SEROLOGICAL AND MOLECULAR DETECTION OF COWPAE MOSAIC VIRUS INFECTING COWPAE IN IRAQ

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ABSTRACT

This study was conducted to detect CPMV in infecting cowpea plantes at Plant Protection Department/College of Agriculture, University of Baghdad. Symptomatic cowpea plants collected from fields in Baghdad and Diyala Provinces were tested by Emzyme-linked immunosorbent assay (ELISA), reverse transcription- polymerase chain reaction RT-PCR and Immuno-capture reverse transcription-polymerase chain reaction (IC-RT-PCR) using commercial kits. ELISA approach could detect *Cowpea mosaic virus* CpMV in tested samples but with virus concentration lower than the positive control supplied with the kit, indicating a unique virus strain. RNA from samples showed the highest CpMV concentration were extracted by direct release and used in IC-PT-PCR using como2 a comovirus specific primer set. cDNA was synthetized using como2 revers primer. RT-PCR confirmed the detection of comoviruses when amplified 200 bp DNA fragments from CpMV infected samples. Sequence analysis confirmed CpMV detection when compared with equivalent GenBank sequences. The sequence obtained shared 94.1% maximum nucleotide identity with RNA dependent RNA polymerase (RdRp) gene of CpMV from Egypt (Acc. No. KT438619 and X00206). Both sequence analysis and IC-RT-PCR indicated that virus isolate detected could be an Iraq divergent strain of CpMV.

Key words: IC-RT-PCR, Phylogeny, ELISA, Sequence analyses, Comoviruses.

INTRODUCATION

Legumes are cultivated worldwide due to their economic and nutritional values. The world legume production is about 27% of total grain yield (Vance et al., 2000; Graham and Vance, 2003). In Iraq legumes ranks 3rd after solanaceous and cucurbitaceous crops based on consumption. According to FAO statistics in 2014 the total production of legumes (including bean, faba bean, cowpea and pea) was 22770 tons. Legumes are attacked by several pests including viruses. Virus infection may cause significant losses up to 95% in the production of some legumes (Albrechtsen, 2006). More than 40 different viruses have been reported to infect legumes worldwide including comoviruses (Makkok et al., 2012). The genus *Comovirus* belongs to the subfamily *Comovirinae,* the family *Secoviridae* within Pecornavirales order (ICTVdB, 2017). Comoviruses are transmitted through seeds and beetles. It was reported that 8 of 15 viruses

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species of comovirus infecting legumes which are *Cowpea mosaic virus* (CpMV), *Cowpea sever mosaic virus* (CpSMV, *Broad bean stain virus* (BBSV), *Broad bean true mosaic virus* (BBTMV), *Bean rugose mosaic virus* (BRMV), *Bean pod mottle virus* (BPMV), *Pea green mottle virus* (PGMV) and *Pea mild mosaic virus* (PMMV) (Maliogka et al., 2004; Sastry, 2013). In Iraq, CpSMV only has been detected based on biological properties (Abd-Alghafor, 1988).

CpMV is one of the most important viruses infecting cowpea that causes serious losses up to 100% (Raheji and Leleji, 1974; Shoyinka et al., 1997). CPMV is seed transmitted by 75% in cowpea. CpMV is about 28 nm dimeter and has bipartite polyhedral particles. Virus genome composed of two ssRNA segments; RNA1 and 2, encapsidated separately to form two similar size particles (Hesketh et al., 2017). RNA-1 is about 6-8 kb and encoding proteins necessary for virus replication. While RNA-2 is 4-7 kb and encodes two capsid proteins and products involved in cell-to-cell movement. The capsid is composed of two 60 units proteins large (L) and small (S) domains (Hulo et al. 2011, ICTVdB., 2017) which involve long and short distance movements, respectively (Steinmetz et al., 2009; Sainsbury et al., 2010).

RT-PCR has extensively been used to detect many plant RNA viruses due to high sensitivity, accuracy and reliability. It is a powerful tool to detect plant viruses and strains compared to ELISA and biological approaches (Menzel et al., 2002; Verma et al., 2006; Deshpande & Joshm 2010). RT-PCR was used to detect legume viruses worldwide including CpMV (Salem et al., 2010). Furthermore, it has been applied to screen potyviruses and caralviruses in infected legume samples using group specific primer sets (Al-Kuwaiti, 2013). Recently, CpMV like symptoms have been viewed in cowpea growing fields located at Baghdad and Diyala Provinces, so this study was initiated to confirm CpMV detection based on serological and molecular approaches.

MATERIALS AND METHODS

ELISA test: Cowpea *Vigna unguiculata* samples (n=24) exhibiting mosaic (Fig. 1) were collected from fields in Kan'aan and Madain distracts in Diyala and Baghdad provinces, repectively. Samples were kept in plastic bags on ice and transferred to laboratory for further examination. ELISA was applied to detect CpMV in samples using Reagent Set for Cowpea mosaic virus (CPMV), 96 test wells commercial kit from (Agdia, USA). DAS-ELISA steps were performed following the manufacturer instructions. Virus concentration was estimated by

absorbance detection at 405 nm wavelength using ELISA microplate reader (Bio Tek, USA).

Fig. 1. Naturally infected cowpea plant exhibits mosaic symptom

RNA extraction: Two methods were performed to extract viral RNA, the first was by direct release of viral RNA using immune-capture approach following Yang et al. (2012). About 300 µl of CpMV specific antibodies diluted in coating buffer (Agdia, USA) was used to coat 0.2 mL thin-wall polypropylene PCR tubes then incubated over night at 4 °C. Three handred microliter of leaf extract sap was added to the pre-coated tubes and incubated for 2-4 hours at 37 °C then washed three times and used for cDNA synthesis. Cowpea samples showed the highest virus concentration in ELISA test were used. The second method was performed by extracting total RNA from infected cowpea samples using commercial RNA extraction kit AccuZolTMTotal RNA Extrection Reagent 100 ml) from (Bioneer, South Korea) following the manufacturer procedure. The resultant RNA was used for cDNA synthesis later on.

$_{C}$DNA synthesis: cDNA was synthesized using commercial kit (RT-Premix) from (Bioneer. South Korea). Eighteen microliter of extracted RNA mixed with 2 µl of the reveres primer como2, a comovirus group specific primer (50 PM concentration) was used for cDNA synthesis (Mahmoud et al., 2010). RT-PCR mixture was prepared by mixing 5 µl of cDNA with 2 µl of each como2 forward and reverse primers (Mahmoud et al. , 2010), then added to Premix PCR tubes (Bioneer, South Korea) volume was adjusted to 20 µl of PCR grade water (Bioneer, South Korea). PCR was performed using the following program: one pre denaturation cycle for 2 min at 94 °C, 35 cycles of denaturation for 1 min at 94, annealing for 2 min at 45 or 55 °C and extension for 2 min at 72 °C and one cycle of final extension for 7 min at 72 °C. PCR products were analyzed using ethidium bromide gel electrophoresis (1.5% agarose) for 45 min at 125 mA. PCR products of the expected DNA fragment size were sent to (Bioneer, South Korea) for sequencing. Sequence analyses were performed using MEGA6 software package.

RESULTS AND DISCUSSIN

ELISA test indicated that symptomatic cowpea samples was CpMV infected when specific antibodies could detect CpMV in 6 out of 34 samples. The highest absorbance values at 405 ranged from 0.965-1.510 compared to 2.184 and 0.275 for positive and negative control respectively (Table 1). The low absorbance values scored for CpMV infected cowpea samples were quite low compared to the positive control. This could be rationalized to the low virus concentration in collected samples or the virus under study could be a serotype or a different strain. So IC-RT-PCR was performed to confirm the detection of CpMV by ELISA was reliable.

Table 1. Absorbance values of cow pea samples detected by ELISA. P: positive control, N: negative control

	1	2	3	4
A	2.184 P	0.560	0.337	0.468
B	0.475	0.544	1.510	0.344
C	0.447	0.482	0.965	0.684
D	0.447	0.630	0.504	0.933
E	0.375	0.623	0.386	1.271
F	0.455	0.432	1.350	0.592
G	0.426	1.293	0.877	0.476
H	0.275 N	0.598	0.490	0.429

$_{C}$DNA amplification

The analysis of RT-PCR on gel electrophoresis showed that RT-PCR using comov2 primer set at 55 °C annealing temperature could detect comoviruses in tested samples when amplify a single band of ~200 bp DNA fragments (Fig. 2A).

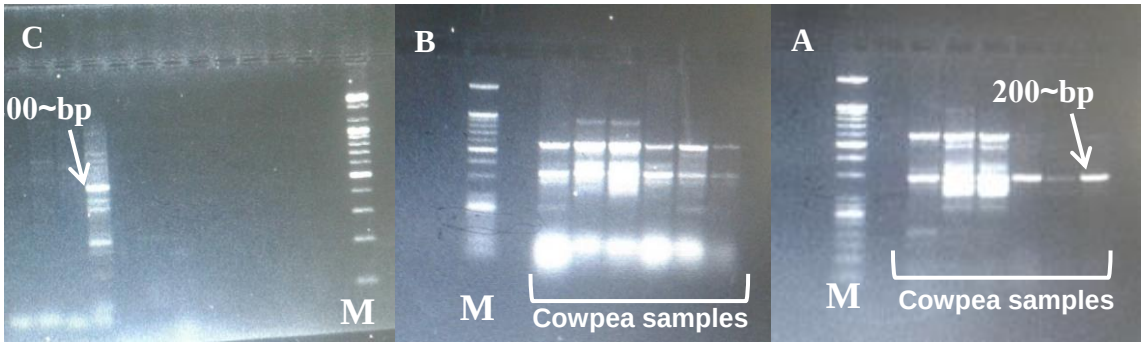

Fig 2. Gel patterns show ~200 bp DNA fragments amplified from symptomatic cowpea samples by como2 primer set at 55°C (A) and 45°C (B) annealing temperature. (C) ~200 bp DNA fragments amplified by IC-RT-RCR at 55°C annealing temperature. M: 20-2000 bp DNA marker (Bioneer, South Korea).

Whereas, nonspecific DNA fragments were amplified, alongside the targeted ~200 bp DNA fragments, when the recommended annealing temperature at

45°C (Mahmoud et al. 2010) was applied (Fig. 2B). IC-RT-PCR using como2 primer set confirmed CpMV detection in tested samples at 55 °C when amplified the ~200 bp DNA fragment (Fig. 2C). Thus, comovirus specific primer set together with IC-RT-PCR approach showed to be a useful tool to detect CpMV.

DNA sequencing

Sequence comparison to equivalent GenBank sequences, confirmed the detection of *Cowpea mosaic virus* when DNA fragments amplified shared 94.1% maximum nucleotide (nt) identity with RNA dependent RNA polymerase (RdRp) gene of CpMV from Egypt (Acc. No. KT438619 & X00206). Similar results were obtained when deduced amino acids (aa) of partial RdRp gene CpMV isolated shared 96.4% maximum identity to equivalent GenBank sequences from Egypt mentioned above (Fig. 3). In contrast, when compared against CpSMV, partial RdRp obtained scored 22.9 and 20% maximum nt and aa sequence identity, respectively (Fig. 3).

KT438619.1 Egypt ---ALF-ISNLVTRTLRFKELLLFCKQQFLEKMQASIVWAPELEQYLQVEG-DAVAQGVSQ-------- [69]
X00206.1 Egypt ---...-.....-.....-------- [69]
CpMV Iraq ---...-.....L.....L..-.....-------- [69]
M83830.1 CpSMV GDDN.LSVQSAI.HVFDGTK.KE.L.LNGITITDGKDKTS.V.N-FRNL.DC.FLKR.FKKESDVVWVG [69]

Fig 3. Duduced amino acid comparison of partial RdRp gene sequences of CpMV

Neighbor Joining (NJ) Phylogenetic tree constructed from partial RdRp nt and aa sequences grouped CpMV isolated to GenBank sequences within one group and separated them from CpSMV (Fig. 4A & B). According to the sequence species demarcation and classification criteria, the sequnce obtained, therefore, belongs to CpMV but not to other comoviruses (ICTVdB, 2017).

Both molecular and serological tests are confirmed that the virus isolated was an isolate of CpMV as these approaches showed to be reliable to detect comoviruses up to specie level (Albrechtsen, 2006). Thus, symptomatic plants grown in cowpea fields at Diyala and Baghdad provinces were CpMV infected. Based on sequence comparison and serological results CpMV isolated could be an Iraqi strain (ICTVdB., 2017). Based on the author's knowledge, this study is the first to investigate and characterize CpMV using molecular and serological approaches in Iraq. As they may impact legume and other crops in Nigeria (Shoyinka et al., 1997), further molecular studies, therefore, are required to

investigate other comoviruses infecting legumes in fields from other Iraqi provinces.

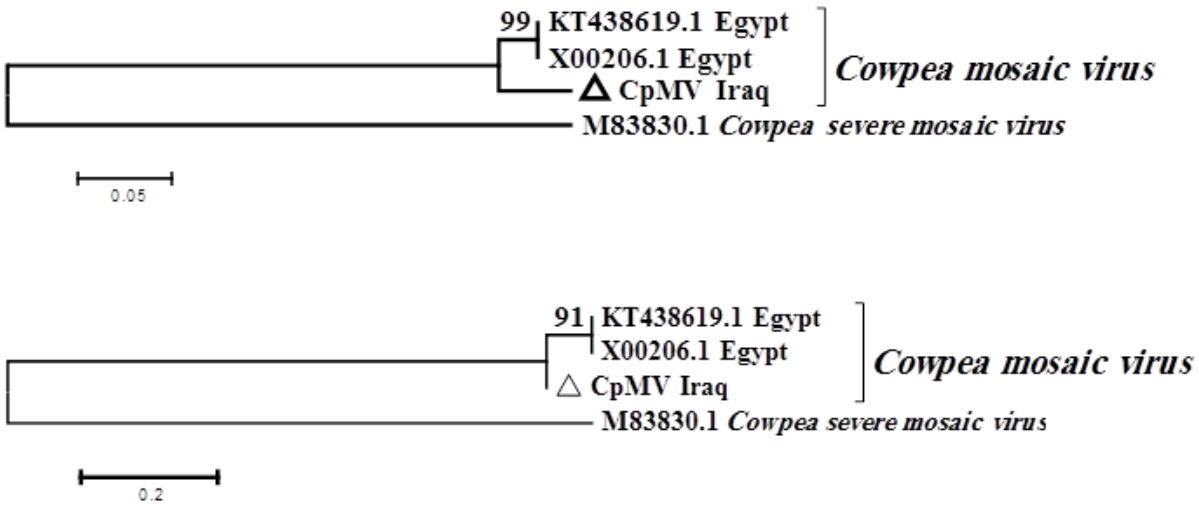

Fig. 4. Phylogenetic relatedness of CpMV

NJ phylogenetic tree constructed from partial RdRp nt (A) and deduced aa (B) sequences of CpMV isolated (indicated with Δ) and equivalent GenBank sequences. Data analyzed by MEGA6 software (Tamura et al., 2013).

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التشخيص المناعي والجزيئي لفايروس موزائيك اللوبياء الذي يصيب نبات اللوبياء في العراق

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المستخلص

اجريت هذه الدراسة في قسم وقاية النبات/ كلية الزراعة–جامعة بغداد اذ جمعت عينات نباتات تظهر عليها اعراض موزائيك من حقول زراعة اللوبيا في مناطق مختلفة من ناحية كنعان في محافظة ديالى وقضاء المدائن في محافظة بغداد، ثم اختبرت مناعيا بوساطه تقانة اليزا (ELISA) Emzyme-linked immunosorbent assay وجزيئياً بوساطة تقانة النسخ الرجعي لانزيم تفاعل البلمرة المعتمده على التقييد المناعي Immuno-capture reverse transcription-polymerase chain reaction (IC-RT-PCR) باستعمال عدة فحص مناعي متخصصة بفايروس موزائيك اللوبيا CPMV. أعطت نتائج الفحص المناعي قراءات مختلفة للعينات كانت اعلاها اقل من العينة الموجبة من عدة الاستخلاص والتي قد تكون سلالات مختلفة لنفس الفايروس، بعدها اختيرت العينات التي اعطت اعلى تركيز في تقانة اليزا وتم استخلاص الحامض النووي الفايروسي عن طريق تحريره مباشرة من جسيمات الفايروس المقيدة مناعياً وصناعه شريط cDNA من شريط RNA الفايروسية من خلال استعمال البادئ الخلفي من طقم بوادئ como2 المتخصص على مجموعة الكوموفايروس بعدها اجري تفاعل البلمرة التسلسلي باستعمال طقم بوادئ como2 وتم الحصول على قطع DNA ذات الوزن الجزيئي 200 زوج قاعدي

دالة على تشخيص فايروس يعود الى مجموعة الكوموفايروس بعدها تم تحديد التتابع النيكلوتيدي للفايروس ومطابقته مع التتابعات النيوكليوتيدية المكافئة المسترجعة من بنك الجينات للتأكد من انه فايروس CpMV والذي اظهر تسلسله الجزئي تطابقاً نيوكليوتيدياً مقداره 94.1 % مع جين RdRp RNA dependent RNA polymerase لفايروس موزائيك اللوبياء والعائد للتسلسلات النيوكليوتيدية من مصر (رموزها البنكية KT438619 و X00206). اشارت النتائج الى ان عزلة الفايروس قيد الدراسة قد تكون سلالة عراقية متباعدة لفايروس موزائيك اللوبياء.

الكلمات المفتاحية: IC-RT-PCR، Phylogeny، ELISA، Sequence analyses.