First report of citrus bacterial canker caused by *Xanthomonas axonopodis* pv. *citri* in iraq

التسجيل الاول لتقرح الحمضيات المتسبب عن البكتريا *Xanthomonas axonopodis* pv. *Citri* في العراق

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Abstract

Collected citrus leaves, with erumpent, callus – like lesions with a water-soaked margin of Tangerine (*Citrus reticulate* Blance), Mexican lemon (*Citrus limon*), and Sweet orang (*Citrus sinensis* Osbec) from different orchards in Diyala, Baghdad and Babylon Governorate of Iraq. Pathogenicity of different isolates was confirmed along with symptoms under laboratory condition on detached leaf using pin prick method, observed that 21 (60 %) isolate *Xac*. was highly virulent to initiate water soaked lesion and fully developed symptoms within 10 to15 days, 14 (40 %) isolate *Xac*. were less virulent. The results of morphological (shape, colony and color) and biochemical characteristic (gram reaction, starch hydrolysis, gelatein liquefaction, KOH test, Indole production, oxidase reaction, acid and gas production, catalase reaction, fluorescent pigmentation test and NaCl tolerant). Molecular identification was performed by PCR using specific primer *J-pth1/ J-pth2* amplicon 198 bp indicated the occurrence of CBC in Iraq. All isolates 35(100%) were harbor *pthA* gene. These represent the first report of CBC in Iraq.

Keywords: citrus, citrus bacterial canker (CBC), *Xanthomonas axonopodis pv.citri*., gene, PCR.

الملخص

جمعت اوراق من اشجار الحمضيات (لالنكي *Citus reticulate* Blance ، الليمون الحامض Citrus limon و البرتقال *Citrus sinensis* Osbec) من عدة بساتين من محافظات بغداد، ديالى وبابل تظهر عليها اعراض بثرات مرتفعة فلينية المظهر ذات حواف مائية. اظهرت نتائج اختبار القدرة الامراضية للعزلات التي جمعت باستخدام طريقة وخز الدبوس (pin prick method) تحت ظروف المختبر ، ان 21 عزلة (60%) من العزلات كانت شديدة الضراوة واستغرقت فترة 10-15 يوم لتطور الاعراض بشكل كامل ، 14 عزلة (40%) من العزلات كانت ضعيفة الضراوة . كما واظهرت نتائج اختبار الصفات المظهرية، الكيمياء الحياتية مثل الشكل، شكل المستعمرة، تفاعل كرام، تحلل النشأ، سيولة الجيلاتين، اختبار هيدروكسيد البوتاسيوم، انتاج الاندول، تفاعل الاوكسيديز، انتاج الغاز والحامض، تفاعل الكتليز، انتاج صبغات لاصفة، تحمل الملوحة والاختبارات الجزيئية باستخدام تقنية (تفاعل البلمرة المتسلسل) باستخدام بادئات متخصصة *J-pth1/ J-pth2* (انتاج حزمة ذات 198 زوج قاعدي) وجود بكتريا تقرح الحمضيات. وان معظم العزلات 35 (100%) احتوت على الجين harbor *pth*A ويعد هذا التسجيل الاول لمرض التقرح البكتيري على الحمضيات في العراق.

كلمات مفتاحية: الحمضيات ، بكتريا تقرح الحمضيات ، *Xanthomonas axonopodis pv.citri* ، الجين ، تقنية (تفاعل البلمرة المتسلسل)- (PCR).

Introduction

Canker is a disease affecting citrus species caused by the bacterium *Xanthomonas axonopodis* pv. citri. Infection causes lesions on the leaves, stems, and fruit of citrus trees, including lime, oranges, and grapefruits [1]. Citrus bacterial canker typically occurs on seedlings and on young and adult trees of susceptible hosts in which there is a flush of actively growing shoots and leaves from late summer through to autumn in most citrus growing areas. Wounds caused by wind, thorns, insects, and physical or mechanical damage facilitate infection of mature tissues [2]. The disease characterized by the occurrence of conspicuous raised necrotic lesions that develop on the leaves, twigs, and fruits. Severe infections can cause a range of symptoms from defoliation, blemished fruit, premature fruit drop, and twig dieback, to general tree decline [3,4], also reported that the pathogen originated in the tropical areas of Asia, such as China, Indonesia and India, where citrus species are presumed to have originated. They also reported that the disease is presently prevalent in Africa, Asia, and Australia, Oceania and South America and stated canker causes heavy losses when the infection occurs at early stage of plant growth. The causal bacterium *Xanthomonas axonopodis* pv. citri has distinct forms (A, B and C) based on geographical distribution and host range [5]. Grapefruit, sweet

oranges like pineapple, Hamlin, Mexican limes, lemons, trifoliate orange and their hybrids are severely affected by *Xac* [6]. Isolated bacterium (*X. axonopodis* pv. citri) for pathogenicity on healthy plants. An aqueous suspension of the bacterium having a concentration of approximately 10^8 cells/ml was prepared by plate count method [7]. On the leaf symptom appears as small (2 to 5 mm), irregular, prominent water soaked spots, which later become necrotic with light to dark brown centre surrounded by prominent water soaked margins. At the advance stage of the disease the individual spots coalesce giving an eventual blighted appearance [8,9]. Isolated *Xanthomonas axonopodis pv.* citri from the canker infected part of citrus seedlings and identified by studies on morphological, biochemical and cultural features of the bacteria. They observed that the bacterium was gram negative; rod shaped and showed positive results in KOH solubility test, starch hydrolysis test, catalase test, citrate utilization test, motility indole urease agar (MIU) test, gelatine liquefaction test and negative result in oxidase test. It produced circular, flattened or slightly raised, yellow to bright yellow colour, mucoid colonies on YDCA medium and light yellow to slightly blue, mostly circular, small, flattened colonies on SX medium [10]. Pathogenicity approach (still the main methodology for detection and identification of *Xac*) and molecular PCR-based methods, in recent years, studies on improved identification and detection of plant pathogens have mostly concentrated on molecular approaches because of their potential increased specificity and sensitivity. PCR-based methods are powerful techniques that have been widely used for *Xcc* detection [11]. To achieve an accurate, fast and reliable detection of Xac, an integrated approach that combines bacterial isolation and conventional PCR has recently been proposed [12]. Features of citrus-attacking *xanthomonads* including X. citri subsp. citri and the genus *Xanthomonas* as a whole have been characterized at the molecular level to develop quick and accurate methods for reclassification and identification. The procedures include DNA–DNA hybridization, genomic fingerprinting, multilocus sequence analysis [13]. Reported primer set, *J-pth1/J-pth2*, based on the *pth* gene, which were reported as the primer that can detect all canker strains [14] and rep-PCR [15].

Materials and Methods

Bacterial isolation

The citrus canker diseased samples were collected from different districts of Diyala\ (AL-DoryeenVillige and AL-Hoiesh District), Baghdad \ (AL-Rashidiya Province) and Babylon\ (AL-Midhatah Province). A total of thirty five isolates of pathogen were obtained from infected leaves, twigs of acid lime showing typical symptoms of citrus canker. The diseased samples were washed under running water. Then the young lesions with green healthy portion of diseased fruits were cut into small pieces. Surface sterilize were done by dipping them in 5% sodium hypochlorite solution for 2-3 minutes. Then they were washed three times with sterile water. After surface sterilization the cut pieces were kept in a test tube containing 3-4 ml of sterile water and kept for 30 minutes for bacterial streaming and getting stock. After prepared different dilution, 0.1 ml of each dilution was spread over nutrient agar plate previously dried (to remove excess surface moisture) with micropipette at three replications. A bacterial suspension of each specimen was grown on nutrient agar plates and incubated at 28-30ºC. The plates were observed after 24-48 hr. Purified cultures were maintained on NA media one of single orange- yellow colony was picked by wire loop and streaked on another media plate for pure culture. After purification of bacteria on nutrient agar plates, it was kept in refrigerator at 4ºC in screw-cap test tubes on NA slant for future use.

Identification of the pathogen

Identification of the pathogen causing citrus canker was determined by conducting studies on morphological, biochemical and cultural features of the pathogen as per standard microbiological procedures.

Morphological studies

Morphological characteristics of the pathogen such as cell shape, gram's reaction and pigmentation were investigated as per the standard procedures described by [16].

Biochemical test

Different biochemical tests such as starch hydrolysis, gelatine liquefaction, KOH test, Indol production, oxidaes test, acid and gas production , catalase reaction, fluorescent pigmentation test and salt tolerant test 3% NaCl were investigated as per the methods described by [17].

Pathogenicity test

Citrus plant grown in pot under greenhouse condition was used for studying the pathogenicity of *Xanthomonas axonopodis* pv. *citri.* The test was conducted by following the method described by [18]. For the preparation of inoculum, bacterial cells were grown overnight in NA broth at 28±°C for 24 hr and it was resuspended in sterile distilled water to a concentration of approximately 1×10^{8} cfu/ml. Then an aliquot of the inoculums suspension was injected into the lower surface of citrus leaf with the help of a sterile syringe. Distilled water was used as a negative control. After that it was observed for 15 days. Visual symptoms were recorded and examined. To confirm Koch's postulates, bacteria were reisolated from the infected area.

DNA extraction

The total DNA was extracted from bacterial cells using a small-scale protocol described by [19,20] with little modification. *Xan.* isolates were cultured on NA medium 48 hr. prior to extraction. Cells were subculture in nutrient broth (NB) and were incubated at 28°C for 24hr. with shaking. Cells were harvested by centrifugation at 10 000 × g for 5min, washed twice with a 1 M NaCl solution and resuspended in 1000 µl of extraction buffer (0.2 M Tris-HCl pH 8.0, 10 mM EDTA, pH 8.0, 0.5 M NaCl, 1% SDS). Following 1 hr at 55°C, 0.5 vol. of 7.5 M ammonium acetate was added to the mixture, gently mixed, and left to stand for 10 min at room temperature. After centrifugation at 13 000 × g for 10 min, the supernatant was transferred into a fresh tube and the DNA was precipitated by adding 1 vol. of cold isopropanol centrifuged. The pellet was washed with 70% ethanol, air dried, and resuspended in a 1X TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and the suspension was used as a template.

Amplification PCR

The screening tests were necessary to prevent the citrus canker outbreak form plant material and fruit transported. Many methods for screening canker pathogen are available for diagnostic test. PCR screening test with specific primers is one of the reliable methods for rapid analysis of suspect samples. Since, high sensitivity of 10^{2}- 10^{3} cfu/ml can be detected [14,21] via specific of primer complementary with only *X. axonopodis* pv. *citri* gene. Primer *J-pth1**J-pth2* target a 198 base pair (bp) fragment of the nuclear localization signal in the virulence gene *pthA* in *Xanthomonas* strains that cause citrus canker symptoms. These strains include *X. citri* subsp. *citri*, *X. fuscans* subsp. *aurantifolii* and the atypical *X. citri* subsp. *citri* strains A* and A^{w} detected in Florida [14]. In situations where the presence of atypical *X. citri* subsp. *citri* strains A* and A^{w} is suspected – for example, where citrus canker symptoms are observed on the hosts *C. aurantiifolia* (Mexican lime) and *C. macrophylla* (Alemow) – both primer sets should be used.

PCR protocol of [14]. The primers are in Table (1):

The PCR mixture was prepared in a sterile tube and consists of 1× Taq buffer, 3 mM MgCl2, 1 µM each primers *J-pth1*\ *J-pth2*, 0.2 mM each dNTP and 1 U Taq DNA polymerase. Extracted DNA sample volume of 2.5 *µl* was added to 22.5 *µl* of the PCR mixture to give a total of 25 *µl* per reaction. The reaction conditions were initial denaturation step of 94 ºC for 5 min followed by 40 cycles of 93 ºC for 30 sec, 58 ºC for 30 sec and 72 ºC for 45 sec, and a final extension step of 72 ºC for 10 min. The amplicon size was 198 bp. After the termination of these programs, the PCR amplification product of DNA were took it and tested on 1.5% (w/v) agarose gel electrophoresis with 70 volt/cm for 90 min the gels were stained with ethidium bromide and visualized under ultraviolet light. Water was used as negative control and *Xac* isolates as positive for the PCR reactions.

Table (1): The Sequence Forward and Reverse Primers of *J- pth1*\ *J- pth2* Genes

Primer Name	Sequence 5' ⟶ 3'	Detected Gene	Product Size (bp)
***J- pth1*(F)**	**5' - CTT CAA CTC AAA CGCC GGA C- 3'**	***pthA***	**198 bp**
***J-pth2*(R)**	**5' - CAT CGC GCT GTT CGG GAG - 3'**		

Results and Discussion

Symptom expression of canker lesions can vary depending on the citrus variety, plant part affected and the age of the lesions. On leaves, first appearance was small, blister-like lesions, usually on the abaxial surface Figure (1).

Fig. (1): Symptoms of citrus necrosis on leaves

Lesions can be irregular in shape and appear atypical if found in association with a wound site. As leaf lesions aged, they turned gray to tan brown with an oily margin, usually surrounded by a yellow halo. In this study, the first report prevalence of citrus canker on fruits varied in respect of different districts of Iraq in Diyala, Baghdad and Hilla Governorate. Similar variation in prevalence of canker on seedlings of citrus in respect of nursery and site was recorded by [22,23]. In the present study, it was also observed that the incidence and severity of canker of citrus varied from location to location. These variations may be due to influence of environment of different agro-ecological zone. Highest incidence and severity of canker of citrus was recorded at Diyala. This high prevalence may be due to environmental influence of that particular agro-ecological zone. The causal agent of citrus canker of *Xanthomonas axonopodis* pv. *citri* was identified by conducting studies on its morphological, biochemical and cultural features as per standard microbiological procedures. In the present study, thirty five isolates were studied according to their colonies color, shape and grams staining reaction. Coloniesof *X. axonopodis* pv. *citri* on NA medium after 48 hr of incubation at 28-30^{0}C appeared as circular, mucoid, convex, yellow to orange color Figure (2).

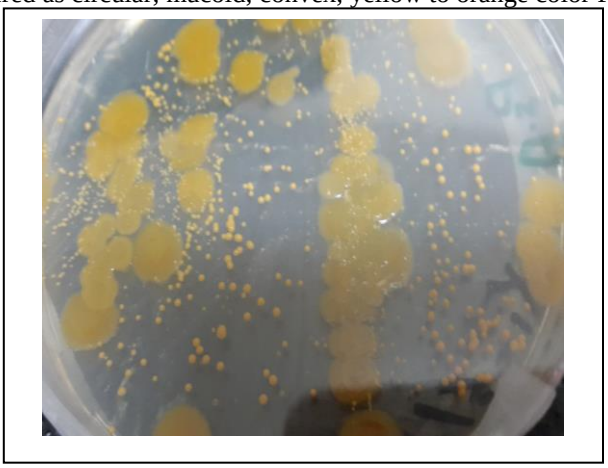

Figure. (2): Pure culture of *Xanthomonas axonopodis* pv. *citri* on (NA) plate color colonies appeared shiny with yellow- orange

The pathogen has also been reported by researchers throughout the world [24,25] . [26] isolated the bacterial pathogen from the canker infected leaves and proved pathogenicity. Reported that *Xanthomonas* produce mucoid, circular, convex, yellow, round, glistening and raised colonies on nutrient agar medium [27]. In case of salt tolerance test, turbidity was formed after 24 hr, 48 hr and 72 hr up to 3% salt concentration in shaker incubator. With the results of present study, twenty one isolate it was observed that *Xanthomonas axonopodis* pv. *citri* was comparatively highly virulent to tolerate up to 3% salt concentration after 72 hours of incubation which is supported by [28]. In addition, from the fourteen isolate poor virulent to salt tolerance test showed lower than 2% salt concentration. After gram's staining under the microscope, observed gram negative (red colour) bacterium was rod shaped, cells appeared singly and also in pairs. A mucoid thread was lifted with the loop in KOH solubility test that backing the result of gram's staining test. Similar result in KOH solubility test was described by [29] and [30] also reported *Xanthomonas axonopodis* pv. *citri* as gram negative, rod shaped bacterium. The biochemical tests for their identification, some of the tests were performed for comparing the characteristics depicted in Burgey's manual of systematic bacteriology. In the present study the bacterium *Xac* pv. *citri* isolates identified biochemically, gave positive results for starch hydrolysis, catalase test, indole test and gelatine liquefaction, but showed negative result in oxidase test (table, 2). Similar results have also been reported by [31].

Table (2: Morphological and biochemical characteristic *Xanthomonas axonopodis* pv. *citri* isolates

No. of Isolate	Shape	Colony Color	Gram Reaction	starch hydrolysis	Gelatine liquefaction	KOH test	Indol production	Oxidaes test	Acid &gas production	Catalase test
Xac 1	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 2	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 3	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 4	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 5	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 6	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 7	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 8	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 9	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 10	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 11	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 12	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 13	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 14	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 15	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 16	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 17	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 18	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 19	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 20	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 21	Rod	Orange-Yellow	-	+++	+++	+++	+++	-	+++	+++
Xac 22	Rod	Orange-Yellow	-	++	++	++	+++	-	+++	+++
Xac 23	Rod	Orange-Yellow	-	++	++	++	+++	-	+++	+++
Xac 24	Rod	Orange-Yellow	-	++	++	++	+++	-	+++	+++
Xac 25	Rod	Orange-Yellow	-	++	++	++	+++	-	+++	+++
Xac 26	Rod	Orange-Yellow	-	++	++	++	+++	-	+++	+++
Xac 27	Rod	Orange-Yellow	-	++	++	++	+++	-	+++	+++
Xac 28	Rod	Orange-Yellow	-	++	++	++	+++	-	+++	+++
Xac 29	Rod	Orange-Yellow	-	++	++	++	+++	-	+++	+++
Xac 30	Rod	Orange-Yellow	-	++	++	++	+++	-	+++	+++
Xac 31	Rod	Orange-Yellow	-	++	++	++	+++	-	+++	+++
Xac 32	Rod	Orange-Yellow	-	++	++	++	+++	-	+++	+++
Xac 33	Rod	Orange-Yellow	-	++	++	++	+++	-	+++	+++
Xac 34	Rod	Orange-Yellow	-	++	++	++	+++	-	+++	+++
Xac 35	Rod	Orange-Yellow	-	++	++	++	+++	-	+++	+++

Note: Negative (-), Weak Positive (++), Strong Positive (+++).

In the current study, it was found all of 35 isolates of different *Xanthomonas axonopodis* pv. *citri* for different physiological and biochemical properties. H_2S production, catalase test and indol production are strong positive. While negative result for oxidase test, confirmed the diagnosis of isolated bacteria and that described by previous study [32]. The cultured showed variation among the isolates of *Xanthomonas axonopodis* pv. *citri.* Similar variation among the isolates has been earlier noted by [33]. For hydrolysis of starch, liquefaction of gelatin and KOH while 14 (40%) isolates are weak positive to the same tests, [22]. In our study canker infected leaves samples from Iraq were collected and *Xac* was isolated and purified. Different biochemical tests such as Gram reaction, starch hydrolysis, oxidase, gelatin liquefaction, fluorescent pigmentation and KOH test characterized the *Xac* as gram negative bacteria. The isolates of *Xanthomonas axonopodis* pv. *citri* for different physiological and biochemical properties of bacterial cells are 21(60%) isolates are strong positive our results confirmed the work of [28] who used several biochemical tests to identify and differentiate different pathotypes of citrus canker bacteria. The tests included hydrolysis of gelatin, in addition to the growth on 3% NaCl. Similarly [29] observed non fluorescent pigmentation occurred in gram negative bacteria on KB media as liken to *pseudomomas syringae* pv. *syringae*. Moreover [34] thorough KOH test to accurately characterized gram negative bacteria of wheat. However, this test is not reliable for *Bacillus megaterium* and some gram positive strains of *Clavibacter*, Therefore it is concluded that biochemical assays can successfully characterized the gram positive and gram negative bacteria. We can formulate different strategies for the management of citrus canker using this biochemical information. All 35 isolates were confirmed to be *Xac* bacterium by causing canker lesions on all four tested citrus species Tangerine, Mexican lemon, and Sweet orange were collected from different districts of Iraq .The DNA was extracted from all isolates of *Xanthomonas axonopodis* pv. *citri* . and then detected by gel electrophoresis. All 35 isolates produced an expected 198 bp band in the PCR amplification using specific primers *J-pth*1\ *J-pth*2 Figure (3).

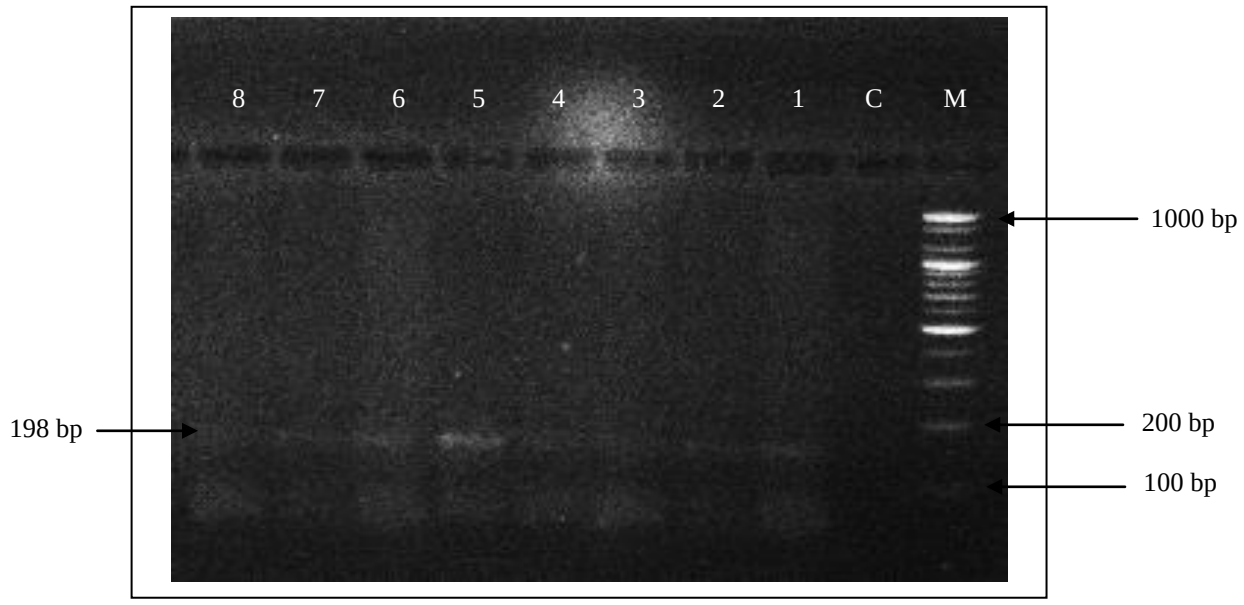

Figure (3): Specific amplification of *XAC* target (198 bp fragment) by *J-pth*1 and *J-Pth*2 specific primers. Using 1.5% (w/v) agarose for 90 min. at 70 v\cm

(1) M: Marker DNA ladder size (100bp).

(2) C: Negative control.

(3) Lanes (1-8) positive for *J-pth1/J-pth2* (198 bp).

For PCR detection we used as extract targets bacterial suspensions in fruit after DNA extraction. Primers *J-pth1* and *J-pth2* allow the amplification of a 198 bp fragment of genomic DNA in all type of isolate [14]. With pure cultures, whereas by PCR with primers *J-pth1* and *J-pth2* was 10^2 and 10^4 cfu\ ml^{-1}. Through information obtained in this study, the isolation of live bacteria demonstrates that *Xac* can survive in lesions of market fruits and retained pathogenicity. Consequently, symptomatic of market fruits represent a risk for the spread of CBC into the citrus producing of the Iraq. This study agree with [35] reported he was concluded of bacterium responsible for the disease was spread from infected trees to healthy trees by wind-driven rain or on contaminated tools and equipment.

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