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Molecular Characterization of Phytoplasma-associated Disease in Tomato (*Lycopersicon esculentum*) in Saudi Arabia

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ABSTRACT

Tomato plants showing yellowing, stunting and malformation symptoms were collected from Eastern Province and Jizan in Saudi Arabia. Molecular methods have been used to detect phytoplasma-associated disease in tomato plants for the first time in Saudi Arabia. Phytoplasmas were detected from all diseased tomato samples by specific amplification of the 16S rRNA gene using nested Polymerase Chain Reaction (PCR). Successful amplification is representing the expected size when DNA extractions from symptomatic samples were used as a template. PCR products of approximately 1.8 kb were visible after direct PCR using primer pairs P1/P7 and 1.2 kb were amplified in the nested-PCR using the primer pairs R16F2n/R16R2n. Also 880 bp was obtained in nested-PCR using primer pairs fU5/rU3 after using primer pairs R16mF2/R16mR2 in the first run. Amplified fragments of nested PCR using primer pairs of U5/rU3 were cloned, sequenced and submitted in GenBank under accession numbers (KF017472, KF017473, KF017474, KF017475 and KF017475) for isolates Hasa1, Hasa5, Jizan7, Jizan10 and Jizan11, respectively. The analysis of the phylogenetic tree revealed that the obtained sequences belong to the cluster of *Candidatus phytoplasma* 16SrII group with identity between 99 and 100%. Also, RFLP putative patterns of obtained sequences with AluI, RsaI, HpaII and TaqI were compared with other sequences from 16SrI, 16SrII and 16SrIII. It expressed that the obtained sequences gave a similar pattern band to 16SrII. However, some different fragments were detected compared with 16SrI and 16SrIII. To our knowledge this is the first report and identification of phytoplasma 16SrII associated with disease in tomato in Saudi Arabia. Further studies are needed to investigate the phytoplasma 16SrII disease throughout the country.

Key words: Phytoplasma, tomato, *Lycopersicon esculentum*, Saudi Arabia, nPCR

INTRODUCTION

Diseases caused by Phytoplasmas occur worldwide in many crops and more than 700 distinct diseases, attributed to phytoplasma, affecting hundreds of plant genera, have been reported (Parrella *et al.*, 2008). Phytoplasmas are plant pathogenic, unculturable wall-less bacteria that are associated with a wide variety of economically important plants (McCoy *et al.*, 1989; Seemuller *et al.*, 2002). In nature phytoplasmas are transmitted by homopterous insect vectors (Chiykowski and Sinha, 1989). Phytoplasma diseases have been determined on the basis of the host range and characteristic symptoms induced in the plant hosts and, nowadays, molecular analysis is used increasingly to identify and differentiate phytoplasmas (Al-Saady *et al.*, 2008).

Phytoplasmas and mycoplasmas are two pathogenic bacteria in the class Mollicutes (Namba, 2011). Recent large-scale phylogenetic studies groups of important using available genome sequences suggested that Mollicutes form a monophyletic clade and are closely related to lineages in the Phylum Firmicutes, such as Bacilli and Clostridia (Wu *et al.*, 2009; Chen *et al.*, 2012). Phylogenetic analysis of the 16S rRNA gene sequence has revealed that phytoplasmas constitute a coherent genus of the Mollicutes (Lee *et al.*, 1998, 2006; Seemuller *et al.*, 2002). Based on RFLP analysis and sequencing of the 16S rRNA gene it has been possible to demonstrate the occurrence of a diversity of phytoplasmas (Bedendo *et al.*, 1998; Montano *et al.*, 2000; Barros *et al.*, 2002; Amaral *et al.*, 2004). Currently they are divided into 18 groups and more than 40 subgroups based upon RFLP analysis of the 16S rRNA gene sequences and a total of 24 members of *Candidatus phytoplasma* have been proposed on 16S rRNA gene phylogeny (Arocha *et al.*, 2005; Valiunas *et al.*, 2006; Lee *et al.*, 2006). The computer-simulated Restriction Fragment Length Polymorphism (RFLP) analysis of the 16S rRNA gene sequences streamlines the classification of phytoplasmas and 28 phytoplasma groups and 50 subgroups have been identified (Li *et al.*, 2012; Wei *et al.*, 2007; Zhao *et al.*, 2009). PCR amplification of ribosomal RNA genes and the 16S-23S rDNA intergenic spacer region (ISR) have become the conventional means of detecting and identifying them because the phytoplasmas are non-culturable (Bosco *et al.*, 2002; Davis *et al.*, 1997; Liu *et al.*, 2011; Smart *et al.*, 1996). This study aimed to characterize five isolates of phytoplasma that are associated with tomato plants (*Lycopersicon esculentum*) in the Eastern Province and Jizan, Saudi Arabia using PCR amplification of ribosomal RNA genes and the 16S-23S rDNA Intergenic Spacer Region (ISR) and nucleotide sequence for the amplified fragments.

MATERIALS AND METHODS

Source of healthy and diseased tomato plants: Tissue samples of tomato variety *L. esculentum* were collected from different locations in the Eastern Province (Alhasa) and Jizan in Saudi Arabia (Fig. 1) showing yellowing, witches broom appearance, stunting and malformations along with symptomless samples that were collected.

Total DNA extraction: Two DNA extraction methods were used to extract the total DNA small scale from symptomatic and healthy tomato plant tissues. The first extraction method used was that described by Rezk (2006). One hundred milligram of leaf tissue was ground in 0.5 mL extraction buffer (50 mM EDTA, 100 mM Tris-HCl and 500 mM NaCl) and incubated at 65°C for 10 min. Centrifugation at 10,000 rpm for 10 min was used and the aqueous phase was transferred to a clean tube and diluted 1:10 in distilled deionized water and kept at -20°C until using PCR. The second method for DNA extraction used a DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions.

Polymerase chain reaction (PCR) amplification of 16S rRNA: DNA from symptomatic and asymptomatic tomato tissues was extracted as described above and amplified using direct and nested PCR. The phytoplasma universal primer pairs P1/P7 (Table 1) for direct PCR were used. Phytoplasma universal primer pairs R16F2n/R16R2n (Table 1) for nested PCR, derived from conserved regions of the 16S rRNA gene sequence (Nejat *et al.*, 2009), were used to amplify the phytoplasma ribosomal RNA gene. Universal primer pairs P1/P7 were used to amplify a ~1.8 kb fragment that encompasses the entire 16S rRNA gene (Table 1), 16S-23S intergenic spacer region and the beginning of 23S rRNA gene. Primer pairs R16F2n/R16R2n were used for nested PCR to



Fig. 1: Samples were collected from the East and South of Saudi Arabia (Alhasa and Jizan)

Table 1: Showing the name, nucleotide sequences and references of the used primers to amplify the 16S rRNA genes

Primer name	Sequences 5'-----3'	Expected size (bp)	Reference
P1	AAG AGT TTG ATC CTG GCT CAG GAT T	1784	Deng and Hiruki (1991)
P7	CGT CCT TCA TCG GCT CTT		Smart <i>et al.</i> (1996)
R16F2n	ACG ACT GCT AAG ACT GG	1239	
R16R2n	TGA CGG GCG GTG TGT ACA AAC CCC G		Gundersen and Lee (1996)
R16mR1	CTT AAC CCC AAT CAT CGA C	1416	
R16mF2	CAT GCA AGT CGA ACG GA		
fU5	CGG CAA TGG AGG AAA CT	876	
rU3	TTC AGC TAC TCT TTG TAA CA		Lorenz <i>et al.</i> (1995)

amplify a ~1240 bp fragment of 16S rRNA gene internal to the P1/P7 priming sites. Also, the phytoplasma universal primer pairs R16mF2/R16mR1 (Table 1) were used for direct PCR (first run) and followed by nested PCR using phytoplasma universal primer pairs fU5/fU3 (Table 1), derived

from conserved regions of the 16S rRNA gene sequence, used to amplify the partial sequence of 16S ribosomal RNA gene. Universal primer pairs R16mF2/R16mR1 were used to amplify a 1416 bp fragment that encompasses the entire 16S rRNA gene. Universal primer pairs fU5/fU3 were used to amplify a 876 bp fragment (Table 1). PCR were performed in all cases, in 25 μ L volumes containing 2 μ L of DNA template (in both extraction methods), 1 μ L of each primer (10 pmol), 2.5 μ L of 2.5 mM dNTPs, 2.5 μ L of $MgCl_2$, 2.5 μ L of 10 \times Taq polymerase buffer and 1.5 units of Taq DNA polymerase (Fermentas). First round amplification with P1/P7 primers was performed in a thermocycler using an initial denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 45 sec, 55°C for 1 min and 72°C for 2 min and final extension at 72°C for 10 min. PCR product of the first round was diluted 1:80 and 1 μ L of diluted P1/P7 reaction product was used as a template in nested PCR using primer pairs ofR16F2n/R16R2n under the same conditions as described in the first round. The same PCR programme at the same previous condition of PCR was used for another two sets of primers R16mF2/R16mR2 in the first run followed by fU5/rU3 for nested PCR. Aliquots of 7 μ L of each reaction mixture of the first round and nested PCR were analysed by 1% agarose gel electrophoresis using TBE buffer (90 mM of Tris-borate, 2 mM EDTA) as a running buffer. Gel was stained with Ethidium bromide and visualized by UV trans-illumination and photographed. DNA size marker (1 kb DNA marker Promiga, USA) was used to estimate the sizes of the PCR products.

Cloning, sequencing and alignment analysis: Obtained fragments of nested PCR using the set of primers fU5/rU3 were purified from agarose gel using the Qiaquick gel extraction kit (Qiagen). The purified PCR products were cloned into pGEM-TEasy vector (Promiga, USA) according to the manufacturer's instructions and sequenced. The ligated DNA was transformed into *Escherichia coli* strain DH5 α . The transformants were plated on Luria-Bertani (LB) agar medium containing ampicillin (100 μ g mL⁻¹) and 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal) and incubated overnight at 37°C. Single white clones were transferred and grown at 37°C in LB broth containing ampicillin (100 μ g mL⁻¹) and the plasmid DNAs were prepared by the Mini preparation kit (Promiga, USA) and analysed by PCR. Plasmid ligated with insert was sequenced by Macrogen Inc., Seoul, Korea. Alignment analysis was performed using the online BLAST service of the National Center for Biotechnology Information (URL: www.ncbi.nlm.nih.gov/BLAST/).

Phylogenetic analysis: The phylogenetic tree was constructed from the multiple alignments using the DNAMAN software (Lynnon, Canada) using the neighbour-joining method (Saitou and Nei, 1987) and the Jukes-Cantor distance correction method (Jukes and Cantor, 1969). Sequences from clones of phytoplasma-associated disease fragments with tomato were aligned and compared among themselves and with sequences from other phytoplasma belonging to 16SrI, 16SrII and 16SrIII groups (Table 2).

In silico enzyme digestion: *In silico* restriction analysis and virtual pattern gel using the DNAMAN programme for the obtained five fragment sequences of KF017472, KF017473, KF017474, KF017475 and KF017475 were compared with AAY as 16SrI group, PYCD as 16SrII group and ESP as 16SrIII group. Each DNA fragment was digested *in silico* with four distinct-restriction enzymes (AluI, HpaII, RsaI and TaqI) that had been previously used for phytoplasma 16Sr rRNA gene RFLP analysis (Wei *et al.*, 2007; Tapia-Tussell *et al.*, 2012). The virtual image and PCR-RFLP pattern were compared.

Table 2: Acronyms GenBank accession No. of phytoplasma 16S rDNA sequences used to construct the phylogenetic tree

Acronym	Phytoplasma strain designation	Group	Accession No.
AAY	'American aster yellows'	16SrI	X68373
AY	Aster yellows phytoplasma	16SrI	AF222063
OY	'Onion yellows'	16SrI	D12569
SPW	<i>Phytoplasma</i> sp. STRAWB1	16SrI	U96614
ACLR-AY	Aster yellows phytoplasma strain ACLR-AY	16SrI	AY265211
PYCD	'Papaya yellow crinkle'	16SrII	YI0097
PnWB	'Peanut witches broom'	16SrII	L33765
TBB	Tomato big bud phytoplasma	16SrII	EF193359
WBDL	' <i>Candidatus</i> Phytoplasma aurantifolia'	16SrII	U15442
CPP	Cotton phyllodyphytoplasma	16SrII	EF186827
MPPT	Montana potato purple top phytoplasma	16SrIII	FJ226075
ESP	Eggplant stunt phytoplasma	16SrIII	EF378627
TBB-BA	Tomato big bud phytoplasma	16SrIII	AY863192
TBB-Br	Tomato big bud phytoplasma	16SrIII	AY863194
YTPP	Yucatan tomato Phytoplasma	16SrIII	FJ951627
Hasa 1	This study	-	KF017472
Hasa 5	This study	-	KF017473
Jizan 7	This study	-	KF017474
Jizan 10	This study	-	KF017475
Jizan 11	This study	-	KF017476

RESULTS

Disease symptoms: The infected tomato plants exhibited symptoms of chlorosis, little leaf witches broom-like symptoms, yellowing, stunting and malformations. Another symptom observed was that some infected plants produced leaf-like structures instead of flowers. Previously described symptoms are the evidence of the most common symptoms associated with the presence of these phytoplasma (Bertaccini and Duduk, 2009).

DNA extraction and PCR amplification: Two DNA extraction methods were used to extract the total DNA small scale from symptomatic and healthy tomato plant tissues. The first extraction method was that described by Rezk (2006) and the second method was that described on Plant Mini Kit (Qiagen). As in Fig. 2a, amplification-PCR products of approximately 1.8 kb were visible after the first round (direct PCR using primer pairs P1 and P7) and 35 cycles in the thermal-cycler machine for the infected tomato samples; however, no amplification was seen with the symptomless samples. Also, PCR products of approximately 1.2 kb were amplified in the nested PCR using the primer pairs R16F2n and R16R2n (Fig. 2a) and fU5/rU3 (Fig. 2b) from the extracted DNA in both extraction methods. It was demonstrated that the PCR products from the DNA extracted using the Qiagen kit were more concentrated than the other method using extraction buffer. Both of the extraction methods were used successfully to extract DNA for PCR amplification but the method described by Rezk (2006) was easier and lower in cost compared with the Qiagen kit method.

Nested PCR with two universal primer pairs R16mF2/R16mR1 and fU5/rU3 was used successfully to amplify 880 bp of 16SrRNA (Fig. 3). DNA extraction from tomato plant samples collected from different locations of Alhasa and Jizan, Saudi Arabia all had the same pattern. No amplification was observed with asymptomatic plant samples. This finding shows that disease-causing phytoplasma has occurred in South West and East areas of the kingdom.

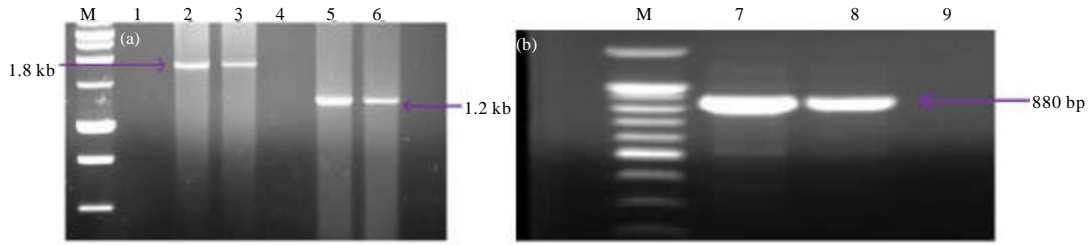


Fig. 2(a-b): (a) Amplification of phytoplasma rRNA gene products using primers P1/P7 with direct PCR and R16F2n/R16R2n primers with nested PCR, (b) Nested PCR using set of primers R16mF2/R16mR2 followed by nested PCR using set of primers fU5/rU3, Lanes (M) 1kb ladder in (a) and 100 bp marker in (b), (1, 4 and 9). Healthy tomato plants extracted with Qiagen Extraction kit; (2, 5 and 7). Amplification of phytoplasma from infected tomato plants using Qiagen DNA Extraction kit with P1/P7 primers (Lane 2), nested PCR with primers R16F2n/R16R2n (Lane 5) and nested PCR with primers fU5/rU3 (Lane 7) and (3, 6 and 8). Amplification of phytoplasma from infected tomato plants using extraction method as described by Rezk (2006), with P1/P7 primers (Lane 3), nested PCR primers R16F2n/R16R2n (Lane 6) and nested PCR with primers fU5/rU3 (Lane 8)

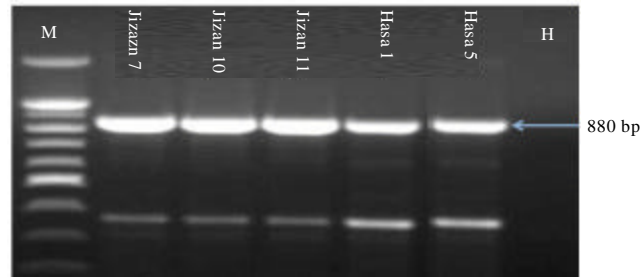


Fig. 3: A 1.5% agarose gel electrophoresis of nested-PCR amplified products using set of primers R16mF2/R16mR1 followed by fU5/rU3, M: 1 kb ladder; Jizan7, Jizan 10 and Jizan 11 are amplified PCR products from extracted DNA from symptomatic tomato plants collected from Jizan, Hasa1 and Hasa 5 are amplified PCR products from extracted DNA from symptomatic tomato plants collected from Alhasa and H: Asymptomatic tomato plant

Sequence analysis of amplified PCR products: PCR product obtained from infected tomato plants that were collected from Jizan and Alhasa using nested PCR with the primer pairs R16mF2/R16mR1 followed by fU5/rU3, were cloned into pGEM T-Easy vector and the sequences of the inserts were determined using primers fU5/rU3 (880 nucleotides). Sequences of the products were submitted into the GenBank under accession numbers KF017472, KF017473, KF017474, KF017475 and KF017476 for Hasa 1, Hasa 5, Jizan 7, Jizan 10 and Jizan 11 respectively. The comparison study of these sequences was carried out with other phytoplasma 16S rRNA genes from existing members in representative strains of 16SrI, 16SrII and 16SrIII groups that were selected

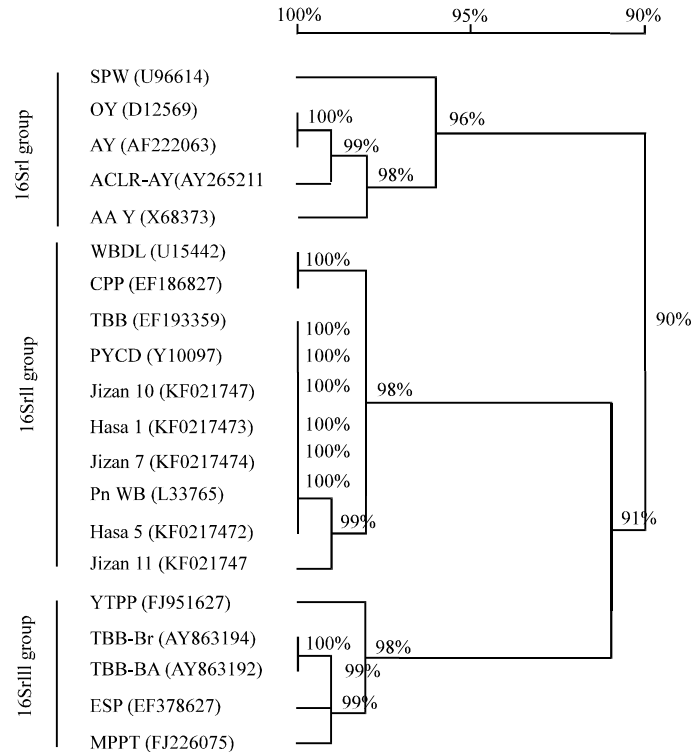


Fig. 4: Phylogenetic relationships among the tomato witches' broom phytoplasmas, (TWB) and selected phytoplasmas referenced in GenBank, including group 16SrII, GenBank Accession numbers are specified in brackets, The 0.01 bar indicates one nucleotide change per 100 nucleotides

from the NCBI database (Table 2). The analysis of the NCBI BLAST programme, followed by DNAMAN software using the tool BLAST, revealed 99-100% matches with *Mollicutes* sp. (associated with papaya yellow crinkle disease) (Y10097), peanut witches broom (L33765), tomato big bud phytoplasma (EF193359), 'Candidatus Phytoplasma aurantifolia' (U15442) and Cotton phyllody phytoplasma (EF186827) (Fig. 4). The obtained data showed that the sequences in this study belong to the cluster of Candidatusphytoplasma16SrII group (Fig. 4). However, the identity with the phytoplasmas located in the16SrI and 16SrIII groups were between 90-92% matches. The phylogenetic tree in Fig. 4 clearly demonstrated that the Hasa1, Hasa5, Jizan7, Jizan10 and Jizan11 isolates of phytoplasma disease associated with tomato belonged to the 16SrII group. Figure 4 shows that the Hasa 1 and Jizan 7 are more related together, but Hasa5, Jizan 10 and Jizan 11 are more related together. This finding leads to the suggestion that the distribution of that disease covers a wide area from South West to East KSA. Moreover, it seems the disease has two types of clusters, which can help the breeding for resistance programme.

RFLP *in silico* enzyme digestion: RFLP putative patterns with AluI, RsaI, HpaII and TaqI were characteristic of 16SrIII,16SrII and 16SrI (Tapia-Tussell *et al.*, 2012; Lee *et al.*, 1998). Virtual PCR-RFLP patterns (Fig. 5) revealed differences between Jizan and Hasa tomato phytoplasma isolates and other phytoplasma strains of group 16SrII as well as with 16SrI and 16SrIII groups used in this study. The *in silico* PCR-RFLP patterns from Jizan (Jizan7, Jizan 10 and Jizan 11)

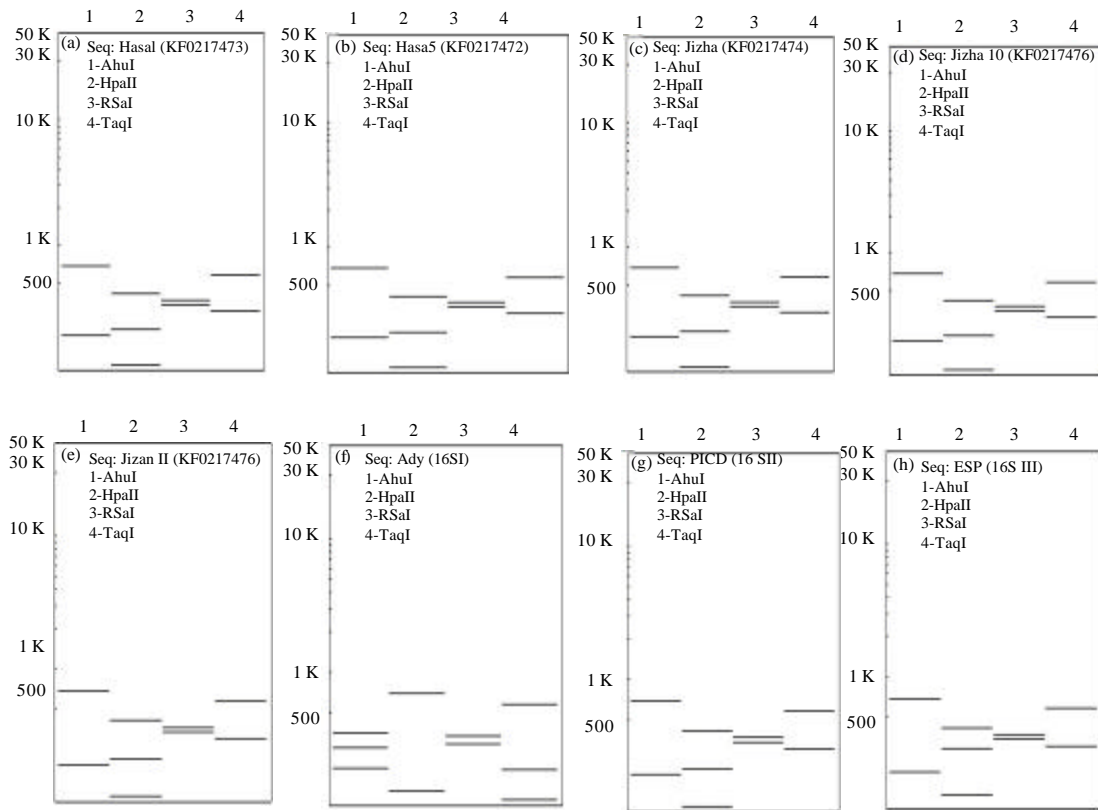


Fig. 5(a-h): Virtual PCR-RFLP patterns from *in silico* digestion of 16SrRNA gene using DNAMAN from obtained five isolates of phytoplasma (Hasa1, Hasa5, Jizan7, Jizan 10 and Jizan 11), a, b, c, d and e, respectively, compared with representing three different groups extracted from GenBank; AAY (16SrI) (f), PYCD (16SrII) (g) and ESP (16SrIII) (h) using enzymes AluI (1), HpaII(2), RsaI(3) and TaqI (4)

and from Alhasa (Hasa1 and Hasa5) tomato phytoplasma were similar to PYCD (Papaya yellow crinkle) that was described by White *et al.* (1998) which belong to the 16SrII group. However, some different fragments were detected in AluI, HpaII and TaqI patterns compared with 16SrI and 16SrIII.

DISCUSSION

Phytoplasma diseases of tomato have been previously reported in many countries including Poland (Krawczyk *et al.*, 2010), China (Xu *et al.*, 2013), Mexico (Tapia-Tussell *et al.*, 2012), Egypt (EL-Banna *et al.*, 2007), Brazil (Mello *et al.*, 2006), Jordan (Anfoka *et al.*, 2003), Turkey (Ozdemir *et al.*, 2009), France (Pracros *et al.*, 2006), Bolivia (Arocha *et al.*, 2007), Lebanon (Choueiri *et al.*, 2007) and Italy (Del Serrone *et al.*, 2001). These previous data mentioned that these reported phytoplasma belonging to groups I, II, III, V, VI and XII of 16SrRNA induce the diseases.

The results of this study indicated that the symptoms of yellowing, chlorotic and malformation of tomato plants were associated with phytoplasma disease in the collected samples from Alhasa and

Jizan, Saudi Arabia. Tapia-Tussell *et al.* (2012) characterized phytoplasma 16SrIII from tomato samples showing similar symptoms; however, in this study we find 16SrII. Among these groups 16SrI and 16SrII groups are the most common infecting tomatoes. Nothing is recorded 16SrII-phytoplasma group except tomato big bud from Australia (Martini *et al.*, 2007). Furthermore, samples were collected from two locations; one in the East (Alhasa) and the other is too distant (more than 1000 km) in the South-west (Jizan). Previous information indicated that the phytoplasma was recorded in some other crops in Saudi Arabia such as lime, Alfalfa and date palm (Alhudaib *et al.*, 2007, 2009). As far as we know this study is the first record of phytoplasma-associated disease that is affecting tomatoes in Saudi Arabia. 16S rRNA groups have been characterized all over the world using DNA-based techniques and sequence analysis (Anfoka *et al.*, 2003; Santos-Cervantes *et al.*, 2007, 2008). Restriction fragment length polymerase *in silico* patterns of enzyme digestion using AluI, HpaII, RsaI and TaqI (Fig. 5) have been used by many references and DNAMAN software was used for sequence analysis in this study. Groups of 16SrI, 16SrII and 16SrIII were used to compare data. The virtual PCR-RFLP patterns (Fig. 5) revealed differences between strains in groups 16SrI and 16SrII. The sequences of Hasa and Jizan phytoplasma-diseased samples were similar to those described as Papaya yellow crinkle (White *et al.*, 1998) which belongs to 16SrII. There was a different pattern between two groups (16SrI and 16SrIII). Also, all the isolated strains (Hasa1, Hasa5, Jizan7, Jizan 10 and Jizan 11) show the same patterns and same size as AluI (1), HpaII (2), RsaI (3) and TaqI (4) that same gave with 16SrII and this referred to the phytoplasmas in this study belong to the 16SrII group.

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