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Comparison of Two Egyptian Isolates of *Spiroplasma citri* by Crossed Immunoelectrophoresis and Polyacrylamide Gel Electrophoresis of Cell Proteins

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Abstract: Differences between two *Spiroplasma citri* isolates were detected by crossed immunoelectrophoresis (CIE) with intermediate gel and polyacrylamide gel electrophoresis. In the homologous reactions of Fewa isolate, eleven precipitin peaks were detected using CIE. Identical homologous reaction of Qualubia isolate was produced. One antigen (a) was specific for the Fewa isolate and one antigen (b) was specific for the Qualubia isolate when CIE with intermediate gel was used. One-dimensional electrophoresis analysis demonstrates very similar patterns of protein with two different protein bands between the two isolates of *S. citri.*

Keywords: *Spiroplasma citri*, crossed immunoelectrophoresis, polyacrylamide gel electrophoresis, CIE

INTRODUCTION

Spiroplasma citri, (helical mollicutes: Bacteria: Firmicutes: Mollicutes: Entomoplasmatales: Spiroplasmataceae) the first spiroplasma to have been cultured, is the causal agent of stubborn disease of citrus[1]. Crossed immunoelectrophoresis (CIE) is an extremely useful technique for quantitative and qualitative analysis of proteins[2]. It has been used to study serological relationships between mycoplasmas[3] and to identify specific proteins[4,5]. Electrophoretic analysis of mycoplasma proteins on polyacrylamide gels has been used previously for taxonomic purposes[6] and comparison among spiroplasmas by one-dimensional SDS-PAGE[7] and by both one and two-dimensional SDS-PAGE[8]. Five groups of *S. citri* strains could be distinguished on the basis of the Electrophoretic mobilities (Ems) of their major proteins using sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the total proteins[9]. Solubilized proteins of five selected spiroplasmas were separated in one-dimensional SDS-PAGE and the results showed that *Spiroplasma citri* strains SP-4 and R8A2 and the honeybee (BC3) Spiroplasma had a very similar patterns, apart from the mobility of the major band of apparent molecular weight between 23000 and 26000 and the homology of some *S. citri* and honeybee (BC3) Spiroplasma antigens was confirmed by crossed immunoelectrophoresis[10]. The purpose of the present study was the differentiate between the two Egyptian isolates of *S. citri* collected from two different locations.

MATERIALS AND METHODS

Two isolates of *Spiroplasma citri* were isolated each from Kafr El-Sheikh (Fewa isolate) and El-Qualubia (Qualubia isolate) governorates. They were cultured at 32°C in C-3G medium[11].

Preparation *Spiroplasma citri* antiserum: Each isolate was grown in 1200 mL volume of C-3G broth medium for 6 days at 32°C. The cells were collected by centrifugation at 32000 g for 15 min at 6°C and washed three times in PBS (8 g NaCl, 0.3 g KCl, 0.730 g Na_2HPO_4, 0.2 g KH_2PO_4 and 950 mL distilled water, pH 7.4).The pellet was suspended in 12 mL of PBS and sonicated for 10 sec at 52 w cm^{-1} and used as antigen[12].Two female New Zealand white rabbits were individually injected intramuscularly with each of the two antigens. The antigen was emulsified with an equal volume of Fruends'complete adjuvant. The rabbits were injected twice every week for four weeks. The dosages used were 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0 and 2.25 mL, respectively. Blood was collected one week after the last injection. It was collected in a sterile test tubes and left to clot for 2 h at 37°C. The clot was loosen from the wall of the tubes with a fine glass rod. The tubes of blood were

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kept at 4°C overnight. The antiserum was centrifuged for 5 min at 2000 g for removal of cell. The collected antiserum was stored in vials at -20°C.

Crossed immunoelectrophoresis (CIE): CIE and CIE with intermediate gel were performed according to published procedures[13]. Agarose (0.85%, Bio-Rad) was dissolved by heating in Tris (hydroxymethyl) aminomethane- barbital- sodium barbital buffer, pH 8.6, ionic strength 0.03, containing 1% (v/v) Triton X-100 and filtered through glass wool and used in both first- and second–dimensional electrophoresis. Electrophoresis in the first dimension was performed on 10×10 cm glass plates. The prewarmed plates (50°C) were covered with 15 mL of agarose gel to give an agarose volume-to-surface area ratio of 0.15 mL cm^{-2}. After gelling and cooling at room temperature, three antigen wells (4 mm in diameter) were punched out at 3.5 cm from the cathodic end and 1 cm apart in the vertical axis of the glass plate. Antigen samples (15 μL $well^{-1}$) were added to each well (two wells) and 0.1% bromophenol blue was mixed with one sample as an indicator for migration and electrophoresis was carried out at 10 v cm^{-1} for 3 h. The slide was removed from the electrophoresis chamber. CIE, in the second dimension, was performed on 5×5 cm glass plates. After the first-dimensional electrophoresis, agarose stripes (5×1.5 cm) containing antigen were cut and transferred on one side of the glass plate. The remaining space on the plate was then covered with agarose containing antiserum (5 mL agarose + 0.4 mL antiserum) of each isolate. For CIE with an intermediate gel, agarose stripes containing the antigen were transferred on 5×7.5 cm slides. A brass barrier was placed 1.5 cm from the first dimension antigen-containing strip and space filled with agarose containing antiserum of isolate Fewa or Qualubia. After gelling, the remaining space was covered with agarose containing antiserum of the Fewa or Qualubia isolate. The agarose volume-to-surface area ratio in the intermediate gel and the second dimension gel was 0.15 cm^{-2}. Electrophoresis in the second dimension was performed at 3 v cm^{-1} for 16 h. Gels were stained with Coomassie brilliant blue (R-250, Bio-Rad)[13].

Polyacrylamide gel electrophoresis: Cells were harvested from 5 mL cultures by centrifugation at 30,000 g for 30 min at 4°C. The pellet was washed three times by resuspension in 2 mL 0.1 M sodium phosphate to 0.33 M sodium chloride buffer and finally resuspended in 0.5 mL of SDS-solubilizing buffer (0.05 M Tris-hydrochloride (pH 6.8), 5% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 15% (v/v) glycerol and 0.1% (w/v) bromophenol blue), the mixture was sealed into a 10 mL glass tube and heated for 5 min in a boiling water bath. Insoluble debris was removed by centrifugation at 10,000 g for 10 min at 20°C[8]. Fifteen microliter proteins were electrophoresed in polyacrylamide (15 cm long, 12 cm wide and 0.15 cm thick) prepared according to Laemmli[14] and the gels were stained with Coomassie brilliant blue[6]. Protein molecular weight standards were run on the same gel slabs as cell protein samples. The standards used were myosin (molecular weight 215 kDa, phosphorylase B (120 kDa), BSA (84 kDa), ovalbumin (60 kDa), carbonic anhydrase (39.2 kDa), trypsin inhibitor (28 kDa) and lysozyme (18.3 kDa).

RESULT AND DISCUSSION

In the homologous reactions of Fewa isolate, eleven precipitin peaks were detected using CIE. Eleven peaks were also detected with Qualubia isolate (Fig. 1A and B).

Reciprocal analysis procedure using CIE with an intermediate gel: In the heterologous reaction between Fewa and Qualubia isolate, ten precipitin peaks were detected in the intermediate gel indicating that these antigens were found common between the two isolates. In the upper gel one antigen was detected when the antigens of Fewa isolate were electrophoresed, while other antigen was detected in the upper gel when Qualubia isolates were electrophoresed indicating that antigens a and b were specific to Fewa and Qualubia isolate, respectively (Fig. 2 and Table 1)

Detection of individual protein antigens by crossed immunoelectrophoresis is very sensitive and has been applied to mycoplasmas[5,10,15,16]. It has been used to study serological relationships between mycoplasmas[3,4]. CIE with an intermediate gel has proved very useful in the identification of membrane protein antigens from different strains of *Acholeplasma laidlawii*[17]. Spiroplasma reacted against its homologous antiserum produce between 20 to 30 precipitin peaks; although the number depends on whether, the antiserum was raised against fixed whole cells, lysed cells or the membranes[18].

Polyacrylamide gel electrophoresis: One-dimensional SDS-polyacrylamide gel electrophoresis was used to determine the differences in proteins patterns and mobility of the major proteins of the two isolates of *S. citri*. Results indicated that the *S. citri* isolates have very similar

Table 1: Reciprocal analysis of Fewa isolate and Qualubia isolate by CIE with an intermediate gel

Antibodies	Antigens used for absorption	Fewa isolate	Qualubia isolate	Heterogous reaction peaks
Fewa isolates	Fewa isolates	0	0	11(10+a)
	Qualubia isolates	a	0	
Qualubia isolates	Fewa isolates	0	b	11(10+b)
	Qualubia isolates	0	0	

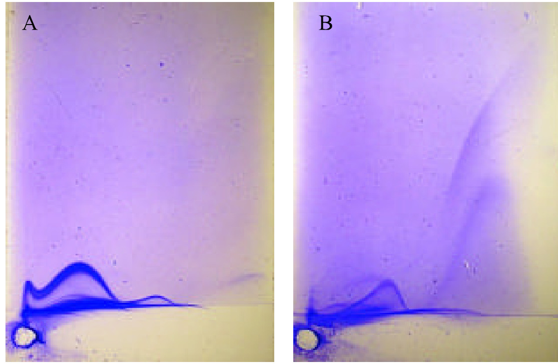

Fig. 1: Two-dimensional crossed immunoelectrophoresis of *S. citri* Fewa isolate with its antiserum A and Qualubia isolate with its antiserum B in homologous reaction. Around eleven individual peaks represents different antigens

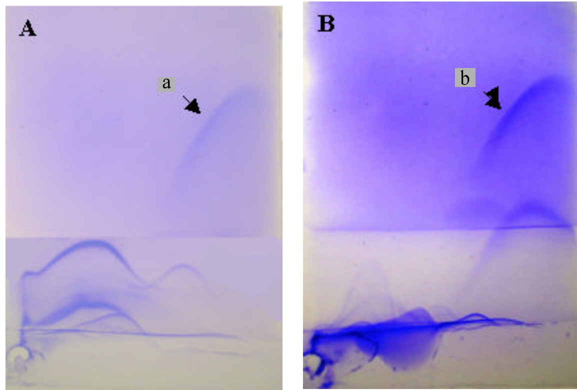

Fig. 2: Crossed immunoelectrophoresis with an intermediate gels. (A). Fewa isolate with antiserum Qualubia isolate in intermediate gel and its antiserum in the upper gel, (B). Antigen Qualubia isolate with antiserum Fewa isolate in intermediate gel and its antiserum in the upper gel. (a) is specific antigen for Fewa isolate and (b) is specific antigen for Qualubia isolate

patterns and more than 40 proteins bands could be obtained. There was no obvious difference between the mobility of the major proteins of apparent molecular weight 26,000 (Fig. 3), but there are two different protein bands between the two isolates (Fig. 3). One-dimensional SDS-polyacrylamide gel electrophoresis was used successfully by several workers to compare among *S. citri* strains and the results indicated that most of *S. citri* strains had very similar protein patterns[7,8,10,19]. However, they could be separated into group and subgroups according to the mobility of major proteins of apparent molecular weight of 23,000 or 25,000[7-9].

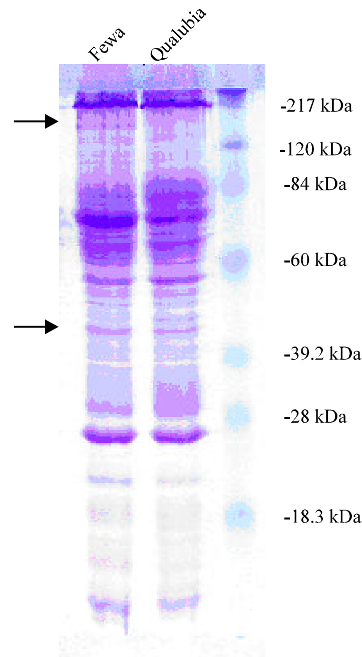

Fig. 3: Polyacrylamide gel electrophoresis of SDS-solubilized protein from *S. citri* Fewa isolate trak 1 and Qualubia isolate trak 2

CONCLUSIONS

Crossed immunoelectrophoresis (CIE) and polyacrylamide gel electrophoresis were used successfully to differentiate between the two Egyptian isolates of *S. citri*. The two isolates are not identical based on each of the specific precipitin peak and minor protein band in PAGE.

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