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Immunocapture Polymerase Chain Reaction (IC-PCR) and Nucleic Acid Hybridization Techniques for Detection of *Spiroplasma citri*

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Spiroplasma citri Saglio *et al* is the type species of the genus spiroplasma. Spiralin gene is the gene responsible for spiralin, which is the major protein of *Spiroplasma citri* (*S. citri* Saglio *et al*), the causal organism of citrus stubborn disease. RecA gene protein is the enzyme primarily responsible for the homologous recombination of chromosomal DNA in bacteria. The Immunocapture- Polymerase Chain Reaction (IC-PCR) technique was applied for detection of *S. citri* Saglio *et al* in which the Spiroplasma was captured with the specific polyclonal antibodies on a solid-phase. A specific primers based on the sequence of the Morocco strains of *S. citri* Saglio (R8A2HP and GII3) were used. Two pairs of primers SC, SC` and SC8, SC9 were used. PCR fragment of correct size 330bp was amplified with primers SC, SC` expressing spiralin gene and 760bp was amplified with primers SC8, SC9 for recA gene. No amplified products were obtained from samples of healthy citrus trees. Nucleic acid hybridization using non- radioactive DNA probes through southern blot hybridization technique revealed the sensitivity of this technique for detection of *S. citri* Saglio *et al* infected citrus trees.

INTRODUCTION:

Citrus stubborn disease caused by *Spiroplasma citri* Saglio *et al* is one of the most important diseases attacking citrus trees causing great loss in their yield (Chaudhary and Embassy, 2002 and Andre *et al.*, 2003). *Spiroplasma citri* Saglio *et al* , is the type species of the genus *Spiroplasma* (*Spiroplasmataceae, Mollicutes*), it belongs to the Spiroplasma group I which is of a great interest, because of the ecological diversity of its members, in addition it contains the only three plant pathogenic spiroplasmas i. e. *S. citri, S. kunkelii* and *S. phoeniceum* (Fletcher and Wayadande, 2003).

S. citri Saglio *et al* is wall-less pleomorphic cells with characteristic spiral morphology. It is restricted to the phloem sieve tubes and transmitted by phloem sap-feeding insects, as is characteristic of the phytopathogenic mollicutes. The minimum size of a helix is 2.0x 0.1-0.2 µm. *S. citri* is one of the very few plant pathogenic mollicutes to have been cultured (Bradbury, 1991 and Bove *et al.*, 2003). The genome size of *S. citri* ranges from 1.6 to 1.780kbp (Ye *et al.*, 1995)

Spiralin was defined as the most abundant protein in the cell membrane of *S. citri*. The name "Spiralin" does not infer any special function (Duret *et al.*, 2003). Spiralin represents more than 20% of the total membrane protein. Several primers were constituted and used for PCR amplification and detection of *S. citri* (Wroblewski *et al.*, 1984 and Saillard *et al.*., 1996).

The recA protein is the enzyme primarily is responsible for the homologous recombination of chromosomal DNA (Berger *et al.* 2001)

Under Egyption conditions citrus stubborn disease was recorded even before its agent was recognized (Nour-Eldin, 1959). On the other hand Abou-Zeid *et al.* (1988) and Sidaros *et al.* (2000) studied this disease and stated that it is existed in many citrus culture Governorates in Egypt.

Since the work of Kirkpatrick *et al.* (1987) DNA probes are also available for certain MLOs. The DNA probes proved a specific, sensitive, rapid and inexpensive means for diagnosis of mycoplasma infections. (Razin *et al.*, 1987). Probes made up of conserved genes, such as 16SrRNA genes; do offer the advantage of identifying and distinguishing multiple species with a single labeled reagent.

Rapid and accurate detection of the causal agent of certain disease is essential for plant health certification. The molecular-based methods for the detection and identification of plant pathogens were developed in the last decade. PCR assays using certain primers presented reliable and sensitive means for detection and characterization of the plant pathogenic mollicutes including Phytoplasma and Spiroplasma (Saillard *et al.*, 1996. Bové and Garnier, 1997 and Bové & Garnier, 2002).

Because crude plant extracts often inhibit PCR and decrease the sensitivity of the test, immunocapture polymerase chain reaction (IC-PCR) has been developed. A new procedure IC-PCR simplifies sample preparation and enhances the specificity and sensitivity of conventional PCR (Orrego, 1990 and Saillard *et al.*, 1993).

Molecular hybridization and PCR can successfully be used for the detection of *S. citri* (Saillard *et al.*, 1993; Bové and Garnier, 1997; Duret *et al.*, 1999 and Whitcomb *et al.*, 1999).

Dot blot hybridization system using digoxigenin-labelled probes and colorimetric visualization for detection of *S. citri* Saglio *et al* from diseased plants is a promising technique (Bové and Garnier, 2002 and Fletcher and Wayadande, 2003)

Up to our knowledge no reports concerning molecular biology based detection methods of *S. citri* Saglio *et al* were published under Egyptian conditions. So this work was carried out to recognize the best molecular methods of detection of *S. citri* Saglio *et al* in infected citrus trees. In the present work, IC-PCR and DNA hybridization techniques were used for detection of an Egyptian isolate of *S. citri* Saglio *et al* in the different species of citrus trees.

MATERIAL AND METHODS

Spiroplasmas isolate and plant materials

In the present work, an Egyptian isolate of *Spiroplasma citri* Saglio *et al* previously isolated from Sweet orange (Citrus sinensis Osb.), Mandarin (Citrus reticulata L.) and Lime (Citrus aurantifolia) (Farag, Azza, 2004) trees was used. Infected navel orange, mandarin and lime were used as source of *S. citri* in IC-PCR technique. Polyclonal antibodies (IgG) against *S. citri* Saglio *et al* were obtained from SANOFI, (Sante Animale, Paris, France)

Oligonucleotide primers.

SC, SC' (spiralin gene) and SC8, SC9 (recA gene) specific primers based on the sequence of Morocco strains (R8A2HP and GII3) of *S. citri* Saglio *et al* were used and are

illustrated in Table (1) according to Marais *et al.* (1996b)

Table (1): Nucleotide sequence of specific primers for spiralin and recA genes region of *Spiroplasma citri* Saglio *et al*

Primer	Nucleotide sequence
SC8	5`-CTCAAACATTACATGAGCC-3`
SC9	5`- TTCACCGTATGATGGTCG-3`
SC. SC	*Kindly provided by Dr Colette Saillard

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Immunocapture Polymerase Chain Reaction (IC-PCR)

Spiroplasma citri Saglio *et al* extracted from different citrus samples were directly captured by polyclonal antibody following the method described by Saillard *et al.* (1996).

Fifty microliters of 6µg/ml-purified anti-*S. citri* Saglio *et al* IgG in coating buffer was added to each tube (0.5ml PCR eppendorf tubes) and incubated overnight at 4°C. The IgG solution was removed and the tubes were washed three times with 100µl of phosphate buffer saline PBS-sorbitol (137 mM NaCl pH 7.2, 2 .7 mM KCl, 1.8 mM KH2PO4, 4 mM NaHPO4, 1 mM Sorbitol). Tissue sample (0.3g) sample was chopped to a fine mince in a sterile Petri dish containing 1ml PBS sorbitol buffer and left at room temperature for several hours. Fifty microliters of the minced tissues was transfered to a pre-coated tube and incubated overnight at 4°C. The minced tissue fluid poured off from the tube and washed three times with 100µl of PBS sorbitol buffer.

Two pairs of primers (SC, SC' and SC8, SC9) were constructed from published conserved sequences of spiralin and recA genes of *S.citri* strains R8A2HP and GII3 (Chevalier *et al.*, 1990 and Foissac *et al.*, 1997).

Fifty microliters of PCR reaction mix. were added to each coated Eppendorf tube contained the following reaction mixture 2.5 untis of the thermostable Taq polymerase (5 u/µl, Promega Corporation U.S.A.), 2mM dNTPs, 5ul of 10X PCR buffer (10mM Tris-HCl pH 8.3, 50 mM KCl, 0.01 % gelatin (w/v)), 2mM MgCl2, 1ul of 10pm of primer SC or SC8, 1ul of 10pm of primer SC' or SC9 and sterile water to up volume of 50µl. The mixtures were over lid with 50ul of mineral oil. For amplification of spiralin gene, DNA was subjected to the following thermal cycler program: 94°C for 45s, 62°C for 45s and 72°C for 30s followed by an extension step 10 min at 72°C for 40 cycles in a DNA Thermal Cycler (Perkin-Elmer Cetus).

For DNA amplification of recA gene. The primers SC8, SC9 were hot started 2 min at 94°C in a DNA thermocycler,1 min at 92°C, 1 min at 42°C and 2 min at 72°C, followed by an extension step 10 min at 72°C, 35 repeated cycles as described by Marais *et al.* (1996b). The amplification was preceded in the thermocycler (Uno II, Biometra, Germany).

The amplified DNA was electrophoresed on 1% agarose gel with 1xTAE buffer, stained with ethidium bromide and photographed using Gel Doc. 2000 Bio-RAD.

Probe preparation

Probes for southern blot and dot blot hybridization were generated by PCR in the presence of digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN). DNA fragments,

which were amplified by IC-PCR from *Spiroplasma citri* Saglio *et al* using primer SC8, SC9 was used as the templates to synthesize a specific S. citri DNA probes. PCR was performed in thin-walled PCR tubes containing the following reaction mixture 1µl PCR product for primers (SC8, SC9), 0.5µl Taq DNA polymearse, 5µl of 10x PCR buffer, 1µl dNTPs labelled mixture (Promega), 1µl of 10pm of primer SC8, SC9 and sterile water to up volume of 50µl. PCR reaction was amplified with the cycling parameters as described by Marais *et al.* (1996b).

Southern blotting hybridization

Southern blot hybridization was used to confirm that PCR amplified fragments were *S. citri* Saglio *et al*.recA gene using the non-radioactive dig labeled probe. The product was then transferred to nitrocellulose membrane according to the method of Southern (1975) and was fixed by UV for 30 sec. The DNA probe specific for S. citri recA gene was added to the PCR products of the tested S. citri and subjected directly to hybridization according to Boehringer Mannheim, Indianapolis, IN for nucleic acid hybridization

Dot blot from plant material extract

About 0.3g leaf tissues of new leaves of Navel orange, Mandarin, and Lime trees infected with *S. citri*, were placed in microfuge tubes with 100µl of extraction buffer (0.2M potassium phosphate, 5 mM dithiothreitol ,0.1%Triton X-100,10 mM, 2-mercaptoethanol, pH 8.3), and then ground using knots pestles. Equal volume of denaturation solution, 1xSSC (15Mm Nacl, 15Mm Sodium citrate, pH 7.0, 50 % Formaldhyde) was added and heated at 60°C for 10 min then centrifuged in microfuge tubes at 10,000 rpm for 5 min. Dot blots were carried out according to Loebenstein *et al.* (1997). Five µl of the supernatant were spotted onto nitrocellulose membrane (Millipore) with 20x SSC. The hybridization was carried out as described by Boehringer Mannheim, manual protocol.

Dot blot from PCR product

Three microliters of PCR product for recA gene were blotted on nitrocellulose membrane (Millipore). The DNA was fixed on the membranes by Ultraviolet (UV) cross- linked for 30sec. The membranes were used directly for hybridization

Hybridization technique

Prehybridization, hybridization and immunological detection were carried out using the GeniusII DNA Labelling and Detection Kit (Boehringer Mannheim, IN System).

RESULTS AND DISCUSSION

Detection of *S. citri* by IC-PCR

The IC-PCR was used for detection of *Spiroplasma citri* in infected citrus samples including (Navel orange, Mandarin, and Lime) collected from different locations in Egypt. PCR fragment of correct size 330bp and 760bp were amplified with the primers SC, SC' for spiralin gene and SC8, SC9 for recA gene. Agarose gel electrophoresis analysis of the amplified PCR products is demonstrated in Fig. (1). No reaction for samples of healthy trees was observed.

Rapid and accurate detection of the causal agent of disease is essential for plant healthy certification. PCR assays using certain primers presented reliable and sensitive means for

detection and characterization of the plant pathogenic mollicutes including *Phytoplasma and Ppiroplasma* (Marais *et al.*, 1996a; Foissac *et al.*, 1997 and Bové & Garnier, 2002). In the present work, IC-PCR was used for detection of *S. citri* in the different species of citrus infected trees

IC-PCR proved to be very sensitive and reliable method. IC-PCR for its simplicity, sensitivity and reproducibility, has some advantage, therefore, IC-PCR can be regarded as a valuable alternative for large-scale testing spiroplasma infected trees (Heinrich *et al.*, 2001). Regarding these advantage, IC-PCR was used in the present investigation for detection of *S. citri* Saglio *et al* in different citrus trees in different locations in Egypt (El-Kanater plantation and the plantation of Horticulture Research Institute, ARC, Giza .

Using IC-PCR the spiroplasmas present in crude plant extracts are captured by polyclonal antibodies coating the walls of the tubes in which the PCR is carried out. After the capture step the plant extracts is decanted and the spiroplasma trapped in the tube are submitted to the PCR reaction without DNA isolation

In the present work, IC-PCR was used for detection of *S. citri* in the different species of citrus infected trees. Polyclonal antibodies of recA and spiralin genes were used. The obtained PCR products were identical to fragments 760bp and 300bp representing recA and spiralin genes.

The procedure of DNA hybridization with specific probes as mentioned by Degorce-Dumas *et al.* (1983); Hull (1984, 1986) and Razin *et al.* (1987) was used to detect *S. citri* in field samples showing symptoms suspected to be caused by Spiroplasma (Bove *et al.*, 1993 and Saillard *et al.*, 1993).

Southern blot hybridization was used as an effective method to confirm the validation of PCR product of recA gene (760bp) Fig.(2) for stubborn disease through the nucleic acid hybridization with *S. citri* -DNA probe spiralin and recA gene(Marais *et al.*, 1996; Foissac *et al.*, 1997 and Bové and Garnier, 2002).

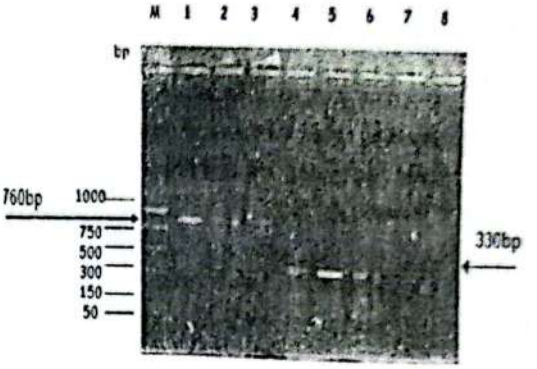

Fig. (1): Agarose gel Electrophoresis of IC-PCR amplification products obtained from *Spiroplasma citri* using primers SC8, SC9 and SC, SC' specific for recA and spiralin genes using polyclonal antibodies for immunocapture. Lanes: (1, 2 and 3) representing the recA gene (760bp) developed from infected Navel orange, Mandarin and Lime respectively and lanes: (4, 5 and 6) the spiralin gene (330bp) developed from infected Navel orange, Mandarin and Lime respectively. Lanes (7, 8): represent healthy citrus samples. M: PCR marker (Promage) (1000, 750, 500, 300, 150, 50).

In the present study a simple, specific, and rapid method for the detection of *S. citri* infected new leaves of citrus plants Navel orange, Mandarin and Lime on dot blotting and southern blotting from samples of infected and healthy trees onto nitrocellulose membrane, followed by hybridization with *S. citri* using DNA probe of recA gene (760bp). No hybridization signals were obtained with healthy citrus plants in southern blot and dot blots. (Southern, 1975;;

Gilbertson *et al.*, 1991; Landridge *et al.*, 1991 and Bove *et al.*, 1993)

The reaction between dig - labeled DNA specific for *S. citri* recA gene region using dot blot and PCR blot was achieved (Fig.3) . Positive strong reaction of dot blot hybridization was observed with extracts of new leaves of Navel orange, Mandarin, and Lime tissues infected with *S. citri*. Also strong blue signal was observed with PCR products blot with the same tested leaves. No signal was observed in healthy control. Strong signal was obtained with *S. citri* recA gene region of DNA used as positive control.

Dot blot hybridization is very sensitive and would be used for detection of a small quantity of viral DNA in plant tissues, but it gives a non-specific couloured background so it can be used in routine (Loebenstein *et al.*, 1997).

Spiroplasma citri Saglio *et al* detection is now available using PCR and dot-blot hybridization for detection of stubborn disease in the field detection of *S. citri* even if it is found with very low concentrations in infected tissues. On the other hand nucleic acid hybridization technique using non-radioactive DNA probe considered one of the modern methods for early detection of the stubborn disease infected plants.

PCR and IC-PCR are the most sensitive and specific techniques used for the detection of *S. citri*. PCR has the capacity to produce million of DNA molecules. One control must contain all PCR components expect the template DNA. PCR or IC-PCR is carried out on field trees, it is obligatory to include healthy trees from the greenhouse as a control. If an amplified DNA fragment is visible on the gel from the healthy greenhouse trees, the presence or absence of *S. citri* in the field trees cannot be inferred. General procedures for minimizing the contamination in the PCR reaction have been described by Saillard *et al.* (1996).

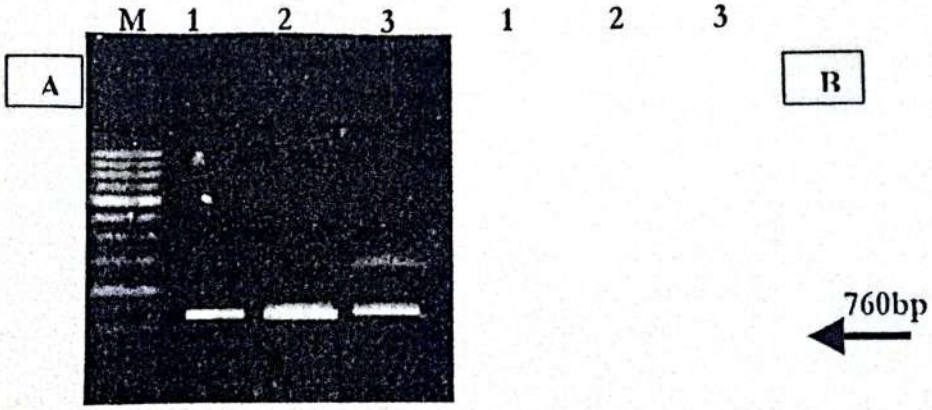

Fig. (2): A: Agarose gel Electrophoresis of IC-PCR amplification products obtained from *Spiroplasma citri* using primers SC8, SC9 specific for recA gene using polyclonal antibodies for immunocapture. Lanes: (1, 2 and 3) representing the recA gene (760bp) developed from infected navel orange, Mandarin and Lime respectively. M: PGEM marker (Promage).

B: Southern hybridization of *Spiroplasma citri* amplified PCR fragment using a specific DNA probe labeled with dig-11-dUTP. The PCR product was transferred from agarose gel to a nitrocellulose membrane using southern technique. The membrane was cross-linked and hybridized with *Spiroplasma citri* specific Dig labeled DNA probe. These products hybridized strongly with their respective non radioactive-labelled probe to identify the *S. citri* recA fragments .

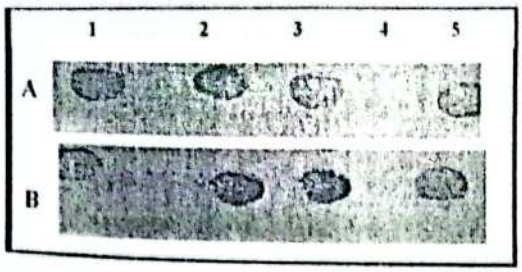

Fig. (3): Dot blots hybridization assays using recA gene region of DNA probe labeled with dig.

(A): The membrane hybridized with specific non-radioactive S. citri probe with dot blots. Lanes (2, 3 and 5) strong signal with infected new leaves of (Navel orange, Mandarin, and Lime) respectively. Lane (4) no reaction with healthy sample (negative control). PCR positive control recA gene in lane (1).

(B): The membrane hybridized with specific non-radioactive S. citri probe with PCR blots. Lanes (3, 4 and 5) strong signal with infected new leaves of (Navel orange, Mandarin, and Lime) respectively. Lane (2) no reaction with healthy sample (negative control). Lane (1) PCR positive control recA gene.

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