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Isolation, Characterization of *Candidatus asiaticus* Causal Agent of Greening Disease in *Citrus spp.* and Evaluation of Its Sensitivity Pattern

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ABSTRACT

The present investigation was carried out to isolation and characterization of Candidatus asiaticus bacteria from citrus greening or Huanglongbing (HLB) disease of Citrus spp. and evaluation of its sensitivity. The pathogen was isolated and cultured on LB liquid media at pH 7.5 and colonies on the nutrient agar media were creamy white in color. Isolated bacteria were gram negative in Gram staining test. The isolated bacteria showed Endospore staining, Indole formation, H_2S production, Urease and Methyl Red test negative. It also showed positive result in case of motility, Simmon's citrate agar, Catalase, MacConey agar, KOH, Gas production, Lactose and Glucose formation in Kligler Iron Agar and Triple Sugar Iron agar test. Highest Optical Density (0.28) was observed in Maltose containing medium of the isolate in carbohydrate utilization test. Antibiotic and antimicrobial activities were screened by disc diffusion method. The highest 27±0.5mm diameter of zone of inhibition was observed by penicillin in 30µg/disc concentration against the isolated bacteria. Momordica charantia showed the highest antimicrobial activity with inhibition zone 20±0.5mm at 30µl/disc concentration. Molecular detection of the isolated bacteria was done by PCR using specific primers 27F (5'-AGA GTT TGA TCC TGG CTG AG-3') and 1492R (5'-GGC TAC CTT GTT ACG ACT T-3') which amplify 1300bp approximately DNA fragment of the citrus greening bacteria.

Key words: *Citrus greening disease, gram negative bacteria, antibiotic sensitivity test, 16S rDNA amplification.*

INTRODUCTION

Citrus is one of the most cultivated fruits in the world. *Citrus* is a genus of flowering trees and shrubs in the rue family, Rutaceae. Plants in the genus produce *citrus* fruits, including important crops like oranges, lemons, grapefruit, and limes. Additionally, it possesses enormous therapeutic qualities. The most recent research indicates an origin of *citrus* in Australia, New Caledonia and New Guinea[1].

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The generic name originated from Latin, where it referred to either the plant known as Citron (*C. medica*) or a conifer tree (*Thuja*). It is somehow related to the ancient Greek word for cedar. This may be due to perceived similarities in the smell of citrus leaves and fruit with that of cedar [2]. Huanglongbing (HLB; citrus greening) is thought to be caused by the bacterium, *C. asiaticus*. HLB has seriously affected citrus production in a number of countries in Asia, Africa, the Indian subcontinent and the Arabian Peninsula, and was discovered in July 2004 in Brazil. The most characteristic symptom of HLB is a blotchy mottle. This mottling is distinct from nutrient deficiency in that HLB induced mottling usually crosses the veins and is asymmetrically displayed on the leaf blade. Mottling is most frequently found on newly mature hardened-off leaves but fades with leaf age. The blotchy mottle will be visible on both sides of the leaf and have multiple hues of yellow and green. *C. asiaticus* is gram-negative bacteria with a double-membrane cell envelope found in the sieve tube elements of phloem. The bacteria are transmitted by psyllids as they feed. *C. asiaticus* and *C. americanus* are transmitted by the adults of the citrus psyllid *Diaphorina citri* Kuwayana. *C. africanus* is transmitted by the adult psyllid *Trioza erytreae* Del Guercio. The bacteria can be acquired by the insects in the nymphal stages and may be transmitted throughout the lifespan of the psyllid[3]. There is a remote possibility that *C. asiaticus* bacteria are transmitted transovarially[4]. HLB affects almost all citrus cultivars and causes substantial economic losses to the citrus industry by shortening the lifespan of trees and making fruit inedible. Gottwald *et al*.[5] reported that almost 100 million trees have been affected and destroyed in many countries of South and Southeast Asia, Indonesia, Philippines, India, Arabian Peninsula, and South Africa, compromising the local citri culture.

The present research work has been under taken to characterize the bacteria of citrus greening disease and to analyze its management by different antibiotics. The goal of this present study was to provide useful data to citrus breeders and to characterize the isolated bacteria using some biochemical tests and to study the effect of some antibiotics against isolated bacteria.

MATERIALS AND METHODS

Collection and processing of infected leaves

The present investigation was conducted during the period of November 2015 at Professor Joarder DNA and Chromosome Research Laboratory in the Department of Genetic Engineering & Biotechnology, University of Rajshahi. Leaves from different sections of the same citrus plant were collected from Rajshahi University area and tested for the presence of *C. asiaticus* as described below by different morphological and biochemical tests.

Isolation of bacteria

Infected plant leaves were surface disinfested using a dilute sodium hypochlorite solution (10%) and rinsed thoroughly. Surface-disinfested tissue was then placed in a LB liquid medium and allowed to grow bacteria into LB liquid medium by overnight. After that, a sterile loop was used to streak the bacteria onto a solid agar medium. The bacteria were allowed to grow for at least 48 hours at room temperature and examined periodically for colony growth.

Biochemical characterization of the isolated bacteria

Isolated bacteria were characterized by some morphological and biochemical tests. Colony morphology of the isolated bacteria on the agar plate was recorded after 24 h of growth on LB agar plate at 37^0C. In order to characterize bacteria a series of biochemical tests were conducted as described by Bergey's Manual of Systematic Bacteriology [6]. Gram (+/-) staining test, Endospore (+/-) staining test, SIM test, Simmons citrate utilization test, Catalase test, Triple Sugar Iron test (TSI), Klinger's Iron Agar test (KIA test), Methyl red test (MR), Urease test, Tween 80 hydrolysis test and Carbohydrate utilization test were carried out using isolated bacterial colonies or broth cultures.

Carbohydrate test

For carbohydrate test five different carbohydrates were used such as-Maltose, Fructose, Lactose, Glucose and Sucrose. At first 10 test tubes were taken with 10 ml D. H_20. Among them 5 test tubes were used as control. Then 100 µl of MS stock were taken in each test tube. Then 0.1 gm carbohydrates (Maltose, Fructose, Lactose, Glucose, sucrose) were taken in the 10 test tubes. Among them 5 test tubes were used as control. Then the test tubes were covered with aluminum foil and autoclaved for 20 minutes. Then added 1 ml of bacterial liquid culture in 5 test tubes (control is not included). Then the test tubes were placed in the incubator for 36 hours.

Antibacterial activity test using different antibiotics

Antibiotic susceptibility test was usually carried out to determine which antibiotic will be most successful to treat this test pathogen[8]. Antibacterial activity test of Gentamicin, Erythromycin, Doxycycline, Tetracycline, Penicillin, Amoxycillin, Chloramphenicol, Clarithromycin, Cefotaxime, Neomycin, Kanamycin, Azithromyin, Ampicillin, Stretomycin and Carbenicillin were done against aused for the test [7]. Antibiotic disk was impregnated on the respective plates and incubated overnight at 37°C. After overnight incubation zones were observed on the plate and measured with the help of millimeter (mm) scale. The entire test was performed manually and enough care was taken for plating, streaking and handling of the test pathogen.

Antibacterial activity test using different plant extracts

Materials of different plant species were taken from different location of University of Rajshahi Campus. In this research, different parts of 10 different plant extracts were used for antimicrobial test[8]. The names of the plants were *Phyllanthus emblica* (amalaki), *Azadirachta indica* (neem), *Terminalia arjuna* (arjun), *Justicia adhatoda* (vasaka), *Momordica charantia* **(**bitter guard), *Ocimum tenuiflorum* (tulsi), *Aloe barbadensis*(Aloe vera), *Allium sativum* (garlic), *Zingiber officinale* (zinger). After preparing LB agar plates, the disc were impregnated in the medium and extracts were applied in different concentrations. The plates were incubation at 37°C for 16 hours. Next day, the zone of inhibition was measured with the help of mm scale.

Molecular characterization of isolated bacteria

Extraction of bacterial genomic DNA

DNA was isolated from fresh cultured colonies by phenol-chloroform extraction method. Bacteria from a saturated liquid culture were lysed and proteins were removed by digestion with proteinase-K. Cell wall debris, polysaccharides and remaining proteins were removed by phenol-chloroform extraction and high molecular-weight DNA was recovered from resulting supernatant by isopropanol precipitation.

16S rDNA amplification by PCR

PCR was performed from the genomic DNA by using 16s rDNA bacterial universal primer set of 27F (5'-AGA GTT TGA TCC TGG CTG AG-3') and 1492R (5'-GGC TAC CTT GTT ACG ACT T-3'). The PCR reaction mixture contained 12.5µl of 10x PCR master mix (100mM Tris-HCl pH 8.3, 500 mM KCl, 11 mM $MgCl_2$ and 0.1% gelatine and 10 mM dNTP mix), 0.4 µl of each primer (10pM), 2µl of genomic DNA template and 0.4 Dream Taq DNA Polymerase mixed in 9.3 µl of PCR grade water formulated a final 25µl reaction volume. Amplification was carried out with the Program Temp Control System.

Statistical analysis

All the above investigations of the present study were repeated trees for consistency of results and statistical purpose. The data were expressed as Mean±SE and analyzed using Microsoft Excel software of 2010 version. $P<0.05$ was considered statistically significant.

RESULTS

Isolation of bacteria

The isolated colonies were creamy white in color. The size and shape of colonies were found to be small, medium, convex and mucoid**.** Purification was done by streaking method.

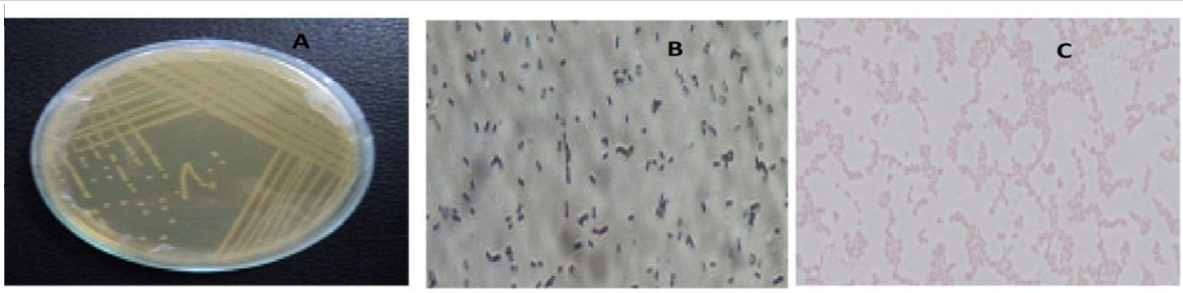

Fig 1: A. Isolated bacterial colony; B. Gram staining test; C. Endospore staining test

Morphological and Biochemical characterization of isolated bacteria

In Gram staining test, the isolated bacteria were gram-negative, rod shaped and pinkish in color when stained with counter-stained by the Safranin which was the indication for gram negative bacterium. In Endospore staining test, the isolated bacteria did not contain any endospore. In SIM-medium test (Sulphide-Indole-Motility medium), bacteria did not produce red/pink color band on the top of tube, after adding kovac's reagent and H_2S was not produced as no black precipitation formed and after incubation of 48 hours isolated bacteria showed motility in SIM medium.. In Simmon's citrate agar test, Simmon's citrate test was positive because the medium color turns Prussian blue. In catalase test, the presence of bubbles resulting from production of oxygen gas clearly indicates a catalase positive result in this study. MacConkey agar test was positive and colonies were lactose fermenting because the isolate produce pink color around the colony. In KIA test, the isolated bacterium was lactose fermenting because slant was yellow and cracks, splits, or bubbles in the medium indicated gas production of the bacteria. In TSI test, the isolated bacteria did not produce Hydrogen sulfide and were lactose, glucose or sucrose fermenting. In KOH test, the test was positive and the bacterium was found to be negative because the bacterium was viscous and formed a mucoid string in 15 sec. In Urease test, isolated bacterium did not hydrolyze urea. So, Urease test was negative for isolated bacterium. In Methyl Red test, bacterium showed negative result because the color of the methyl red not changed from yellow to red when added into the broth culture. In Tween 80 hydrolysis test, the isolated bacteria were tween 80 hydrolysis test positive because it produced milky white Precipitate. The results of the biochemical tests are summarized in **Table 1**.

Table 1: Characterization of the isolated bacteria in different biochemical test media

Test Name	Response	Appearance	Remarks
Gram staining	-(ve)	Small, rod shaped, pink color colony	Gram staining results conforms gram negative
Endospore staining test	-(ve)	No endospore formation	the isolated bacteria did not capable of producing endospore
SIM	-(ve)	Motile, no H_2S and indole production	Gram negative bacteria showed motile and did not produce any indole and H_2S
Simmon's citrate agar	+(ve)	Color changed from green to the royal blue	Citrate metabolizing gram negative bacteria
Catalase	+(ve)	Presence of oxygen bubbles	Gram negative bacteria formed bubbles resulting from production of O_2 gas
MacConkey agar	+(ve)	Pink color around the colony	Gram negative bacteria showed pink color confirming lactose fermenting
Kligler Iron Agar	+(ve)	Yields yellow slants and acidic butt , Cracks, splits, or bubbles in the medium	Gram negative bacteria yield yellow slants confirmed lactose fermenting and gas forming bacteria
Triple Sugar Iron	+(ve)	Color changed from red to yellow	Gram negative bacteria did not produce H_2S and confirming slant and butt both were yellow, so the bacteria were glucose, lactose and or sucrose fermenting
KOH	+(ve)	Thread like slime	Gram negative bacteria formed thread like slime
Urease	-(ve)	Slant remains yellow	Gram negative bacteria did not hydrolyze urea
Methyl Red	+(ve)	Color changed from yellow to red ring	Gram negative bacteria had the ability to utilize glucose
Tween 80 hydrolysis test	+(ve)	Milky white Precipitation	Gram negative bacteria had the ability to hydrolyze the tween 80

Carbohydrate utilization test

To find out the utilization of carbohydrates by isolate, five different carbohydrates were used. Utilization observed in nutrient broth with respective sugars, at 35°C for 72 hours. Isolated bacteria revealed positive result in carbohydrate utilization. The overall results of carbohydrate utilization test of the screened bacterial strain are summarized in the Table 2 and Figure 2.

Table 2: Carbohydrate utilization test

Name of Carbohydrates	Result	Optimum density (OD)
Maltose	Positive	0.28
Fructose	Positive	0.10
Lactose	Positive	0.10
Sucrose	Positive	0.12
Glucose	Positive	0.12

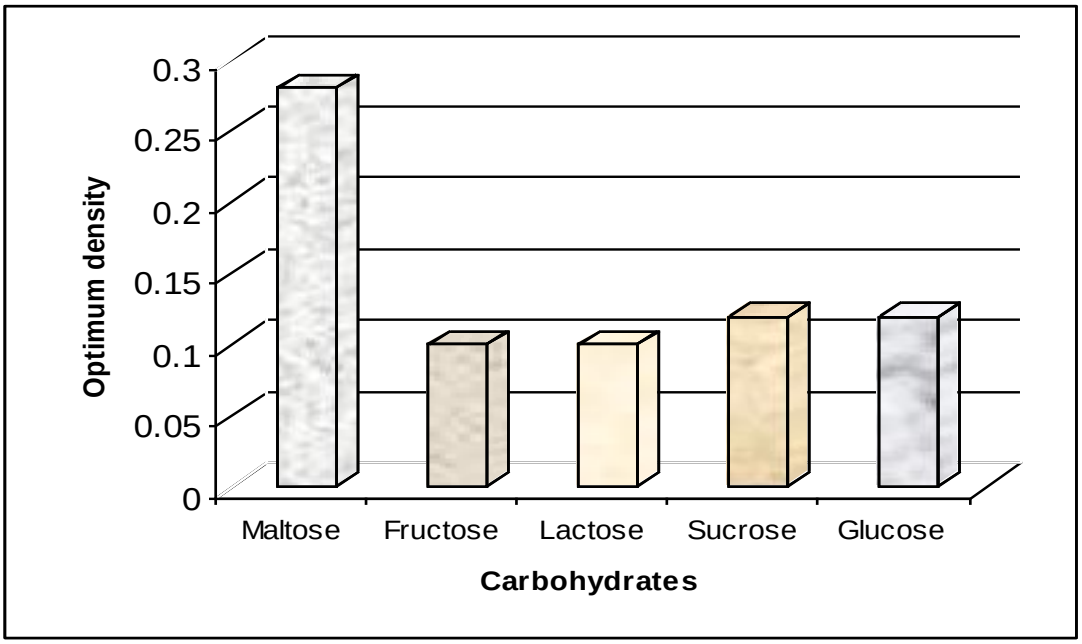

Fig 2: Graphical presentation of carbohydrates utilization of isolated bacteria

Antibacterial activity of some antibiotics against isolated bacteria

The antibacterial activities of fifteen commercial antibiotics against isolated bacterium were determined. The standard Penicillin revealed highest antibacterial activity with 27±0.5mm diameter of zone of inhibition at 30µg/disc concentration followed by Streptomycin and Neomycin with 23±0.5mm and 22±0.5mm diameter of zone of inhibition at 10µg/disc and 30µg/disc concentration respectively. On the left hands, the standard Amoxycillin and Carbenicillin showed the lowest zone of inhibition with 6±0.0mm at 10µg/disc and 100µg/disc concentration respectively against the isolated bacterium. The result of antibiotic sensitivity test is summarized in **Table 3 and figure 3.**

Table 3: Results of antibiotic sensitivity test of isolated bacteria

Name of antibiotic	Disc concentration µg/disc	Zone diameter (mm)	Sensitivity pattern of isolated bacteria
Tetracycline	30 µg	19±0.5 mm	Susceptible
Doxycycline	30 µg	18±0.5 mm	Susceptible
Erythromycin	15 µg	17±0.5 mm	Susceptible
Gentamicin	10 µg	17±0.5 mm	Susceptible
Clarithromycin	15 µg	15±0.5 mm	Intermediate
Chloramphenicol	30 µg	10±0.5 mm	Resistant
Penicillin	10 µg	27±0.5 mm	Susceptible
Amoxycillin	10 µg	6±0.0 mm	Resistant
Neomycin	30 µg	22±0.5 mm	Susceptible
Kanamycin	30 µg	17±0.5 mm	Susceptible
Cefotaxime	30 µg	17±0.5 mm	Susceptible
Azithromycin	15 µg	15±0.5 mm	Intermediate
Carbenicillin	100 µg	6±0.0 mm	Resistant
Streptomycin	10 µg	23±0.5 mm	Susceptible
Ampicillin	10 µg	7±0.5 mm	Resistant

Note: Resistant=<10 mm; Intermediate =10-15 mm; Susceptible=>15 mm[8]

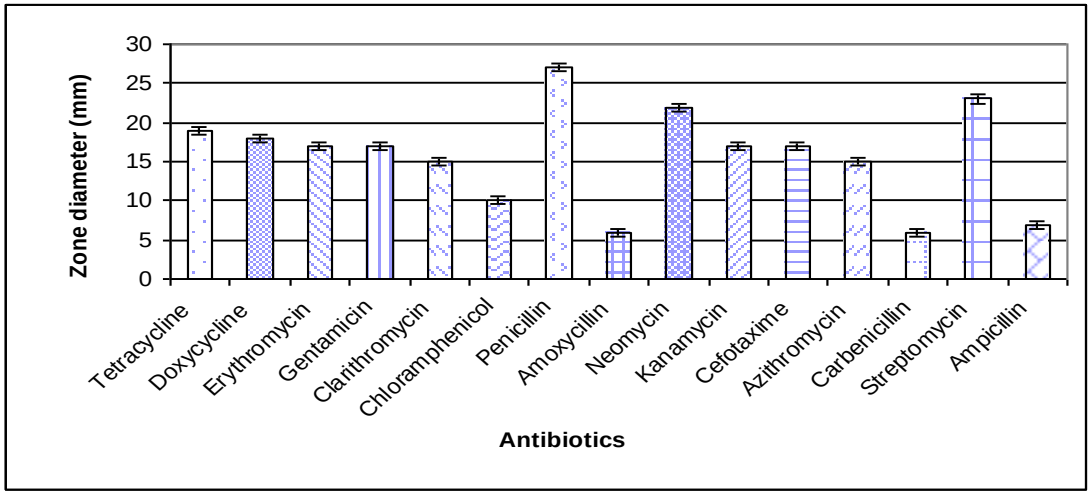

Fig 3: Graphical presentation of antibacterial activity of some antibiotics against isolated bacteria

Antibacterial activity of some plant extracts against isolated bacteria

Antibacterial activities of 10 different plants extracts were determined against the isolated bacterium. The extract of *Momordica charantia* **(**bitter guard) showed highest antibacterial activity with 20±0.5mm diameter of zone of inhibition at 30µl/disc concentration followed by 9±0.5mm diameter of zone of inhibition at 30µl/disc concentration by *Allium sativum* plant extract. On the left hands, the extracts of *Azadirachta indica* (neem), *Ocimum tenuiflorum* (tulsi) showed lowest 6±0.0mm inhibition zone against the isolated bacteria at 30 µl/disc concentration respectively. The results are presented in **Table 4 and figure 4.**

Table 4: Antibacterial activity of some plant extracts against isolated bacteria

Name of plant extract	Dose of plant extract (zone in mm) µl/disc			Sensitivity pattern against isolated bacteria
	10 µl	20 µl	30 µl	
Phyllanthus emblica (amalaki)	6±0.0	6±0.0	7±0.5	Resistant
Azadirachta indica (neem)	6±0.0	6±0.0	6±0.0	Resistant
Terminalia arjuna (arjun)	7±0.5	8±0.5	8.5±0.5	Resistant
Justicia adhatoda (vasaka)	7±0.5	7.5±0.5	8±0.5	Resistant
Momordica charantia **(**bitter guard)	12±0.5	15±0.5	20±0.5mm	Susceptible
Ocimum tenuiflorum (tulsi)	6±0.0	6±0.0	6±0.0	Resistant
Aloe barbadensis (Aloe vera)	7±0.5	7.5±0.5	8±0.5	Resistant
Allium sativum (garlic)	8±0.5	9±0.5	9±0.5	Resistant
Zingiber officinale (zinger)	6±0.0	7±0.5	7.5±0.5	Resistant
Allium cepa (onion)	6±0.0	6±0.5	7±0.5	Resistant

Note: Resistant=<10 mm; Intermediate =10-15 mm; Susceptible=>15 mm[8]

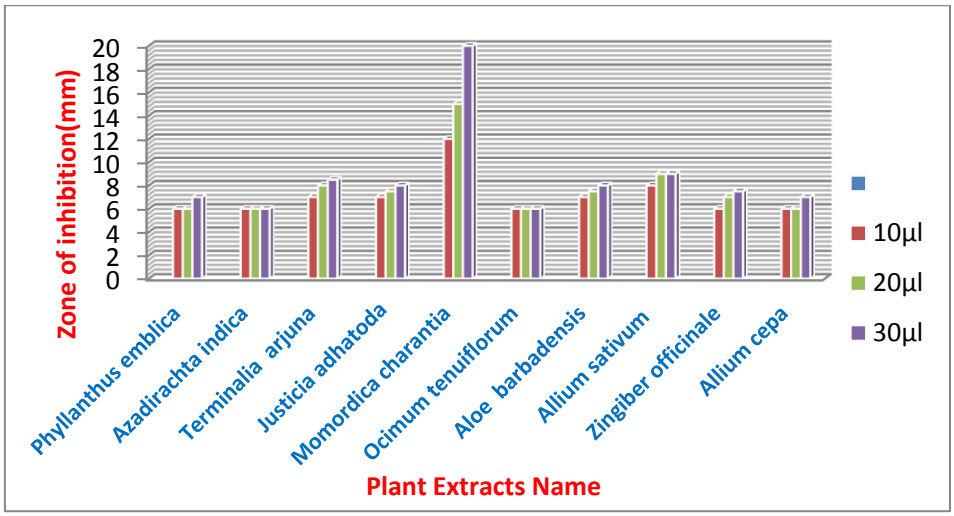

Fig. 4: Graphical presentation of antibacterial activity of some plant extracts against the isolated bacteria

Amplification of 16S rDNA Extracted from Isolated Bacterial Strain

Electrophoretic analysis of the isolated DNA from bacterial strain (citrus greening bacteria) using 2% agarose gel followed by observation on UV-transilluminator revealed sharp high molecular weight bands of DNA that indicates the DNA was of good quality and suitable for PCR analysis. The 16S rDNA of the isolated bacterial strains was then amplified using bacteria specific universal primers 27F and 1492R. Electrophoretic analysis of amplified 16S rDNA using 1.5% agarose gel followed by observation on gel documentation system indicates that the 16S rDNA of bacterial strain was amplified up to 1300 bp which was confirmed by 1 kb DNA ladder **(Figure 5).**

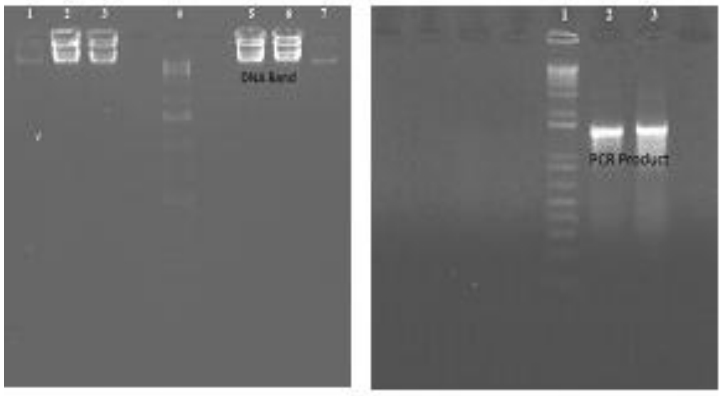

Fig. 5: Agarose gel electrophoresis of Genomic DNA and PCR products of isolated bacteria

DISCUSSION

Citrus is a delectable, seedless and juicy fruit having great nutritional value[9]. Although citrus crop is kept in great esteem, yet its present status is threatened by a number of problems, including low production caused by diseases. Citrus plant is attacked by a number of diseases like citrus canker, gummosis, citrus decline, Citrus tristeza virus (CTV), greening etc. *C. asiaticus* is the bacterium implicated as a causal agent of the economically damaging disease of citrus called huanglongbing (HLB)[10]. Bove[11] reported that HLB symptoms are virtually the same wherever the disease occurs. Creamy color colonies of isolated bacteria on LB medium were found in the present investigation. In Gram staining, result showed that the isolated bacteria were gram negative bacteria. Our result was confirmed by the different biochemical characteristics of gram negative bacteria and similar result also found by Kottle[12]. The isolated bacteria showed endospore staining test negative because it did not produce any endospore. SIM test showed H_2S, indole negative and motile on the medium. On contrast, Baron and Finegold[13] reported a positive indole test and it gave red color on top of the agar medium within a seconds when Kovacs reagent was added. Isolated bacterium showed positive result to Simmon citrate agar medium and it gave blue color. Brown and Smith[14] found that some microbes grow on Simmons citrate agar medium. They are capable of using citrate as the sole carbon source, the ability to metabolize the ammonium salt in the medium and change the color into blue. This result confirmed our present finding. Catalase test was used to differentiate that bacterium that produces an enzyme catalase. Here, positive result was found when H_2O_2 was added to the isolated bacterium and it produced air bubbles. Our work was confirmed by the work of Facklam and Elliott[15]. The lactose fermenting capability of these strains was detected from the MacConkey agar test. Bacteria produce color around the colony so it was lactose fermenting. In Kligler Iron Agar test, yellow slant confirmed lactose fermenting and cracks confirmed gas production positive. TSI test is most frequently used in the identification of the *Enterobacteriaceae*, although it is useful for other gram-negative bacterium. Isolated bacterium was glucose, lactose and or sucrose fermenting. Different biochemical tests such as, Starch hydrolysis, Tween 80 hydrolysis, Kovacs' oxidase, Gelatin liquefaction, Fluorescent pigmentation and KOH characterized the *Xac* as gram negative bacteria[16]. According to Verniere *et al.*[17], citrus greening bacteria is a gram negative bacteria. In KOH test, threads like slime were found. All isolates responded positively to loop test by forming a thread when uplifted gently. The loop formation was indicated the isolated strain was gram negative and similar results were also observed by Halebian *et al.*[18]. Each isolate was tested twice and same results were revealed. Moreover, Suslow *et al.*[19] performed that KOH test to accurately characterized gram negative bacteria of wheat. The isolates showed differences in cell sizes indicating some variability among isolates collected from different localities[20]. Different sizes of the bacteria were observed that may be

due to the difference in age of the bacteria. All the isolates found catalase positive result and gave off H_2O_2 in the petriplates which is similar with the result of Farah-Naqvi *et al*[21]. In urease test, the isolated bacterium showed negative result because no color change was found. Similar result was found by Bailey and Scott[22] and Christensen[23]. The MR test was used to identify mixed acid fermenting bacteria that yield a stable acid as end product. So, the isolated bacterium was positive in methyl red test. Our result was confirmed the work of Crown *et al*.[24]. In Tween 80 hydrolysis test, the isolated bacteria showed positive result because it formed milky white precipitate which confirmed by the result of Mubeen *et al*[25].

In antibiotic sensitivity test, the isolated bacteria were highly sensitive against Penicillin with inhibition zone 27±0.5mm at 10µg/disc concentration. Hasan and Sikdar[8] found the similar results by standard Kanamycin against *Pseudomonas sp.* Bharathi *et al*.[26] reported a similar diameter of zone of inhibitions by Erythromycin against *Pseudomonas aeruginosa.* These results support our present findings. Different types of antibiotics, such as Tetracycline and Penicillin were injected into infected citrus trees to temporarily relieve HLB symptoms and decrease Las bacterial titer [27]. So this result appreciated our research findings. Injecting antibiotics was recommended as a part of the integrated management program in India[28]. The use of medicinal plant as traditional medicine has been started several 1000 years ago[29]. The highest zone of inhibition 20±0.5mm was found in the extracts of *Momordica charantia* against the isolated bacteria. Similar results was found by Ozusaglam and Karakoca[30]. In this investigation, *Azadirachta indica, Ocimum tenuiflorum* showed lowest antibacterial activity against isolated bacteria with inhibition zone 6±0.0mm at 30 µl/disc concentration respectively. Total genomic DNA was isolated from infected citrus leaves following Phenol-chloroform extraction method and amplified using primers 27F and 1492R. Approximately 1300bp DNA band was found for citrus greening bacteria in agarose gel electrophoresis. Three species of the pathogen responsible for citrus greening have been identified by their 16S rDNA sequences. *C. asiaticus* (Las), the most prevalent species in Asia and America[31], *C. africanus* (Laf) in Africa [5] and *C. americanus* (Lam) in South America [32]. Development of specific primers and DNA probes for identification and detection has been reported for a number of plant pathogenic bacteria [33] including *C. asiaticus*. Successful detection of the pathogens using PCR techniques depends upon the specificity of primers. In this study, the specificity and sensitivity of PCR assays were evaluated through the detection of the pathogen in naturally infected plants. PCR conditions such as primers, template, concentration of Mg^{2+} [34], thermocyclers, and thermostable polymerase origin[35] have been shown to affect amplification. In this study, all these parameters were optimized to avoid artifacts and to ensure reproducibility of amplification.

CONCLUSION

In the present research, it was found that the citrus greening bacteria were gram and endospore negative from different morphological and biochemical tests. Citrus greening bacteria were highly sensitive against Penicillin, Tetracycline, Doxycycline, Neomycin, Streptomycin antibiotics and the susceptibility zones were 27±0.5, 19±0.5, 18±0.5, 22±0.5, 23±0.5 mm respectively. Thus, this study confirms the efficacy of some antibiotics and suggests the possibility of employing them as drugs for treatment of infectious diseases caused by the test pathogens which may contribute in our agricultural sector. In molecular analysis, it was found that 16s rDNA of isolated bacteria was detected by PCR using specific forward and reverse primers which amplify 1300 bp DNA fragment approximately of the citrus greening bacteria.

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Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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