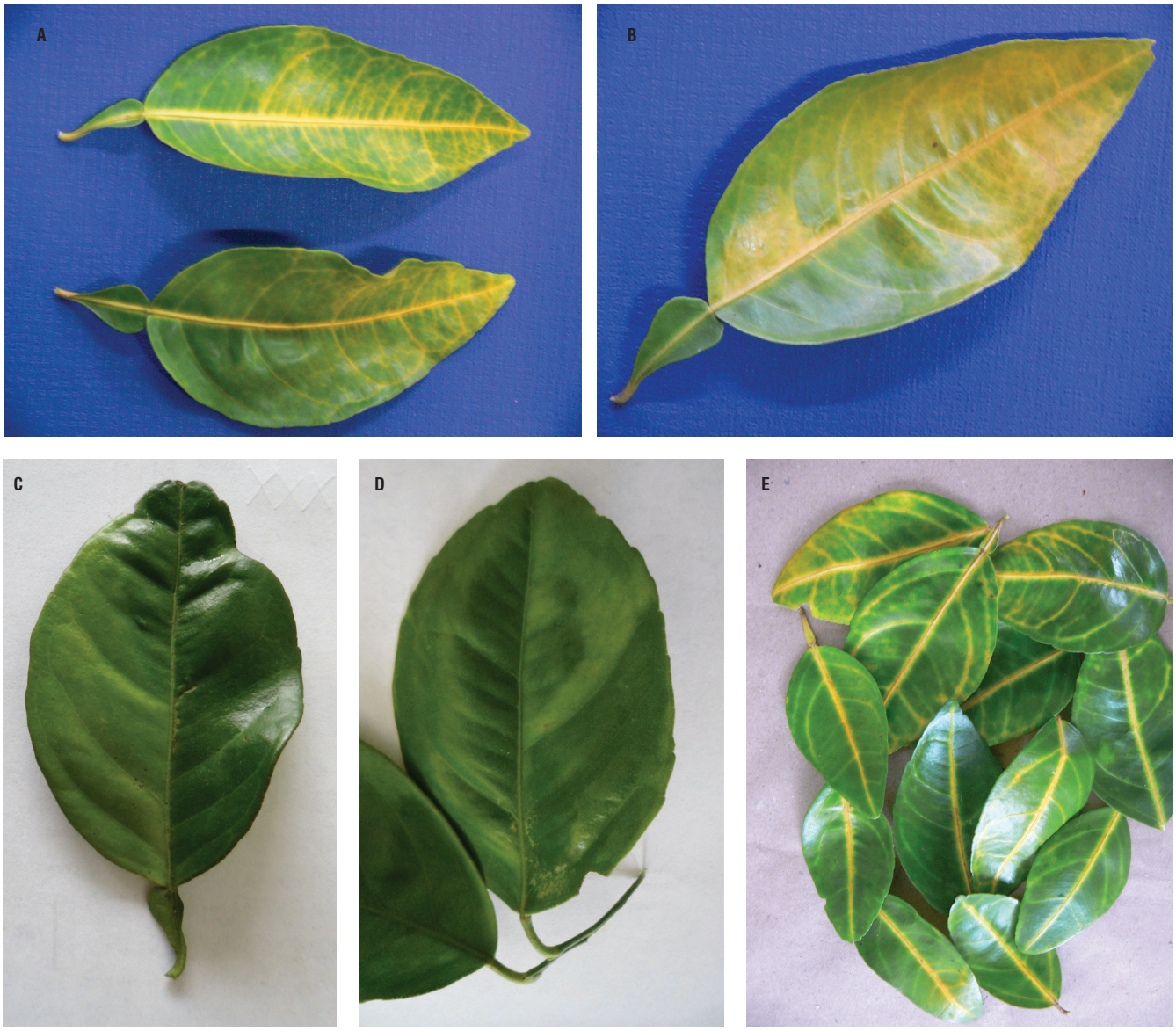

FIGURE 3. Leaf tissue with apparent symptoms of HLB. A and B, Tahiti lime (*Citrus aurantifolia*); C, D, and E, mandarin (*Citrus reticulata*).

Plant sample

Branches from asymptomatic adult trees and from adult trees with apparent HLB symptoms that were found with different phenological stages (vegetative, flowering and harvesting) of the following species: orange (*Citrus sinensis*), mandarin (*Citrus reticulata*), Tahiti lime (*Citrus*

aurantifolia), grapefruit (*Citrus paradasi*), satsuma mandarin (*Citrus unshiu*), Volkamer lemon (*Citrus volkameriana*), orange jessamine (*Murraya paniculata*) and Tabog (*Swinglea glutinosa*), were collected from the same plants where the presence of psyllids was observed. A total of 168 samples of 10 to 20 leaves were collected, which were saved in paper bags and stored at -20°C until the implementation of the DNA extraction process (Fig. 3).

Detection of *Ca*. L. asiaticus and *Ca*. L. americanus on psyllids and leaf tissue with PCR

The methods used for the DNA extraction were those previously reported for both leaf tissues and psyllids. For the detection of HLB on psyllids, the protocol reported by Manjunath *et al*. (2008) was used, and for the detection on leaf tissue, the method reported by Murray and Thompson (1980) was used. For the detection of the bacteria in psyllids, qPCR with the specific primers Cit 295 – Cit 298 tested for the Asian variant, Cit 297 – Cit 298 for the American variant, and a combination with the probe Cit 409-FAM in both cases was used (Li *et al*., 2006). For the internal control of the reaction while monitoring the quality of the DNA extraction of the psyllids, the primers Cit 418 – Cit 419 were used, which amplify a fragment of the wingless gene (wg) of *D. citri*, which codes for a secreted, diffusible glycoprotein, in addition to the probe Cit 420-HEX (Manjunath *et al*., 2008). On the plant tissue, the detection of these bacterial variants was done with the same primers and specific probe, and the internal control of the reaction was done with the primers Cit 315 – Cit 317, which amplify a fragment of the cytochrome oxidase gene, together with the probe Cit 316-Cy3 (Li *et al*., 2006) (Tab. 1). The reactions in the real-time PCR to determine the presence of the Asian and American variants were carried out with a reaction volume of 25 µL, according to the conditions of amplification reported by Li *et al*. (2006).

TABLE 1. Sequence of primers and probes utilized in the qPCR for the detection of *Ca*. L. asiaticus and *Ca*. L. americanus and the internal controls.

Primer/Probe	Sequence
Primer Cit 295 F	5'- GTCGAGCGCGTATGCAATAC-3'
Primer Cit 298 R	5'- TGCGTTATCCCGTAGAAAAAGGTAG-3'
Primer Cit 297 F	5'- TCGAGCGAGTACGCAAGTACTAG-3'
Probe Cit 409	5' FAM-AGACGGGTGAGTAACGCG-3' BHQ1
Primer Cit 418 F	5'- GCTCTCAAAGATCGGTTTGACGG-3'
Primer Cit 419 R	5'- GCTGCCACGAACGTTACCTTC-3'
Probe Cit 420	5' HEX-TTACTGACCATCACTCTGGACGC-3' BHQ1
Primer Cit 315 F	5'- GTATGCCACGTCGCATTCCAGA-3'
Primer Cit 317 R	5'- GCCAAAACTGCTAAGGGCATTC-3'
Probe Cit 316	5' CY3-ATCCAGATGCTTACGCTGG-3' BHQ2

Additionally, an alternative verification method of conventional PCR technique was done through a preliminary test with positive controls provided by Fundecitrus (Brazil), in which region 16S of the bacterial DNA of *Ca*. L. americanus and *Ca*. L. asiaticus was been inserted in the plasmid TOPO TA cloning (Invitrogen, Carlsbad, CA). This method was carried in a reaction volume of 25 µL, in which, in the case of the controls for the American variant primers, GB1 - GB3 were used along with the conditions reported by Teixeira *et al*. (2005). Likewise, the procedures used for the Asian variant were done with the primers OI1 – OI2c and conditions according to Jagoueix *et al*. (1996) (Tab. 2).

TABLE 2. Sequence of the primers GB1 - GB3 and OI1 – OI2c used for the amplification of the positive controls of *Ca*. L. americanus and *Ca*. L. asiaticus, respectively.

Primers	Sequence 5´→ 3´	Size (bp)
GB1	AAGTCGAGCGAGTACGCAAGTACT	1,027
GB3	CCAACTTAATGATGGCAAATATAG	
OI1	GCGCGTATGCAATACGAGCGGCA	1,160
OI2c	GCCTCGCGACTTCGCAACCCAT	

Validation of the real-time PCR protocol

Preparation of the samples for the validation of the model

Due to an absence of insects and leaf tissue infected with HLB in Colombia and the difficulty of importing this infected material stemming from bio-security and preventing a possible HLB bacterial infection in Colombia, negative insect and leaf tissue material, previously tested by qPCR, were inoculated with DNA plasmid containing the sequence of the 16S rDNA gene of *Ca*. L. asiaticus or *Ca*. L. americanus with the objective of obtaining positive samples in order to conduct the different experiments during the validation method. For this purpose, bacterial DNA fragments of approximately 1,500 pb that corresponded to the sequence of the 16S gene of *Ca*. L. asiaticus and *Ca*. L. americanus were independently cloned using TOPO® TA Cloning Kits (Invitrogen, Carlsbad, CA) and with competent cells using One Shot® TOP10 chemically competent *E. coli* (Invitrogen, Carlsbad, CA). Subsequently, 10 dilutions were done for each variant from the plasmid DNA that was positive for *Ca*. L. asiaticus and *Ca*. L. americanus, for which NanoDrop ND 1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE) was used with a starting concentration of 1,500 ng μL^{-1} until reaching 1.5 fg μL^{-1}. The number of copies present in the different concentrations of plasmid DNA was determined with the equation reported by Lee *et al*. (2006) (Tab. 3).

$$\text{DNA copy number} = \frac{(6.023 \cdot 10^{23} \text{ copy mol}^{-1}) \text{ (DNA amount (g))}}{\text{DNA lenght (bp) x 660 (g mol}^{-1}) \text{ bp}^{-1}}$$

TABLE 3. Concentrations and number of copies of positive plasmid DNA used to inoculate the plant and insect samples.

Dilution	Concentration	Number of copies
1	1,500 ng μL^{-1}	$2.5 \cdot 10^{11}$
2	150 ng μL^{-1}	$2.5 \cdot 10^{10}$
3	15 ng μL^{-1}	$2.5 \cdot 10^{9}$
4	1.5 ng μL^{-1}	$2.5 \cdot 10^{8}$
5	0.15 ng μL^{-1}	$2.5 \cdot 10^{7}$
6	15 pg μL^{-1}	$2.5 \cdot 10^{6}$
7	1.5 pg μL^{-1}	$2.5 \cdot 10^{5}$
8	0.15 pg μL^{-1}	$2.5 \cdot 10^{4}$
9	15 fg μL^{-1}	$2.5 \cdot 10^{3}$
10	1.5 fg μL^{-1}	$2.5 \cdot 10^{2}$

Ten midrib plant samples with 100 mg of *Citrus aurantifolia* (Thaiti lime) that were negative for HLB were used in order to obtained positive samples, with ten replicates for each one. The first replication of each sample was inoculated with 5 µL of the first dilution of the positive plasmid dilution, thereby independently obtaining a 10 x 10 matrix for each of the two variants (Tab. 4). Afterwards, the sample was soaked and the DNA extraction was carried out through the method described by Murray and Thompson (1980) and the respective detection of bacteria with real-time PCR using the specific indicators reported for *Ca*. L. asiaticus and *Ca*. L. americanus (Li *et al*. 2006; Manjunath *et al*. 2008).

Establishment of the 90% detection limit of Candidatus *Liberibacter sp.*

The 90% detection limit was calculated using the formula for the final-point method of Spearman-Kärber, $LD_{90\%} = m\text{-}d\ (S\text{-}0.5)$, (NordVal, 2009), in which m is the highest dilution dose with 100% presence (ng μL^{-1} or No. of copies/ μL^{-1}); d is the distance between the doses or dilution factor (0.1); S is the sum of fractions P (presence) from the highest dilution with 100% P to 0% P, divided by 100; and 0.5 is a constant. This procedure was carried out with the plant and insect samples in order to detect the bacterium.

Determination of the sensitivity, specificity, efficiency, false positives, false negatives, and selectivity of the real-time PCR method

The sensitivity, specificity, efficiency, alse positives, false negatives and selectivity of the technique on insect and plant tissue for *Ca*. L. asiaticus and *Ca*. L. americanus were determined by the inoculation of the previous dilution obtained in the detection limit for each variant with the different possible sample combinations. Three different midrib samples of plant leaf and three samples of insect tissue (six psyllids in each sample) were soaked in liquid nitrogen. Four replicates of 100 mg were taken from each soaked sample, which were used to obtain the four possible sample combinations using inoculation with five µL of plasmid DNA of the previous dilution used in obtaining the detection limit of each variant (Tab. 5). Subsequently, the DNA extraction was done using the protocol described by Manjunath *et al*. (2008) for insects and by Murray and Thompson (1980) for foliar tissue, with the respective analysis with real-time PCR for the detection of the Asian and American variants; the results were registered in a matrix for the presence or absence in the plant tissue and insect tissue (Tab. 6).

Results and discussions

Taxonomic identification of the psyllids

The presence of the insect vector was confirmed in the Andean and Orinoquia geographical regions of the sampled departments of Cundinamarca, Tolima, Santander, Quindio and Meta. In 66% of the visited sites, *D. citri* was

TABLE 4. Matrix of the inoculated samples in order to obtain the detection limit in the plant and insect tissue, where P (presence) or A (absence) was revealed after the real-time PCR analysis.

Sample	1,500 (ng μL^{-1})	150 (ng μL^{-1})	15 (ng μL^{-1})	1.5 (ng μL^{-1})	0.15 (ng μL^{-1})	150 (pg μL^{-1})	15 (pg μL^{-1})	1.5 (pg μL^{-1})	0.15 (pg μL^{-1})	150 (fg μL^{-1})
Sample 1	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A
Sample 2	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A
Sample 3	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A
Sample 4	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A
Sample 5	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A
Sample 6	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A
Sample 7	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A
Sample 8	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A
Sample 9	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A
Sample 10	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A	P/A

found in different stages of development on the young growth of Rutaceus plants (Tab. 7); however, it was only during the new shoots season, after the first rains, that the different stages of development were observed on the same plants (Figs. 1 and 2). During the dry season, only the adult stage was observed on the observed citrus plantations, except on *Swinglea glutinosa* plants (Blanco), used as live barriers, and on Orange Jessamine, *Murraya paniculata* (L.) Jack., used as an ornamental plant. These observations are consistent with those made by Gómez (2009) and Ebratt *et al*. (2011a).

TABLE 5. Preparation of the four possible sample combinations, where + is the inoculated variant and - is the non-inoculated variant.

Sample combination	Preparation of the sample combinations for the plant and insect tissue	
	***Ca.* Liberibacter asiaticus**	***Ca.* Liberibacter americanus**
1	+	-
2	-	+
3	-	-
4	+	+

TABLE 6. Matrix for the presence and absence of the parameters of sensitivity, efficiency, false positives, false negatives and selectivity utilized for *Ca*. L. americanus and *Ca*. L. asiaticus.

PCR method for validatione	Reference value of inoculated positive control		Total
	Positive samples	Negative samples	
Positive samples for Real Time PCR	A(True+)	B(False +)	A+B
Negative samples for Real Time PCR	C(False -)	D(True-)	C+D
Total	A+C	B+D	N= A+B+C+D

The sensitivity or percentage of correctly assigned positives was determined using the formula [A/(A+C) * 100]; the specificity or percentage of correctly assigned negatives was determined with [D/(B+D) * 100]; the efficiency or percentage of the following were also determined: correctly detected variation [(A+D)/N * 100(%)], false positives [B/(A+B) * 100], false negatives [C/(C+D) * 100] and selectivity [Log [(A+B)/N)]], where N is the total number of samples, A is a true positive, B is a false positive, C is a false negative and D is a true negative (NordVal, 2009; ISO, 2011).

Of the 239 psyllid samples, 598 adults were obtained, of which 58% were female, and 2,695 were nymphs (Tab. 5). The semi-quiesence of the nymphs and the presence of buds on the rutacea plants facilitated the capture of the nymphs. Cundinamarca, Valle del Cauca and Meta presented the higher number of captured individuals at 1,086, 673, and 580, respectively; *D. citri* predominated in the municipalities that had characteristics of a dry, tropical forest (Ebratt, *et al*., 2011b).

Detection of Ca. *L. asiaticus and* Ca. *L. americanus on psyllids and leaf tissue using qPCR and conventional PCR*

All of the 168 samples of leaf tissue with apparent symptoms (Fig. 3) and the 239 samples of psyllids collected in the departments of Cundinamarca, Santander, Valle del Cauca, Meta and Quindio were diagnosed as negative for the presence of *Ca*. L. asiaticus and *Ca*. L. americanus using real-time PCR; the negative controls did not generate an amplification curve while the positive controls did with a Ct that started amplification between 15 and 22 for the two variants in the foliar and insect tissues (Figs. 4A, 4B, 5A and 5B). Likewise, all of the DNA samples from the leaf tissue

TABLE 7. Samples of leaf tissue and psyllids collected in the citrus-producing municipalities for departments of Cundinamarca, Meta, Quindio, Santander and Valle del Cauca.

		Department of Cundinamarca				
Municipalities		La Mesa (635-959 m a.s.l.)	Nilo (356-1,066 m a.s.l.)	Sasaima (1,184-1,515 m a.s.l.)	Tibacuy (730-1,185 m a.s.l.)	Tocaima (364-557 m a.s.l.)
		4.6919N;74.4627W	4.3036N;74.6261W	4.5600N;74.2500W	4.3015N;74.4704W	4.4176N;74.7093W
		4.6474N;74.4627W	4.3242N;74.6070W	4.5618N;74.2459W	4.3021N;74.4696W	4.4951N;74.6518W
		4.6318N;74.5166W	4.3455N;74.5943W	4.5757N;74.2625W	4.3006N;74.4655W	4.4017N;74.5654W
		4.6319N;74.5246W	4.3546N;74.5751W	4.5757N;74.2625W	4.3019N;74.4627W	4.4477N;74.5804W
		4.6473N;74.5381W	4.3632N;74.5782W	4.5758N;74.2612W	4.3018N;74.4625W	4.4716N;74.6378W
		4.6468N;74.5396W	4.3572N;74.5803W	4.5758N;74.2612W	4.1817N;74.3119W	4.4956N;74.6478W
		4.6503N;74.5382W	4.3396N;74.5535W	4.5758N;74.2612W	4.1803N;74.3196W	4.4923N;74.6462W
		4.6497N;74.5379W	4.3513N;74.5411W	4.5711N;74.2552W	4.1644N;74.3276W	4.4931N;74.6455W
		4.6476N;74.5371W	4.3571N;74.5294W	4.5705N;74.2601W	4.1653N;74.3264W	4.4970N;74.6521W
		4.6454N;74.5499W	4.3581N;74.5319W	4.5708N;74.2523W	4.1644N;74.3268W	4.4944N;74.6507W
		4.6452N;74.5347W		4.5702N;74.2603W		
		4.6469N;74.5347W		4.9339N;74.4135W		
Month of sampling		**February (2013)**	**June, July and December (2013)**	**December (2012)**	**March and April (2013)**	**February and May (2013)**
Leaf tissue of species collected (Samples)		Orange, lemon, tangerine (16)	Orange, lemon, tangerine, myrtle (10)	0	Orange, lemon (6)	Orange, lemon, tangerine, myrtle (7)
Psyllids collected	Samples	18	13	11	14	12
	Adults	73♀; 59♂	12♀; 3♂	16♀; 5♂	4♀; 4♂	33♀; 26♂
	Nymphs	194	81	237	154	185

CONTINUACIÓN TABLE 7.

Departament of Meta						
Municipalities		**Acacias (479-554 m a.s.l.)**	**Granada (316-458 m a.s.l.)**	**Guamal (533-613 m a.s.l.)**	**Lejanias (472-610 m a.s.l.)**	**Villavicencio (259-378 m a.s.l.)**
		3.9412N;73.7640W	3.4730N;73.7362W	3.8720N;73.7791W	3.4954N;73.9543W	4.0596N;73.4585W
		3.9358N;73.7585W	3.4780N;73.8912W	3.8675N;73.7801W	3.4923N;73.9339W	4.0310N;73.5874W
		3.9373N;73.7554W	3.4857N;73.8614W	3.8591N;73.8000W	3.4949N;73.9699W	4.0235N;73.5761W
		3.9483N;73.7572W	3.4845N;73.8664W	3.8548N;73.8078W	3.5056N;73.9779W	4.0284N;73.5753W
		3.9283N;73.7258W	3.4775N;73.8150W	3.8622N;73.8074W	3.5048N;73.9767W	4.0762N;73.5848W
		3.9820N;73.7072W	3.4788N;73.8237W	3.8789N;73.7812W	3.5041N;73.9760W	4.0942N;73.5653W
		3.9982N;73.7406W	3.4780N;73.8145W	3.8840N;73.8026W	3.4621N;73.8812W	4.0883N;73.5660W
		3.9502N;73.7672W	3.4615N;73.8331W	3.8813N;73.8019W	3.4803N;73.9551W	4.0103N;73.4535W
		3.9988N;73.7334W	3.4702N;73.7681W	3.8896N;73.7743W	3.4822N;73.9586W	4.0082N;73.4568W
		4.0352N;73.7703W	3.4782N;73.8132W	3.8880N;73.7744W	3.4796N;73.9537W	4.0028N;73.4506W
						4.0133N;73.4625W
						4.0527N;73.3475W
Month of sampling		**June and July (2013)**	**April (2013)**	**July (2013)**	**February (2013)**	**February, April and June (2013)**
Leaf tissue of species collected (Samples)		Lemon, tangerine, swinglea (4)	Orange, lemon (8)	Orange, tangerine, myrtle (9)	Orange, lemon, tangerine (7)	Orange, lemon (9)
Psyllids collected	Samples	5	13	10	3	12
	Adults	1♀; 1♂	18♀; 11♂	9♀; 8♂	3♀; 0♂	12♀; 12♂
	Nymphs	59	175	87	12	172
Departament of Quindio						
Municipalities		**Buenavista (1,136-1,378 m a.s.l.)**	**Calarca (1,112-1,337 m a.s.l.)**	**Montenegro (1,187-1,291 m a.s.l.)**	**Quimbaya (1,144-1,291 m a.s.l.)**	**La tebaida (1,150-1,443 m a.s.l.)**
		4.3663N;75.7546W	4.3854N;75.7778W	4.6404N;75.7917W	4.3854N;75.7779W	4.4638N;75.7645W
		4.3406N;75.7583W	4.4360N;75.7085W	4.5384N;75.8108W	4.6165N;75.7665W	4.4525N;75.7997W
		4.3407N;75.7583W	4.4365N;75.7141W	4.4730N;75.8102W	4.6409N;75.7911W	4.4455N;75.8568W
		4.3644N;75.7773W	4.4346N;75.7136W	4.5121N;75.8444W	4.6409N;75.7911W	4.4367N;75.8032W
		4.3796N;75.7418W	4.3710N;75.7819W	4.5403N;75.7855W	4.5698N;75.7970W	4.4358N;75.7966W
		4.3824N;75.7404W	4.4166N;75.7243W	4.5151N;75.8002W	4.5900N;75.7836W	4.4393N;75.7959W
		4.3893N;75.7391W	4.4252N;75.7344W	4.5155N;75.8057W	4.5740N;75.7762W	4.4497N;75.8053W
		4.3928N;75.7457W	4.4143N;75.7252W	4.5145N;75.8361W	4.5739N;75.7761W	4.4501N;75.8143W
		4.3929N;75.7407W	4.4570N;75.7113W	4.5135N;75.8388W	4.5870N;75.7803W	4.4426N;75.8407W
		4.3762N;75.7591W	4.4591N;75.7069W	4.5559N;75.7845W	4.6068N;75.7909W	4.4487N;75.8321W
				4.5613N;75.8101W	4.5954N;75.8199W	4.4025N;75.7819W
					4.5954N;75.8200W	
Month of sampling		**September (2013)**	**July and september (2013)**	**April, June and July (2013)**	**April, June and July (2013)**	**April and June (2013)**
Leaf tissue of species collected (Samples)		Orange, lemon, tangerine (6)	Orange, lemon, tangerine (9)	Orange, lemon, tangerine (6)	Orange, lemon, tangerine, satsuma mandarin (7)	Orange, lemon, tangerine, satsuma mandarin (7)
Psyllids collected	Samples	9	8	10	10	9
	Adults	10♀; 5♂	7♀; 3♂	12♀; 16♂	25♀; 17♂	11♀; 12♂
	Nymphs	51	100	71	80	100

Department of Santander						
		Giron	**Lebrija**	**Pinchote**	**Rionegro**	**San Gil**
		(320-892 m a.s.l.)	**(988-1,171 m a.s.l.)**	**(1,473-1,722 m a.s.l.)**	**(520-680 m a.s.l.)**	**(1,178-1,698 m a.s.l.)**
		6.9898N;73.1726W	7.1526N;73.1649W	6.5144N;73.1557W	7.2533N;73.1512W	6.5242N;73.1197W
		6.9894N;73.1712W	7.0850N;73.0850W	6.5256N;73.1492W	7.2546N;73.1501W	6.5173N;73.1182W
		6.9884N;73.1724W	7.1476N;73.2066W	6.5217N;73.1593W	7.3710N;73.1776W	6.5150N;73.1158W
		6.9843N;73.1703W	7.1550N;73.2148W	6.5240N;73.1462W	7.3720N;73.1807W	6.5152N;73.1146W
		6.9859N;73.1709W	7.1373N;73.1948W	6.5258N;73.1625W	7.2683N;73.1484W	6.5821N;73.1604W
		6.9796N;73.1665W	7.1405N;73.1944W	6.5216N;73.1644W	7.2679N;73.1485W	6.5801N;73.1609W
Municipalities		6.9805N;73.1594W	7.1451N;73.2149W	6.5255N;73.1686W	7.2548N;73.1440W	6.5892N;73.1746W
		6.9779N;73.1599W	7.1116N;73.2214W	6.5266N;73.1676W	7.2542N;73.1442W	6.5884N;73.1723W
		6.9774N;73.1629W	7.1110N;73.2263W			6.5990N;73.1695W
		6.9813N;73.1662W	7.0923N;73.2311W			6.5920N;73.1656W
			7.0919N;73.2345W			
			7.1422N;73.2260W			
			7.1438N;73.2282W			
			7.1482N;73.1848W			
			7.1446N;73.2326W			
Month of sampling		**May (2013)**	**May and June (2013)**	**December (2013)**	**June (2013)**	**December (2013)**
Leaf tissue of species collected (Samples)		Orange, lemon (10)	Orange, lemon, swinglea, Volkamer lemon (6)	0	0	0
Psyllids collected	Samples	16	9	0	0	0
	Adults	42♀; 25♂	11♀; 8♂	0♀; 0♂	0♀; 0♂	0♀; 0♂
	Nymphs	255	93	0	0	0

Departament of Valle						
		Alcala	**Bugalagrande**	**Caicedonia**	**La victoria**	**Sevilla**
		(1,207-1,346 m a.s.l.)	**(938-964 m a.s.l.)**	**(1,138-1,229 m a.s.l.)**	**(957-1,424 m a.s.l.)**	**(1,094-1,251 m a.s.l.)**
		4.6971N;75.8139W	4.2377N;76.1621W	4.3233N;75.8715W	4.5133N;75.8931W	4.3414N;75.8809W
		4.6655N;75.7819W	4.2375N;76.1636W	4.3283N;75.8308W	4.5134N;75.8932W	4.3688N;75.8594W
		4.6570N;75.7892W	4.2309N;76.1618W	4.3253N;75.8294W	4.5150N;75.8898W	4.3620N;75.8614W
		4.6628N;75.8055W	4.2381N;76.1581W	4.3234N;75.8270W	4.5170N;75.9166W	4.3815N;75.8645W
Municipalities		4.6611N;75.8018W	4.2456N;76.1827W	4.3256N;75.8227W	4.5195N;75.9170W	4.3694N;75.8741W
		4.6645N;75.7802W	4.2420N;76.1814W	4.3227N;75.8344W	4.5064N;75.9176W	4.3182N;75.8768W
		4.6826N;75.7916W	4.2430N;76.1831W	4.3582N;75.8269W	4.5079N;75.9819W	4.3333N;75.8751W
		4.6940N;75.8223W	4.2247N;76.1945W	4.3675N;75.8314W	4.5072N;75.9841W	4.3254N;75.8791W
		4.6858N;75.8137W	4.2240N;76.1982W	4.3795N;75.8240W	4.4996N;75.9899W	4.3320N;75.8746W
		4.6689N;75.7526W	4.2283N;76.2120W	4.3820N;75.8235W	4.5000N;75.9905W	4.3358N;75.8902W
				4.3208N;75.8709W	4.4324N;75.8901W	
Month of sampling		**October (2013)**	**November (2013)**	**April and October (2013)**	**October (2013)**	**October and November (2013)**
Leaf tissue of species collected (Samples)		Orange, lemon, tangerine (9)	Orange, lemon, tangerine, grapefruit (8)	Orange, tangerine (10)	Lemon, tangerine, myrtle (7)	Orange, lemon, tangerine, myrtle (7)
Psyllids collected	Samples	13	12	14	8	10
	Adults	14♀; 6♂	15♀; 14♂	11♀; 3♂	2♀; 4♂	7♀; 8♂
	Nymphs	139	116	140	114	80

as well as from the insects amplified their respective DNA quality internal control, which demonstrated the absence of an inhibition of the reaction (Figs. 4C and 5C). The absence of the HLB disease in the analyzed citrus farms in Colombia indicated that the variants of the bacteria have not yet been found in these analyses. However, due to the large insect populations that are closely associated with the citrus plants and the fact that the disease is already present in neighboring countries, it is necessary to maintain permanent surveillance programs in order to take measures that avoid the establishment of the disease in this country or to avoid rapid propagation throughout the national territory the moment the presence of any of the bacterial variants that cause the disease is detected. The conventional PCR methodology was established as an alternative with the use of GB1 – GB3 indicators, which amplified a fragment of 1,027 bp in the positive control of the Americana variant, and OI1 – OI2c indicators, which amplified a fragment of 1,160 bp for the positive control of the Asian variant were used (Figs. 6 and 7). These results facilitate complimentary analysis when a sequence examination is required for the two variants.

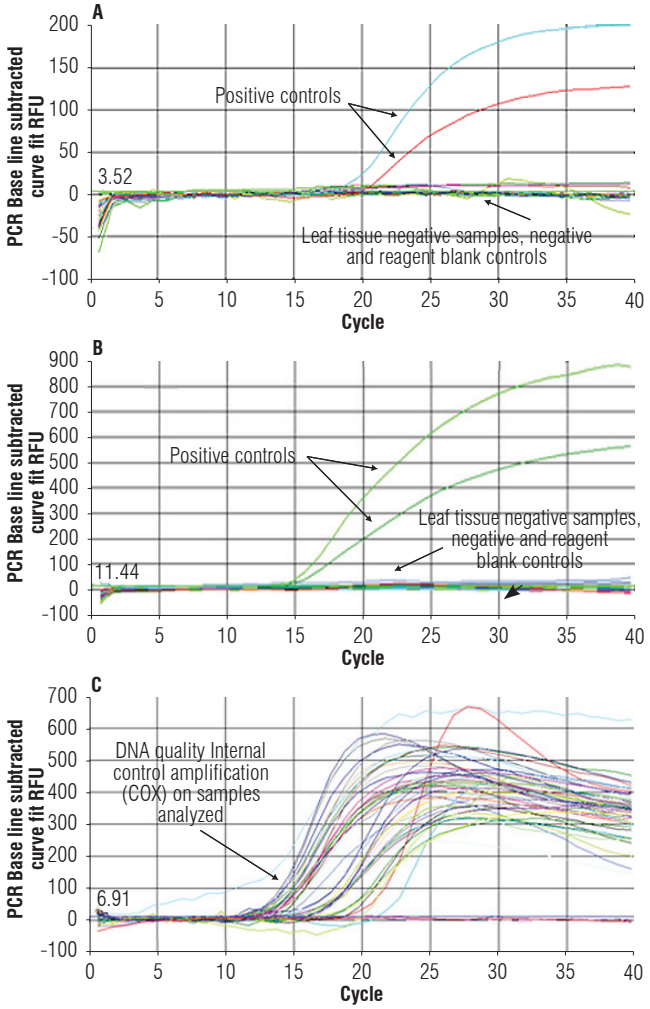

FIGURE 4. Amplification curves using real-time PCR of positive, negative and reagent blank controls for the detection on citrus leaf tissue. A, *Ca*. L. asiaticus; B, *Ca*. L. americanus; C, DNA quality of the internal control of the reaction (COX).

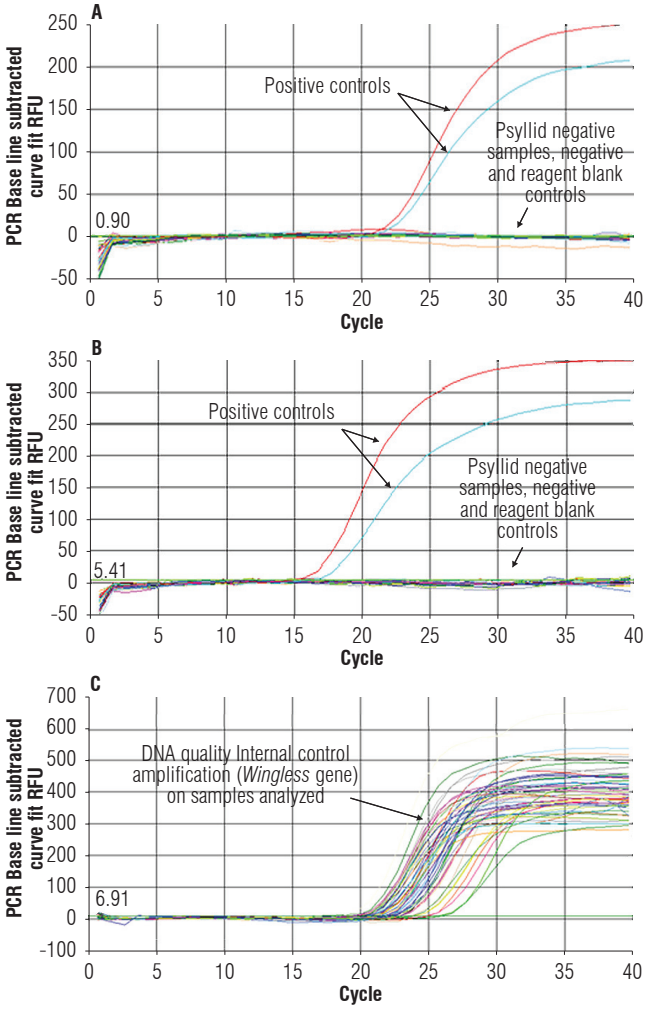

FIGURE 5. Amplification curves using real-time PCR of positive, negative and reagent blank controls for the detection on psyllids. A, *Ca*. L. asiaticus; B, *Ca*. L. americanus; C, DNA quality of the internal control of the reaction (wingless gene).

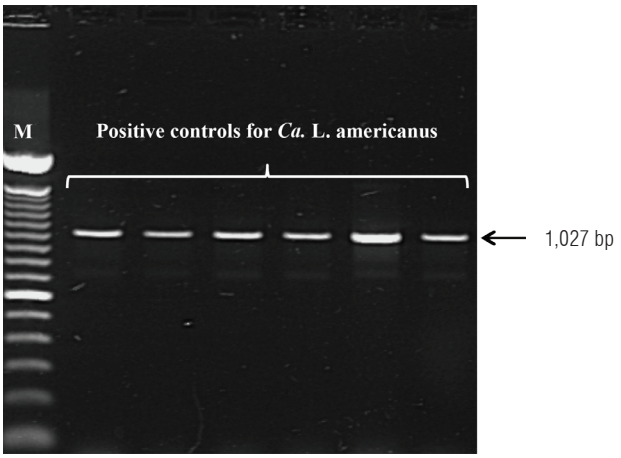

FIGURE 6. Amplification of positive controls of *Ca*. L. americanus through conventional PCR using the specific primers GB1 and GB3. M, marker for a 100 bp weight.

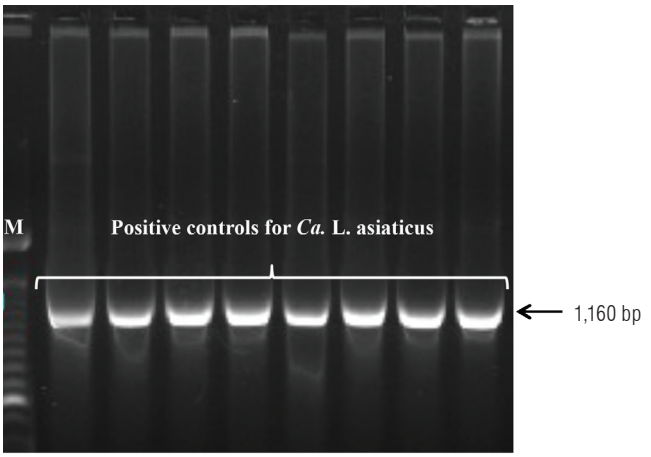

FIGURE 7. Amplification of positive controls of *Ca*. L. asiaticus through conventional PCR using the specific primers OI1 and OI2c. M, Marker for a 100 bp weight.

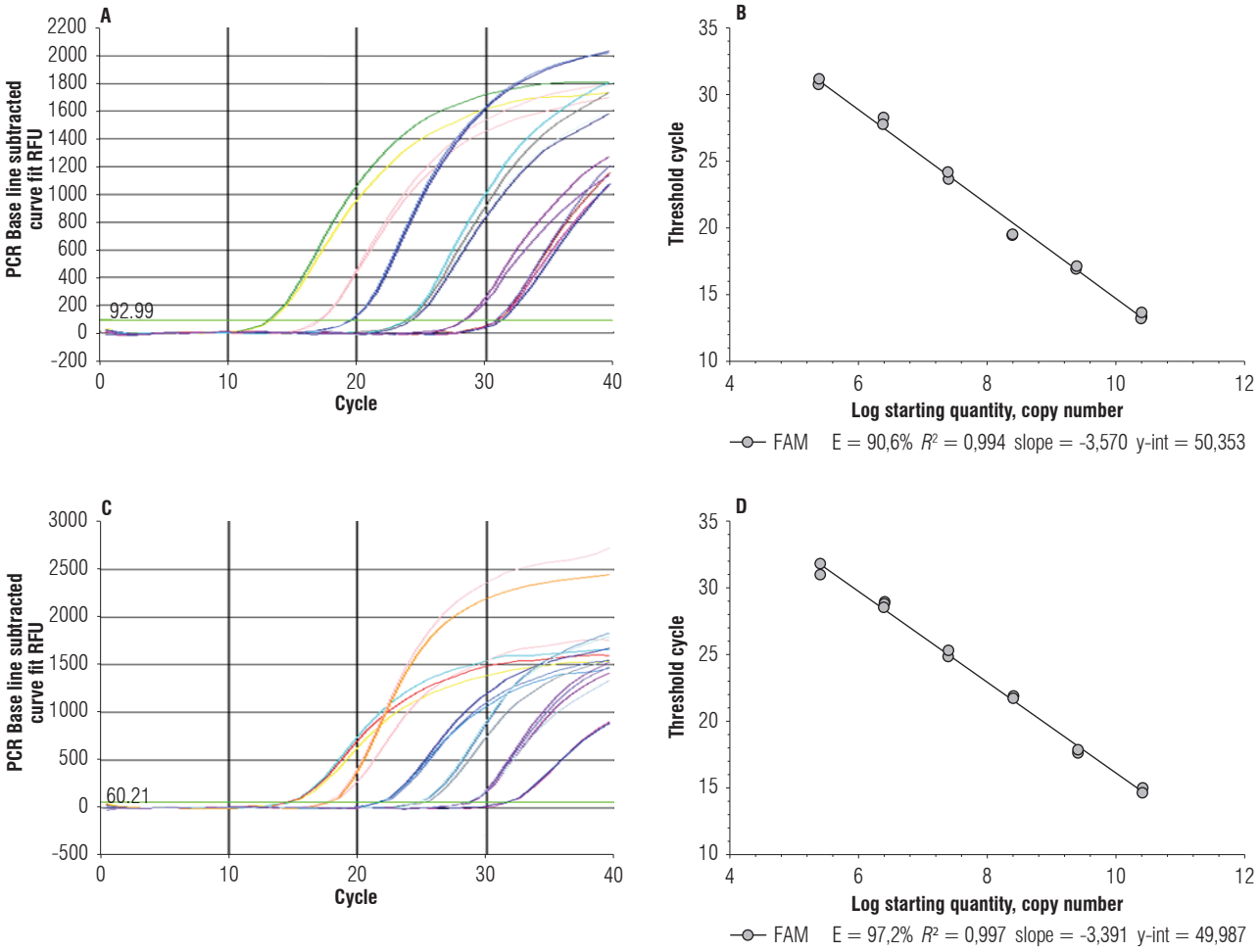

FIGURE 8. Standard curves obtained with real-time PCR. The primers and probes used were specific for *Ca*. L. asiaticus (A and B) and *Ca*. L. americanus (C and D) and the serial dilutions (10^{-1} - 10^{-6}) from an initial plasmid concentration of 1,500 ng μL^{-1}.

Validation of the detection method for *Ca*. L. asiaticus and *Ca*. L. americanus using real-time PCR

The standard curves for *Ca*. L. asiaticus and *Ca*. L. americanus were made by using dilutions 2 to 7 of the plasmid DNA for each variant, as described in Tab. 3. The standard curve for *Ca*. L. asiaticus obtained a correlation coefficient of 0.994 and an efficiency of 90.6%. Likewise, the standard curve for *Ca*. L. americanus obtained a correlation coefficient of 0.997 and an efficiency of 97.2% (Fig. 8). These data demonstrated the effectiveness, efficiency, and linearity of the method employed for the detection of the two analyzed variants.

In terms of the absence of positive samples in Colombia, which were sought for in the present study, it resulted from the approximation of the conditions of a real sample, carrying out an inoculation of negative tissues with positive plasmid DNA, as discussed in the materials and methods section. The inoculated samples were analyzed through real-time PCR with indicators specific for each variant, with the obtained results registered in the presence-absence matrixes (Tab. 8). Using the Spearman-Kärber formula for final points, it was determined that the 90% detection limit for *Ca*. L. asiaticus was 14.91 ng μL^{-1}, equivalent to $2.49 \cdot 10^9$ copies, in the case of the insect material and 14.89 ng μL^{-1} ($2.49 \cdot 10^9$ copies) for the citrus leaf tissue. The detection limit for *Ca*. L. americanus was 1.36 ng μL^{-1} ($2.49 \cdot 10^8$ copies) in the insect material and 1.25 ng uL^{-1} ($2.49 \cdot 10^8$ copies) in the plant tissue (Tab. 9).

Three plant samples and three insect samples were inoculated in each of the possible sampling combinations starting from the previous dilution until the detection limit (150 ng uL^{-1}) with the positive controls of *Ca*. L. americanus and *Ca*. L. asiaticus, for a total of 12 plant samples and 12 insect samples, on which DNA extraction was performed with subsequent detection using real-time PCR, wherein only true positives and true negatives were found (Tab.

TABLE 8. Presence-absence matrix of the results obtained with real-time PCR for the detection limit.

Sample		*Ca*. L. asiaticus										*Ca*. L. americanus									
		Dilution										Dilution									
		1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
Plant	S1	P	P	P	A	A	A	A	A	A	A	P	P	P	P	P	P	A	A	A	A
	S2	P	P	P	P	A	A	A	A	A	A	P	P	P	P	P	P	A	A	A	A
	S3	P	P	P	A	A	A	A	A	A	A	P	P	P	P	P	P	A	P	A	A
	S4	P	P	P	A	A	A	A	A	A	A	P	P	P	P	A	P	P	A	A	A
	S5	P	P	P	P	A	A	A	A	A	A	P	P	P	P	P	A	A	A	A	A
	S6	P	P	P	P	A	A	A	A	A	A	P	P	P	P	P	A	P	A	A	A
	S7	P	P	P	P	A	A	A	A	A	A	P	P	P	P	P	P	P	A	A	A
	S8	P	P	P	P	A	A	A	A	A	A	P	P	P	P	P	A	A	A	A	A
	S9	P	P	P	P	A	A	A	A	A	A	P	P	P	P	P	A	A	P	A	A
	S10	P	P	P	A	A	A	A	A	A	A	P	P	P	P	P	P	A	A	A	A
Insect	S1	P	P	P	P	A	A	A	A	A	A	P	P	P	P	A	A	A	A	A	A
	S2	P	P	P	A	A	A	A	A	A	A	P	P	P	P	A	A	A	A	A	A
	S3	P	P	P	P	A	A	A	A	A	A	P	P	P	P	P	A	A	A	A	A
	S4	P	P	P	A	A	A	A	A	A	A	P	P	P	P	P	A	A	A	A	A
	S5	P	P	P	A	A	A	A	A	A	A	P	P	P	P	P	A	A	A	A	A
	S6	P	P	P	A	A	A	A	A	A	A	P	P	P	P	P	A	A	A	A	A
	S7	P	P	P	A	P	A	A	A	A	A	P	P	P	P	P	A	A	A	A	A
	S8	P	P	P	A	A	A	A	A	A	A	P	P	P	P	P	A	A	A	A	A
	S9	P	P	P	A	A	A	A	A	A	A	P	P	P	P	P	P	A	A	A	A
	S10	P	P	P	P	A	A	A	A	A	A	P	P	P	P	P	A	A	A	A	A

P, presence; A, absence.

TABLE 9. Detection limit of Ca. L. asiaticus and CA. L. americanus.

	Insect tissue	Plant tissue
Ca. L. asiaticus	$DL_{90\%\ (conc.)} = 15.00\ ng\ \mu L^{-1} - 0.1(1.4 - 0.5)$ $DL_{90\%\ (conc.)} = 15.00\ ng\ \mu L^{-1} - 0.1(0.9)$ $DL_{90\%\ (conc.)} = 15.00\ ng\ \mu L^{-1} - 0.09$ $DL_{90\%\ (conc.)} = 14.91\ ng\ \mu L^{-1}$	$DL_{90\%\ (conc.)} = 15.00\ ng\ \mu L^{-1} - 0.1(1.6 - 0.5)$ $DL_{90\%\ (conc.)} = 15.00\ ng\ \mu L^{-1} - 0.1(1.1)$ $DL_{90\%(conc.)} = 15.00\ ng\ \mu L^{-1} - 0.11$ $DL_{90\%(conc.)} = 14.89\ ng\ \mu L^{-1}$
Ca. L. americanus	$DL_{90\%\ (conc.)} = 1.50\ ng\ \mu L^{-1} - 0.1(1.9 - 0.5)$ $DL_{90\%\ (conc.)} = 1.50\ ng\ \mu L^{-1} - 0.1(1.4)$ $DL_{90\%\ (conc.)} = 1.50\ ng\ \mu L^{-1} - 0.14$ $DL_{90\%\ (conc.)} = 1.36\ ng\ \mu L^{-1}$	$DL_{90\%\ (conc.)} = 1.50\ ng\ \mu L^{-1} - 0.1(3 - 0.5)$ $DL_{90\%\ (conc.)} = 1.50\ ng\ \mu L^{-1} - 0.1(2.5)$ $DL_{90\%\ (conc.)} = 1.50\ ng\ \mu L^{-1} - 0.25$ $DL_{90\%\ (conc.)} = 1.25\ ng\ \mu L^{-1}$

conc., concentration.

10). A sensitivity of 100%, a specificity of 100%, an efficiency of 100%, an assignment of false positives of 0%, an assignment of false negatives of 0%, and a selectivity of -0.3 for the two variants of the disease, both in the plant and insect tissue, were determined, which indicated the reliability and robustness of the real-time PCR technique utilized in the present study for the detection of region 16S of *Ca*. L. americanus and *Ca*. L. asiaticus, in both in the leaf and insect tissues.

Through this validation process, it was possible to determine that the real-time PCR technique for the detection of the American and Asian variants in the citrus-producing regions of Colombia is reliable and highly sensitive and can be used for further monitoring of HLB, with all its controls, both on citrus leaf tissues and on the insect vector.

Conclusions

The HLB insect vector *Diaphorina citri* was found in the 25 tested municipalities of Cundinamarca, Santander, Valle del Cauca, Meta and Quindio, in the egg, nymph and/or adult stages.

During the development of this project, using real-time PCR, it was confirmed that all of the insect and leaf tissue samples were negative for the presence of *Ca*. L asiaticus and *Ca*. L americanus, which indicates the absence, to date, of this disease in the sampled citrus-producing regions.

It was determined that the detection limit for the real-time PCR technique for region 16S of *Ca*. L. asiaticus was 14.89 ng μL^{-1} for the leaf tissue and 14.91 ng μL^{-1} for the insect

TABLE 10. Results of the amplification of the possible sample combinations through real-time PCR.

Sample		Plant tissue		Insect tissue	
		A	B	A	B
Sample 1	CM1	+	-	+	-
	CM2	-	+	-	+
	CM3	-	-	-	-
	CM4	+	+	+	+
Sample 2	CM1	+	-	+	-
	CM2	-	+	-	+
	CM3	-	-	-	-
	CM4	+	+	+	+
Sample 3	CM1	+	-	+	-
	CM2	-	+	-	+
	CM3	-	-	-	-
	CM4	+	+	+	+

CM, sample combination; A, amplification with indicators specific for *Ca*. L. americanus; plus(+), a positive amplification result through real-time PCR; minus(-), a negative amplification result through real-time PCR.

tissue; and 1.25 ng μL^{-1} for the leaf tissue and 1.36 ng μL^{-1} for the insect tissue for region 16S of *Ca*. L. americanus.

The results for sensitivity, specificity, efficiency, false positives, false negatives and selectivity for the real-time PCR technique used for the detection of *Ca*. L. asiaticus and *Ca*. L. americanus on plant and insect tissues presented reliability and robustness for the molecular detection of the two variants of the disease known as citrus HLB.

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