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Identification and Molecular Characterization of *Candidatus* Liberibacter *asiaticus* (Citrus Huanglongbing Disease Pathogenic Agent) and Its Control by Plant Extracts in Bangladesh

Habiba[1,2], Md. Firose Hossain[1], Junaite Bin Gias Uddin[2], Asura Khanam Lisa[3], Md. Asadul Islam[1] and Biswanath Sikdar[1*]

[1]Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi-6205, Bangladesh.
[2]Molecular Cell and System Biology Lab, College of Life Science, Fujian Agriculture and Forestry University, China.
[3]Bangabandhu Sheikh Mujibur Rahman Science and Technology University, Gopalganj, Gopalganj 8100, Bangladesh.

Authors' contributions

This work was carried out in collaboration between all authors. Authors Habiba and MFH designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors JBGU and AKL managed the analyses of the study. Authors MAI and BS managed the literature searches. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: To identify and characterize Citrus Huanglongbing disease causing pathogen *Candidatus* Liberibacter *asiaticus* and to evaluate its biological control using medicinal plant extracts.
Study Design: The study was designed based on standard laboratory protocol.
Place and Duration of Study: Professor Joarder DNA and Chromosome Research Lab,

**Corresponding author: E-mail: sikdar2014@gmail.com;*

Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi-6205, Bangladesh between June 2015 and September 2016.

Methodology: Causal pathogen of Citrus Huanglongbing disease was isolated from infected leaves. Different types of biochemical and morphological characterizations of *Candidatus* Liberibacter *asiaticus* were done. 16S rDNA primers (27F and 1391R) were used to amplify genomic DNA of *Candidatus* Liberibacter *asiaticus*. Sequencing of 16S rDNA sequence of *Candidatus* Liberibacter *asiaticus* were performed. Sensitivity pattern of *Candidatus* Liberibacter *asiaticus* against several standard antibiotics were done. Antimicrobial activity test was observed using two solvents extracts of four medicinal plants by disc diffusion method *in vitro* condition.

Results: *Candidatus* Liberibacter *asiaticus* showed positive and negative response to different biochemical test. *Candidatus* Liberibacter *asiaticus* showed gram negative in gram staining test. *Candidatus* Liberibacter *asiaticus* showed highest 35±0.5 mm and lowest 8±0.2 mm zone of inhibition against amoxycillin and kanamycin respectively. Approximately 1300bp band was found in PCR amplification and phylogenetic tree analysis of 16S rRNA gene sequence of *Candidatus* Liberibacter *asiaticus* showed 75% similarity with *Candidatus* Liberibacter *asiaticus* strain 374.15. *Candidatus* Liberibacter *asiaticus* showed highest 16±0.5 mm diameter of zone of inhibition at 60 µg/ml concentration for ethanol extract of *Cuscuta reflexa.*

Conclusion: This study will be helpful for proper identification, characterization and control of Citrus Huanglongbing disease causing pathogen *Candidatus* Liberibacter *asiaticus* in an eco-friendly way.

Keywords: Citrus Huanglongbing; Candidatus Liberibacter *asiaticus; biological control; disc diffusion; antimicrobial activity.*

1. INTRODUCTION

Citrus is one of the most widely cultivated fruits in the world. The genus *Citrus* belongs to family Rutaceae and subfamily Aurantioideae. Citrus is believed to have originated in the part of Southeast Asia bordered by Northeast India, Myanmar (Burma) and the Yunnan province of China. Citrus fruit has been cultivated in an ever-widening area since ancient times; the best-known examples being the oranges, lemons, grapefruit, and limes. *Citrus* is the main fruit tree crop in the world and therefore has a tremendous economical, social and cultural impact in our society. It is a delectable, juicy and seedless fruit having great nutritional significance [1]. Diets rich in fruits and vegetables have been strongly associated with numerous health benefits and lower risk of disease [2,3]. There is considerable evidence that citrus fruits have antioxidant and antimutagenic properties and positive associations with bone, cardiovascular and immune system [4]. Citrus provides important source of vitamin-C and minerals [5]. In Bangladesh, most of the people live under the poverty line and about 91% people are suffering from the deficiency of vitamin-C [6]. Large varieties of citrus fruits are cultivated in Bangladesh. In 2010, the production of citrus fruit worldwide was estimated as 122.5 million tones with ~8.7 million hectares harvested; oranges were 50%–62% of the total area harvested and total production [7]. However production of citrus fruits is seriously hampered by different types of diseases. Bacterial diseases of citrus fruit are most common. Bacterial Leaf Spot, Citrus Canker, Bacterial Blast, Citrus Variegated Chlorosis and Citrus Huanglongbing (Citrus greening) are commonly occurred in Bangladesh. Among them Citrus Huanglongbing is most serious disease of citrus plant.

Huanglongbing (HLB), previously called greening disease, is one of the most severe diseases of citrus in Asia, Africa, the Arabian Peninsula and the islands of Mauritius, Reunion and Madagascar [8,9]. It is caused by *Candidatus* Liberibacter *asiaticus* bacteria. It is an uncultured obligate, rod-shaped, gram negative phloem restricted bacterium [10]. Greening pathogen occurs in two forms, Asian form and African form. They can be distinguished according to their temperature sensitivity. Symptoms of African greening are more pronounced in cooler regions; Asian greening is more severe than Africans [11]. Two forms were named L. *asiaticus* and L. *africanus*; they can be distinguished as separate species according to sequence homology. The new species recently found in Brazil has been referred to as L. *americanus* [12]. In Asia HLB has damaged probably 60 million citrus trees. Major symptoms of HLB diseases are blotchy mottling leaves, yellow shoots, chlorosis and twig dieback soon after. Trees are shortened in

height, have reduced growth, have lop-sided and have small-sized fruits that are imperfectly in color. The Citrus Huanglongbing (HLB) disease causing pathogen is transmitted by grafting and vectors named psyllids *Diaphorina* citri and *Trioza erytreae*. Asiatic citrus psyllid (Diaphorina citri Kuwayama [Hemiptera: Psyllidae]) was recognised as a major pest of citrus in subtropical and tropical Asia, initially in India and then elsewhere in the region [13,14]. The available evidence suggests that it is indigenous to the Indian subcontinent [15] and has spread from this region to other citrus-producing regions of Asia. *Trioza erytreae* is mainly found in Africa. Transmission of Citrus Greening Disease happens in persistent manner, i.e. bacteria multiply in the psyllids. The greening bacterium can be found in the haemolymph of the vectors. Under laboratory conditions, Asian citrus psylla can transmit both Asian and African forms of citrus greening bacteria simultaneously [16].

Citrus Huanglongbing causes huge damage of yield that leads to loss of economic value. Proper identification and management of this disease can only reduce the yield loss. In Bangladesh there is no effective way for identification and control of this disease. HLB associated infectious pathogen is hard to detect due to its nonspecific nature of disease symptoms. HLB bacterial disease symptoms are more or less similar with nutrient (Zn) deficiency [17]. Some chemical pesticides are commonly available in the local market for the control of this disease but these are hazardous to humans and other animals. Biological control can be alternative way for the control of this disease in safety manner.

Hence, the aims of this present study were to identify the causal pathogen of Citrus Huanglongbing (Greening) disease through 16S rDNA PCR amplification, sequencing and evaluate its biological control using medicinal plant extracts which are available in Bangladesh.

2. MATERIALS AND METHODS

2.1 Plant Materials

In the present study, symptomatic HLB diseased leaves (Fig. 1A) were collected from Pabna district of Bangladesh and symptoms were identified by Bangladesh Council of Scientific and Industrial Research (BCSIR), Binodpur, Rajshahi, Bangladesh on the basis of diseased symptoms. Symptomatic leaves were also confirmed by the Bangladesh Fruit Research Institute, Regional Office, Binodpur, Rajshahi. Citrus Huanglongbing disease infected leaves were used as plant material.

2.2 Isolation of *Candidatus* Liberibacter *asiaticus* of Citrus Huanglongbing Disease

Citrus Huanglongbing disease-infected leaves were washed by distilled water and then disinfected using dilute sodium hypochlorite solution (10%) and rinsed thoroughly. Then the infected area was cut and placed on Luria and Bertani (LB) liquid media [18] which is composed of 1 g peptone, 0.5 g yeast extract and 1 g NaCl per 100 ml. Then incubated at 37°C for 12-16 h. After the bacteria had grown into LB liquid medium, bacteria were streaked onto a solid nutrient agar plates and incubated at 37°C for 12-16 h. Single colony was picked by wire loop and streaked on another media plate for pure culture.

2.3 Biochemical Characterization of *Candidatus* Liberibacter *asiaticus*

A series of biochemical tests were performed for the proper biochemical characterization of *Candidatus* Liberibacter *asiaticus*. All the morphological, physiological and biochemical tests including gram staining test were done by standard microbiological techniques according to Bergey et al. [19]. Potassium hydroxide (KOH) solubility test is special test for gram negative bacteria. For the KOH solubility test, bacteria were aseptically removed from petridishes with an inoculating wire loop, mixed with 3% KOH solution on a clean slide for 1 min and observed for formation of a thread-like mass. KOH test was performed according to Halebian et al. [20]. SIM medium is a combination of differential medium that tests three different parameters, which are represented by the three letters in the name Sulfur Reduction, Indole production and Motility (SIM). Using a needle, strains were introduced into test tubes containing SIM medium and were incubated at room temperature until the growth was evident according to Kirsop and Doyle [21]. Turbidity away from the line of inoculation was a positive indicator of motility. Indole production from tryptophan was tested using the method of Clarke and Cowan [22]. We used SIM medium manufactured by High Media Lab. Pvt. Ltd. from India. 36.23 gm medium were suspended in 1 L distilled water following manufacturer's instructions. Catalase test determine the ability of

bacteria to produce catalase enzyme. The catalase test was done by adding the H_20_2 (3% v/v) to a bacterial culture and the presence of catalase indicated by bubbles of free oxygen gas [23]. The citrate test was done according to Simmons [24] method. It was performed to determine the ability of organism to utilize carbon as energy sources. For Kovac oxidase test, a loopful inoculum from pure culture was picked up by sterilized loop. The inoculum was smeared over the area of filter paper containing oxidase reagent to develop deep blue or purple color within ten seconds indicating the oxidation of the reagent [25]). MacConkey agar test was performed for isolation of gram negative enteric bacteria and the differentiation of lactose fermenting from lactose non-fermenting gram negative bacteria [26]. MacConkey agar was inoculated with bacteria using streak plate technique and incubated the test tubes at 37°C for 16h. Bacteria were inoculated into the MR broth medium in test tubes for methyl red test. Then Test tubes were incubated at 37°C for 16h. After incubation 2-3 drops of Methyl red reagent was added into bacterial suspension. KIA medium was prepared by using usable amount in 1 liter distilled water and sterilized at 121°C for 20 minutes [27]. The tube cooled in a slanted position to obtain a butt of 1.5-2.0 cm. The 24 h of old culture of each isolate were stabbing the butt and streaking the surface of the tube. Tubes were incubated aerobically at 37°C for 16h. Production of H_2S was observed after 7 day incubation at 28°C in triple iron salts agar.

2.4 Isolation and Purification of *Candidatus* Liberibacter *asiaticus* Genomic DNA

For the isolation of bacterial genomic DNA, a single colony of *Candidatus* Liberibacter *asiaticus* was cultured in LB liquid medium at 37°C for 16 h. The culture was then taken in an Eppendorf tube and centrifuged and the liquid was discarded from the upper portion of the tube. The total genomic DNA were isolated from bacterial mass by heat lysis and selective precipitation of cell debris and polysaccharides with CTAB (Cetyltrimethyl Ammonium-Bromide) and the procedure was done according to Ausbel et al. [28]. The DNA was then re-suspended in TE buffer and quantified using a spectrophotometer then electrophoresed on 1% agar gel by comparison with DNA samples of known concentration.

2.5 PCR Analysis of *Candidatus* Liberibacter *asiaticus* Genomic DNA

The amplification of 16S rDNA gene from the *Candidatus* Liberibacter *asiaticus* genomic DNA was done by PCR reaction in a thermo cycler (Nyx, Technic, Inc., USA), using the primers 27F (5′-AGAGTTTGATCCTGGCTC-3′) and 1391R (5′-GACGGCGGTGTGTRCA-3′). PCR was done in total volumes of 25 µl, containing nuclease free ddH_2O 15 µl, dNTP mix 1.0 µl, forward primer 1.0 µl, reverse primer 1.0 µl, DNA template 1.5 µl, $MgCl_2$ 2.5 µl, *Taq* buffer B 2.5 µl and *Taq* polymerase (Takara, Japan) 0.5 µl. The procedure for PCR analysis of *Candidatus* Liberibacter *asiaticus* genomic DNA was as following: initial denaturation at 95°C for 5 min; 35 cycles of denaturation for 40 s at 95°C, annealing for 1min at 65°C, and extension for 2min at 72°C; the final extension at 72°C for 10 min, followed by cooling to 4°C until the sample was recovered. Gel electrophoresis was used to visualize the PCR products lengths. 0.5x TBE buffer was used in agar gel and visualized under a UV transilluminator. DNA was purified by agar gel electrophoresis method using AccuPrep® Gel Purification, Bioneer kits.

2.6 Sequencing and Phylogenetic Analysis of *Candidatus* Liberibacter *asiaticus* 16S rDNA

Genomic DNA was isolated from the *Candidatus* Liberibacter *asiaticus* and purified. Then the purified products were sequenced in sequencing service laboratory, Invent Biotechnology Ltd. Dhaka, Bangladesh. Two samples were sequenced. Sequencing was performed using *Candidatus* Liberibacter *asiaticus* 16S rDNA gene specific primers 27F (5′-AGAGTTTGATCCTGGCTC-3′) and 1391R (5′-GACGGCGGTGTGTRCA-3′). All sequences were compared with their related strains using BLASTN program in the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic trees were constructed at Professor Joarder DNA and Chromosome Research Lab., Bangladesh based on the Maximum Likelihood method.

2.7 Antibiotics Sensitivity Test

Antibiotics sensitivity test was done according to Bauer et al. [29]. We used fifteen different types of antibiotic against *Candidatus* Liberibacter *asiaticus*. *Candidatus* Liberibacter *asiaticus* was

cultured in LB liquid medium and incubated at 37°C for 12-16 h with continuous shaking at 150 rpm. LB agar medium was prepared in sterile conical flasks, cooled down to 40°C and placed in 90 mm petridish. 20 ml of liquid medium was poured in each petridish and left the airflow cabinet for solidification. Commercially available antibiotics disc were placed centrally on agar plate and incubated at 37°C for 12-16 h. After incubation, zone of inhibition was measured with help of mm scale.

2.8 Screening of Antimicrobial Activity of Medicinal Plant Extracts against *Candidatus* Liberibacter *asiaticus*

Screening of antimicrobial activity of selected medicinal plant extracts was done by moderate disc diffusion technique according to Hossain et al. [30]. We used four different types of medicinal plants which are more or less available in Bangladesh and have antimicrobial and medicinal value. *Cassia fistula, Curcuma longa, Cuscuta reflexa* and *Aloe barbadensis* plants were used for antimicrobial screening based on their medicinal value [31,32,33]. We collected these medicinal plants from Botanical garden, University of Rajshahi. We used ethanol and methanol as solvent for the preparation of plant extracts. The collected plant materials were washed, air-dried and grinded in a fine powder by a grinding machine. 20 g of dried powder from each plant was soaked in 200 ml ethanol and methanol, respectively in round bottom flask at room temperature for seven days with occasional shaking. The extracts were filtered by cotton white cloth followed by Whatman No.41 (Whatman, UK) filter paper. The filtrate was evaporated at 45°C to dryness and the dried substance was kept in sterile bottle under refrigerated condition until use. An inoculum suspension was swabbed uniformly to solidified 20 mL LB agar media for bacteria and the inoculum was allowed to dry for 5 minutes. 6mm diameter paper discs were used. Extracts from each solvent were used at 20 µg/ml, 40 µg/ml and 60 µg/ml concentrations and added onto each disc on the seeded medium and allowed to stand on the bench for 1 hour for proper diffusion and thereafter incubated at 37°C for 24 h. The resulting diameter of zone of inhibition was measured by mm scale.

2.9 Statistical Analysis

All experiments were performed at least three times. The data were expressed as mean and standard error (Mean±SE) and analyzed by one way analysis of variance (ANOVA). $P<0.05$ was considered statistically significant. The data were analyzed using Microsoft Excel 2010 software.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Isolation of *Candidatus* Liberibacter *asiaticus* of Citrus Huanglongbing disease

After incubation at 37°C for 16 h liquid medium turned into turbid liquid that confirmed the presence of bacteria isolated from infected leaves. Subculture was done on LB agar medium in 90 mm petridish by streaking for the isolation of pure culture. Visual observation was performed by the colony morphology of the bacteria. Colonies of the *Candidatus* Liberibacter *asiaticus* were found to be creamy white in color, small in size, round in shape (Fig. 1B) and showed pink color in gram staining (Fig. 1C).

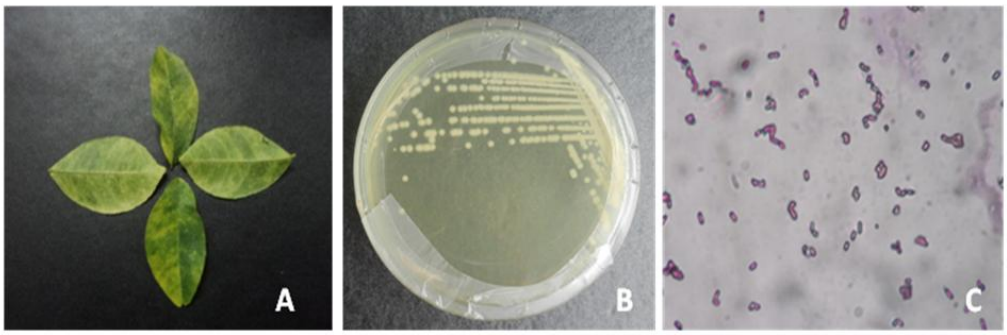


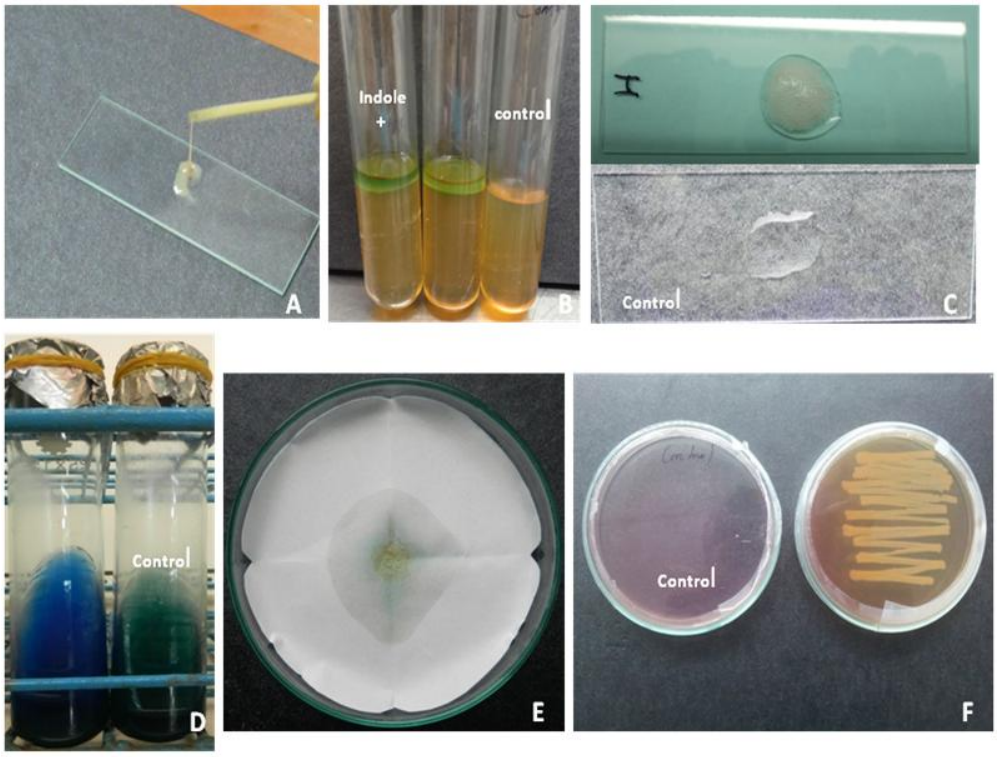
Fig. 1. Showing the infected plant sample, causal pathogen and gram staining. (A) Huanglongbing disease infected leaves, (B) Colony of causal pathogen and (C) Gram staining of causal pathogen

3.1.2 Biochemical characterization of the *Candidatus* Liberibacter *asiaticus*

Several types of biochemical tests were performed to characterize the *Candidatus* Liberibacter *asiaticus*. *Candidatus* Liberibacter *asiaticus* showed thread like viscous appearance in KOH solubility test (Fig. 2A). All gram negative bacteria showed thread-like sticky appearance in Potassium hydroxide (KOH) test. In SIM medium test, no motility was found and indole ring was formed (Fig. 2B). Bubbles were produced in catalase test (Fig. 2C), these indicated that *Candidatus* Liberibacter *asiaticus* can produce oxygen by breaking down hydrogen peroxide (H_2O_2). Deep blue color was formed in citrate medium (Fig. 2D) but no color was changed in kovac oxidase test after adding the bacteria with kovac reagent (Fig. 2E). The test organism grew well in MacConkey agar and absorbed its color (Fig. 2F). A red ring was formed in methyl red test (Fig. 2G). Nevertheless no H_2S was produced in Kligler Iron Agar or in SIM medium. On the other hand butt and slant turned into yellow color and this indicated that acid was produced in test tube (Fig. 2H). Biochemical characterization of *Candidatus* Liberibacter *asiaticus* with results are shown in Fig. 2 and Table 1.

3.1.3 Molecular identification of *Candidatus* Liberibacter *asiaticus*

Genomic DNA from *Candidatus* Liberibacter *asiaticus* was extracted using CTAB method [28]. Electrophoretic analysis of the isolated DNA from bacterial strain revealed sharp high molecular weight bands of DNA in lane 1, 2 and 3 (Fig. 3A). Comparisons with DNA samples of known concentration indicate the DNA was of good quality and suitable for PCR analysis. The 16S rDNA of the *Candidatus* Liberibacter *asiaticus* strain was then amplified using bacteria specific universal primers 27F and 1391R. Electrophoretic analysis of amplified 16S rDNA using 1.4% agarose gel followed by observation on gel documentation system (AlphaInnotech) indicated that the 16S rDNA bacterial strain was amplified up to 1300 bp (Fig. 3B) which was confirmed by 1 kb DNA ladder (Invitrogen). For phylogenetic analysis, partial 16S rDNA gene sequences were PCR amplified from *Candidatus* Liberibacter *asiaticus* strain, which was classified in the same class as the *Candidatus* Liberibacter *asiaticus* strain 374.15 (Fig. 3C). The amplified sequence of 16S rDNA of Citrus Huanglongbing disease causing pathogen *Candidatus* Liberibacter *asiaticus* showed approximately 75% similarity with *Candidatus* Liberibacter *asiaticus* strain 374.15.



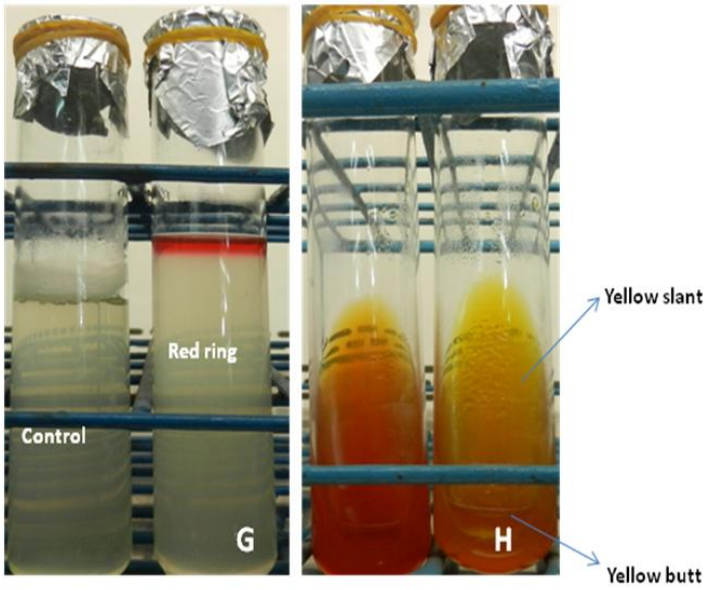


Fig. 2. Showing biochemical characterization of *Candidatus* Liberibacter *asiaticus*. (A) KOH solubility test, (B) SIM medium test, (C) Catalase test, (D) Citrate test, (E) Kovac oxidase test, (F) MacConkey agar test, (G) Methyl red test, (H) Kligler Iron agar test

3.1.4 Antibiotic sensitivity test

In antibiotic susceptibility test against *Candidatus* Liberibacter *asiaticus*, commonly available antibiotic discs were used. *Candidatus* Liberibacter *asiaticus* showed broad spectrum of zone of inhibition against all antibiotic discs. *Candidatus* Liberibacter *asiaticus* showed highest 35±0.5 mm zone of inhibition against amoxycillin. While it showed lowest 8±0.2 mm zone of inhibition against kanamycin. Results of sensitivity pattern of *Candidatus* Liberibacter *asiaticus* are given in Table 2.

Table 1. Results of biochemical characterization of *Candidatus* Liberibacter *asiaticus*

Name of the test	Reaction	Appearance	Remarks
Potassium Hydroxide (KOH) solubility test	+(ve)	Thread like viscous appearance	Thread like sticky appearance confirmed KOH positive bacteria
SIM medium test	+(ve)	No H_2S was produced, no motility was found and indole ring was formed	*Candidatus* Liberibacter *asiaticus* were non motile
Catalase test	+(ve)	Bubbles were produced	*Candidatus* Liberibacter *asiaticus* bacteria can breakdown H_2O_2 into O_2.
Citrate test	+(ve)	Deep blue color was produced	*Candidatus* Liberibacter *asiaticus* bacteria used carbon as sole source of energy
Kovac oxidase test	-(ve)	No purple color was produced	*Candidatus* Liberibacter *asiaticus* bacteria showed negative response to Kovac oxidase test
MacConkey agar test	+(ve)	*Candidatus* Liberibacter *asiaticus* grew in the MacConkey agar.	*Candidatus* Liberibacter *asiaticus* showed positive response to MacConkey agar
Methyl red test	+(ve)	Red ring was formed	*Candidatus* Liberibacter *asiaticus* were methyl red positive.
Kligler Iron agar test	+(ve)	No gas was produced, butt and slant turned into yellow color	Acid was produced in test tube

Notes: KOH= Potassium Hydroxide, SIM= Sulfide, Indole, Motility, +(ve)= Positive, -(ve)= Negative

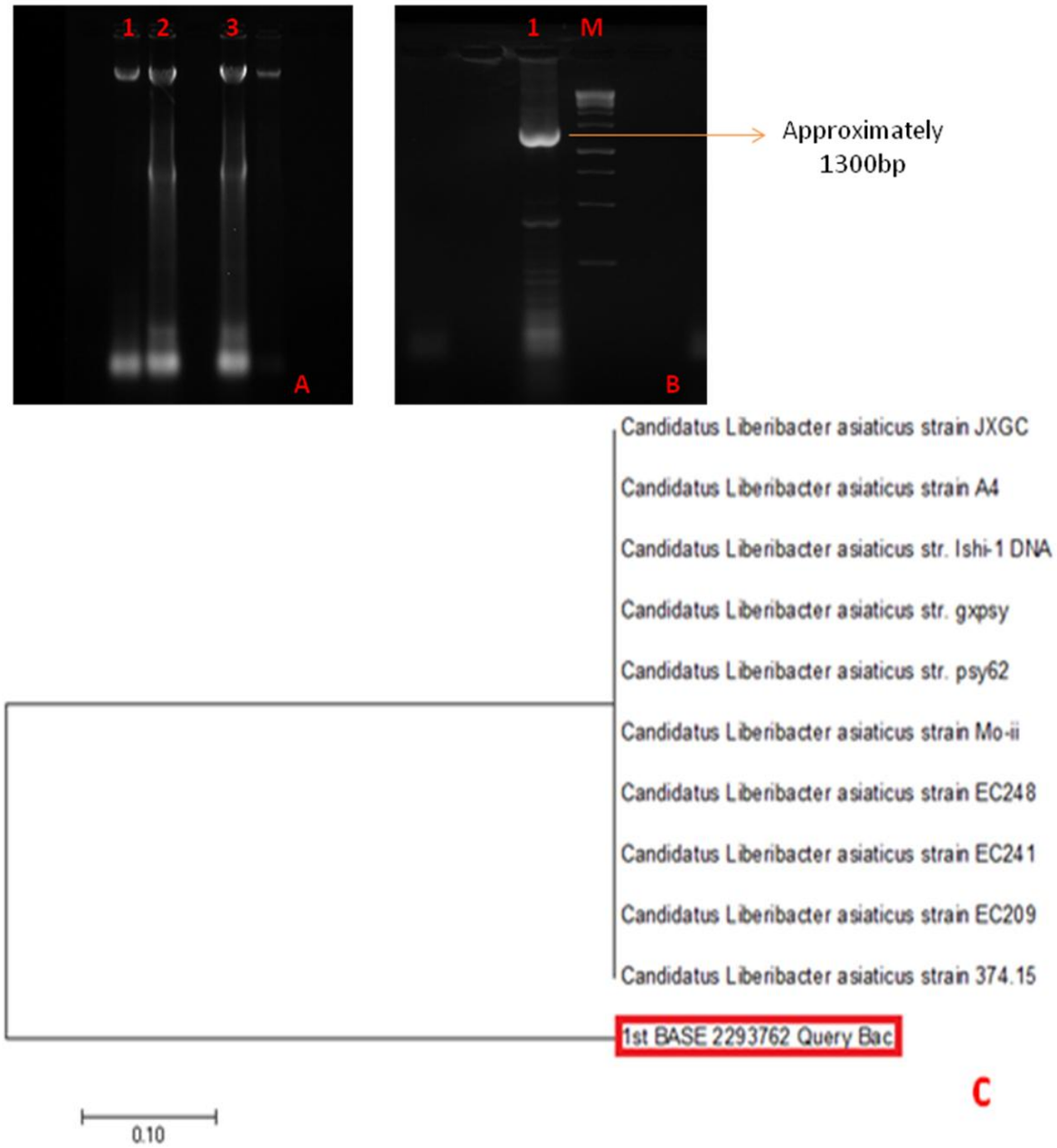


Fig. 3. Molecular characterization of *Candidatus* Liberibacter *asiaticus*. (A) Total genomic DNA, (B) PCR amplification and (C) The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [34]. The tree with the highest log likelihood (-660.2533) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 253 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [35]. M= Molecular marker. 1, 2, 3= clones of *Candidatus* Liberibacter *asiaticus*

3.1.5 Screening of antimicrobial activity of medicinal plant extracts against *Candidatus* Liberibacter *asiaticus*

Antimicrobial activity of selected medicinal plants against *Candidatus* Liberibacter *asiaticus* were done using two solvents. *Candidatus* Liberibacter *asiaticus* showed different sensitivity pattern against different types of extracts from selected plant. *Candidatus* Liberibacter *asiaticus* showed highest 16±0.5 mm diameter of zone of inhibition for ethanol extract of *Cuscuta reflexa. Candidatus* Liberibacter *asiaticus* showed 12±0.5 mm zone of inhibition against methanol extract of *Aloe barbadensis*. Lowest 7±0.3 mm zone of inhibition was found for *Candidatus* Liberibacter *asiaticus* against ethanol extract of *Curcuma longa.* Results of antimicrobial activity of medicinal plant extracts against *Candidatus* Liberibacter *asiaticus* are given in Table 3.

3.2 Discussion

Citrus Huanglongbing disease is very serious disease of citrus fruit. It causes great economic damage all over the world [36]. In Bangladesh, this disease greatly hampers the production of citrus [37]. Different types of conventional cultural and mechanical practices are used to control this disease in Bangladesh. Various chemicals are also used which are harmful to animal diversity. So biological control can be applied as an alternative control system for this disease. In the present study *Candidatus* Liberibacter *asiaticus* was isolated from infected leaves. *Candidatus* Liberibacter *asiaticus* showed white creamy color colony on agar plate. *Candidatus* Liberibacter *asiaticus* showed gram negative in gram staining test. Different biochemical characteristics of gram negative bacteria confirmed our work. Similar result regarding biochemical characteristics of gram negative bacteria was also found by Kottle [38]. *Candidatus* Liberibacter *asiaticus* showed thread-like sticky appearance in KOH solubility test. Halebian et al. [20] found all isolates respond positively to loop test by forming a thread when uplifted gently which confirmed our findings regarding KOH solubility test. Similar result was found by Suslow et al. [39] who performed KOH test to accurately characterized gram negative bacteria of wheat. No motility and H_2S was formed but indole ring was formed when Kovac reagent was added into bacteria inoculated with SIM medium. Baron and Finegold [40] found similar indole ring formation for SIM medium test. *Candidatus* Liberibacter *asiaticus* showed positive response in catalase and citrate test. Naqvi et al. [41] found positive response for all the isolates in catalase test which confirmed our work. Baron and Finegold [40] observed similar result for gram negative bacteria regarding citrate test. Kovac oxidase test, Methyl red test, MacConkey agar test and Kligler Iron agar test showed that *Candidatus*

Table 2. Sensitivity pattern of *Candidatus* Liberibacter *asiaticus* against some standard antibiotics

Name of Antibiotic	Symbol	Disc potency (µg/disc)	Zone of inhibition (M±SE) (mm)	Response	ANOVA
Amoxycillin	AML	10 µg	35±0.5 mm	Susceptible	P<0.05
Erythromycin	E	10 µg	31±0.3 mm	Susceptible	P<0.05
Gentamycin	GEN	10 µg	18±0.3 mm	Susceptible	P<0.05
Chloramphenicol	C	30 µg	10±0.2 mm	Resistant	P<0.05
Clarithromycin	CLR	15 µg	9±0.5 mm	Resistant	P<0.05
Ciprofloxacin	CP	5 µg	26±0.4 mm	Susceptible	P<0.05
Tetracycline	TE	30 µg	25±0.3 mm	Susceptible	P<0.05
Carbenicillin	CB	100 µg	10±0.4 mm	Resistant	P<0.05
Neomycin	N	30 µg	20±0.5 mm	Susceptible	P<0.05
Streptomycin	S	10 µg	20±0.5 mm	Susceptible	P<0.05
Azithromycin	AZM	15 µg	10±0.4 mm	Resistant	P<0.05
Kanamycin	K	30 µg	8±0.2 mm	Resistant	P<0.05
Doxycycline	DO	30 µg	10±0.4 mm	Resistant	P<0.05
Cefotaxime	CTX	30 µg	11±0.5 mm	Intermidiate	P<0.05
Penicillin	P	10 µg	30±0.5 mm	Susceptible	P<0.05

Note: Resistant =<10 mm; Intermediate = <10-15 mm; Susceptible = >15 mm, M ±SE= Mean and standard error, AN0VA= Analysis of Variance

Table 3. Summarized results of antimicrobial activity of medicinal plant extracts against *Candidatus* Liberibacter *asiaticus*

Name of plants	Family name	Plant parts used	Diameter of zone of inhibition(mm)				ANOVA
			Solvent	Concentration			
				20 µg/ml	40 µg/ml	60 µg/ml	
Cassia fistula L.	*Leguminosae*	flower	Ethanol	8 ±0.2	9±0.5	11±0.6	P<0.05
			Methanol	10±0.4	8±0.2	12±0.5	P<0.05
Curcuma longa L.	*Zingiberaceae*	plant	Ethanol	7±0.3	8±0.2	10±0.5	P<0.05
			Methanol	11±0.4	9±0.5	12±0.3	P<0.05
Cuscuta reflexa Roxb.	*Convolvulaceae*	Plant	Ethanol	12±0.4	14±0.2	16±0.5	P<0.05
			Methanol	11±0.2	13±0.3	9±0.4	P<0.05
Aloe barbadensis Mill.	*Xanthorrhoeaceae*	Leaf	Ethanol	10±0.2	11±0.4	8±0.3	P<0.05
			Methanol	9±0.2	12±0.5	10±0.5	P<0.05

Note: Resistant =<10 mm; Intermediate = <10-15 mm; Susceptible = >15 mm, M ±SE= Mean and standard error, AN0VA= Analysis of Variance

Liberibacter *asiaticus* are gram negative bacteria. Visual symptoms and biological indexing have been the historical means of diagnosis of HLB [42,43]. Further detection systems were developed using electron microscopy, HLB specific fluorescent substance and enzyme-linked immunosorbent assay (ELISA). Based on the 16S rDNA and other regions of the bacterial genome PCR based detection methods were developed [44]. In our present study we extracted genomic DNA of *Candidatus* Liberibacter *asiaticus* using CTAB method [28] then purified it. PCR amplification technique amplified approximately 1300bp and showed clear band in agarose gel electrophoresis. In sequencing and phylogenetic analysis, sequenced result of 16S rDNA sequenes of *Candidatus* Liberibacter *asiaticus* showed 75% similarity with the *Candidatus* Liberibacter *asiaticus* strain 374.15. Deng et al. [45] found 1160bp amplified 16S rDNA sequence of *Candidatus* Liberibacter *asiaticus* from pummel which confirmed our present findings.

Because of unprecedented epidemics of citrus HLB in citrus growing regions in the world, chemotherapy, including the use of antibiotics against *Candidatus* Liberibacter *asiaticus* is urgently needed for the survival of the citrus industry. In our present study we used different types of antibiotic to evaluate the sensitivity pattern. Antibiotic sensitivity test was done to determine the response of *Candidatus* Liberibacter *asiaticus* to different antibiotics. *Candidatus* Liberibacter *asiaticus* showed highest zone of inhibition against amoxycillin and lowest against kanamycin. Different types of antibiotics, such as tetracycline and penicillin, were injected into infected citrus trees to temporarily relieve HLB symptoms and decrease the bacterial titers [46]. So this result appreciated our research findings. Hossain et al. [30] also found zone of inhibition for gram negative bacteria against tetracycline, doxycycline, kanamycin, azithromycin. Amoxycillin would be best for decreasing *Candidatus* Liberibacter *asiaticus.* Antibiotic sensitivity test will be helpful for proper management of the disease [47].

In present study antimicrobial screening of ethanol and methanol extract of different medicinal plants were performed for establishing effective biological control. Ethanol extract of *Cuscuta reflexa* showed maximum inhibitory action against *Candidatus* Liberibacter *asiaticus*. Ethanol extract of *Curcuma longa* showed lowest inhibitory action while ethanol and methanol extract of *Cassia fistula* and *Aloe barbadensis* showed moderate inhibitory action against *Candidatus* Liberibacter *asiaticus*. To the best of our knowledge, there are no previous data about the antimicrobial activity of these selected plants against Citrus Huanglongbing disease causing pathogen. Monirujjaman et al. [48] found inhibitory action of ethanol extract of *Cuscuta reflexa* against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Salmonella typhi.* Lupeol isolated from *Cuscuta reflexa* is pharmacologically active tri- terpenoids and posse's active antimicrobial, anti inflammatory, antiprotozoal and chemoprotective properties [49]. Vimalraj et al. [50] found antibacterial effect of ethanol extract of *Cassia fistula* against *Staphylococcus aureus.* Irshad et al. [51] found zone of inhibition for ethanol extract of *Aloe barbadensis* against *E.coli, Bacillus subtilis* which appreciated our work.

4. CONCLUSION

Citrus Huanglongbing (HLB) disease is one of the most damaging disease in the world. The disease is caused by gram negative *Candidatus* Liberibacter *asiaticus* bacteria. In the present study, *Candidatus* Liberibacter *asiaticus* showed positive and negative response to different biochemical test. *Candidatus* Liberibacter *asiaticus* was detected by PCR technique. Sequencing and phylogenetic tree analysis of 16S rDNA sequence of *Candidatus* Liberibacter *asiaticus* showed 75% similarity with *Candidatus* Liberibacter *asiaticus* strain 374.15. Different extracts from selected medicinal plant showed broad spectrum of inhibitory action against *Candidatus* Liberibacter *asiaticus*. Chromatographic bio-guided fractionation of ethanol extract of *Cuscuta reflexa* and structure characterization of bioactive compounds could be done by LC or NMR from this research work in future. So present investigation would be helpful for proper identification and biological control of *Candidatus* Liberibacter *asiaticus* of Citrus Huanglongbing disease.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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