TESTING TWO TYPES OF MOLECULAR METHODS FOR THE DETECTION OF CANDIDATUS LIBERIBACTER, THE CAUSATIVE AGENT OF HUANGLONGBING (HLB) IN THE GREENING OF CITRUS FRUITS IN ECUADOR

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ABSTRACT

Citrus greening disease, caused by the pathogenic bacterium *Candidatus Liberibacter*, is a looming problem in Ecuador, as it is present in neighbouring countries. The damage caused by this disease disrupts phloem function and severely impairs the translocation of assimilates in the host plant, often leading to death. To accurately identify the causative bacterium of citrus greening using molecular techniques and a rapid-action protocol, clustered sampling was conducted on symptomatic plants in four provinces of Ecuador, where the vector is already present and citrus-producing areas exist. Two methodologies, CTAB DNA extraction and the DNA-easy plant mini kit, were employed to extract DNA for subsequent verification of the presence/absence of this microorganism through PCR, followed by electrophoresis and cost analysis. Results showed similar DNA extractions using both methods, with quantification through electrophoresis and spectrophotometry indicating a positive correlation of r2=0.9402. PCR reaction with the 16S rRNA molecular markers of the 22 samples did not detect the presence of the bacterium in any of the areas. The total diagnostic cost for PCR of 18 samples was determined to be $84.93, with a unit value of $4.71. Using the Qiagen® DNA-easy plant mini method, the unit value per sample was $3.36, resulting in a total cost of $60.65 for the CTAB method.

Keywords: *Candidatus Liberibacter*, HLB, DNA, Electrophoresis, citrus.

INTRODUCTION

In the quest for new alternatives for the detection of pathogens hazardous to our country, the topic titled "Use of molecular markers for the detection of *Candidatus liberibacter* causing 'Huanglongbing' (HLB) citrus greening in Ecuador" is proposed, which aims to utilize molecular markers to detect this condition in our country. The most reliable method so far for identifying HLB is the quantitative polymerase chain reaction (PCR) test Puttamuk *et al*., (2016) (Monzó et al., 2015).

El cultivation of citrus fruits worldwide is highly significant in agriculture. It is grown in 140 countries, and one of the limitations for its production are diseases, which cause considerable damage to orchards Zhou, Powell, Li, Irey, & Duan, (2016). As a result, they decrease production and lead to significant losses in the citrus sector. (Sáenz Pérez et al., 2019). The cultivation of citrus fruits is not only important in terms of generating employment and income, but it also contributes to fulfilling nutritional requirements in many low-resource countries. (Rodríguez Cabrera et al., 2016). In Ecuador, the key lime (Citrus aurantifolia Swingle) is the most cultivated species, followed by the Tahiti lime (Citrus latifolia Tan), together totaling approximately 4,400 hectares under cultivation. (Santistevan Méndez et al., 2017).

Huanglongbing (HLB), also known as citrus greening or yellow shoot disease, is currently considered the greatest threat to citrus farming worldwide (Granados-Ramírez & Hernández-Hernández, 2018). *C. liberibacter,* the causative agent, has primarily impacted Asia, South Africa, and Brazil. The most reliable method so far is early detection of HLB through quantitative real-time polymerase chain reaction (qrt-PCR), which is considered costly and time-consuming (Garza-Saldaña, Varela-Fuentes y Gómez-Flores, 2017).

Mora (2016), Commentary states that the alphaproteobacteria *C. liberibacter* is the causative bacterium of citrus HLB, it is a Gram-negative bacterium restricted to the sieve tubes of the phloem through which it moves via pores (Graça & da Graça, 2016). Nowadays, three species of this bacterium are known: 1) *Candidatus liberibacter* asiaticus, which is widely distributed in citrus plantations in Asia, and recently in Brazil, Florida, Mexico, and the Caribbean. 2) *Candidatus liberibacter* africanus, a species recorded in some citrus regions of Africa; and 3) *Candidatus liberibacter* americanus, which was detected in Brazil and recently in Asia (Bové) as cited by (Robles-González et al., 2013). The HLB causes disorder in the phloem and severely damages the translocation of assimilates by the host plant (Samaniego et al., 2015); (Santivañez, Mora, Díaz, López, & Vernal, 2016).

The general objective of this research was to evaluate the presence of *Candidatus liberibacter spp*. Huanglongbing (HLB), the causative agent of citrus greening, in Ecuador using molecular markers. The specific objectives were to: Quantify the amount of DNA extracted from three citrus species using a commercial extraction kit; Detect the presence of the CL bacteria in oranges (*Citrus × sinensis*), mandarins (*Citrus reticulata*), and lemons (*Citrus × limon*) using the Polymerase Chain Reaction (PCR) technique; and perform a cost analysis for the molecular detection of CL.

METHODOLOGY

The present research was conducted in the provinces of Los Ríos, Guayas, Manabí, and Santa Elena, from which citrus-producing areas were selected, specifically the orange (*Citrus × sinensis*), mandarin (*Citrus reticulata*), and lemon (*Citrus × limon*) species, which are representative vulnerable crops to the pathogen under study. Both the field sampling phase and the laboratory work were carried out from July to December 2020 and the first quarter of 2021.

Population and Sample

The selected population consists of branches and shoots from orange (*Citrus × sinensis*), mandarin (*Citrus reticulata*), and lemon (*Citrus × limon*) trees that exhibited similar symptoms (appearance of irregular light green or yellowish spots, thickening, and clearing of veins) to those caused by the phloem bacteria *C. liberibacter*, the causative agent of Huanglongbing (HLB), or citrus greening, in Ecuador. The samples were collected in georeferenced areas, as shown in **Table 1**, which include: Guayas Province (Guayaquil and Milagro), Los Ríos Province (Quinsaloma and Ricaurte), Manabí Province (Portoviejo and Chone), and Santa Elena Province. The samples were taken to the laboratory of the Biotechnology Department at the National Institute of Agricultural Research Experimental Station in Pichilingue, located at Km. 5 on the Quevedo-El Empalme road, in Mocache Canton, Los Ríos Province, where their DNA was extracted.

Amplification

The extraction of nucleic acids from symptomatic HLB samples was performed using two methodologies: one of them being the QIAGEN DN-easy Plant Mini kit for purification, and the other known as the CTAB method (Cetyl Trimethyl Ammonium Bromide) followed by the electrophoresis process to determine the presence or absence of the *C. liberibacter* bacteria within the samples collected during the research and data collection process (**Figure 1**).

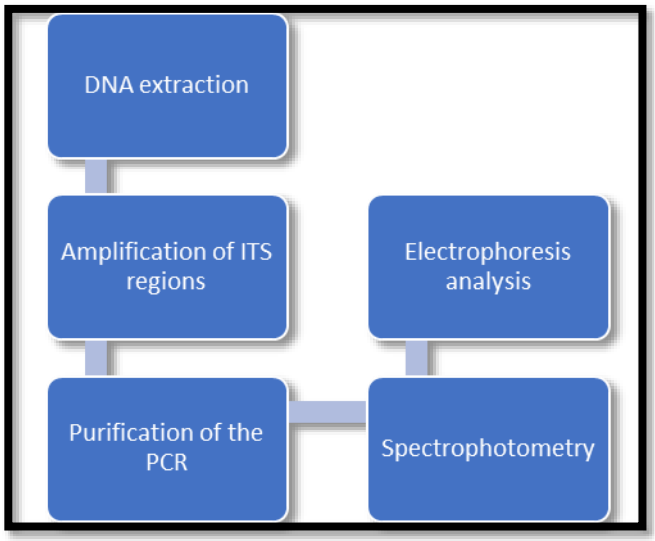

Figure 1. Process flow for DNA extraction and spectrophotometry and electrophoresis used in the research "molecular markers for the detection of *C. liberibacter* causing huanglongbing (HLB) citrus greening in Ecuador".

Table 1. Georeferencing of potential areas with the presence of HLB disease in orange (*Citrus × sinensis*), mandarin (*Citrus reticulata*), and lemon (*Citrus × limon*) citrus crops in various provinces of Ecuador, with a total of 24 samples.

N.	Geographic Coordinates	Code	DNA	Province Locality	Type of Sample	Symptom
01	S -1.186672, W -79.337006	LQN	+	Los Ríos Quinsaloma	Naranja	Leaves with yellowish colorations
02	S -1.207368, W -79.339782	LQM	+	Los Ríos Quinsaloma	Mandarina	Leaves with yellowish colorations
03	S -1.213215, W -79.343799	LQL	+	Los Ríos Quinsaloma	Limón	Leaves with yellowish colorations
04	S -1.563965, W -79.484334	LRN	+	Los Ríos Ricaurte	Naranja	Leaves with yellowish colorations
05	S -1.562238, W-79.480767	LRM	+	Los Ríos Ricaurte	Mandarina	Leaves with yellowish colorations
06	S -1.574640, W -79.482307	LRL	+	Los Ríos Ricaurte	Limón	Leaves with yellowish colorations
07	S -1.041662, W -80.352788	MPN	+	Manabí Portoviejo	Naranja	Fruits with discolorations
08	S -1.038876, W -80.360854	MPM	+	Manabí Portoviejo	Mandarina	Leaves with yellowish colorations
09	S -1.026516, W -80.396432	MPL	+	Manabí Portoviejo	Limón	Leaves with yellowish colorations
10	S -0.720801, W -80.102094	MCHN	-	Manabí Chone	Naranja	Leaves with yellowish colorations
11	S -0.725482, W -80.094702	MCHM	+	Manabí Chone	Mandarina	Leaves with yellowish colorations
12	S -0.724092, W -80.125677	MCHL	+	Manabí Chone	Limón	Fruits with discolorations
13	S-2.329621, W-80.266640	GCN	+	Guayas Cerecita	Naranja	Leaves with yellowish colorations
14	S-2.340869, W-80.252200	GBM	-	Guayas Balzar	Mandarina	Leaves with yellowish colorations
15	S-2.329621, W-80.266640	GCL	+	Guayas Cerecita	Limón	Leaves with yellowish colorations
16	S-2° 6' 0", W-79° 29' 0"	GML	+	Guayas Mariscal	Naranja	Leaves with yellowish colorations
17	S-1.400274, W-79.943047	GBM	+	Guayas Balzar	Mandarina	Leaves with yellowish colorations
18	S-2° 6' 0", W-79° 29' 0"	GML	+	Guayas Mariscal	Limón	Leaves with yellowish colorations
19	S-2.01667, W-80.6668	SCN	+	Santa Elena Coloncheo	Naranja	Fruits with discolorations
20	S-2.01667, W-80.6668	SCM	+	Santa Elena Coloncheo	Mandarina	Leaves with yellowish colorations
21	S-2.00230, W -80.339000	SLL	+	Santa Elena Limonsito	Limón	Leaves with yellowish colorations
22	S-2.00230, W -80.339000	SLN	+	Santa Elena Limonsito	Naranja	Leaves with yellowish colorations
23	S-1.5000, W-80.4400	SMM	+	Santa Elena Manglar	Mandarina	Leaves with yellowish colorations
24	S-1.5000, W-80.4400	SML	+	Santa Elena Manglar	Limón	Leaves with yellowish colorations

LQN: *Los Ríos Quinsaloma Naranja ;* ***LQM:****Los Ríos Quinsaloma Mandarina;* ***LQL:*** *Los Ríos Quinsaloma Limón;* ***LRN:*** *Los Ríos Ricaurte Naranja;* ***LRM:*** *Los Ríos Ricaurte Mandarina;* ***LRL:*** *Los Ríos Ricaurte Limón;* ***MPN:*** *Manabí Portoviejo Naranja ;* ***MPM:*** *Manabí Portoviejo Mandarina;* ***MPL:*** *Manabí Portoviejo Limón;* ***MCHN:*** *Manabí Chone Naranja ;* ***MCHM:*** *Manabí Chone Mandarina;* ***MCHL:*** *Manabí Chone Limón ;* ***GCN*** *Guayas Cerecita Naranja;* ***GBM*** *Guayas Balzar Mandarina;* ***GCL*** *Guayas Cerecita Limón;* ***GMN*** *Guayas Mandarina Naranja;* ***GBM*** *Guayas Balzar Mandarina;* ***GML*** *Guayas Mariscal Limón;* ***SCN*** *Santa Elena Coloncheo Naranja;* ***SCM*** *Santa Elena Coloncheo Manglar;* ***SLL*** *Santa Elena Limoncito limón ;* ***SLN*** *Santa Elena Limoncito Naranja;* ***SMM*** *Santa Elena Manglar Mandarina;* ***SML*** *Santa Elena Manglar Limón*

Molecular process

The collected samples were subjected to DNA extraction using a DNA easy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The concentration and quality of DNA were verified by visualizing it on a 1.5% agarose gel after electrophoresis.

Total cost analysis of treatments

It was obtained by adding up fixed costs (analysis, supplies, reagents, etc.) and variable costs (PCR - AMPLIFICATION). It was calculated and analysed using the following formula: CT = X + PX, CT = Total Costs, X = Fixed Costs, PX = Variable Costs.

Descriptive and Inferential Statistics

For the following research, a modified cluster sampling method was used to combine the samples that go into each Polymerase Chain Reaction (PCR) tube.

Experimental Design

For the following research, a non-experimental descriptive design was used for the PCR diagnosis. Cluster sampling will be used with samples of 3 symptomatic leaves, fruits, and young branches per tree per locality with a minimum of replicates due to costs, for diagnostics, two replicates, comprised of georeferenced locations.

RESULTS

DNA quantification using spectrophotometry and visualization on gel

Regarding **Table 2**, we observed DNA quantification using spectrophotometry and gel visualization. We confirmed that for samples 1 to 8, the CTAB methodology was used, while for samples 9 to 22, the extraction kit (DN-easy plant mini) from Qiagen® was employed. DNA quality, determined by the 260/280 ratio, was 1.62 and 1.52, respectively, for each methodology.

Below are the results of DNA extraction using the CTAB method from orange (Citrus × sinensis), mandarin (*Citrus reticulata*), and lemon (*Citrus × limon*) citrus fruits, visualized through gel electrophoresis at the Laboratory of the Department of Biotechnology of the National Institute of Agricultural Research Experimental Station

Tropical Pichilingue. The standard concentrations, also known as lambda (λ), showed concentrations greater than 100 ng/µl in all CTAB method extractions, while kit extractions ranged between 25 and >100 ng/µl (**Table 2**).

Additionally, visualization on a 1.5% agarose gel by electrophoresis showed DNA fragmentation in samples 1 to 8 (**Figure 2**) compared to DNA extraction with the kit in samples 9 to 22 (**Figure 3**).

Table 2. DNA quantification by spectrophotometry, pure DNA with an OD 260 /OD 280 ratio of ~1.8; pure RNA has an OD 260 /OD 280 ratio of ~2.0.

Code	Sample	Gel Lambda	Absorbance 260nm	DNA quality 260/280	DNA Quantity ng/ul
LQN	1	>100	0,085	1,87	212
LQM	2	>100	0,078	1,85	195
LQL	3	>100	0,072	1,82	180
LRN	4	>100	0,052	1,78	130
LRM	5	25 - 50	0,03	1,41	75
LRL	6	>50	0,029	1,46	72
MPN	7	>50	0,15	1,17	375
MPM	8	>50	0,045	1,61	112
MPL	9	10 - 25	0,05	1,39	125
MCHM	11	100	0,125	1,67	312,5
MCHL	12	100	0,086	1,37	215
GCN	13	100	0,063	1,4	157,5
GCL	15	25 - 50	0,031	1,18	77,5
GML	16	100	0,085	1,94	212,5
GBM	17	25	0,03	2,07	75
GML	18	50	0,102	1,35	255
SCN	19	100	0,118	1,33	295
SCM	20	25	0,026	1,55	65
SLL	21	50	0,131	1,36	327,5
SLN	22	25 - 50	0,127	1,57	317,5
SMM	23	50	0,051	1,45	127,5
SML	24	100	0,065	1,58	162,5
X̄ CETAB	-	-	**0,0676**	**1,62**	**168,9**
X̄ Qiagen®	-	-	**0,0779**	**1,52**	**194,6**

**Dilution factor 50; *dsDNA Constant 50.*

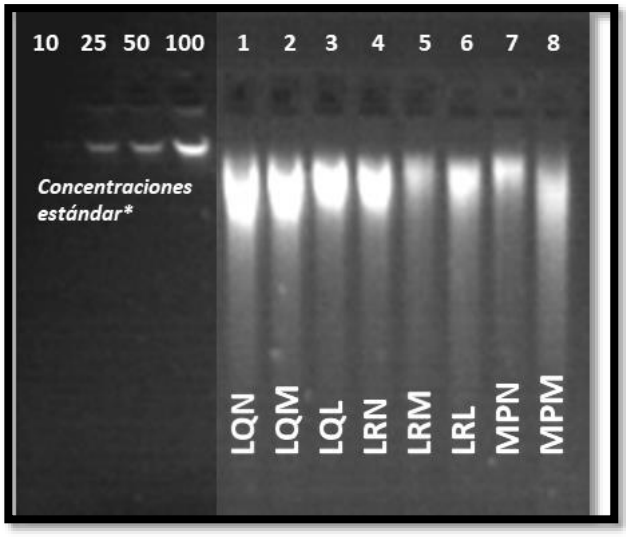

Figure 2. 1.5% Agarose gel electrophoresis of DNA extraction using the CTAB method. Lanes from the left end: 4 lanes are standard concentrations of 10, 25, 50, and 100. Lanes 1 to 8, DNA from orange (*Citrus × sinensis*), mandarin (*Citrus reticulata*), and lemon (*Citrus × limon*) citrus fruits in the study areas of the country.

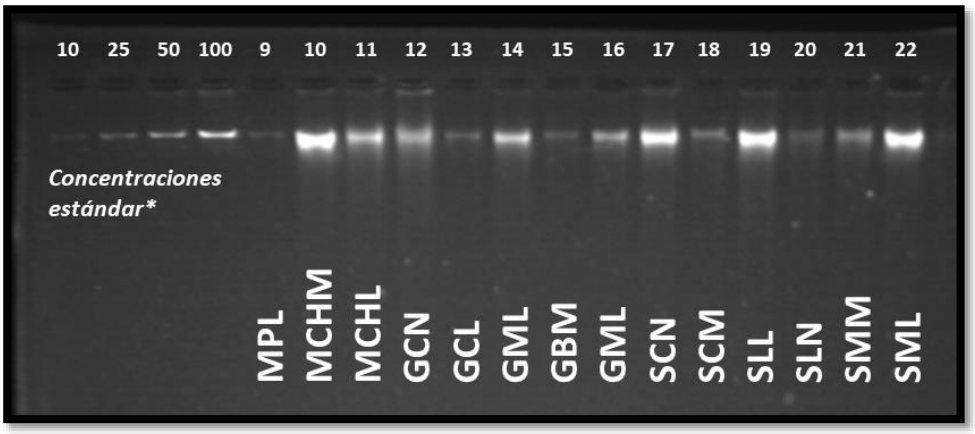

Figure 3. 1.5% Agarose gel electrophoresis of DNA extracted using the QIAGEN kit. Lanes from the ends: 4 lanes are standard concentrations of 10, 25, 50, and 100. Lanes 9 to 22 DNA from orange (Citrus × sinensis), mandarin (*Citrus reticulata*), and lemon (*Citrus × limon*) citrus fruits in the study areas of the country.

The quantification of DNA from symptomatic citrus samples with HLB using spectrophotometry showed a range of 65 to 327 ng/µl with an average of 168.87 ng/µl for extractions using the CTAB method and 194.64 ng/µl for extractions using the kit (Qiagen®). The comparison of DNA quantification by visual Lambda method and spectrophotometry showed a positive correlation r2=0.9402 (**Figure 4**).

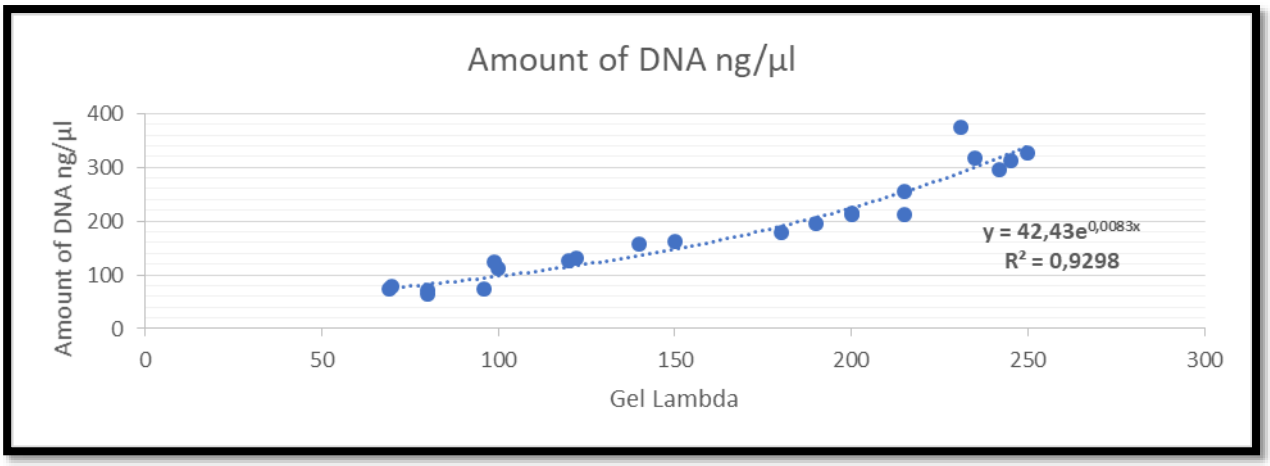

Figure 4. Correlation Graph Regarding Lambda Gel and DNA amount ng/µl in DNA quantification through spectrophotometry and visualization in 1.5% agarose gel concentration.

Presence - absence of the *C. liberibacter* bacteria.

To identify the presence or absence of *C. liberibacter*, diagnostic amplifications for HLB were carried out using conventional PCR with the primers OI1-OI2c and A2-J5. A positive control was used for each amplification, employing DNA from *C. liberibacter* as a reference for the process of determining the presence or absence of the bacterium (**Table 3**).

Below, the results of the amplification with molecular markers OI1-OI2c visualized through agarose gel electrophoresis at 1.5%, carried out in the Biotechnology Department laboratory of INIAP, are presented. **Figure 5** of the gel shows positive amplification for the control and the absence of amplification for the 22 HLB samples from orange (*Citrus × sinensis*), mandarin (*Citrus reticulata*), and lemon (*Citrus × limon*) in all locations.

In **Figure 6**, each of the points where sampling was conducted in the field is shown, indicating georeferenced points in the provinces of Los Ríos, Manabí, Guayas, and Santa Elena. Blue denotes presence, and red indicates the absence of the pathogen Candidatus liberibacter causing HLB.

Table 3. Percentage of observed symptoms expressed as a percentage in the molecular markers research for the detection of *C liberibacter* causing citrus greening (HLB) in Ecuador.

N.	Code	Sample Type	Symptom	% de symptom
1	LQN	Naranja	Leaves with yellowish colorations	25%
2	LQM	Mandarina	Leaves with yellowish colorations	52%
3	LQL	Limón	Leaves with yellowish colorations	33%
4	LRN	Naranja	Leaves with yellowish colorations	15%
5	LRM	Mandarina	Leaves with yellowish colorations	20%
6	LRL	Limón	Leaves with yellowish colorations	32%
7	MPN	Naranja	Fruits with discolorations	62%
8	MPM	Mandarina	Leaves with yellowish colorations	32%
9	MPL	Limón	Leaves with yellowish colorations	25%
10	MCHN	Naranja	Leaves with yellowish colorations	65%
11	MCHM	Mandarina	Leaves with yellowish colorations	41%
12	MCHL	Limón	Fruits with discolorations	58%
13	GCN	Naranja	Leaves with yellowish colorations	59%
14	GBM	Mandarina	Leaves with yellowish colorations	45%
15	GCL	Limón	Leaves with yellowish colorations	24%
16	GML	Naranja	Leaves with yellowish colorations	36%
17	GBM	Mandarina	Leaves with yellowish colorations	57%
18	GML	Limón	Leaves with yellowish colorations	45%
19	SCN	Naranja	Fruits with discolorations	47%
20	SCM	Mandarina	Leaves with yellowish colorations	15%
21	SLL	Limón	Leaves with yellowish colorations	23%
22	SLN	Naranja	Leaves with yellowish colorations	42%
23	SMM	Mandarina	Leaves with yellowish colorations	45%
24	SML	Limón	Leaves with yellowish colorations	23%

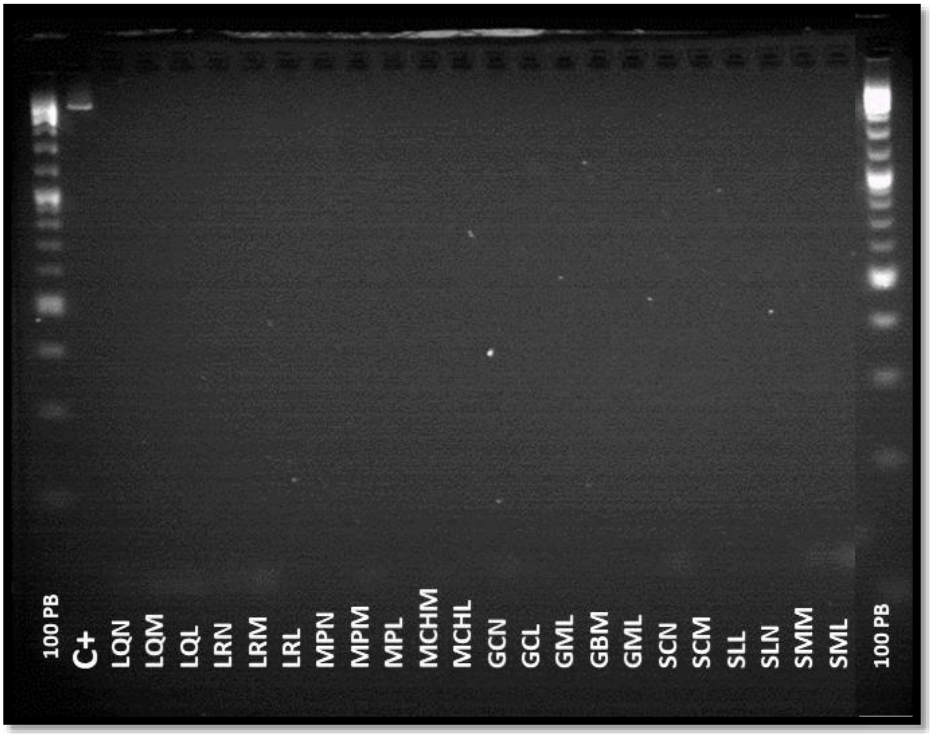

Figure 5. 1.5% agarose gel electrophoresis of DNA. End lanes with 1Kb molecular ladder (100bp), C+ with DNA from C. liberibacter, lanes with abbreviations referring to samples from orange (Citrus × sinensis), mandarin (Citrus reticulata), and lemon (Citrus × limon) in the study areas of the country.

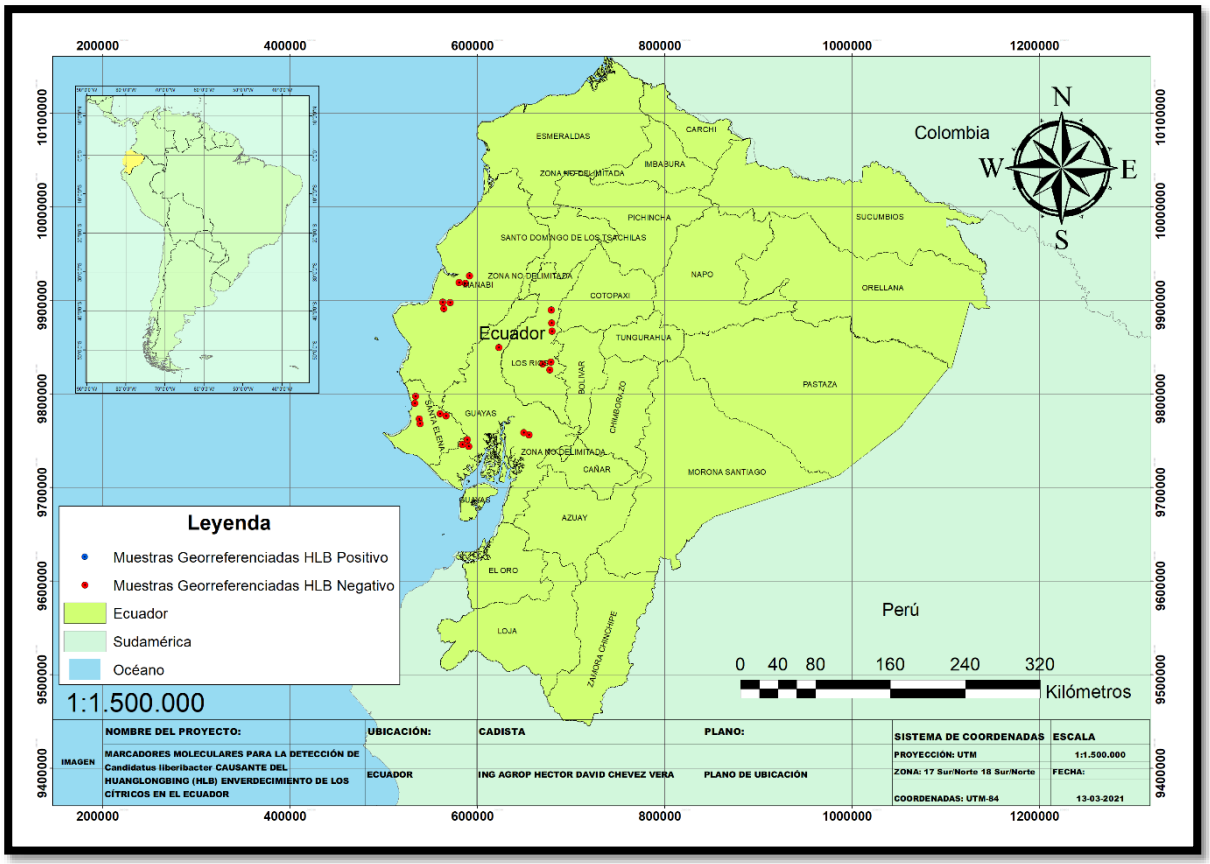

Figure 6. Shows the georeferencing, codes, and symptoms present in the research on Molecular Markers for the Detection of *Candidatus liberibacter*, the causative agent of huanglongbing (HLB) or citrus greening in Ecuador.

Cost analysis

Below in the **Table 4** and **Table 5** are the results of the cost analysis after carrying out data collection activities and processing the samples in the laboratory of the Biotechnology Department of the National Institute of Agricultural Research, Tropical Experimental Station Pichilingue, in the various areas defined in Ecuador. The following unit and total costs for the analysis of *C liberibacter*, the causative agent of Huanglongbing (HLB) Citrus Greening in Ecuador, were obtained.

DISCUSSION

DNA Quantification Using Spectrophotometry and Gel Visualization

Through spectrophotometry and the gel visualization technique, DNA quantification was carried out by visualization in 1.5% agarose gel, which indicates that pure DNA has an optical density (OD) ratio of OD 260 /OD 280 of ~1.8; pure RNA has an OD 260 /OD 280 ratio of ~2.0. Averages of 1.62 for the CTAB method and 1.52 for extraction (DN-easy plant mini) by Qiagen® were obtained, respectively. Lower ratios could be caused by contamination with proteins or phenol. A dsDNA sample was diluted 50 times. The diluted sample gave a reading of 0.65 on a spectrophotometer at OD 260. (Ruíz Sesma et al., 2010) comment that a ratio of 0.6 for the (A260/A280) ratio corresponds to the unique presence of proteins; and a ratio between 1.8–2.0, corresponds to a 90% - 100% purity of nucleic acids, furthermore, (Checa Rojas, 2018) mention that values greater than 2.0 for the ratio indicate an excess of RNA in the sample and for the (A260/A230) ratio, values less than 2.0 indicate the presence of carbohydrates, proteins, or phenols. (M. Somma, 2015) in their DNA Extraction and Purification protocol mention that if the available amount of nucleic acids is scarce, the agarose plate method with ethidium bromide can be used, which allows for the calculation of the amount of nucleic acids from the intensity of the fluorescence emitted by the DNA Extraction and Purification.

As can be observed in the 1.5% agarose gel electrophoresis performed at the laboratory of the Department of Biotechnology of the National Institute of Agricultural Research Tropical Experimental Station Pichilingue, results of the DNA extraction from orange citrus (Citrus × sinensis), mandarin (Citrus reticulata), and lemon (Citrus × limón) using the CTAB method are shown in **Figure 2**, as seen in the first 8 lanes, the initial 4 lanes refer to standard concentrations of 10, 25, 50, and 100, respectively. Regarding **Figure 4**, similarly to **Figure 3** in the 1.5% agarose gel electrophoresis performed at the

laboratory of the Department of Biotechnology of the National Institute of Agricultural Research Tropical Experimental Station Pichilingue, results of the DNA extraction from orange citrus (Citrus × sinensis), mandarin (Citrus reticulata), and lemon (Citrus × limón) using the extraction kit method (DN-easy plant mini) by Qiagen® are presented.

Regarding the correlation analysis between Lambda Gel and DNA quantity ng/µl, methods to quantify DNA, we achieved a positive relation close to zero, thus, with a value of (Y) 0.42.42 there is a high relationship between these two factors under study with a value of r^2 0.9402. **Figure 4**, this could be the result of the difference between quantitative and qualitative data produced by the methodologies under study.

Table 4. Cost analysis based on the Qiagen® DN-easy plant mini method and PCR amplification for the detection of *C. liberibacter*, the causative agent of Huanglongbing (HLB) Citrus Greening in Ecuador, 2020.

Material	Extent	Amount	Cost U$
Mobilization	day	10	5,00
Laboratorian	hour	1	3,00
Gloves	pair	1	0,50
Micro tips 0-200 µL	unit	7	0,15
Barrier-free colorless micro tips	unit	10	0,80
Microtube 1.5 mL	unit	1	0,02
Microtubo 0.2 mL	unit	10	1,53
dNTPs 10 mM	unit	5	3,11
direct oligonucleotide 18 pmol	µL	5	3,07
reverse oligonucleotide 18 pmol	µL	5	3,07
ADN polimerasa Platinum Taq	µL	2	2,65
Sterilized distilled water per c/1000 mL	µL	325	0,00
Electrophoresis for 18 samples	samples	18	13,26
Gel stain for 18 samples	samples	18	6,40
extraction kit	equipment	1	31,25
Losses (10%)			6,20
Indirect costs			4,92
TOTAL (18 samples)			84,93

Total PCR cost for 18 samples. Which in this case is considered equivalent to the use of 100% of the capacity.

Table 5. Cost analysis based on the CTAB method and PCR amplification for the detection of C. liberibacter, the causative agent of Huanglongbing (HLB) Citrus Greening in Ecuador, 2020.

Material	Extent	Amount	Cost U$
Mobilization	day	10	5,00
Laboratorian	hour	1	3,00
Gloves	pair	1	0,50
Micro tips 0-200 µL	unit	7	0,15
Barrier-free colorless micro tips	unit	10	0,80
Microtube 1.5 mL	unit	1	0,02
Microtubo 0.2 mL	unit	10	1,53
buffer CTAB 3 %	unit	5	2,15
Beta-mercaptoetanol	µL	6	1,07
RNA'sa	µL	15	1,00
ADN polimerasa Platinum Taq	µL	2	2,65
Sterilized distilled water per c/1000 mL	µL	325	0,00
Electrophoresis for 18 samples	samples	18	13,26
Gel stain for 18 samples	samples	18	6,40
chloroform	µL	60	12,00
Losses (10%)			6,20
Indirect costs			4,92
TOTAL (18 samples)			60,65

Total PCR cost for 18 samples. Which in this case is considered equivalent to the use of 100% of the capacity.

Presence - absence of *C. liberibacter* bacteria

Regarding the analysis of the 1.5% agarose gel electrophoresis of DNA in **Figure 5**, a positive control with the presence of DNA from *C. liberibacter* is observed for each of the samples and their respective codes, indicating that there was no presence of the bacteria under study causing HLB at each of the georeferenced sites after the relevant sampling. On the other hand, in the Data Collection, symptoms on leaves were observed, particularly asymmetric discolorations between green and yellow as shown in Annex 1. It is noteworthy that the presence of the vector *Diaphorina citri* was not observed; however, its presence was found by Navarrete et al., (2016) In that year, the presence of the bacteria was ruled out by the PCR technique and its subsequent electrophoresis; the results are in agreement with con (Cornejo, 2016) who carried out the collection of samples from various locations on the Ecuadorian coast, Cuadros, Vélez, Velasquez, & Chirinos, (2020) also managed to detect the vector *D. citri* in the province of Manabí in Murraya paniculata trees. Chavez *et al*., (2019) state that there are natural enemies of *D. citri* which could counter a possible spread of it, such as coccinellids, *Cheilomenes sexmaculata*.

Cost Analysis

The initial stage involved pricing techniques based on a global evaluation, unit and total cost analysis, encompassing not only the cost of reagents and instruments and equipment but also qualified personnel and indirect costs as previously mentioned. The cost description was based on a single site. For example, **Tables 4** and **Tables 5** present the estimated cost of the technique performed, polymerase chain reaction (PCR) with the extraction kit method (DN-easy plant mini) from Qiagen® and the CTAB method. The average cost to detect *C liberibacter* causing huanglongbing (HLB) citrus greening in Ecuador in the extraction - PCR - electrophoresis process of a single sample is $4.71. For the total of 18 samples, a value of $84.93 was obtained using the (DN-easy plant mini) method from Qiagen®; however, using the CTAB method, a value of $3.36 per sample was obtained with a total value of $60.65. Schlatter, Matte, Polanczyk, Koehler-Santos, & Ashton-Prolla, (2016) in their research titled "Costs of genetic testing: Supporting Brazilian Public Policies for the incorporation of molecular diagnostic technologies" obtained unit values per sample of $1.58 in the extraction process. This could be explained by the fact that the cost of reagents and equipment is more expensive in the country since most are imported.

CONCLUSIONS

Regarding the absence/presence of the pathogenic bacterium *C liberibacter* causing citrus greening (HLB), it is not present at the sites where sampling has been conducted, thus, the hypothesis suggesting its presence in the study areas of the country is dismissed. Both methodologies used, the (DN-easy plant mini) method from Qiagen® and the CTAB method, are viable for DNA extraction and for the diagnosis of bacterial diseases since their accuracy percentage is quite high, making them reliable tests when determining or discarding dangerous pathogens. The current research recorded a cost per sample for molecular analyses of $4.71 for the (DN-easy plant mini) method from Qiagen® and $3.36 for the CTAB method, obtaining a minimal numerical difference of $1.35, which makes it accessible for citrus producers in Ecuador in case they present symptoms of the pathogen under study.

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