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Biological, Serological and Molecular Characterization of Papper MildMottle Virus Isolated from Weast Region of Kingdom of Saudi Arabia

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Abstract

In this paper, The virus was identified based on host range, symptomatology, and stability in crude extracts, modes of transmission, serological reaction, inclusion bodies, electron microscopy and molecular biology. Study ofthe host range which inoculated different plant species belonging to twelve families revealed that the reactions different hosts. Data concerning stability of virus in crude sap were TIB95C^{0},DEP10^{-7}-10^{-8} and LIV 28 days. Modes of transmission showed that PMMoV transmitted mechanically and by infected seeds but cannot be transmitted byany of the tested aphid species.A one-step reverse-transcription polymerase chain reaction (RT-PCR) assay was used for the detection and identification of the isolated virus from nucleic acid extracts of infected pepper plants .Using specific oligonucleotide primer PMMoV-F/ PMMoV-R, which amplified the coat protein (CP) gene for detection of *Pepper mild mottle virus* (PMMoV). A major product of approximately 387bp PMMoV-CP gene was produced. DNA-DNA hybridization assays of viral genome have been used for detection of the present virus isolate using specific cDNA probe prepared for PMMoV. A one-step reverse-transcription polymerase chain reaction (RT-PCR) assay was used for the detection and identification of the isolated *Pepper mild mottle virus* (PMMoV) from nucleic acid extracts of infected pepper plants.Using specific oligonucleotide primer PMMoV-F/PMMoV-R, which amplified the coat protein (CP) gene for detection of *Peppermild mottle virus* (PMMoV). A major product of approximately 387bp PMMoV-CP gene was produced. A high reliable sensitive immunocapture reverse transcription-polymerase chain reaction (IC-RT-PCR) technique was applied for detection of PMMoV in which the in infected pepper plant. PCR fragment of correct size 387bpwas amplified with primer specific coat protein gene. DNA-DNA hybridization assays of viral genome have been used for detection of the present virus isolate using specific cDNA probe prepared for PMMoV. This study of PMMoVis carried out for the first time in Taif Saudia Arabia.

Keywords: Pepper, mild mottle virus, antibodies, purification, UV-absorption spectrum

Introduction

Pepper is grown for its fruits are eaten fresh, a green before fully ripe or after completion of maturity and colored, and the fruits of the richest pepper vegetables in vitamin C as it is relatively rich in vitamin A and also contain moderate amounts of niacin and iron [27]. Pepper (*Capsicum spp*.) is one of the most diverse vegetable species and is considered to be a high value crop. Viral diseases in field-grown pepper (Capsicum annuurn L.) plants are a major concern for the pepper growers and the processing industry. Pepper growing areas have been affected by several viruses, including *Cucumber mosaic virus* (CMV), *Pepper mottle virus* (PepMoV), *Tobacco etch virus* (TEV) *Tomato spotted wilt virus* TSWV), *Alfaalfa mosaic virus* (AMV), and *Potato mosaic virus'* (PVY) [2]. Tobamoviruses are rigid rod-shaped plant viruses that are very easily transmitted to healthy plants by mechanical inoculation and contact between plants. They

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are exceptionally stable viruses that are known to survive for years in plant debris in the soil and for weeks or months on greenhouse structures, pots and horticultural tools. They persist on clothing and, most importantly, on workers hands. TMV is known to survive in cigarettes and cigars made from infected tobacco leaves and to be transmitted from the hands of a smoker to susceptible plants. In addition, TMV, ToMV and PMMoVcan be transmitted on the surface of seeds harvested from infected plants. There is no known insect vector for these viruses. The predominant method of spreading a tobamovirus is the handling of plants. This makes human beings the main vector of these viruses, Symptoms consist of various degrees of mottling, chlorosis, curling, distortion and dwarfing of leaves, flowers, fruits and entire plants .In some cases necrotic areas develop on leaves and fruit Symptoms are most easily seen on new growth and are usually more pronounced on plants infected when young than on those infected when mature [**4**,**8**,**51**] reported that *Pepper mild mottle virus* (PMMoV) was detected in 27 pepper (*Capsicum* spp.) plants of 3000 tested and found to be present two regions in Turkey. Results of reverse transcription-polymerase chain reaction (RT-PCR) using prim capsid protein (CP) genes confirmed those of ELISA by amplifying all PMMoV-infected plants. This is the first molecular information on PMMoV isolates present in Turkey, and Tunisia for which this information could have guiding significance in future pepper resistance breeding in theses country [**24**,**51**]. reported that a new method to discriminate tobamoviruses and *Pepper mild mottle virus* (PMMoV) infect green pepper (*Capsicum annuum* L.) was developed using IC -RT-PCR and (RT-PCR) method were developed based on results from this study, which facilitateddetection of *Tobamoviruses* in pepper plants, seeds, and field soils. More recently, the immunocapture polymerase chain reaction (IC-PCR) has been shown to be a more sensitive method of identification than either ELISA or unmodified PCR. In fact, IC-PCR was developed for simultaneous detection of two pepper-infecting RNA viruses, *Pepper mild mottle virus* (PMMoV) and *Tobacco mild green mosaic virus* (TMGMV) [**35**]. PMMoV induces symptoms that are not readily distinguishable from those of other tobamoviruses infecting peppers. It is necessary, therefore, to use specific diagnostic procedures to identify the virus.Among peppers (*Capsicum baccatum*), the cultivar 'Aji Amarillo', native to the Andes, South America and Brazil. Plants of pepper 'Aji Amarillo' from a commercial crop in, showing leaf mosaic, were subjected to molecular tests, RT-PCR and nucleic acid hybridization [**11**].

The aim of the current study

Isolation and identification of PMMoV on the basis of host range, stability in sap, modes of transmission, inclusion bodies, serological studies and electron microscopy,detection of PMMoV V-RNA by antiserum production using in direct ELISA, detection of PMMoV using more sensitive and advanced methods including RT-PCR, IC-PCR and nucleic acid hybridization.

Materials and methods

Isolation and propagation of the virus isolate

Different samples from naturally infected pepper plants (*Capsicumannum*) during the growing season of 2011-2012. The observed symptoms were due to virus infection, were collected open field West region of Saudi Arabia and were used for isolation and identification of the virus under investigation. tar and pestle, then the juice was expressed through a double layer of chees cloth into sterilized container, was then used in mechanical inoculation (*Capsicum annum* L. cv. California Wonder) as described by [**46**].

Host range and symptomatolgy

The tested plant species were (twenty nine) different families were mechanically inoculated by the virus isolate to study host range. The classification, identification and nomenclature of the wild host plants. Inoculum was prepared as mentioned above. Ten, young seedlings of each species and cultivar were inoculated with the virus isolate in addition ten seedlings were left as control treatment. Five weeks later, symptomless plants were assessed for latent infection onto plants of *C. amaranticolor* and tested using ELISA.

Virus stability in crude sap

Using *Capsicum annum L.* as source plant and *Ch.amaranticolor* for at the 4-5 leaf stage as an indicator host plant forthe virus isolate.

Modes of transmission

Mechanical transmission

Ten healthy seedling of each tested species and cultivars were inoculated with pepper isolate. The inoculated plants were kept in the greenhouse under observation for external symptoms and away of any infection with other viruses up to 30 days.

Insect transmission

Myzusprasicae Sulz. and *Aphis craccivora. Koch* were tested to find out whether or not they transmit the virus isolate. Aphids were reared on cabbage seedlings under insect proof cage for a long period of experimental time. Filial generation were used in subsequent experiments. Virus-free aphids starved for one hour, were allowed to feed on virus-infected *C.annum.* L California Wonder plants for acquisition feeding period of 5 min. Viruliferous aphids (five aphids/plant) and then transferred to ten healthy seedlings (for each aphid species) of pepper plants for inoculation feeding period of 24 hours. The seedlings were used as control received the same numbers of non virusliferous insects which were feeding on healthy seedlings. The aphids were killed using systemic insecticide (Tafaban 0.15%). Symptoms and percentage of transmission were recorded for a period of one month.

Seed transmission

An experiment seed transmission carried out to study the

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transmission of the virus isolate through seeds of pepper cultivars (*Capsicum annum* L. cv. California Wonder). For each cultivars were sown with five seed in each of 20 cm- diameter pots containing sterilized soil and kept in an insect-proof greenhouse. Half of the result seedlings were mechanically inoculated as mentioned before with the virus isolate. The remainder of the seedlings were kept without inoculation to serve as controls plants were left tillmaturity. Later on, seeds were harvested from healthy and infected plants representing different treatments, separately at a rate of 400 seeds per each. The seeds were sown in pots and kept in an insect proof greenhouse. Emerged plants were observed for virus symptoms development and the percentage of seed transmission were calculated.

Examinations of inclusion bodies by light micro scopy

Light micro scopic examination was used for the determination of chemical nature of inclusion bodies, epidermal. Stripes taken from the lower surface of (*Capsicum annum cv.* California Wonder)Leaves inoculated mechanically with virus isolate (after 20 days from inoculation). Samples of healthy plants were also examined as acheck.Epidermal stripes were prepared and dipped in 5% Triton x-100 for 10 minutes to disrupt the plastids and facilitate the observation of inclusions. Theen the strips were stained by immersing in stain containing 10 mg bromophenol blue and 10 gm mercuric chloride in 100 ml distilled water for 15 min. The treated strips were then placed in 0.5% acetic acid for 15 min,then tap washed in waterfor 15 min and mounted in water [15]. Finally, the strips were examined with light microscope model Olympus Japan and photographs were picked up using camera model Olympus C-35AD-4, Japan. To detect the protein contents of inclusion bodies.

Serological detection of the isolated virus using direct ELISA

Enzyme linked immunosorbent assay (ELISA) Technique (DAS-ELISA) was used for rapid and sensitive serological detection method for pepper mild mottle virus(PMMoV). It was provided by SANOFI, sante, Animals, Paris, France, as described by [12].

Varietal susceptibility for PMMoV infection

Susceptibility of four pepper cultivars to PMMoV infection under greenhouse conditions was tested. Ten young potted seedling of four tested plants in this study were pepper cultivars wereAnahem,California wonder Marconi and yolo wonder inoculated mechanically with sap expressed from infected pepper leaves. An equal number of healthy seedlings of the same cultivars and egg were left without inoculation as controls. Healthy and inoculated seedling werekept in insect proof greenhouse and examined for external symptoms. Symptomless plants were checked for the presence of the virus using direct ELISA.

Molecular Biology Studies

The total RNA extraction and Reverse transcriptase -Polymerase chain reaction (RT-PCR)

Total RNA was extracted from both infected pepper leaves (*Capsicum annum* (collected from the field and pepper leaves that have been mechanical inoculated with the virus, using RNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. For isolation of PMMoV coat protein (CP) gene, a set of primers (PMMoV-F/PMMoV-R) was designed according to [33]. The forward primer (PMMoV-F) was 5′-TACTTCGGCGT-TAGGCAATC-3 and the reverse one (PMMoV-R) was 5′-GGAGTT-GTAGCCCAGGTGAG-3′. For first strand cDNA synthesis, an RT mix (One step RT-PCR kit, Qiagen), 25µl, consisting of 5µl of 5X first strand RT buffer, 1µl each of each primer (PMMoV-F and PMMoV-R), 1µl 10 mM dNTPs,1µl of Qiagen one step RT-PCR enzyme, 5µl of 5x Q-solution, and 8 µl nuclease-free water, was added to 3µl of the total RNA extracted from test tissue. The reaction mix was then incubated at 50°C for 30 min followed by treatment at 94°C for 2 min to inactivate the enzyme. The PCR program (a thermocycler of Uno II, Biometra, Germany) was as follows: 94°C, 2 mm; 35 cycles of 94°C, 30 sec; 54°C, 30 sec; 72°C, 1 min; and one cycle of 72°C, 8 min.

Detection of PMMoV using Immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR)

Immunocapture PCR (IC–PCR) has been applied according to [12,35]. In this technique PMMoVpresents in crude plant extracts of pepper was captured by polyclonal antibodies coating the walls of the PCR tube in which the PCR is carried out after the capture step. Plant extracts were decanted and the PMMoVtrapped in the tube was submitted to the PCR reaction without RNA isolation. IC-PCR technique has been performed as follows: the micro tubes were coated with IgG antibodies of PMMoV, 100µl, (kindly provided by [34] and incubated overnight at 4°C. The tubes were then washed three times with 300µl of phosphate buffered saline PBS (8g NaCl, 0.2g KH_2PO_4, 2.9 g $N_2HPO_4.12\ H_2O$ and 2g KCL). 300µg of infected pepper leaves were exposed to PBS buffer. Fifty microliter of the tissue extract were transferred to the pre-coated tubes and incubated overnight at 4°C. The tissue was washed again three times each 5 min with 100µl of PBS buffer and followed by a final wash with deionized water. Finally, RT-PCR reaction mix was added to the micro tubes after washing (50µl reaction mix/tube). RT-PCR was performed using a One-step RT-PCR kit (Qiagen) as described before.

Agarose gel electrophoresis

One and half gram of agarose was dissolved in an appropriate 100ml of Tris-acetate- EDTA buffer (TAE) (0.04 M Tris acetate and 0.001 M EDTA, pH 8.0). After cooling, gel was poured into the tray that has been prefitted with comb. The gel was immersed into the electrophoresis tank containing TAE buffer. The comb was then removed to expose the wells formed. The gel was stained with 1µl of 1% ethidium bromide to stain the

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DNA.Loading buffer, 1µl, bromophenol blue was added into each comb well in the gel with 8µl amplified nucleic acids (RT-PCR or IC-RT-PCR). A standard 100 bp (Super ladder-Low 100 bp ladder –AB gene)was used and treated in similar manner. The gel was run at 80 volt and the DNA band in the gel was observed under ultra violet light to specify its position according to the used fractions of the DNA molecular marker. The expected band size is about 387bp.

Nucleic acid hybridization -Non-radioactive technique

The obtained insert from PCR products was labeled according to the following PCR technique using the Genius™ System (Boehringer Mannheim Corp.).

Preparation of cDNA PMMoV probe

The IC-RT-PCR amplified fragments were used in this step as template to synthesize a specific PMMoV probe. Amplification was performed in thin-walled PCR tubes containing the following reaction mixture: 1 µl of IC-RT-PCR products, 0.5µl *Taq*DNA polymerase, 5µl of 10x PCR buffer [1x PCR buffer, 10 mMTris-Hcl, pH 8.3, 50 mMKcl, 0.01% gelatin (W/V), 1.5mM $MgCl_2$, 1µl dNTPs (dig labeled mixture), 1µl of 10pm of each primer and nuclease free water to a final volume of 50µl. The mixture was hot started for 2 min at 94°C in the DNA thermocycler then the PCR reaction was performed using the cycling parameters as described by [33]. The resulting probes were used in dot blot hybridization processes.

Dot blotting hybridization

300mg of pepper leaf tissues for both PMMoV infected and healthy plants were placed in microfuge tubes with 100µl of extraction buffer (0.2M potassium phosphate, 5 mM dithiothreitol, 0.1% Triton X-100, and10 mM 2-mercaptoethanol, pH 8.3), and then grounded using knots pestles. Equal volume of the denatured solution, 1X SSC (150mM NaCl, 15mM Sodium citrate, pH 7.0 and 50% formaldhyde), was added and heated at 60°C for 15 min then centrifuged in microfuge tubes at 10,000 rpm for 5 min [41]. Five µl of this dot blot supernatant and the previously extracted RNA were spotted onto nitrocellulose membrane. The PCR product of CP gene was also added to the nitrocellulose membrane as a positive control. Prehybridization, hybridization and immunological detection were carried out using the Genius II DNA Labelling and Detection Kit according to Boehringer Mannheim, IN System instructions.

Experiment result

Isolation and propagation of the virus isolate

Samples of naturally infected pepper plants were collected from western region of Saudi Arabia showing mottling, veinbanding, leaf malformation and plant stunting were used to prepare infectious crude sap for mechanically inoculated to the test plants. The virus isolated from such plants was biologically purified and inoculated onto pepper plants (Cupcicum annum) which developed the symptoms as those have seen in naturally infected plants. After isolation and biological purification, the identify of the isolate was confirmed serological.

Virus identification

The virus isolated was identification as *Pepper mild mottle virus* (PMMoV) basis on host range, symptomatolgy, virus stability, modes transmission, inclusion bodies, serological tests, electron micro scoby and molecular biology studies.

Host range and symptomatolgy

Reactions of twenty seven plants species and cultivars belonging to twelve families different to virus infection are summarized in (**Table1**). The presence or absence of the virus isolated was confirmed serologically using direct ELISA the tested plants could be divided according to their action into the following groups.

a-Plants reacted only with local symptoms

PMMoV production chlorotic local lesions on the inoculated leaves of chonopodiumamaranti color and ch.quinun, 4-7 days after inoculation by (PMMoV) (**Figure 1**).

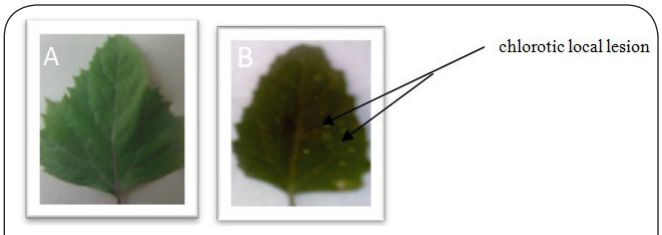

Figure 1. Chlorotic local lesion caused by PMMoV on chenopodium amaranticolor.
(**A**) healthy. (**B**) Infected leaf.

b-Plants reacted only with systemic symptoms

Systemic mottling occurred on pepper cultivar *Capsicum annum L.cvs.* California Wonder, Anaheim, yolo wonder and Marconi14-21 days after inoculation by *Pepper mild mottle virus* (**Figure 2-5**).

c-Plants species could not be infected with the isolated virus

Capsicum fratescens L.cv Chilli, Solanum lycopersicum Solanum melongena, S. tuberosum, Radish, Brassica, Allium, Allium cepa L oleracea Phaseolus vulgaris, Coriandrumsativum Petroselinum, Daucuscarota, Abelmoschusesculentus, Lactucasativa, *Menth, a* Origanummajorana, *Ocimumbasilicum,* camellia sinensis, Galiumodoratum.

1-2-2- Virus stability in crude sap

Thermal in activation point(TIP), Dilution end point (DEP) and longevity in vitro point were determined separately for PMMoV. Data in (**Table 3**) indicated that thermal inactivation point was $95C^{0,}$ dilution end point was 10^{-7} -$10^{-8}$and longevity

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Table 1. Reaction of different hosts inoculated mechanically with the virus isolate.

Fam	Tested plant	Symptoms	ELISA Reading
Chenopodiaceae	***Chenopodium quinoa wild***	CLL	0.947+
	C.amaranticolor	CLL	0.995+
Solanaceae	*Capsicum annum* L. cvs. California Wonder	SM,VB	0.822+
	Anaheim	SM,VB,Stu	0.955+
	Marconi	SN,DE	0.882+
	Yolo wonder	SM,VB	0.791+
	*Capsicum fratescens*L.cv Chilli.	0	0.322-
	*N. tabacum*L. cv. Samsun	SL	0.689+
	Solanum lycopersicum	0	0.244-
	Solanum melongena	0	0.298-
	S. tuberosum	0	0.301-
Cucurbitaceae	Cucmissativus L. cv. S. striped American	SL	0.642+
Cruciferae	Radish	0	0.222-
	Brassica oleracea	0	0.201-
Alliaceae	Allium	0	0.322-
	Allium cepa L	0	0.251-
Leguminosae	Phaseolus vulgaris	0	0.242-
Umbelliferae	Coriandrumsativum	0	0.303-
	Petroselinum	0	0.251-
	Daucuscarota	0	0.271-
Compositae	Lactuca sativa	0	0.252-
Malvaceae	Abelmoschusesculentus	0	0.311-
Lamiaceae (Mint Family)	*Mentha*	0	0.235-
	Origanummajorana	0	0.301-
	Ocimumbasilicum	0	0.324-
Theaceae	camellia sinensis	0	0.282-
Rubiaceae	Galiumodoratum	0	0.245-

Abbreviations for symptoms
DE: Deformation; Stu: Stunting; NLL: Necrotic local lesions
SL: Symptomless; M: Mosaic or mottle systemic; 0: No infection
CLL: Chlorotic local lesion; VB: Vein banding; -: Negative Reaction +: Positive;
*Control of direct ELISA absorbance at 405nm=232

Figure 2. Mottling, chlorosis, curling and deformation symptoms caused by PMMoV on Capscium annum cv. Anaheim.

Figure 3. Stunting ,vein banding and leaf rolling curling, and distortion symptoms developed in California Wonder due to PMMoV infection.

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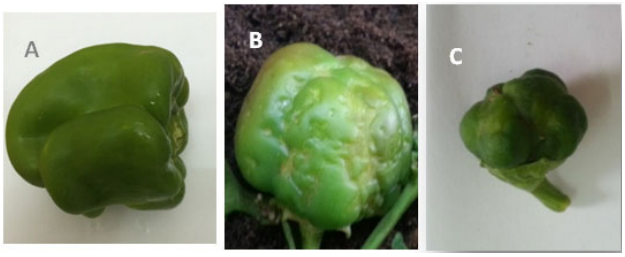

Figure 4. Severe symptoms such asdistortion, chlorotic spots, white areas, large necrotic blotches on fruits and small fruit of infected pepper plantscapsicum annum cv. yolo wonder with the virus isolate.
A: healthy **B** and **C**: Infected leaf.

Figure 5. Sever symptoms, deformation,clorosis, chlorotic spots on fruits of infected pepper plants with PMMoV.
A: infected fruit of PMMoVand **B**: healthy fruit.

in vitro point was 28 days at room temperature.

Results obtained from the study stability in crude sap of PMM ovare as follows (**Table 2**).

Modes of transmission

Mechanical transmission

Result showed that PMMoV was readily transmitted by mechanical inoculation by sap extracted from infected Pepper of *Capsicum annum.cvs*. California wonder, Marconi, Anahem and Yolo wonder were (40-70-100)% respectively.

Table 2. Results obtained from the study stability in crude sap of PMMov.

Characters	Result
(T. I. P)	95 °C
(D. E. P)	10^{-7}-10^{-8}
(L. I. V)	28 days

Table 3. The percentage of seed transmission.

Capsicum annum.cvs.	Tested plans infection
California wonder	0
Anaheim	30%
Marconi	10%
Yolo wonder	20%

Insect transmission

The virus under investigation was not transmitted by either *Myzuspersicaeor Aphis craccivora*. Non of tested plants produced any characteristic symptoms.

Seed transmission

Result revel that the isolated virus was transmitted through seeds of pepper plants. The percentage of seed transmission ranged from10%-30%. According to pepper plants. (California wonder, Marconi, Anahem) and these results were confirmed by ELISA tests (**Table 3**).

Examinations of inclusion bodies by light micro scopy

Amorphous cytoplasmic inclusions induced by PMMoV was observed with light microscopy in effected epidermal strips of capsicum annum cv. California wonder infected with PMMoV 21 day spot inoculation revealed amorphous inclusions (X bodies)′ whereas inclusions were not observed in *Capsicum annum*. (**Figures 6** and **7**).

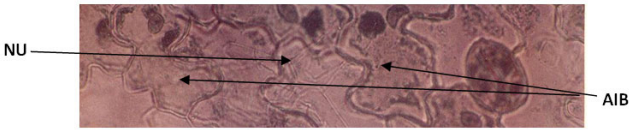

Figure 6. Light microscopy of epidermal strips of capsicum annum leaf infected with PMMoV showing amorphous inclusions(AIb) bodies (X-400). Nu=nucleus. AIB=amorphous inclusions.

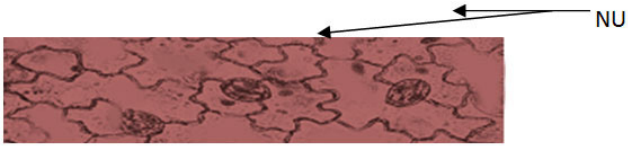

Figure 7. Healthy epidermal cells of capsicum annum leaf (X-400), Nu=Nucleus.

Serological detection of the isolated virus using direct ELISA

The virus isolate was identified byDAS-EL1SA. Against PMMoV using ELISA kits supplanted from SANOFI, Sante Animal, Paris, France. Positive reaction was obtained only with the virus isolate and its corresponding antiserum but it did not react with sap from healthy plants as illustrated by absorbance at 405nm.

Varietal susceptibility for PMMoV infection

Four varieties of capsicum annum were tested to evaluate. Their susceptibility to infection with PMMoV. Results in table demonstrate that, pepper cultivars were susceptible Anaheim and yolo wonder 100% following by Marconi 70%, the other hand California Wonder cultivar was the more resistant one 40% (**Table 4**).

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Table 4. Response of four cultivars to artificial infection.

Cultivar	%of infection
California Wonder	40
Anaheim	100
Marconi	70
Yolo wonder	100

Molecular Biology Studies

Reverse transcriptase -Polymerase chain reaction (RT-PCR) and Immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR).

This study was conducted to develop an RT-PCR and IC-RT-PCR assays for simultaneous detection of PMMoV in pepper that would improve detection sensitivity and specificity compare to the ELISA systems currently in use. RT-PCR and IC/RT-PCR assays successfully and simultaneously detected PMMoVtobamovirues. Electrophoresis analysis for the RT-PCR products showed clear bands at the expected size, from amplification of the coat protein (CP) gene of PMMoV infected pepper samples. RT-PCR and IC-PCR products are corresponding to 387bp (lanes1, 2 and 3) (**Figure 8**). No virus was detected in either healthy sap negative control (lane 4).

Preparation of digoxigenin-labeled PMMoV-cDNA probe

The diagnosis of PMMoV in infected pepper plant using Dig-11-dUTP labeled PMMoV- probe was performed directly on dot blot as a tool for nucleic acid hybridization technique.

Nucleic acid hybridization -Non-radioactive technique

Nucleic acid dot blot hybridization with PMMoV-CP probe was also used to detect the virus in infected pepper tissues. (**Figure 9**) Showed RNA extracted from pepper infected PMMoV, and pepper plant extract using dot blot buffer, respectively.

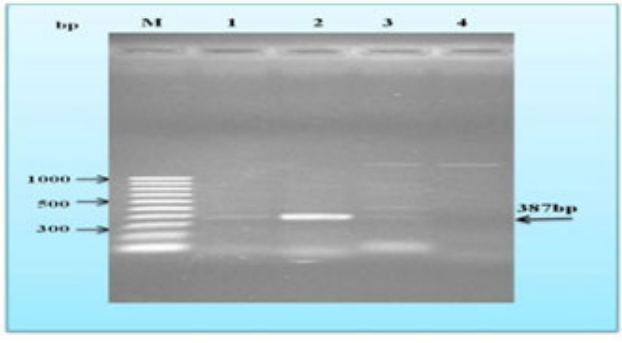

Figure 8. 1.5 % agarose gel electrophoresis showed RT-PCR amplification of the PMMoV-*cp* using PMMoV-F/ PMMoV-R primers. M: 100 bp marker, L1: RT-PCR for RNA extracted from infected pepper plants in filed, L2: RT-PCR for RNA extracted from infected pepper plants by mechanically inoculated PMMoV-*cp*, and L3: IC-PCR of PMMoV-*cp*. L4= RT-PCR for RNA extracted from Healthy plants.

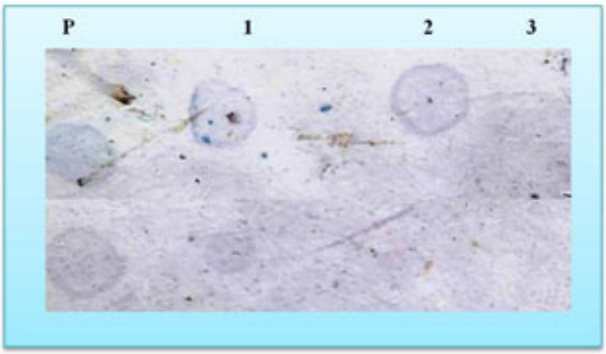

Figure 9. Dot blots hybridization assay using coat protein gene region of cDNA probe labeled with dig. The membrane hybridized with specific non-radioactive PMMoV probe with dot blots. Lanes 1 and 2: strong signal with RNA extracted from infected leaves of pepper and pepper plant extract using dot blot buffer, respectively. Lane 3: healthy sample (negative control). Lane P: PCR positive control of PMMoV-cp gene.

A strong blue reaction with the specific probe **(**Lane 1, 2**)** can be observed. The results also illustrated that there was no reaction with the healthy plants, which was used as a negative control of pepper tissues **(**Lane 4**)**. PCR dot blot PMMoV-CP gene was used as a positive control **(**Lane P**)**.

Discussion

Accurate identification is the first step in successful management of any pathogen. This is especially important for previously unknown pathogens like the *Pepper mild mottle virus*. Although several basic diagnostic methods are in use for many previously characterized plant virus new virus. In this work, *Pepper mild mottle virus* (PMMoV) was isolated from naturally infected pepper plants grown in Western Region of Saudi Arabia, Which showed symptoms mottling , chlorosis, curling, distortion and dwarfing of leaves. The isolated virus was identified on the basis of symptomatology, host range, stability in crude, modes of transmission cytological studies, serologically using DAS-ELISA, particle morphology and molecular studies, the virus was identified as *Pepper mild mottle virus* (PMMoV). The symptoms produced on the inoculated host range by PMMoV consisting of mottling, mild to severe mosaic, stunting, chlorosis and leaf malformation, especially if the plants were infected when young leaves and fruits were small malformed and mottled. These symptoms were similar to those that were described previously for the infection of pepper by PMMoV [**43**,**44**]. PMMoV of the genus *Tobamovirus*occurs worldwide and can drastically reduce fruit yield in peppers [**3**,**20**].

These result appear to be the line with other workers [**43**] PMMoV of the genus tobamovirus occurs wordwide and can drastically reduce fruit yield in peppers [**3**,**20**]. That described by several investigators [**7**,**21**,**36**,**42**]. Host range of

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PMMoV isolate reacted positively with only six plant species of twenty nine. The systemic symptoms produced on tested. *Capsicum annum L.cvs.* California Wonder 40% Marcon 70% and the highest percentage were Yolo wonderand Anaheim 100% Respectively, these virus does not effect tomato and eggplant witch are in the same family, it produsedchlorotic local lesions on the inoculated leaves *of Chenopodium quinoa* and *ch.amaranticolor.*

Ingeneral, the present results were in agreement with the finding of others [**7**,**17**,**20**,**36**,**44**,**54**] Results showed that PMMoV was easly to transmit mechanically and by infected seeds of different *Capsicum annum* cultivars. lowest percentage was recorded in seeds of *Capsicum annum L.cvs.* California Wonder 0%, Marcon10%, Yolo wonde 20% and the highest percentage was recorded in cv Anaheim 30%,on the other hand PMMoV was cannot be transmitted by aphids *Myzuspersicae or Aphis caraccivora* were used to study the transmission of PMMoV the results indicated that PMMo. These results are in line with agreed with those reported by other workers [**1**,**19**,**37**].

In this study PMMoV has a dilution end point 10^{-7} to 10^{-8}, thermal inactivation in 95^{0}C. These results agreed with the results reported by [**1**] but I do not agree with him about 30 days [**1**]. Showed that an amorphous cytoplasmic inclusion induced by PMMoV was observed with light microscopy in infected epidermal strips of *N.celevelandi* leaf but have never been observed in epidermal strips of healthy leaves. In these work examination of epidermal strips of *Capsicum annum cv.* California wonder with PMMoV two weeks after inoculation with light microscopy revealed amorphous inclusions (X bodies),' whereas inclusions were not observed in epidermal strips of healthy leaves. ELISA and RT-PCR are two basic methods commonly used for the detection of PMMoV. RT-PCR is highly sensitive and specific, but it is time consuming and prone to contamination. DAS-ELISA is used widely in routine testing, but it is less sensitive than RT–PCR and needs a specific antibody [**13**,**16**,**29**,**52** and Kamenova and Adkins, 2004).

The importance of PCR lies in its ability to amplify a specific DNA or cDNA transcripte in vitro from trace amount of a complex template. It is possible to amplify specific DNA or cDNA sequences, from as short as 50bp to over 10000bp in length, more than a million fold in a few hours, in a reaction that is carried out in an automated DNA thermal cycler [**22**,**23**]. Amplified DNA was detected by staining the agarose with etheidium bromide [**39**]. Utilization of PCR technology for the detection and identification of plant viruses and mycoplasma appears to be promising technique because of its great sensitivity [**9**]. The molecular-based methods for the detection and identification of plant pathogens were developed in the last decade. These methods have replaced the traditional methods based on biological properties. PCR assays using certain primers presented reliable and sensitive means for detection and characterization of the plant pathogenic [**6**,**49**].

Detection and quantification of PMMoV in total nucleic acid isolated from pepper leaves by RT-PCR was based on sequence-specific priming and subsequent amplification of reverse transcribed viral RNA. The annealing conditions and the primers PMMoV-F/PMMoV-R were supplied to allow amplification of a 387bp fragment of the CP (coat protein) gene of PMMoV which was absent in uninfected tissue extracts. Our primers were designed to amplify CP gene. The size of amplified PMMoVcDNA,isolated from pepper, was 387bp as expected, whether, the cDNA was amplified by RT-PCR or IC-RT-PCR. This result confirms those obtained by [**52**] using total RNA extraction from pepper plants in RT-PCR test. A product of approximately 387 bp for CP gene was amplified from pepper leaves infected with PMMoV. Similar results were obtained with identification of the virus using RT-PCR of the CP gene of PMMoV [**25**]. They indicated that the 387 bp of the CP fragment was identical to those isolated from PMMoV strains from Brazil and Japan as reported by [**14**,**25**], respectively. Using IC-RT-PCR the PMMoV 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 pepper plant extracts is decanted and the PMMoV trapped in the tube are submitted to the PCR reaction without DNA isolation.

In the present work, IC-RT-PCR was used for detection of PMMoV in the pepper infected plant. Polyclonal antibodes of CP gene was used. The obtained PCR product was identical to fragments 387 representing CP gene. IC-RT-PCR technique was used for amplification of PMMoV, because crude plant extracts often inhibit PCR and decrease the sensitivity of the test. A new procedure immunocapture polymerase chain reaction (IC-RT-PCR) has been developed to simplify sample preparation and enhance the specificity and sensitivity of conventional PCR [**48**,**55**,**56**]. The IC-PCR approach has chosen rather than RT-PCR test because of its high sensitivity due to the initial immunocapture enrichment step followed by PCR amplification, high specificity due to the combination of the virus specific antibody capture and primer specificity, and virus detection directly in crude plant extracts rather than in partially or highly purified nucleic acid preparations. Studies on the detection of economically important fruit viruses have documented the potential of IC-RT-PCR to overcome limitations for virus detection in crude tissue extracts of woody plants [**10**,**31**,**55**]. Direct detection sensitivity with ELISA in the presence of PMMoV was not possible as the ELISA assay could not reliably distinguish PMMoV. The IC/RT-PCR detection for other viruses [**10**] reported the presence for apple chlorotic leaf spot virus (ACLSV), Plum pox virus (PPV), Watermelon mosaic virus (WMV) and Prune dwarf virus (PDV).IC-PCR proved to be more sensitive and reliable than DAS-ELISA, when compared to ELISA tests or DNA extraction procedures and subsequent PCR detection. IC-PCR, for its simplicity, sensitivity and reproducibility, has some advantages, therefore, IC-PCR can be regarded as a valuable alternative for large-scale testing infected trees [**28**,**45**] mentioned that a modified

procedure involving immunocapture (IC) followed by reverse transcription polymerase chain reaction (RT-PCR) in a single step reaction was developed for detection of PMMoV (Tunisia isolate of infected pepper).

In the present study a simple, specific, and rapid method for the detection of PMMoV infected pepper plants on the dot blotting onto nitrocellulose membrane, followed by hybridization with a PMMoV using cDNA probe of CP gene (387bp) was used. The dot blot procedure provides a specific, rapid and simple mean of using molecular hybridization techniques to detect infected plant tissue. The nucleic acid hybridization succeeded in detection of PMMoV infected pepper leaves with cDNA probe. Moreover, dot blot hybridization is now the most commonly used procedure for testing large numbers of samples [**26**]. Although dot blot hybridization is very sensitive than squash blot and would be used for detection of a small quantity of viral DNA in plant tissues, it gives a non-specific couloured background so it can be used in routine [**41**]. Dot blot hybridization was more sensitive than ELISA, but less sensitive than RT-PCR in detection of PMMoV on pepper plants. The sensitivity of the dot blot hybridization was similar to the results obtained by others [**5**,**18**,**53**].

Conclusion

PMMoV can be easily detected using PCR and dot blot hybridization, even if it is found in low concentration in the field of infected pepper plants. In addition, these techniques are modern and suitable for detection of PMMoV early infected pepper plants.PMMoV is an emerging tobamovirus that infects pepper plant in taif, KSA. Accurate identification and detection of the virus are the first steps in successful management of the viral diseases. The present study describes a non –radioactive hybridization dot-blot procedure to detect PMMoV. According to our knowledge this approach has not been applied by other researchers to detect that virus. Dot blot hybridization using nucleic acid probes has been used by several research groups to detect and diagnose other plant viruses in the world, and its advantages were established [**18**,**30**,**32**,**38**,**40**] stated that plant virus identification and characterization can be accomplished by several methods involving their morphological, physical, biological, cytological, serological and molecular properties. The use of molecular techniques is increasing worldwide, and some have been developed for identification and characterization of plant viruses. Reverse transcription polymerase chain reaction (RT-PCR) has been shown to be a suitable method for research with RNA plant viruses.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Authors' contributions	ASA	EAK	AGF
Research concept and design	✓	✓	✓
Collection and/or assembly of data	✓	✓	✓
Data analysis and interpretation	✓	✓	✓
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