Polymerase Chain Reaction for Detection and Quantitation of Liberobacter asiaticum, the Bacterium Associated with Huanglongbing (Greening) of Citrus in China

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ABSTRACT. The polymerase chain reaction (PCR) using primers designed based on the sequence information reported (Genbank accession number M94319) was used for detection and quantitation of Liberobacter asiaticum, the bacterium associated with citrus Huanglongbing (Greening disease). By this procedure, the presence of L. asiaticum were detected in samples of infected citrus, periwinkle and the psylla vector fed on infected plants. To compare the amount of the bacteria present in different samples, a PCR-based quantitation was developed in which a competitor DNA fragment was serial-diluted and co-amplified with the sample DNA in the same reaction tube. The competitor shares the primer binding sites with the liberobacter DNA and competes for the primers and the limited substrates during the PCR reaction. The PCR products were separated by agarose gel electrophoresis and the banding intensity was recorded by densitometry. The amount of PCR products from the liberobacter DNA is inversely proportional to that of the competitor, making it possible to construct a standard curve. The quantity of liberobacter was deduced from the standard curve by linear regression analysis.

Citrus Huanglongbing (Greening disease) is a limiting factor of citrus production in some areas of Southeast Asia and Africa. In endemic orchards, trees are decimated and the productive duration of fruit-bearing is shortened. The etiologic agent of the disease has been identified as psylla-borne, graft-transmissible and phloem-restricted bacterium (2, 5, 6, 7, 8). This bacterium has recently been characterized as a new genus in the proteobacteria with the trivial name "*Candidatus* Liberobacter", with "*Candidatus* L. asiaticum" being associated with the Asian form of the disease (4). Monoclonal antibodies (MAbs) against the liberobacter have been developed and different serotypes have been found according to their reactivities with the MAbs generated (3). Biochemical characterization of the liberobacters is hampered by the fact that no cultures of the liberobacter are available.

Recently, cloned DNA fragments of the liberobacter have been obtained and characterized (10, 11). The information of the liberobacter DNA sequences made it possible to design PCR primers for liberobacter detection by PCR amplification. In this study, we sought to detect its presence in samples of citrus plants and psylla vectors by PCR. In addition, we developed a competitive PCR procedure for quantifying the amount of liberobacter in different samples. The procedure for generating competitor was modified from the method described by Überla et al. (9). In this method, a mixture of random PCR products using gene-specific primers is generated under a low annealing temperature. Because all products contain primer sequences at the ends, suitable fragments of the products can be isolated and used as the competitor. The quantitation is achieved when the competitor is serial-diluted and co-amplified with sample DNA. The amount of the PCR products of target DNA is inversely proportional to that of the competitor. After separating PCR products by agarose gel electrophoresis, the banding intensity is recorded by densitometry and the standard curve can be constructed. The amount of liberobacter DNA can be calculated based on linear regression analysis.

MATERIALS AND METHODS

Chemicals and reagents. The restriction enzymes, 100 bp molecular size marker and PCR reagents were purchased from Gibco BRL (Gaithersburg, MD). The GENECLEAN kit was obtained from Bio101 (La Jolla, CA), TA cloning vector (pCRII) from Invitrogen (San Diego, CA), [γ-^{32}P]dATP from Amersham Corporation (Arlington Heights, IL). Oligonucleotides were synthesized by Oligo Etc. Inc. (Wilsonville, OR). PCR primers used in detection and quantitation were OL1 (TCTGTTTTCTT-CGAGGTTG-GTGAG) and OL2 (ACCGCAA-GACTCCTTACCAGGAAG) corresponding to bases of 37 to 60 and 577 to 600 of the cloned liberobacter sequence (Genbank accession number M94319), respectively. OL3 (TCCCATCCCAACTACTGTGACT-TG), which anneals to bases 462 to 485, was used in Southern hybridization to confirm the identity of PCR products.

Plant material and insect vector. Citrus plants (Ponkan and mandarin) tested in this study were either grown in a greenhouse at 25 to 30°C or collected from local orchards near Fuzhou, Fujian Province, China. L. asiaticum was transmitted to periwinkle from infected citrus by dodder in the greenhouse. Psyllid vectors (*Diaphorina citri* Kuwayama) were kept in a cage in a greenhouse in Fuzhou.

Generation of DNA competitors. The competitors were generated in a PCR reaction under the following amplification conditions: 1 × PCR buffer, 200 µM dNTP, 0.1 µg mouse genomic DNA, 2.5 units Taq polymerase, 0.2 µM of each primer; the PCR amplification cycles were 94°C for 40 sec, 37°C for 60 sec and 72°C for 45 sec; and each cycle was repeated 40 times. The PCR products were separated on a 1.2% agarose gel and the products in the range of 200 to 700 bps were isolated using a GENECLEAN kit following the manufacturer's instructions. The isolated products were subcloned into a TA cloning vector. After transforming into *Escherichia coli* (competent cells), clones were grown up and recombinant plasmids were purified using a Quiagen miniprep. The concentrations of the DNA constructs were measured by UV-spectroscopy and serial-diluted for use as the DNA competitor.

Isolation of DNA and detection of L. asiaticum. DNA was isolated from plant samples following the procedure describe by Villechanoux et al. (11). The procedure for isolating DNA from the insect vector was modified from the plant DNA isolation procedure as follows: 10 insects were homogenized in 0.5 ml P1 solution (50 mM Tris/HCl, 10 mM EDTA, pH 8.0), the extract was cooled to 4°C before 100 µl lysozyme (1 mg/ml) was added and then incubated at 4°C for 30 min. The resulting solution was then treated with proteinase K (50 µg/ml) at 37°C for 30 min and the SDS was added to the solution at end of incubation. The DNA was extracted with TE buffer-saturated phenol/chloroform/isoamylic alcohol (25/24/1). The aqueous phase was separated from the organic solvent by centrifugation in a microfuge. The DNA was precipitated from the aqueous solution by addition of 1/10 vol of 3 M sodium acetate, 2 vol of absolute ethanol and chilled at -20°C overnight. The DNA pellet was collected by centrifugation at 16,000 *g* in a microfuge and washed with ice cold 70% ethanol. The pellet was air-dried and rehydrated with 20 µl TE buffer (pH 8.0). For PCR detection, about 0.4 to 1.0 µg DNA was used in PCR amplification using the specific primers. The PCR conditions were as follows: 1 × PCR buffer; 100 µM dNTP, 10 pmol of each PCR primer; Taq polymerase 2.5 units; total reaction volume 100 µl. The reaction (56°C; 30 sec; 94°C; 45 sec; 72°C 30 sec) was repeated 45

times. The PCR products were separated by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Competitive PCR. Equal aliquots of sample DNA were co-amplified with serial-diluted competitor in the PCR reaction containing 1× PCR buffer, 200 μM dNTP, 0.5 μg sample DNA, 1 unit Taq polymerase; the total reaction volume was 50 μl. The PCR reaction (56°C, 30 sec; 94°C, 45 sec; 72°C, 30 sec) was repeated 45 times in a thermal cycler. The resulting PCR products were separated in 2% agarose gel. The DNA in the gel was visualized by staining with ethidium bromide staining. The results were recorded on Polaroid Positive/Negative film and the values of banding intensity were obtained by scanning with a densitometor (Bioimage, MI).

RESULTS

Detection of the Huanglongbing liberobacter. By using PCR amplification, products of 563 bp were detected in DNA samples isolated from the infected citrus, periwinkles and psyllid vectors that had been fed on infected plants, but no PCR products were detectable from healthy controls (Fig. 1). About 0.5 to 1 g of leaf midrib tissue was used for the isolation of DNA. DNA samples from psylla were isolated from a pool of 10 insects. The PCR products were confirmed to be those of the liberobacter by Southern hybridization with radiolabeled oligonucleotide (OL3) and/or confirmed by sequence analysis (data not shown).

Competitive PCR for quantitation. Under the conditions of low annealing temperature (37°C), mixtures of PCR products were generated (Fig. 2). A number of subcloned PCR products were tested for their ability to compete with the liberobacter PCR products. Fig. 3 shows the pattern of competition with one of the clones chosen for the study.

Fig. 1. Detection of the Huanglongbing liberobacter agent by PCR. PCR products of expected size (563 bp) were obtained from infected periwinkles (lane 2, 3), psylla fed on the infected plants (lane 5), infected mandarin (lane 7), two samples of graft-transmitted Ponkan (lanes 10, 11), field-collected sample of Ponkan (lane 12), and two samples of psyllid-transmitted Ponkan (lanes 13, 14). No PCR products were detectable from samples of healthy plants (lane 4, periwinkle; lane 8, symptomless mandarin) nor psyllid vectors that had been fed on healthy plants (lane 6). Lane 1 contains the 100 bp molecular size markers.

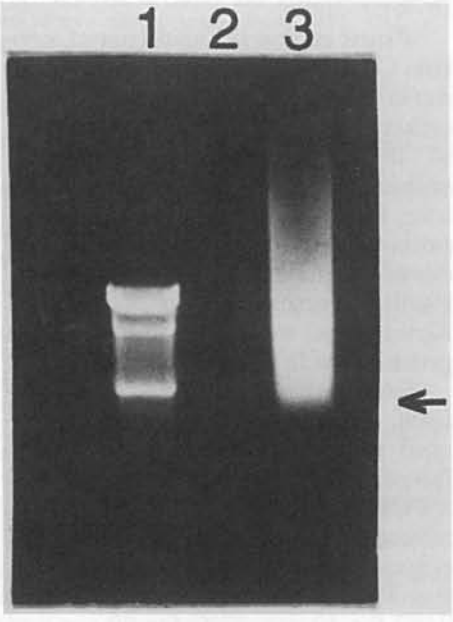

Fig. 2. Generation of DNA fragments for the competitive PCR. A mixture of DNA fragments was generated by PCR amplification under the low annealing temperature (lane 3). Lane 1 contains the 100 bp molecular size markers and lane 2 is a blank lane.

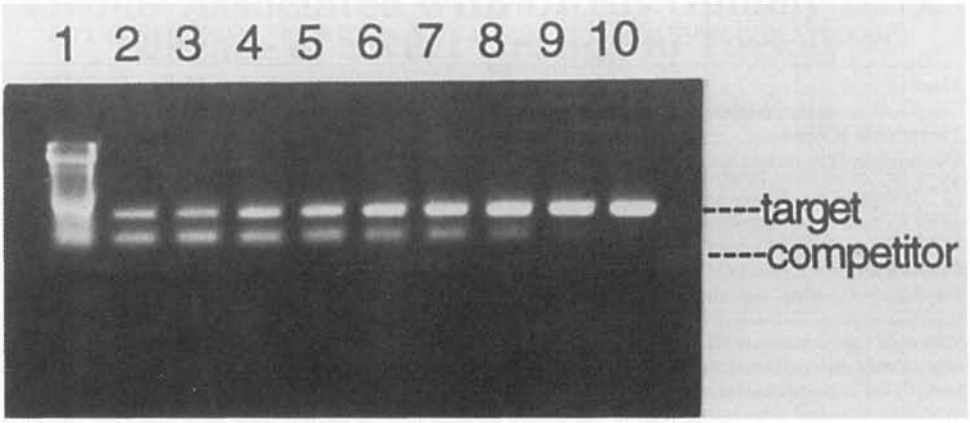

Fig. 3. Quantitation of liberobacter by competitive PCR. A serial-diluted competitor (lane 2 to 10 represents 100, 64, 32, 16, 8, 4, 2, 1, and 0.1 pg) was co-amplified with target liberobacter DNA). The PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Lane 1 contains the 100 bp molecular size markers.

The ratios of the competitor and target versus the amount of the competitor show a linear relationship (Figs. 3 and 4). The point where the ratio of the competitor to target equals one represents the quantity of the target. The amount of liberobacter DNA could be deduced from the linear curve (Fig. 4). By using this method, the amount of liberobacter (in molecules) was determined for the infected Ponkan, mandarin, periwinkle and insect vector carrying the liberobacter (Table 1).

DISCUSSION

Citrus Huanglongbing is a serious problem affecting the production of citrus in many areas of the world. Successful management of the disease requires effective detection of the liberobacter agent and monitoring of the progress of epidemics. In this study, we used PCR to detect the liberobacter in plants and insect vectors. The primers used were designed on the basis of the sequence information reported, which is conserved among Asian strains of the liberobacter (10). By PCR amplification, the organism can be detected in samples from diseased plants and insect vectors, but not in the healthy plants or liberobacter-free vectors. Thus, the PCR primer pair used was specific for the liberobacter strain used in this study. Liberobacter strains infecting citrus were shown to be different in their

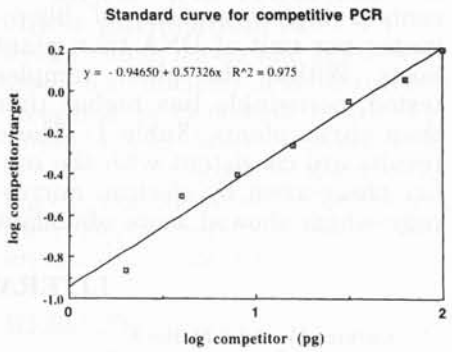

Fig. 4. Standard curve for quantitation of liberobacter. The banding intensity of PCR products described in Fig. 3 was recorded on Polaroid film and digitized after scanning the negative of the photograph using a densitometer. The ratio of the competitor/target were plotted against the amount of the competitor added in each tube. Linear regression analysis was used to define the linear curve. The amount of liberobacter DNA in the sample was defined as the amount of competitor present where the ratio was equal to 1.

TABLE 1
QUANTITATION OF HUANGLONGBING LIBEROBACTER BY COMPETITIVE PCR

Host	Total DNA (μg)	liberobacter DNA (fg)	Mol/μg DNA(10^4)[y]
Periwinkle (China)	1.18	38.0	5.4
Periwinkle (Thailand)	0.86	43.5	8.5
Mandarin	2.11	10.7	0.85
Ponkan 1	0.51	12.4	4.1
Ponkan 2	1.15	14.9	2.2
Ponkan 3[z]	1.66	18.4	1.9
Psyllid	0.512	44.7	14.6

[z]Infected by vector-carrying the HLB agent under greenhouse conditions.
[y]Assuming one cell contains one DNA molecule (bacterial chromosome), then the number of DNA molecules is equal to the number of bacterial cells.

sequence homology. For example, according to Southern blot analysis, L. asiaticum strains differ in sequence homology from that of African strains (L. africanum) (11). Therefore, the suitability of these primers for detecting Liberobacter strains other than the ones used in the present study needs to be tested.

In order to quantify the titer of the liberobacter in plant and insect hosts, we developed a PCR-based procedure to measure the amount of the bacterium present. Results of the quantitation show that psyllids contain higher amount of liberobacter per unit of DNA than plant hosts. Within the plant samples tested, periwinkle has higher titer than citrus plants (Table 1). These results are consistent with the earlier observation by electron microscopy which showed more abundant bacteria in periwinkles than in citrus plants (1, 5). Needless to say, care has to be taken in interpreting this comparison because hosts vary in their total DNA content and within the same host DNA content varies from tissue to tissue.

In this study, we demonstrated that PCR-based methods are highly sensitive and effective in detecting the presence of liberobacter in plants and insect vectors. Furthermore, the amount in samples of plants and insect vectors could be quantified by competitive PCR. The method for creating DNA competitor described in this study is universal and can be applied to quantify many pathogens. The PCR-based detection and quantitation should be useful in managing and monitoring epidemics of citrus HLB.

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