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Genomic Variability in Alfalfa Mosaic Virus Isolated from Alfalfa Plant (*Medicago sativa*) in Saudi Arabia and the Development of RT-PCR Procedures for Accurate Virus Detection

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Alfalfa mosaic virus **(AMV), a pathogen of a wide range of plant species, including *Medicago sativa* (Alfalfa) is considered as one of the most widely distributed viruses in Saudi Arabia. The molecular procedure, reverse transcription - polymerase chain reaction (RT-PCR) was developed in this study for the detection and identification of AMV in Alfalfa plants from Diyrab, Riyadh, Saudi Arabia. Alfalfa leaves were firstly screened for the presence of AMV by a standard double antibody sandwich ELISA and dot blot hybridization. AMV RNAs were easily detected in composite samples of 20 to 30 alfalfa leaves and the sequences of the coat protein (CP) gene of the isolates were determined. The primers designed on the basis of published sequences were applied for amplification of AMV RNA fragments in reverse transcription-polymerase chain reaction (RT-PCR) using infected plants with AMV. The results from this study were characterized AMV at the molecular level and their genetic relationships with other known AMV isolates were documented. Phylogenetic analysis indicated that all detected AMV isolates fell into two groups with the identified isolates from different origins. Similarity among one group ranged between (96 - 100%) and between the two groups was 42%. Evaluations showed that RT-PCR was sensitive and specific for AMV detection in Alfalfa leaf samples. The sequencing and alignment of the RT-PCR amplified fragments deposited in NCBI Gen Bank for eight different AMV strains (accession no. KF487083 - KF487090). Sequence comparison showed that these isolates of AMV shared 42% to 100% sequence similarity with seven AMV obtained from GenBank. This is the second report on the genetic variation of AMV isolates infecting alfalfa plant in Saudi Arabia. Further epidemiological studies into AMV spreading over the crops and the effect on the crop product quality are virtually needed.**

Key words: RT-PCR, Alfalfa Mosaic virus (AMV), *Medicago sativa*, Saudi Arabia.

Medics and clovers including forage grasses Alfalfa (*Medicago sativa* L.) have constituted the backbone of forage production crops in Saudi Arabia. Alfalfa (*Medicago sativa* L.) is one of the most important forage crops grown over a wide range of sod and climatic conditions. It has an ability to produce high yields without nitrogen fertilization with a high level of digestible protein[1]

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Unfortunately, in Saudi arabia Alfalfa (*Medicago sativa* L.) plant faces a very serious infection with Alfalfa mosaic virus (AMV) which has been well documented in many previous studies [2,3,4,5,6]. AMV has been found naturally infecting Black night-shade (*Solanum nigrum*), Datura (*Datura stramonium* L.), Eggplant (*Lycopersicon esculentum* Mill.), Goosefoot (Chenopodium *spp*), Potato (*Solanum tuberosum* L.)[4] generally AMV naturally infects many herbaceous and some woody plant hosts, and causes diseases of many economically important crops including the families Solanaceae and Leguminosae[7]. AMV is a species of the genus

Alfamovirus, family *Bromoviridae*[8] that infects more than 400 plant species, including several vegetable and woody crops[9]. The genome of AMV consists of three single stranded RNA molecules (RNA 1, 2, 3). RNA 1 and 2 encode viral replicas proteins P1 and P2, respectively. The viral coat protein (CP) gene located downstream of the MP gene in RNA 3 which translated via a subgenomic RNA, RNA_4 [10,11,12]. Viral movement protein (MP) is translated directly from RNA 3 [13,14]. The three genomic RNAs are not infective. Infection starts only in the presence of RNA4 or its translation product (CP)[15].

Bright yellow mottle or mosaic is common Symptoms of AMV. Seeds of alfalfa, pepper and weeds (*Datura stramonium* L., *Solanum nigrum* L.,) are preserve and transmit AMV. At least 15 aphid species are known to transmit the virus [16]. Polymerase chain reaction (PCR) is an exceptionally sensitive and a particular technique for the detection and identification of plant pathogens [17,18]. RT-PCR was used to confirm the presence of AMV in the seeds of naturally infected pepper and Alfalfa plants and potato samples respectively, using primers that direct the amplification of the cp gene [19, 20, 21]. These studies confirmed that, the RT- PCR was a more sensitive method than Enzyme-linked immunosorbent assay (ELISA) for the detection of AMV isolates in all tested materials.

Identification and characterization of Saudi AMV alfalfa isolates and their comparison to AMV strains from other countries will lead to a better understanding of the similarities and differences between AMV alfalfa isolates and will help to design a proper approach for the detection and management of this virus in Saudi Alfalfa plants. The main objective of this work was to build up reverse transcription - polymerase chain reaction (RT-PCR) assays for the detection and quantification AMV directly from Alfalfa plants; and evaluate this method as an alternative detection assay for AMV.

MATERIAL AND METHODS

Source of Virus Isolates, Serological and Biological Identification

Leaves of Alfalfa plants exhibited alteration and mottling symptoms assumed to be virus were collected from Dirab (North of the Riyadh region), Saudi Arabia in 2011. Samples were tested serologically using Direct ELISA against AMV (Agdia Co., France). Samples were chosen for further plant host range categorization. *Chenopodium amaranticolor* Cost and Reyn, C. quinoa, *Vigna unguiculata*, *Pisum sativum*, *Vicia faba*, *Nicotiana tobacum*, *N. benthamiana*, *Capsicum annum*, *Cucumis sativus*, *N. rustica* and *S. tuberosum* were chosen. The sap was extracted and ground separately in phosphate buffer (1:3 w/v, 0.1 M, pH 7.2), passed through a double layer of cheese cloth and mechanically inoculated on to carborandoum (600 mesh)-dusted leaves by gently rubbing the freshly prepared inoculums[22]. Resulting symptoms were taken and inoculated in to Alfalfa plant. Appeared symptoms were expressed, described and recorded.

Dot Blot Hybridization

The total clarified sap prepared from infected plant materials was diluted ten-folds with 6X SSC buffer, heated to 95ºC for 10 min, and chilled on ice. A volume of 5 µL of total RNA extracted from each of the symptomatic and healthy samples were collected, prepared and directly applied to nitrocellulose membranes according to established methods [23,24]. Pre-hybridization, hybridization, and colorimetric detection with a single digoxigenin labeled probe were carried out using a hybridization oven (Amersham Biosciences, Piscataway, NJ, USA) following the protocol recommended by Boehringer Mannheim. The results were documented by photography.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted from twelve expressed as positive for AMV by ELISA. These samples were extracted using the Isolate Plant RNA Mini Kit (Bioline). The oligonucleotide primers designed according to [22] were as follows, the upstream primer AMV-coat-F: 5' - CCATCATGAGTTCTTCA CAAAAG-3'] and downstream primer AMV-coat-R: 5' - TCGTCACGTCATCAGTGAGAC-3'] were used. RT-PCR was performed using a thermal cycler (Eppendorf, Germany) according to [22]. DNA was visualized and photographed using DNA documentation gel analysis (IN GENIUS, Syngene Bio Imaging, UK).

Nucleotide Sequence and Phylogenetic Analyses

Virus-specific RT-PCR products were

sequenced using two directional sequencing with the AMV-coat-F and AMV-coat-R primer specific for AMV-CP[22] using an Applied Biosystem AB3730xI DNA analyzer (Life Technologies, USA). The partial nucleotide sequence of the CP gene was subjected to a Blast N search for comparison with published AMV-CP gene sequences retrieved from GenBank. The multiple sequence alignment and phylogenetic relationships for the AMV isolates available in GenBank (Table 1) were analyzed and reconstructed using Lasergene DNASTAR, V5-05.

RESULTS

Serological and Biological Identification

Positive indication to AMV by ELISA was observed in the tested samples. Isolates were inoculated mechanically onto various plants to determine the host range. Variable symptoms and chlorotic lesions were observed.

Dot Blot Hybridization Assay

Dot blot hybridization was used to detect the virus in infected Alfalfa plant tissues. Fig. 1 shows a moderate reaction (blue signal) resulting from dot blot hybridization of the DIG labeled probe with nucleic acids extracts from all Alfalfa infected leaves collected from Dirab. However, hybridization was not observed i the probe and total nucleic acids from uninfected Alfalfa leaf tissue.

RT-PCR

Fig. 2 shows electrophoresis analysis of the RT-PCR products of AMV-CP gene generated from leaf samples from infected Alfalfa plants collected from Dirab, Riyadh, SA (lanes 3,4,5,7,8,9,11,12,13) regions. No fragments were amplified from the RNA extracted from healthy plants. Syrian isolate represented the positive control.

Table 1. References, source and origin of the eight AMV isolates isolated from different countries

AMV	Reference	Source	Origin
K02703	25		USA
DQ314750-CP	22	potato	Canada
FJ858265.1	26	*Carica papaya*	Brazil
FN667967.1	27	*Lavandula stoechas*	Italy
U12509.1	unbublished	Alfalfa *(Medicago sativa)*	New Zealand
AF215663.1	28	Peas	New Zealand
Y09110.1	unbublished	*Lycopersicon esculentum*	Italy
Jehan 1 KF487082	6	Alfalfa *(Medicago sativa)*	Saudi arabia

Table 2. The GenBank accession numbers of the AMV Saudi Arabian isolates from alfalfa leaves

Accession number	Names
KF487083	Alfalfa mosaic virus isolate Jehan2 capsid protein gene, partial cds.
KF487084	Alfalfa mosaic virus isolate Jehan3 capsid protein gene, partial cds
KF487085	Alfalfa mosaic virus isolate Jehan4 capsid protein gene, partial cds.
KF487086	Alfalfa mosaic virus isolate Jehan5 capsid protein gene, partial cds.
KF487087	Alfalfa mosaic virus isolate Jehan6 capsid protein gene, partial cds.
KF487088	Alfalfa mosaic virus isolate Jehan7 capsid protein gene, partial cds.
KF487089	Alfalfa mosaic virus isolate Jehan8 capsid protein gene, partial cds
KF487090	Alfalfa mosaic virus isolate Jehan9 capsid protein gene, partial cds.

Nucleotide Sequence and Phylogenetic Analyses

Total RNAs extracted from Alfalfa leaves were confirmed to be positive for AMV by ELISA, dot blot hybridization and RT-PCR, which flank the entire CP gene of AMV, resulting in PCR amplicons of (310 – 603 bp). Sequence comparisons showed that the percentage of similarity between AMV from Saudi isolate and eight reported isolates of AMV obtained from GenBank ranged from 96 to

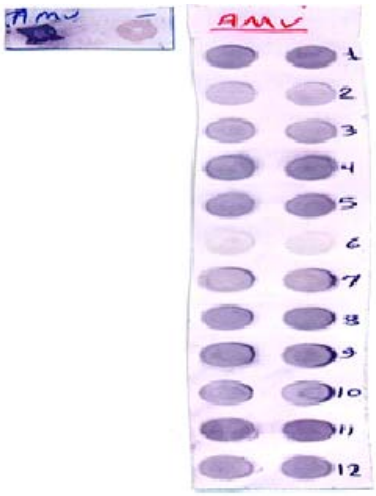

Fig. 1. Dot blot hybridization of the AMV DIG-cDNA probe with the total RNA extracted from the infected Alfalfa leaf samples. Row: (1-12) represent samples collected from the Dirab. Weak hybridization reaction was observed with Alfalfa samples (Row 2, 6).

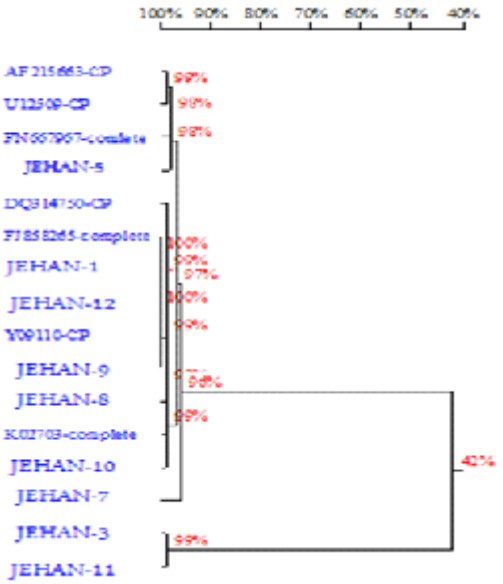

Fig. 2. 1.5% Agarose gel electrophoresis of Reverse-Transcription Polymerase Chain Reaction (RT-PCR) products obtained from RNAs extracted from *Alfalfa Mosaic Virus* (AMV) - infected Saudi Alfalfa plant leaves. All RT-PCR tests were performed using primer set AMV-F/AMV-R. Lanes M, DNA Ladder….. Lanes (3, 4, 5, 7, 8, 9, 11 and 12) are AMV infected A*lfalfa* tissue. Total RNA extract for Syrian isolate was used in the RT-PCR a control. The size of PCR products was between (310 - 351 bp)

100%, similarity. Table 1 shows the references, source and origin of the isolates from the genbank. The AMV Jehan 3 was closely related (99%) to Jehan 11 and both were related (42%) to the seven isolates from database (genBank). Furthermore, Jehan 5 was closely related (98%) to AMV (AF215663.1), New Zealand (U12509.1) isolate isolated from Alfalfa and Italy (FN667967.1) isolate isolated from *Lavandula stoechas*. Jehan 10 shared a high sequence identity (99%) with isolate (DQ314750-CP), Canada isolated from potato. Moreover, Jehan 9 had similarity (100%) with (FJ858265.1) isolated in Brazil from *Carica papaya*, Saudi isolate (Jehan 1, KF487082) isolated from Alfalfa plant and (Y09110.1) isolated from tomato. Jehan 7 had similarity (96%) with all genbank isolates. Jehan 8 shared (99.1%) sequence identity with (K02703) USA isolate and Canadian isolate (DQ314750-CP) as shown in Fig. 3.

The partial nucleotide sequences containing the CP genes of the AMV-Saudi Arabian isolates were deposited in the GenBank under the accession number no. KF487083 to KF487090 (Table 2).

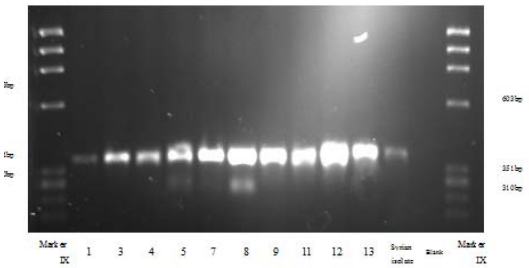

Fig. 3. Phylogenetic dendrogram shows the relationship of the Saudi *Alfalfa Mosaic Virus* (AMV) isolates and other known AMV strains based on alignment of nucleotide sequence of the coat protein gene. The bar indicates the relative evolution distance

DISCUSSION

AMV is a world-wide distributed virus with a very wide host range[9], considered as one of the most widespread viruses in the Saudi Arabia and other countries [22,29,30,31].

In the current investigation AMV was detected in Alfalfa samples collected from an experimental farm in Dirab, Riyadh, Saudi Arabia. AMV isolates were characterized by ELISA, RT-PCR and sequence analysis of the CP gene

detection of AMV isolates was confirmed based on biological test, serological reactions, Dot blot hybridization, RT-PCR and sequencing. Host range studies and serological reactions with AMV antisera showed that isolated isolates from Alfalfa were of the Alfamoviruses.

Dot blot hybridization showed that the DIG-labelled cDNA probe was very sensitive for detection of AMV in infected Alfalfa plants and are more convenient for testing of large numbers of samples the. Detection of AMV and other viruses and viroids in infected plants using dot blot hybridization was documented [32].

RT-PCR product from infected Alfalfa tissue was matching to that of the (310 – 603 bp) CP gene of AMV. According[22] the primers for detection (AMV coat-F and AMV coat-R) were specific for AMV RNA. RT-PCR assays have been successfully utilized to detect viruses from infected Alfalfa leaves which were early confirmed [6].

Dot blot hybridization showed sensitivity for detection of AMV in infected Alfalfa plants which was more sensitive and convenient than ELISA. Data generated from sequence analysis expressed that Saudi Arabian isolates of AMV isolated from Alfalfa plants shared 96% to 100% sequence similarity with the seven reported isolates of AMV obtained from GenBank. These slight differences in the nucleotide sequences in the CP gene could be attributed to the fact that it might be a different strain because only partial CP gene was detected.

RT-PCR was used to detect the AMV in Alfalfa seeds, paper, Lilium plants of various species and potato tuber [19,20,21,22,31] which confirmed that RT-PCR is more sensitive method than ELISA or dot blot hybridization or for detection of the presence of AMV.

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