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Detection of "*Candidatus Liberibacter asiaticus*" Using Conventional PCR and Nested-PCR and Identification of Primer Sensitivity through Q-PCR in HLB Infected Mandarin Orange and Acid Lime Plants in Tamil Nadu

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

One of the most destructive citrus diseases in the world is called citrus Huanglongbing (HLB). For effective HLB associated '*Candidatus liberibacter* spp.' prevention, sensitive and precise diagnostics are essential. *Candidatus Liberibacter asiaticus* (CLas), *Candidatus* Liberibacter africanus (CLaf), and *Candidatus Liberibacter americanus* (CLam) are among the '*Candidatus liberibacter* spp.' that infect citrus. The most common of these is the species known as Las. To assess the impact of citrus greening disease in the citrus orchard, leaf samples exhibiting greening

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symptoms were picked up from 210 mandarin orange trees from nine localities in four districts and among them 189 plants showed HLB positive and leaf samples are picked up from 216 acid lime trees from nine localities in six districts of Tamil Nadu where citrus is cultivated in a larger area and among them 202 plants showed HLB positive. A “conventional singleplex polymerase chain reaction (PCR) Nested PCR and Q-PCR (Quantitative PCR)” was performed on the extracted DNA samples to detect *Ca. Liberibacter asiaticus*. Among all the mandarin orange growing localities from “Kanalkadu and Thandikudi in Dindigul district” and among the acid lime fields, the samples from “Sankarankoil from Tirunelveli district and Kallar from Nilgiris district” showed consistent amplification of 1160 base pairs fragment by using specific primers OI1 and OI2C and also 703 base pairs fragment with gene-specific primers A2 and J5 targeting beta-operon (rpiKAJL-rpoBC) gene which is a ribosomal protein gene of *Ca. Liberibacter asiaticus*, these results showed that these districts are exclusively positive to Clas . The nested PCR method significantly increased the sensitivity to identify Las up to 10 times and 100 times, respectively, compared to qPCR and conventional PCR in this study. A set of nested PCR primer pairs were examined to diagnose Las. Nested PCR was used to analyse 14 samples from 2 different citrus cultivars in 2 different districts. Among those again kanalkadu orchard for mandarin orange and Sankankoil orchard for acid lime showed more disease incidence and consistent amplification. The findings indicate that all the samples tested positive for HLB; the samples of blotched and chlorotic leaves from the acid lime field at Sankarankoil (thirunelveli district) and mandarin orange field at kanlakaadu (Dindigul district) had the highest positive detection rate (100%). The outcomes show that the nested PCR primer pairs are capable of detecting Las in a variety of symptomatic tissues, citrus cultivars, and geographical locations. The set of nested PCR primers used in the current work will be a very helpful addition to the existing methods for Las detection.

Keywords: Huanglongbing; primer sensitivity; phytopathology; nested-PCR; citrus greening; Candidatus Liberibacter asiaticus.

1. INTRODUCTION

Citrus greening is one of the most devastating diseases to threaten the citrus sector in Asia, Africa, and America is citrus Huanglongbing (HLB), [1]. Citrus trees that have been severely infected by HLB exhibit yellow shoots, foliar blotchy mottle that may resemble the symptoms of zinc deficiency, vein corking that may resemble the symptoms of Citrus Tristeza virus infection, poor blooming, and stunting [2]. Citrus trees that have HLB infection are chronically foliated sparsely, have significant twig or limb die-back, and typically die within three to five years [3,4]. Citrus with HLB infection has a 30% to 100% reduction in yield, and the degraded fruit quality [1,2,5]. HLB is caused by "*Candidatus* Liberibacter spp," gram-negative, unculturable and phloem-restricted organisms that fall within the Proteobacteria division. *Candidatus Liberibacter asiaticus* (Las), *Candidatus* Liberibacter africanus (Laf), and *Candidatus* Liberibacter americanus (Lam) are *Candidatus* Liberibacter spp. linked with HLB [3]. The most common species, Las, is also the one that causes the most of the rising economic losses [6,7]. Budding, dodder, grafting, and psyllid vectors were all used to spread HLB. HLB is spread very fast that the spread distance might reach 193 km (120 miles) each year [1,8]. Regrettably, there is currently no cure for this illness that can be used to control it, and there are no cultivars that are resistant to this pathogen. The most common forms of HLB management are eradicating affected trees and controlling the insect vector. As a result, sensitive and precise diagnosis is a requirement for studying and managing HLB. Koch's hypotheses on HLB were not tested since it was impossible to culture the HLB bacteria [9]. Several molecular detection methods based on PCR are currently in use to detect HLB associated bacteria, including conventional PCR [10,11], SSR [3], droplet digital PCR [12], LAMP [13,14], immune capture PCR [15], qPCR [3,16], and nested PCR [17]. Nested PCR has been shown to have higher sensitivity than other molecular detection methods for diagnosing disorders like HLB [18,19], as the first round of PCR products are diluted and used as the template for the second round of amplification. Hence, in this investigation, nested PCR was employed.The F1/B1 and F3/B3 results of Las isolates from various geographic locations revealed positive, indicating that the sequence locus was preserved and shared by these Las isolates (Figs. 3,4,5 and 6). Thus, the selected primers are substantially conserved and species-specific.

2. MATERIALS AND METHODS

In Tamil Nadu, two citrus species, Citrus (mandarin orange)-(experiment 1) and Citrus (acid lime)-(experiment 2), were surveyed and tested for the presence of *Ca*. Liberibacter asiaticus.

I. Experiment 1

Survey for Huanglongbing in Tamil Nadu (Mandarin orange): surveys were carried out in key mandarin orange-growing regions throughout Tamil Nadu from March 2018 to March 2021, including Yercaud (Salem district), Kolli hills (Namakkal district), Conooor, Katteri, Ooty (Nilgiris district), Kanalkadu, Patlankadu, Thandikudi, and Thadiyankudisai (Dindigul district). The PDI, latitude, longitude and altitude were noted to identify the pathogen surveillance in various epidemiological factors. To prevent DNA deterioration, samples showing symptoms such as chlorosis, mottling and blotching were brought from the field were gathered and put right away in a refrigerated box. The gathered samples were taken to the lab and either processed right away for DNA extraction or put into cold storage at -80 °C for later use (Table 1).

II. Experiment II

Survey for huanglongbing in Tamil Nadu (Acid lime): Comprehensive surveys were carried out Between March 2018 and March 2021 in key acid lime-growing regions in Tamil Nadu, including Pollachi (Coimbatore district), Sankarankoil (Thirunelveli district), Kolli Hills (Namakkal district), Thadiyankudisai, Ayyampalayam (Dindigul district), Coonoor, Katteri, and Ooty (Nilgiris district). Acid lime symptom manifestations were observed and noted during the survey. In order to prevent DNA deterioration, samples that were throught to have HLB infection were gathered, stored right away in a cold box, and either processed right away for DNA extraction or preserved at -80 °C for later use. Latitude, Longitude, and Altitude of the specific orchards and fields, together with the percentage disease incidence (PDI), were recorded. The percentage of disease incidence was calculated by the formula given by Ahmad K B. 2008 [20] (Table 2).

% disease incidence = (Total infected citrus trees / Total number of trees evaluated) × 100

Detection of CLas bacterium associated with huanglongbing of citrus in Tamil Nadu:

DNA extraction: Each sample's leaf midribs were individually used to extract total DNA using the CTAB procedure suggested by Doyle *et al*. [18]. One gram of mandarin orange midribs are separated using a clean surgical blade. The midribs were then broken up into little pieces, macerated in a pestle and mortar using liquid nitrogen, and put into an Eppendorf tube. The macerated material was mixed with two millilitres of CTAB buffer and heated to 65 °C in a water bath for 20 minutes (Scientech, India). The samples were centrifuged at 10000 RPM for 15 minutes after being inverted for a brief period of time every 10 minutes. A fresh Eppendorf tube was used to collect the supernatant. Equal quantities of 24 : 1 chloroform : isoamyl alcohol were then added to that and thoroughly blended by vertexing. The sample was then centrifuged for ten minutes at 10000 RPM. A fresh Eppendorf tube was used to transfer the aqueous phase into, and the process was repeated with an equal volume of 24:1 chloroform:isoamyl alcohol. A new eppendorf micrcentrifuge tube was used to collect the aqueous phase and isopropanol was added in an equivalent volume. After three hours incubation at -20°C, the microcentrifuge tube was centrifuged at 10000 RPM for 20 minutes to precipitate the DNA. After washing the DNA pellet with 70% ethanol and centrifuging it at 5000 RPM for five minutes, the supernatant was discarded. The DNA pellet was then air-dried after being washed with ethanol, and the supernatant was discarded. The DNA pellet was finally dissolved in 50µl of sterile water and kept at -20°C. A Nanodrop Spectrophotometer was used to measure the OD values at 260 nm and 280 nm in order to evaluate the quantity and quality of the isolated DNA (Nanodrop, Biodrop, UK). For PCR analysis, DNA samples with OD values between 1.8 and 2.0 were collected.

Pcr amplification: In order to target the bacterial 16s r RNA gene in the collected samples of mandarin orange and acid lime, amplification was carried out in a Thermal cycler (Eppendorf) using the universal primers fd1 and rp2. The most specific region of the CLas genome, partial 16S rvDNA, was the target of PCR using the primer pair OI1F/OI2CR [21], with an amplicon size of 1,100 base pairs, and the beta-operon (rpiKAJL-rpoBC) of the ribosomal protein gene as the target, with an amplicon size of 703 base pairs, using the primer pair A2/J5 (Figs. 1 and 2). The reaction mixture (25 µl) contained 0.3 µl of Taq polymerase (5 units/l, GeneiTM), 0.5 l of dNTPs (10 mM), 2.5 µl of 10x buffers, 2.0 µl of forward and reverse primers (10 mM), 5 µl of

DNA template (100-200 ng/l), and the remaining volume was made up of nuclease-free water. The following were the thermal cycle conditions set for the primer combination OI1F/OI2CR: Step 1: a cycle at 95 degrees Celsius for two minutes, followed by 35 cycles of 95 degrees Celsius for 40 seconds in step 2, 60 degrees Celsius in step 3, and 72 degrees Celsius in step 4, and a 72 degrees extension for 10 minutes [21]. The following are the PCR settings for the A2/J5 primer pair: Step 1: 94 °C for 3 minutes, Step 2: 94 °C for 1 minute, Step 3: 58.5 °C for 1 minute, Step 4: 72 °C for 1 minute, and Step 5: 72 °C for 10 minutes of extension. In 1.2% agarose gel with ethidium bromide in 1x Tris-acetate EDTA buffer, the amplification product was analysed. With the aid of a gel documentation system, the amplicons were seen. The PCR amplified DNA fragments were cleaned in the Eurofins lab using a DNA clean-up kit to get rid of any remaining primers, polymerase, and buffer (Bangalore, India).

Nested PCR amplification:

DNA preparation: Among the HLB positive samples from various districts, the Kanalkadu and Thandikudi orchard mandarin orange leaf samples from Dindigul and Sankarankoil acid lime field from Thirunelveli district were choosen by observing the maximum PDI when compared to the other fields. The mandarin orange and acid lime leaves were cleaned under running water from the faucet and dried with paper towels. Midribs were removed. The CTAB method was then used to extract DNA from each sample after it had been pulverised to a 100 mg size in liquid nitrogen [14]. TE buffer (50 µl) was used to dissolve the isolated DNA. Using a Nanodrop (Biodrop, UK.), DNA quality and concentration were examined [14]. The HLB pathogen was identified using the Las specific primers targeting OMP (outer membrane protein) amplified fragments of 1318 bp for the first set of primers and 443 bp for the second set of primers. (Fig. 3,4,5 and 6)

PCR conditions: The 2x Easy PCR master mix (smart prime) was used to generate the nested PCR mixture (20 µl), and the following settings were used for the amplification process: 94°C for 5 min, then 25 cycles of 30 seconds at 94°C, 30 seconds at 58°C, and 70 seconds at 72°C for the first round of PCR, and 35 cycles of 30 seconds at 94°C, 62°C, and 30 seconds at 72°C for the second round of PCR [22]. All of the findings are in line with the theory that nested PCR was more sensitive and useful for detecting Las at very low titers [22]. The nested primers and Q- PCR primers used in this research was targeting OMP gene in CLas (21). Compared to traditional RT-PCR, nested RT-PCR has better sensitivity and specificity, Nested PCR [10] uses two rounds of PCR, which increases the sensitivity. For bacteria to maintain their regular structure and function, the outer membrane protein (OMP) is essential. OMPs participate in interactions between plants and pathogenic microorganisms as well as exchanges with the outside environment. The nucleotide sequences of OMPs from Las showed high similarity and high species specificity (99%), and their three-dimensional architectures were largely conserved [23,5,2]. In HLB bacterial assays for detection, OMPs have been employed as target genes [5]. OMPs were frequently employed to make antigens and to analyze the variance between different geographic isolates. In this research,. This new HLB diagnosis approach uses nested PCR to amplify this area. The technique significantly increased the sensitivity in the detection of Las by up to 10 times when compared to qPCR and 100 times when compared to conventional PCR.

Identifying the sensitivity of Q-PCR for conventional PCR, first primer set of nested PCR, second set of nested PCR: The primary objective was to quantity the bacterial titre in the sample of which the amount of DNA quantity was unable to identify through conventional PCR. Usually, the determination of primer sensitivity was based on different templates or different concentrations of the same template. Firstly, three suspected samples were amplified by conventional PCR primer (OI1/OI2) and a single sample of mandarin orange and acid lime were run through nested PCR primer pairs (F1/B1 and F3/B3). Then we detected the concentration of the conventional PCR positive product and nested PCR positive product by a Nanodrop (Biodrop, UK)), and adjusted to 100 ng/µl as the dilution template. The PCR product was serially diluted in a range of 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}, which represented 10^{-5} to 1 pg/µl Las DNA, and served as the templates to evaluate the sensitivity among nested PCR, conventional PCR and qPCR. The PCR product from Fig. 1 line 8 and 9 was diluted as the template for conventional PCR, and the PCR product from Fig. 2 line 3 and 6 was diluted as the templates for the nested PCR and qPCR. To evaluate the amplification efficiency of qPCR, Las DNA serially diluted in a range of 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-}

^{4} and 10^{-5} served as the template for Q-PCR (Figs. 5, 6, 7, 8 and 9)**.** The DNA was adjusted to the same concentration for conducting the Q-PCR experiment and both sets of Nested PCR, 'primer pair (forward GGTTA TGCTGCCGTTAAAGTGTC and reverse B1: AACCAGCCCTTTCAGGAACAAG) that was purified by chloroform extraction followed by isopropanol precipitation, and quantified by averaging three replicate A260 absorbance determinations conducted on two spectr*-photometers. A second 443 bp amplicon was produced by pairing of another primers F3/B3 (forward F3: TCTGAGGGTG AGCGTAAA ACAACTG and reverse B3: TTGGGAAAT AGAAT GGCTGCTGAAT). The primer pair combinations of F1/B1 and the nested F3/B3 thus allowed the production of two different sized amplicons (1318 and 447 bp, respectively) using the same DNA standard dilution series [24]. The qPCR mixture (20 µl) was prepared using qPCR Sybr Green (Takara)"., and amplification was proceeded using the following parameters: 94˚C for 30 s and followed by 40 cycles at 94˚C for 5 s and 60˚C for 30 s, and followed by a melt curve (60˚C to 90˚C, 0.3˚Cs-1). Whole Run was completed with a melting curve analysis to confirm the specificity of amplification. Ct values were determined by the Roche software using a fluorescence threshold manually set to 0.0160 and exported into a MS Excel workbook (Microsoft office) for analysis.

The mathematics of Quantitative PCR was described by the protocol given by Rutledge 2003. Irrespective of many runs , Here a single run consisting of original DNA concentration and 10^{1} to 10^{5} dilutions were prepared for conventional PCR, Nested PCR first set of primers and Nested PCR Second set of primers. The calculation was made with. r^{2} (Regression coefficient) Es (ES slope derived estimate of amplification efficiency and Nt (Number of amplicon molecules at fluorescent threshold). After the PCR product was adjusted to 100 ng/µl as the dilution template. Q- PCR at various dilutions were prepared for the conventional PCR amplified product and Cycle threshold (Ct) value of first set of readings were noted and standard curve were constructed. The Ct value readings for conventional PCR is 30.96, 28.98, 26.54, 24.24, 21.28 and 18.84 which occurred through 1 to 10^{5} dilutions. The Q- PCR at various dilutions were prepared and Ct value of first set of nested PCR amplified PCR product were adjusted to 100ng and QPCR were performed with specific primers and Ct values were noted and standard curve were constructed. The Ct value readings for first set of nested PCR is 29.26, 26.21, 22.88, 19.42, 15.22 and 11.24 which is 1 to 10^{5} dilutions. After that with the same quantity of PCR product of first set of nested PCR which was used as a template DNA for second set of Nested PCR were adjusted to 100ng through Nanodrop (Biodrop, UK) and the second set of Nested PCR was performed, Later Q-PCR was performed, "the Ct value" readings for second set of nested PCR is 26.54, 23.98, 21.00, 17.21, 13.44 and 8.98 .All the samples after running the PCR were run in gel electrophoresis and purified through gel elution kit (Takara) and adjusted the DNA in Nanodrop (Biodrop, UK) and quantified through Q-PCR (Roche instrument) at various dilutions. The primary objective of purification is to avoid the remaining the other components except DNA.

2.1 Statistical Analysis

The statistical analysis was performed using OPSTAT and DMRT (Duncan Multiple Range Test) and analysed through SPSS software package.Q-PCR calculations such as Q-PCR calculations such r2, Eff and Nt, Mt, log factors, Average Ct and standard curves were done through MS-Excel.

3. RESULTS

3.1 Huanglongbing Disease Survey

The survey was carried out during different seasons, including winter (December to February), summer (March to May), monsoon season (June to September), and a post-monsoon period (October and November). The samples collected in the summer displayed more symptoms, and the concentration of CLas bacteria was higher during this time, as noted by visual observation as well as by the results of PCR and Q-PCR. The leaf samples were obtained by observing a variety of symptoms, including yellowing of the leaves and branches, the emergence of leaves that resemble rabbit ears, uneven mottling on the leaf lamina (blotchy mottle shoot with yellow areas), and symptoms similar to vitamin shortages (a regular pattern of yellowing or vein yellowing or clearing on leaf lamina).

3.2 Detection of CLas in Mandarin Orange

The CLas in acid lime and mandarin orange were found through the course of two different tests.

One task was to identify samples from mandarin oranges, and the other involved acid lime samples. All the samples of mandarin oranges collected that were thought to be infected with putative CLas isolates initially produced an amplicon of about 1500 bp DNA fragment after a PCR experiment using the universal primers fd1 and rp2 targeted the 16s r RNA gene. These DNA fragments were then confirmed as uncultured CLas by sequencing (Figs. 1 and 2). Additionally, samples were taken from the Nilgiris, Nammakal, Dindigul, and Salem districts for the purpose of detecting CLas in mandarin oranges and from the Nilgiris, Nammakal, Dindigul, Salem, Coimbatore, and Tirunelveli districts for the purpose of detecting CLas in acid Lime samples were examined utilising pairs of OI1 and OI2C primers for amplification of a 1160 base 16s rDNA fragment. The outcomes demonstrated that all samples collected from distinct mandarin orange orchards had amplified partial 16S r RNA (the most conserved portion of the CLas bacteria genome) at position 1160. The mandarin orange orchards that displayed higher PDI also displayed steady CLas amplification. Gene-specific primers A2 and J5, which demonstrated amplification of the 703 bp targeting (rpiKAJL-rpoBC) beta operon gene, further verified the Clas infection. Each field's samples overwhelmingly tested positive for CLas. Mandarin orange sequences for the 16 s r RNA gene and the beta operon gene were entered into the NCBI GenBank database and given accession numbers. Tables 1 and 3 list the primers used and their accession numbers. Mandarin orange samples were drawn at random from each field during the study and combined. Samples from Kanalkadu displayed the highest incidence of HLB illness among the various localities (100%). Out of the 25 samples, every one accurately displayed clinical symptoms and used particular primers to amplify the CLas 1160bp 16s rDNA fragment. The gene-specific primer pair A2 and J5, which amplified at a 703 bp nucleotide fragment, was used to further confirm the samples from Kanalkadu (100% PDI) and Thandikudi (92% PDI), which showed the highest disease incidence (Figs. 3, 4 and 7). The sequenced amplified DNA samples were entered into the NCBI database to receive accession numbers. (Table 1), samples taken from the Patlankadu orchard from the Dindigul district also showed PDI of 91.66% which is followed by Kanalkadu and Thandikudi .

3.3 Detection of CLas in Acid Lime

Acid lime samples from the three investigated localities Kallar in Niligirs district, Amayapalem in Dindigul district, and Sanakrankoil in Tirunelveli district showed the highest frequency of HLB illness (100%). Out of the 25 samples, every one amplified 1160 bp fragments of 16s r DNA using particular primers and displayed accurate symptoms. Also, the samples were amplified using the particular A2/J5 primer pair, yielding a 703 bp DNA fragment (Fig. 5, 6 and 7). The sequenced amplified DNA samples were entered into the NCBI database to get accession numbers after being sequenced. 25 samples were taken during the survey of the 18 fields in the Tirunelveli district, and all 25 samples tested positive for CLas. The majority of them are from the Tirunelveli district's Sankarankoil field. In the Kallar region of the Nilgiris district and the Amayapalam region of the Dindigul District, all 25 samples (out of 25) were positive. This is the first report of CLas being found in the midribs of acid lime and mandarin oranges in Tamil Nadu. By employing mid-ribs, this technique may demonstrate that the CLas inoculum can transfer from all sources, including source and sink through phloem.

3.4 Comparing Nested PCR Amplification and Conventional PCR Amplification

The nested PCR method greatly enhanced the sensitivity to detect Las up to10 times and 100 times compared to qPCR and conventional PCR. . The PCR product was serially diluted in a range of 1, 10, 10^2, 10^3, 10^4 and 10^5, which represented 10^5 to 1 pg/µl of Las DNA, and served as the templates to evaluate the sensitivity among nested PCR, conventional PCR and qPCR. The PCR product from **Sankarakoil** was diluted as the template for conventional PCR (Fig. 3), and the PCR product from **Kanalkaadu** was diluted as the templates for the nested PCR (Fig. 4) and qPCR. To evaluate the amplification efficiency of qPCR, Las DNA (Fig. 2) serially diluted in a range of1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}served as the templates . Analysis of the dilution series of the DNA showed that nested PCR (Table 3 and 4) detected much lower template DNA concentrations than conventional PCR (Table 5). The amplification efficiency (AE) of qPCR for second set of nested PCR was therefore estimated to be 0.91 based on the equation AE = [10−1/slope−1] [19].

3.5 Identifying the Sensitivity of Q-PCR for Conventional PCR, First Primer Set of Nested PCR, Second Set of Nested PCR

. The purified sample was performed with Q-PCR and the CT value obtained at various dilutions were as follows 25.680, 20.248, 16.440, 12.280, 10.440, 8.982. The r^2 (Regression coefficient) Es (ES slope derived estimate of amplification efficiency and Nt (Number of amplicon molecules at fluorescent threshold). Mostly for every Q-PCR run the peak become raised at 24 to 28 cycles. The conventional PCR run the peak become raised at 16 to 24 cycles. Where as for nested PCR the peak raised between 27 to 31 cycles, second set of nested PCR the peak raised between 25 to 27 cycles. The melting peak increased at 79ºC for all the dilutions of the conventional PCR product band in gel electrophoresis which initiate the peak at 0.002 dF/dT and end between 0.006 DT to 0.008 DT. The melting peak increased at 72 to 81 ºC for all the dilutions of the nested PCR first set of primers product band in gel electrophoresis which initiate the maximum peak was initiated at 0.008 and end at 0.0013 DF/DT. The melting peak increased at 79ºC for all the dilutions of the Nested PCR second set of primers product band in gel electrophoresis which initiate the maximum peak was initiated at 0.005 and end at 0.0014 DF/DT. The r^2 (Regression coefficient) Es (ES slope derived estimate of amplification efficiency and Nt (Number of amplicon molecules at fluorescent threshold) was 0.997108, 88.56 % and 15637388142.32 obtained for first set of nested PCR , where as 0.991053469, 91.21 % and 5197936724.43 obtained for second set of nested PCR. Ultimately the amplicon molecules amplification efficiency were high for the second set of Nested PCR (Tables 6 and 7; Plates 1-6). The nested PCR primers sensitivity and conventional PCR primers sensitivity in terms of amplification was confirmed through Q-PCR, and amplification efficiency (Eff) was calculated, among all nested PCR second set of primers showed 91.21 % Eff.

4. DISCUSSION

4.1 Huanglongbing Disease Survey

In the field, strange signs including erratic blotching called mottling and chlorosis (24) have been seen.Our findings suggested that Tamil Nadu was home to many CLas isolates. The symptoms of acid lime and mandarin orange species are comparable to those described by Garnier and Bove in 1993 [25-28].

4.2 Detection of CLas in Mandarin Orange and Acid Lime

The identification of Ca. Liberibacter spp. from an HLB sample always should be based onanalysis of the 16S rDNA locus. Primer set OI1/OI2c amplifies the signature sequence for ‘Ca. Liberibacter asiaticus’ and shouldbe used for the species determination. Our data is similar to the results of previous studies (Xialong). A band of 703 bp was obtained from all infected plants. No amplification was observed from water or healthy citrus plants. The band of 703 bp was sequenced and compared with data from GenBank. The results indicated that the sequence was closely related to the record of *Candidatus Liberibacter asiaticus*, since they were a part of the ribosomal protein gene (rplJ). It can be concluded that the causal agent of citrus Huanglongbing (greening) disease [19].

4.3 Nested PCR, Q-PCR Conventional PCR and their Sensitivity

The primer sensitivity was inversely correlated with the template concentration, the detection system sensitivity order was nested PCR>qPCR> conventional PCR, which made to understand that nested PCR was 10 times more sensitive than that of qPCR and 100 times more sensitive that of conventional PCR [18]. The amplification efficiency (AE) of qPCR for second set of nested PCR was therefore estimated based on the equation AE = [10−1/slope−1 based on the equation given by Rutledge *et al*. ([19]. The efficiency of amplification affected the sensitivity of qPCR. In this study, the efficiency of amplification of second set of nested pcr purified DNA from qPCR was 91.21%. The results showed that the efficiency of amplification was not a key factor for the sensitivity of the assay. Ahmad 2009. The results of Q-PCR showed that amplification effciency and Nt are independent entities. In reality Nt is determined solely by the fluorescent threshold(Ft), and as such its value is independent of the parameters impacting PCR amplification [29]. Indeed, this interrelationship between Nt and Ft has important practical implications, basedon the principle that Ft does not directly reflect the number ofamplicon molecules, but rather DNA mass at fluorescent threshold (Mt). This in turn dictates that Mt could be used to predict Nt for any amplicon of known

size, if it is assumed that amplicon size and base composition do not significantly impact DNA fluorescence. The calculated results in excel sheet were supported the above mentioned equations which was given Rutledge et al. [19].

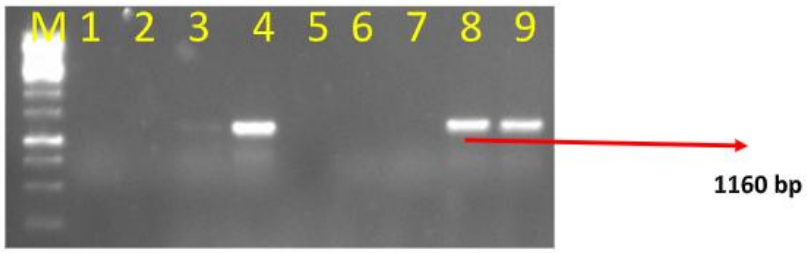

Fig. 1. Gel picture of OI1 and OI2C Primers PCR amplified Mandarin orange midrib samples showing 1160 base pairs similar to *Candidatus Liberibacter asiaticus*; M –1 kb Ladder; Lane 1,2,3- Negative control; 4- Kanalkadu (Dindigul) mandarin orange sample ; Lane 5,6,7 – Negative control; Lane 8- Thandikudi (Dindigul); Lane 9 – Sankarankoil (Thirunelveli) acid lime sample

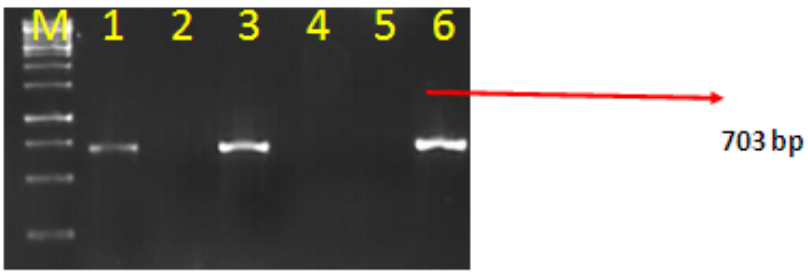

Fig. 2. Gel picture of A2 and J5 Primers (rpiKAJL-rpoBC) gene PCR amplified mandarin orange mid-rib and acid lime midrib samples showing 690 base pairs similar to *Candidatus Liberibacter asiaticus*; M –1 kb Ladder; Lane 1- Kanalkadu (mandarin orange); Lane 2- Negative control; Lane 3 - Thandikudi (mandarin orange);Lane 4,5 –Negative control; 6- Sankarankoil (acid lime)

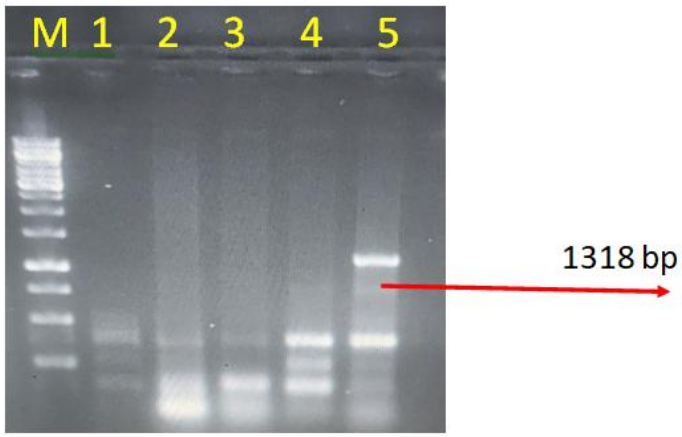

Fig. 3. Nested PCR amplification with first set of primers (F1 and B1) at annealing temp- 58°c showing 1318 bp amplification. M- 1Kb Ladder; Lane 1,2,3,4 – Negative control ; Lane 5 - Mandarin sample (kanalkadu , Dindigul district)

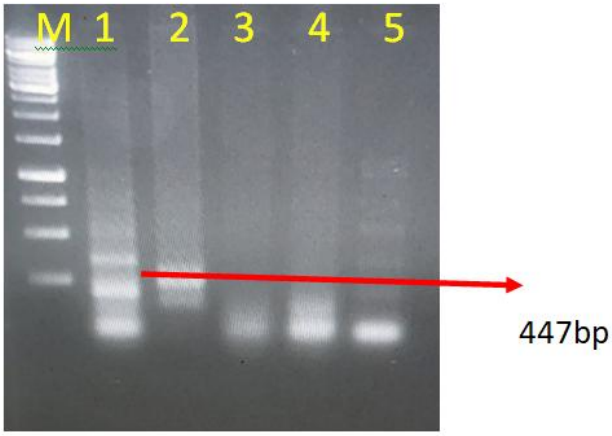

Fig. 4. Nested PCR amplification with second set of primers (F3 and B3) at annealing temp- 62°c showing 443 bp amplification. M- 1Kb Ladder; Lane 1 – Mandarin sample (kanalkadu , Dindigul district) fnested PCR first set PCR product as a DNA template amplified with second set of nested PCR primers; Lane 2,3,4,5 – Negative control

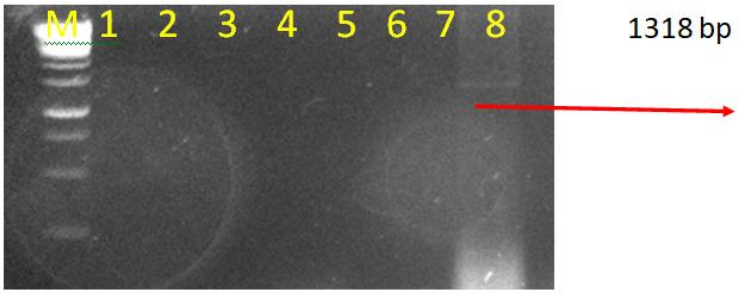

Fig. 5. Nested PCR amplification with first set of primers (F1 and B1) at annealing temp- 58°c showing 1318 bp amplification. M- 1Kb Ladder; Lane 1,2,3,4,5,6,7 – Negative control ; Lane 8 - Acid lime sample (Sanarankoil , Thirunelveli district)

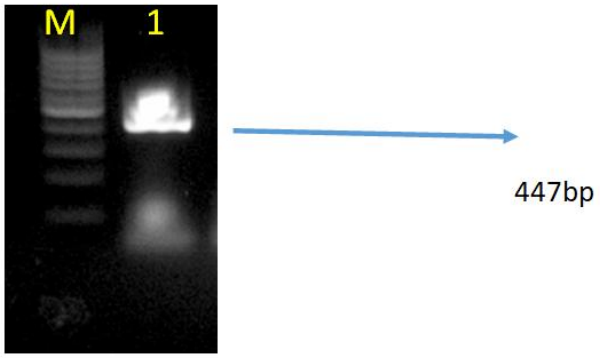

Fig. 6. Nested PCR amplification with second set of primers (F3 and B3) at annealing temp- 62°c showing 443 bp amplification. M- 100bp Ladder; Lane 1 – acid lime sample (Sankarankoil , Thirunelveli district) nested PCR first set PCR product as a DNA template amplified with second set of nested PCR primers

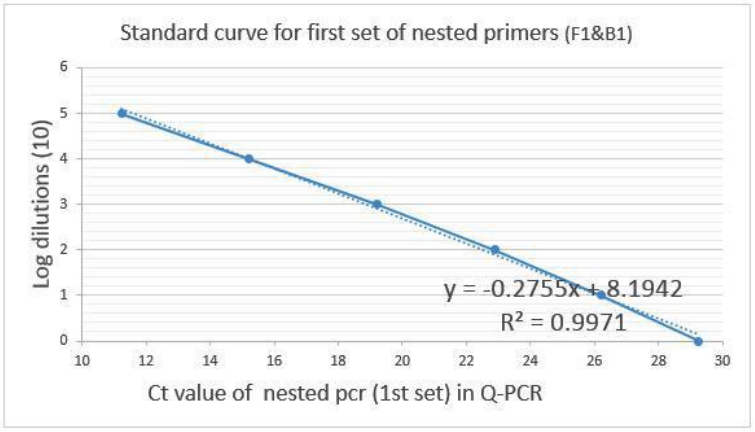

Fig. 7. Standard Curve constructed through the Log dilutions 0 to 5 with the average Ct Values of Q-PCR formed through first set of nested PCR primers (F1 and B1). The linear regression value obtained through the standard curve is 0.9971

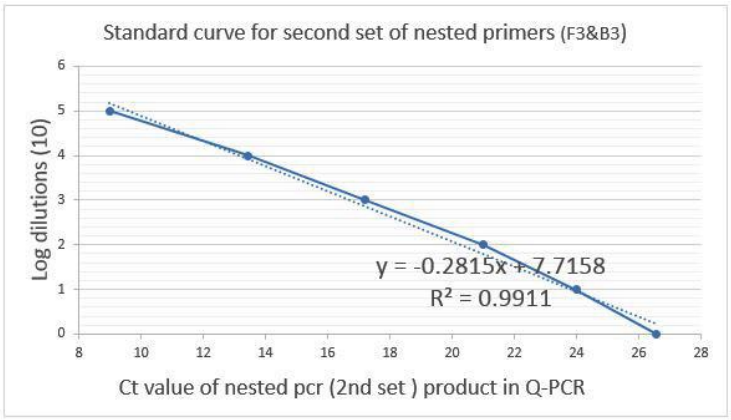

Fig. 8. Standard Curve constructed through the Log dilutions 0 to 5 with the average Ct Values of Q-PCR formed through second set of nested PCR primers (F3 and B3). The linear regression value obtained through the standard curve is 0.9911

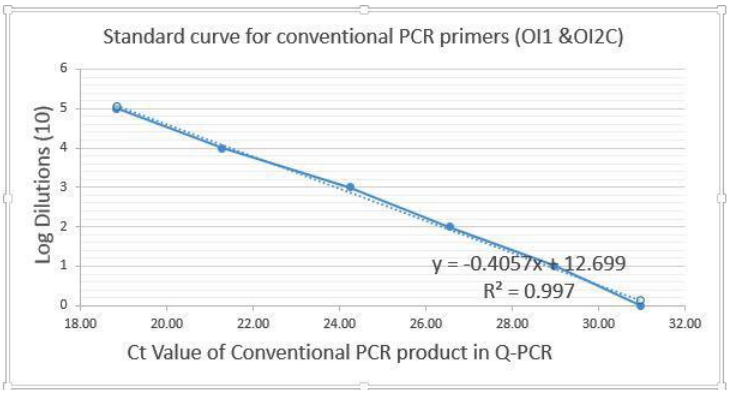

Fig. 9. Standard Curve constructed through the Log dilutions 0 to 5 with the average Ct Values of Q-PCR formed through Conventional primers (OI1 and OI2C). The linear regression value obtained through the standard curve is 0.9911

Table 1. Survey and detection of CLas bacterium in mandarin orange plants at various locations in Tamil Nadu, , PDI (%)represent the mean of three replicates, ± represent standard deviation, The treatment means are compared using Duncan's Multi ple Range Test (DMRT). In a column, means followed by a common letter (s) are not significantly different (*p*=0.05)

S.NO	Locations	District	Latitude(°N)	Longitude(°E)	Altitude (metres)	Number of trees evaluated per field	Number of trees infected	PDI (%)	HLB infection
1.	Yercaud	Salem	11.7748	78.2097	1623	24	22	88.66±2.345^{bc} (70.389)	+VE
2.	Kolli hills	Namakkal	11.2485	78.3387	1400	22	20	86.54±2.292^{bc} (68.525)	+VE
3.	Conoor	Nilgiris	11.3502	76.7961	1850	23	20	90.00± 3.740^{bc} (71.995)	+VE
4.	Katteri	Nilgiris	12.4382	78.1764	1850	22	20	88.90±2.142^{bc} (70.737)	+VE
5.	Ooty	Nilgiris	11.4102	76.6950	2240	20	16	84.00±1.455^{c} (66.435)	+VE
6.	Kanalkadu	Dindigul	10.3022	77.6911	1292	25	25	100.00±0.00^{a} (87.134)	+VE
7.	patlankadu	Dindigul	10.3093	77.6435	1325	25	22	91.66±0.465^{b} (73.839)	+VE
8.	thandikudi	Dindigul	10.3093	77.6435	1500	25	23	92.00±1.457^{b} (73.784)	+VE
9.	Thadiyankudisai	Dindigul	10.2368	77.7088	1098	24	21	90.00±1.670^{bc} (71.823)	+VE
	Total plants					**216**	**189**		
	CD(0.05)							4.409	
	SE(m)							1.428	
	SE(d)							2.019	
	CV							3.502	

Table 2. Survey and detection of CLas bacterium in acid lime plants at various locations in Tamil Nadu, PDI (%) represent the mean of three replicates, ± represent standard deviation, The treatment means are compared using Duncan's Multiple Range Test (DMRT). In a column, means followed by a common letter (s) are not significantly different (*p*=0.05)

S.NO	Locations	District	Latitude (°N)	Longitude (°E)	Altitude (metres)	Number of Trees evaluated per field	Number Of trees infected	PDI (%)	HLB infection
1.	Pollachi	Coimbatore	10.6609	77.0048	293	28	26	92.85±2.461^{bc} (74.655)	+VE
2.	Sankarankoil	Thirunelveli	9.168902	77.5413	154	28	28	100±0.000 ^{a} (87.134)	+VE
3.	Kolli hills	Namakkal	11.2485	78.3387	1400	24	21	94.00 ± 2.311^{bc} (76.989)	+VE
4.	Thadiyankudisai	Dindigul	10.2368	77.7088	1098	20	18	96.42 ±2.483^{ab} (79.498)	+VE
5.	Ayyampalayam	Dindigul	10.2271°	77.7482	281	25	25	100±0.000^{a} (87.134)	+VE
6.	Conoor	Nilgiris	11.350208	76.7961	1850	20	18	90.00±2.381^{cd} (71.642)	+VE
7.	Kallar	Nilgiris	11.3360°	76.8617°	372	25	25	100±0.000^{a} (87.134)	+VE
8.	Ooty	Nilgiris	11.4102	76.6950	2240	24	21	87.50±2.567^{d} (69.394)	+VE
9.	Yercaud	Salem	11.7748	78.2097	1623	22	20	96.90±2.025^{ab} (80.295)	+VE
	Total plants					**216**	**202**		
	C.D.(0.05)							3.580	
	SE(m)							1.184	
	SE(d)							1.674	
	C.V.							2.152	

Table 3. Mandarin orange Clas DNA sample at various dilutions and each sample is having four replicates and dilutions is at 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}, The Nested PCR first set of primers (F1,B1 primers PCR amplified sample at annealing temperature 58 °C and run through Q-PCR F and R primers and Ct value and SD was noted

	No	Rep#1	Rep#2	Rep#3	Rep#4	Av. Ct	SD
10^5	10000000	11.1276	11.0152	11.5772	11.24	11.24	0.242811752
10^4	1000000	14.9156	15.6766	15.0678	15.22	15.22	0.328789578
10^3	100000	18.84344	19.61256	19.03572	19.42028	19.228	0.351053645
10^2	10000	21.96864	23.11284	23.57052	22.884	22.884	0.673686719
10^1	1000	25.676	26.462	26.462	26.2	26.2	0.370523953
1	100	28.3822	29.26	29.5526	29.8452	29.26	0.632088243

Table 4. Mandarin orange Clas DNA sample at various dilutions and each sample is having four replicates and dilutions is at 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}, The Nested PCR second set of primers (F3,B3 primers PCR amplified sample at annealing temperature 62°C and run through Q-PCR F and R primers and Ct value and SD was noted

	No	Rep#1	Rep#2	Rep#3	Rep#4	Av. Ct	SD
10^5	10000000	8.89218	8.80236	9.25146	8.982	8.982	0.194033377
10^4	1000000	13.1712	13.8432	13.3056	13.44	13.440	0.290337183
10^3	100000	16.8658	17.5542	17.0379	17.3821	17.210	0.314210174
10^2	10000	20.16768	21.21808	21.63824	21.008	21.008	0.618458774
10^1	1000	23.5004	24.2198	24.2198	23.98	23.980	0.339128412
1	100	25.7438	26.54	26.8054	27.0708	26.540	0.573329527

Table 5. Mandarin orange Clas DNA sample at various dilutions and each sample is having four replicates and dilutions is at 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}, The conventional PCR primers (OI1 and OI2C primers PCR amplified sample at annealing temperature 60.5°C and run through Q-PCR F and R primers and Ct value and SD was noted

	No	Rep#1	Rep#2	Rep#3	Rep#4	Av. Ct	SD
10^5	10000000	18.6516	18.4632	19.4052	18.84	18.84	0.406990516
10^4	1000000	20.8544	21.9184	21.0672	21.28	21.28	0.45970054
10^3	100000	23.7552	24.7248	23.9976	24.4824	24.24	0.442559826
10^2	10000	25.4784	26.8054	27.3362	26.54	26.54	0.781316445
10^1	1000	28.4004	29.2698	29.2698	28.98	28.98	0.40983909
1	100	30.033625	30.9625	31.272125	31.58175	30.9625	0.668866446

Table 6. The r2 (Regression coefficient) Es (ES slope derived estimate of amplification efficiency and Nt (Number of amplicon molecules at fluorescent threshold) were derived through Log factor and Average ct value derived through Q-PCR of first set nested PCR diluted PCR sample (1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions. The amplification efficiency is 88.56% for first set of nested PCR primers

Dilutions:	No	Log (No)	nested pcr primers 1
10^5	10000000	7.000	11.240
10^4	1000000	6.000	15.220
10^3	100000	5.000	19.228
10^2	10000	4.000	22.884
10^1	1000	3.000	26.200
1	100	2.000	29.260
		r2:	0.997108345
		Eff:	0.885614852 (88.56%)
		Nt:	15637388142.32

Table 7. The r2 (Regression coefficient) Es (ES slope derived estimate of amplification efficiency and Nt (Number of amplicon molecules at fluorescent threshold) were derived through Log factor and Average ct value derived through Q-PCR of second set nested PCR diluted PCR sample (1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions. The amplification efficiency is 91.21 % for second set of nested PCR primers

Dilutions	No	Log (No)	nested pcr primers 2
10^5	10000000	7.000	8.982
10^4	1000000	6.000	13.440
10^3	100000	5.000	17.210
10^2	10000	4.000	21.008
10^1	1000	3.000	23.980
1	100	2.000	26.540
		r2:	0.991053469
		Eff:	0.912189811 (91.21%)
		Nt:	5197936724.43

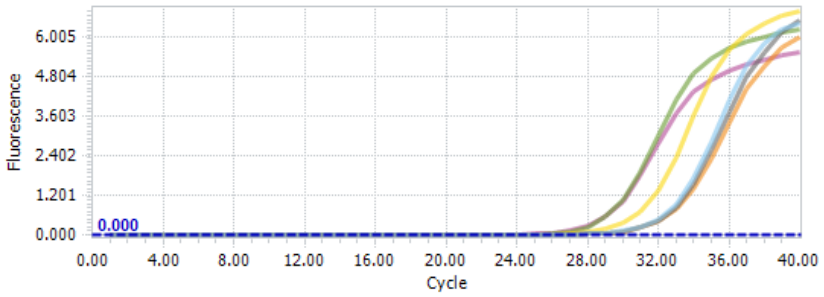

Plate 1. Amplification curve shown raised curves at 27th to 31 cycle for mandarin orange samples and 28th cycle for 10^5 dilution and curves became stationary at 4.824 to 6005 fluorescence level for

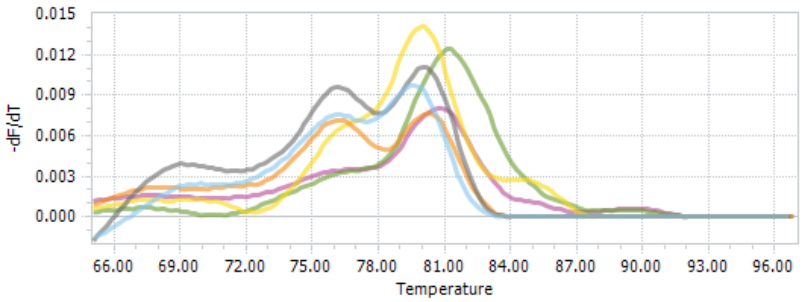

Plate 2. Melting peaks of first set nested PCR amplified PCR product run through Q-PCR primers shown the change in fluorescence level (df/dt) at 0.001 to 0.002 with the increase of peak at temperature 79.0°C (yellow) for mandarin orange sample at Various dilution peaks (1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} (sky blue ,violet, yellow, pink, blue red), the yelow peak at 10^5 dilution showed the minimum ct value 11.24 and 0.013 is the maximum peak at a temperature of 80 °C

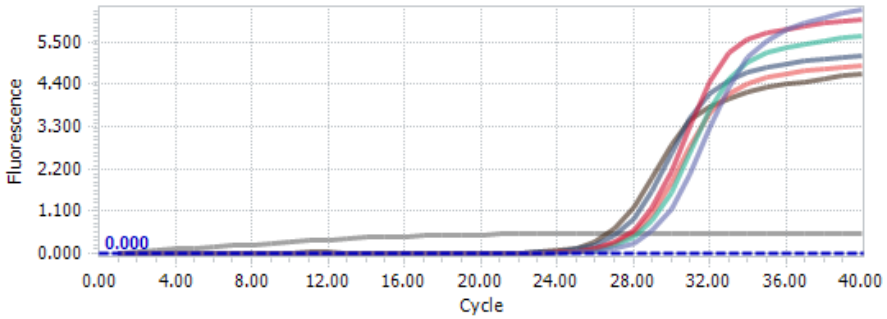

Plate 3. Amplification curve shown raised curves at 24th cycle for mandarin orange (black) and 27th cycle for mandarin orange (light blue) 10^5 dilution and curves became stationary at 3.300 to 4.400

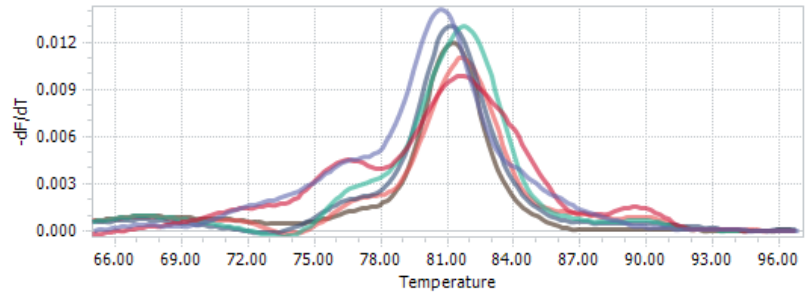

Plate 4. Melting peaks of second set nested PCR amplified PCR product run through Q-PCR primers shown the change in fluorescence level (df/dt) at 0.001 with the increase of peak at temperature 80.0°C (light blue) for mandarin orange sample at Various dilution peaks (1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10-^{5} (dark blue ,pink, blue, red, sky blue), the light blue peak at 10^{5} dilution showed the minimum ct value 8.982 and 0.013 is the maximum peak at a temperature of 80 °C

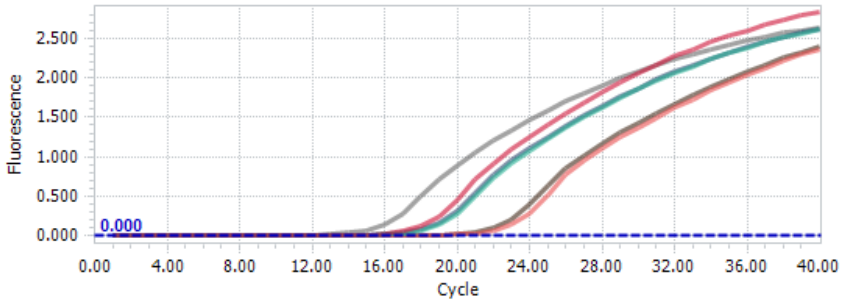

Plate. 5 Amplification curve shown raised curves at 16th to 21st cycle for mandarin orange samples and 18 th cycle for 10^{5} dilution and curves became stationary at 2.000 to 2.400 fluorescence level

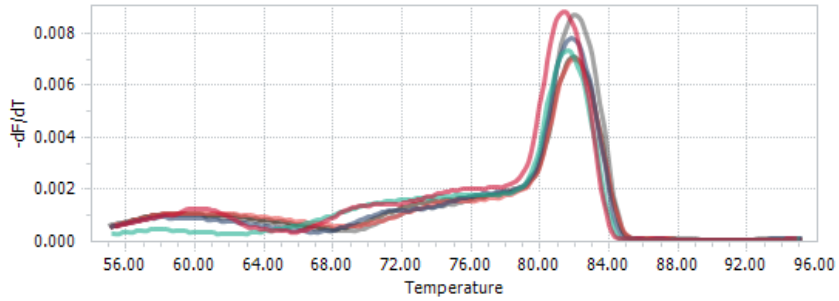

Plate 6. Melting peaks of Conventional PCR amplified PCR product run through Q-PCR primers shown the change in fluorescence level (df/dt) at 0.002 with the increase of temperature at 78.0°C for mandarin orange sample at Various dilution peaks (1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} (Red,skyblue,blue,kight blue and dark red), the dark red peak at 10^{5} dilution showed the minimum ct value 18.84 and 0.008 is the maximum peak at a temperature of 85°C

5. CONCLUSION

The disease prevalence revealed that 189 samples out of 210 samples showed HLB disease incidence of CLas, accounting for 90% of the disease incidence in mandarin oranges throughout the entire Tamil Nadu, and 202 samples showed positive out of 216 samples,

accounting for 93.52 percent of acid lime samples in Tamil Nadu. With the exception of Nilgiris, Dindigul, and Thirunelveli, all field samples were positive, but the incidence varied across each district. Research has shown unequivocally that the PCR approach may be used to detect HLB illness, citrus greening, and to validate the presence of the pathogen CLas. The survey has provided a thorough insight of the highest incidence of HLB occurrence in Tamil Nadu's citrus-growing regions. The low concentration and asymmetrical distribution of CLas in citrus presented challenges for other detection methods, but the Nested PCR and Q-PCR based assay proved to be the most effective method for the pathogen's detection. Excellent HLB diagnosis by Nested PCR was required for identifying infected plants and developing citrus nurseries free of the illness. Using Nested PCR for HLB detection should make epidemiological research easier and contribute to HLB control. The citrus belts of Tamil Nadu can benefit greatly from these discoveries in terms of HLB diagnosis, as well as the provision of HLB free seedlings to registered nurseries for mass multiplication. The data imply that the nested PCR primer pairs were able to identify Las in a variety of symptomatic tissues and geographical locations. The nested PCR primer pairs are suitable for numerous citrus cultivars and geographical regions as a consequence. The nested PCR primers will be a very valuable addition to the current Las detection techniques.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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