RAISING EFFECTIVE POLYCLONAL ANTIBODIES AGAINST PURIFIED PEPPER LEAF CURL VIRUS PARTICLES

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Pepper *(Capsicum annuum*) plant is a species of the genus *Capsicum* native to Southern North America. Bell peppers are a great source of antioxidants (Sinha *et al*., 2011).

Several whitefly-transmitted geminiviruses infect peppers, including Chino Deltomate Virus (CdTV), Pepper Mild TigreVirus (PMTV), Serrano Golden Mosaic Virus (SGMV), Sinaloa Tomato Leaf Curl Virus (STLCV), Texas pepper virus (TPV) all of these viruses have similar symptoms but are biologically and genetically distinct. Seedlings may be infected without showing any symptoms until after transplanting. Symptoms depend upon the virus strain and the pepper variety. Common symptoms are stunting, curling, or twisting of the leaves, bright yellow mosaic, distortion of leaves and fruit, and yield reduction (Arogundade *et al*., 2012). Recently it was proved that pepper infected with pepper leaf curl virus (PeLCV) causing leaves curling, deformation of new leaves and stunting of infected plant (El-Gaied *et al*., 2014). Geminiviruses, belonging to the family *Geminiviridae*, constitute a very important class of viruses transmitted by either leafhoppers or whiteflies. Their single stranded DNA is encapsidated in 20-30 nm geminate particles (Goodman, 1977; Harrison *et al*., 1977).

Virus detection and identification is important for preventing the spread of the viral infection and is needed to implement quarantine regulations. Molecular techniques specifically target the genetic material of plant viruses and the availability of genome sequences for many plant viruses allows molecular techniques to be more sensitive and specific than serologically based techniques. Although ELISA is not as sensitive as molecular techniques, it is still widely used because of low technical skill requirements, cost effectiveness and portability making it ideal for large scale field testing and detection by laboratory methods. Serology in particular, is a primary requirement for large-scale surveys such as those carried out for sanitary selection in the framework of certification programmes (Garcia *et al*., 1997; Alioto *et*

al., 1999; Potere *et al*., 1999; D'Onghia *et al*., 2001).

This investigation was aimed to produce effective diagnostic antibodies specific for PeLCV through raising and evaluation polyclonal antibodies against PeLCV purified particles.

MATERIALS AND METHODS

Virus purification from infected pepper plants

Virus was purified from PeLCV inoculated pepper plants after symptoms manifested according to Hammond *et al*. (1983) with some modification as described by Essam *et al*. (2004). Purification steps were carried out at 4°C after initial homogenization of tissues. Concentration of the purified virus particles was measured and the purity of the virus was measured at $A_{260}/_{280}$ as described by Abdel-Salam (1991).

Biological and molecular characterization of the purified virus particles

Healthy pepper plants 30 days old were injected with purified virus mixed with 0.1 M phosphate buffer, pH 7. Plants left for 15 -20 days in insect proof wooden cage, then tested by PCR using whitefly transmitting geminivirus specific primers (Abdallah *et al*., 1993). The primers position, direction and sequence are listed in Table (1). Total DNA was extracted using Qiagen DNA Extraction kit according to the protocol supplied. The PCR mixture (25 µL) contained primers (2 µM/L each), 1X PCR buffer [750 mM/L Tris HCL (pH 8.8 at 25°C), 200 mM $(NH_4)_2SO_4$, 0.1% Tween-20], 1.5 mM/L $MgCl_2$, 160 µM/L dNTPs and 1.5 units Taq DNA Polymerase and 5 µL of extracted DNA as template. Thermal cycling parameters were 94°C for 5 minutes and 35 cycles of each 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C followed by final extension at 72°C for 10 minutes. As a negative control in molecular and biological characterization, healthy pepper plant injected only with the phosphate buffer and all injected plants symptoms were recorded weekly.

DNA sequencing

Sequencing was carried out for both strands of the viral genome fragment using the same specific primers, DNA band was excised from the agarose gel and DNA fragment was purified using High Pure PCR Product Purification Kit (Cat# 1 732 676, Roche). Purified DNA was determined by cycle sequencing using the fluorescent dideoxy chain terminator technology. The determined sequence data were subjected for analysis by blast and alignment with sequences available in the GENBANK database of the National Centre of Biotechnology Information (www.ncbi.nlm.nih.gov).

Antiserum production

Raising antiserum against the purified virus was carried out according to the method described by Ball *et al*. (1990) using white mice 'Balb-C' (6-8 weeks-old). Purified virus particles were admin-

istered in about 0.1 ml intravenous injection with concentration 1 mg/ml as a primary response; followed by weekly five intraperitoneal injections of 0.2 mg/ml containing 0.1 ml incomplete Freund's adjuvant. The collected blood after immunization was left to clot at 37°C for 1hr then the separated antiserum was centrifuged at 3000 rpm for 1min at 4°C. The serum was filtered through a 0.2 µm Millipore, 0.025% Sodium azide was added and stored at 4°C until use.

Evaluation of raised antibodies by ELISA

Numerous variations of the ELISA procedures were described, one of these is plate-trapped antigen indirect (PTA-I) ELISA, in this approach we trap the antigen on the plastic surface, then the trapped antigen react with an unlabelled intermediate antibody (IA). The IA is then detected using an enzyme-labelled antibody (LA) specific to the IA. In our experiment we used (PTA-I) ELISA with the plant extracts from infected and healthy pepper plants in two different dilutions 1:10 and 1:15, all extractions were diluted 1:1 in coating carbonate and bicarbonate buffer pH 9.6 and overnight trapped on ELISA plate at 4°C. After washing three times for 5 min each using PBST, plates were blocked with 4% milk PBS and incubated for 3 hr at 37°C, then washed three times with PBST and raised antiserum was added to the wells in double-fold dilutions and incubated overnight at 4°C. After washing, anti-mouse universal antibody was added and incubated for 2 hr at 37°C. Plates were washed as previous and subjected for detection by adding substrate buffer containing 0.5 mg/ml p-nitrophinyl phosphate as a substrate for the alkaline phosphatase enzyme.

Antibody trapped -indirect (AT-I) ELISA, was also to evaluate the raised antibodies as trapped. Plate was coated with the raised antibody in double-fold dilutions for overnight at 4°C, then antigen was added in two different dilutions and polyclonal antibody specific for geminivirus was used as intermediate antibody, then anti-rabbit universal antibody was added and subjected for detection by adding substrate buffer containing 0.5 mg/ml p-nitrophinyl phosphate as a substrate for the alkaline phosphatase enzyme. Color development was measured at a wavelength of 405 nm using Bio-Rad ELISA reader model 3550

Evaluation of raised antibodies by western blotting

Similarly, 12% SDS-PAGE was prepared as described by Laemmli (1970). The polypeptides of total extracted proteins from PeLCV infected plants and healthy pepper plants were transferred onto a membrane (immobilon® PVDF membrane, millipore cooperation, Bed ford, MA 01730) using a trans-blot apparatus (Bio-Rad). The membrane was blocked in TBS containing 5% BSA, then the blocking buffer was replaced with the TBS containing raised antibodies diluted 1:1000. After incubation, membrane was washed with TBST, and incubated in TBS

containing anti-mouse universal antibodies and detection done by NBT/BCIP in alkaline phosphatase buffer.

RESULTS AND DISCUSSION

Purification and characterization of PeLCV

PeLCV particles were purified from the inoculated pepper plants after symptoms manifestation. The purified virus particles were yielded 0.8 mg obtained from 100g pepper plants. The yield was measured according to the equation $A_{260}/7.7$ (extension coefficient) multiplied by the dilution factor. The purity of purified virus determined 1.2 at $A_{260/280}$, which proved that the method used for virus purification able to produce a good yield and immunogenic virus particles for raising antibodies against PeLCV.

Virus detection and identification is important for preventing the spread of the viral infection and to implement quarantine regulations. It is also important in disease epidemiology research and for designing new control strategies. Before the development of laboratory testing, field and biological indexing were used to detect and identify viral pathogens using morphological criteria; however this method is time consuming and requires an extensive knowledge in taxonomy and disease symptomology (Liebenberg, 2008).

In our study PeLCV purified particles were biologically characterized by injection in healthy pepper plants which exhibited typical infection symptoms including curling and downward cup shape of the leaves after 45-50 days post infection as depicted in Fig. (1) in comparing with the healthy pepper plants, which didn't develop any noticeable symptoms during the same period.

Because procedures of biological indexing are slow and costly, require adequate facilities and trained personnel and cannot be used for large-scale indexing; clearly, quick and reliable diagnosis methods based on detection of the pathogen are urgently needed. The PCR is a powerful technique, which is sensitive and highly specific and has been used to detect plant viruses. PCR is usually considered the most sensitive method for virus detection (Martin *et al.*, 2004). So, the maintaining of PelCV in pepper plants was characterized by PCR using specific primers. Positive signals as a 659 bp fragment were revealed with DNA extracted from infected pepper plants and no signals appeared with DNA extracted from healthy pepper plants as shown in Fig. (2).

Isolated DNA fragment was subjected to sequencing and determined nucleotide sequences revealed ~659 bp (Fig. 3), which blasted in the Gene bank database and alignments confirmed that the isolated fragment belong to the core region of C1 gene for the geminiviruses. The relationship sequences tree was built by cluster analysis for the isolated fragment which confirmed that it belongs to the geminiviruses by showing the closed relation at the molecular level to geminiviruses different isolates (Fig. 4),

also the result showed that the nucleotide sequences of the PCR fragments was clustered with other members of Begomoviruses.

Raising and evaluation polyclonal antibodies against PeLCV

Serological techniques are extremely useful for the identification, quantitative assay and routine diagnosis of plant viruses. The first breakthrough in the development of a laboratory diagnostic testing techniques came in 1977 with the development of the ELISA (Clark and Adams, 1977). The most important criteria in evaluating diagnostic techniques are reliability, sensitivity, and cost. The sensitive, reliable and rapid identification of plant viruses is essential for effective disease control (Zein and Miyatake, 2009). Polyclonal antibodies were raised against the purified PeLCV virus particles and were evaluated in double-fold dilutions as a trapped and intermediate antibody by ELISA. ELISA readings in Table (2) showed the high reactivity of the raised antibodies as intermediate antibodies against the PeLCV infecting pepper plants in antigen trapped ELISA method. The high detection capability for the raised antibodies were depicted in Fig. (5) which showing the ability of the raised antiserum diluted 1:3200 in detection low concentrations of the antigen (1:10 dilution).

Detection abilities of the raised antibodies were also tested as trapped antibodies in ELISA (antibody trapped method), that demonstrated very high efficacy where, able to detect PeLCV even with very low concentration of antiserum which diluted 1:51,200 times (Table 3), these results were summarized in Fig. (6).

These results confirmed the importance of ELISA as a serological tool for diagnosis of plant viruses as recommended by Clark and Adams (1977).

Evaluation the raised polyclonal antibodies via western blotting

Antibodies against viruses have also been used in other serological diagnostic techniques including western blots, dot-blot immune-binding assay, immunodiffusion assays, immuno-strip tests, DTBIA and SSEM (Schaad *et al.*, 2003). The raised polyclonal antibodies were evaluated by western blotting against infected and healthy pepper plants. Positive reaction signals were developed at a molecular weight of approximately ~30 KDa which distinctive for the coat protein of geminiviruses (Aref *et al*., 1995; El-Gaied *et al*., 2008) with total soluble proteins extracted from pepper PeLCV infected plants and it was absent with total soluble proteins extracted from healthy pepper plants, as depicted in Fig. (7). Our obtained results verify the efficiency of the raised polyclonal antibodies in PeLCV detection by different serological methods even in low virus titers.

SUMMARY

Sensitive diagnostic tools are urgently needed for rapid and specific virus detection. Pepper Leaf Curl Virus

(PeLCV) particles were purified from the injected pepper plants after symptoms manifestation. PeLCV particles were characterized by injection into healthy pepper plants which exhibited typical infection symptoms including curling and downward cup shape of the leaves in 45-50 days post infection. Maintaining PelCV in pepper plants were characterized by PCR using whitefly transmitting geminivirus specific primers which revealed positive signals with DNA extracted from infected pepper plants as a ~659 bp DNA fragment. PCR isolated DNA fragment was subjected to sequencing and the determined nucleotide sequences were blasted in the Gene bank databases. Alignments confirmed that the isolated fragment belong to the C1 gene of geminiviruses (core region). Polyclonal antibodies were raised against the purified virus particles and evaluated by ELISA as a trapped and intermediate antibodies as well as western blotting analysis.

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Table (1): Designed primers used in PCR detection.

Primer	Nucleotides Sequence	Additional coding site	Position	Expected size (bp)
HD-3	5'CGGGATCCAAACAGGTCAGCACATTTCC3'	*Bam*H1	391-1049	659
HD-4	5'GGGGTACCTATATGAGGAGGTAGGTCC3'	*Kpn* I		

Table (2): Evaluation of the raised polyclonal antibodies as intermediate antibody in double-fold dilutions through antigen trapped ELISA method against two different concentrations of the antigen and healthy pepper plants.

Serial dilutions of antiserum	Infected pepper diluted (1:10)	Infected plant diluted (1:15)	healthy pepper diluted (1:10)
1:200	2.017	1.834	0.109
1:400	1.781	1.500	0.110
1:800	1.383	1.110	0.108
1:1600	0.862	0.567	0.112
1:3200	0.315	0.298	0.110
1:6400	0.190	0.157	0.109
1:12800	0.119	0.140	0.111
1:25600	0.108	0.106	0.108
1:51200	0.089	0.084	0.109

Table (3): Evaluation of the raised polyclonal antibodies as trapped antibody in double-fold dilutions through antibody trapped ELISA method against two different concentrations of the antigen and healthy pepper plants.

Serial dilutions of antiserum	Infected pepper diluted (1:10)	Infected plant diluted (1:15)	healthy pepper diluted (1:10)
1:200	1.598	0.976	0.123
1:400	1.500	0.944	0.123
1:800	1.440	0.930	0.122
1:1600	1.354	0.890	0.111
1:3200	1.296	0.860	0.107
1:6400	1.266	0.838	0.108
1:12800	1.212	0.802	0.109
1:25600	1.054	0.642	0.111
1:51200	0.974	0.598	0.109

Fig. (1): Symptoms developed on PeLCV inoculated pepper plants. A and B: symptoms after 45-50 days post inoculation with purified virus exhibited curling, downward cup shape and deformation of new leaves. C: pepper plants inoculated with phosphate buffer without symptoms.

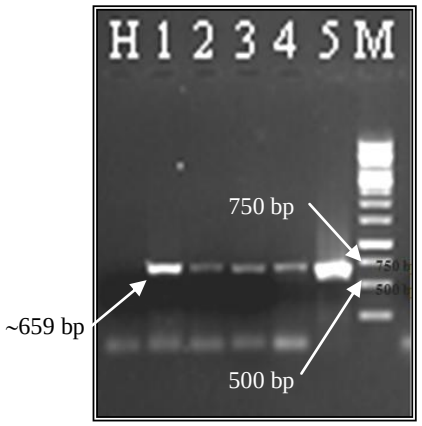

Fig. (2): PCR analysis for injected pepper plants using geminivirus specific primers. 1-5: infected pepper plants. H: negative control (pepper infected with phosphate buffer). M: 1 Kb ladder.

CCCGTATTTTGTGTTGCTTTGCCGGTCCCTCTGGGCCCCCATGAATTCCTTGAAGTGCTTT
AAATAATGCGGGTCTACGTCATCAATGACGTTGTACCACGCATCATTACTGTACACCTTTG
GGCTTAGGTCTAGATGTCCACATAAATAGTTATGTGGGCCTAGGGACCTGGCCCACATTGT
TTTGCCTGTTCTGCTATCACCCTCGATGATAATACTATTAGGTCTCCATGGCCGCGCAGCG
NAAGACACGACGTTCTCGGCGACCCACTCTTCAAGTTCATCTGGAACTTGATTAAAAGAAG
AAGAAAGAAATGGAGAAACATAAACTTCTAAAGGAGGACTAAAAATCCTATCTAAATTTGA
ACTTAAATTATGAAATTGTAAAATATAGTCCTTTGGGGCCTTCTCTTTTAATATATTGAGG
GCCTCGGATTTACTGCCTGAATTGAGTGCTTCGGCATATGCGTCGTTGGCAGATTGCTGAC
CTCCTCTAGCTGATCTGCCATCGATTTGNAAAACTCCAAAATCAATGAAGTCTCCGTCTTT
CTCCACGTAGGTCTTGACATCTGTTGAGCTCTTAGCTGCCTGAATGTTTGNATGNAAATGT
GCTG

Fig. (3): Determined nucleotide sequences of the amplified PCR DNA fragment.

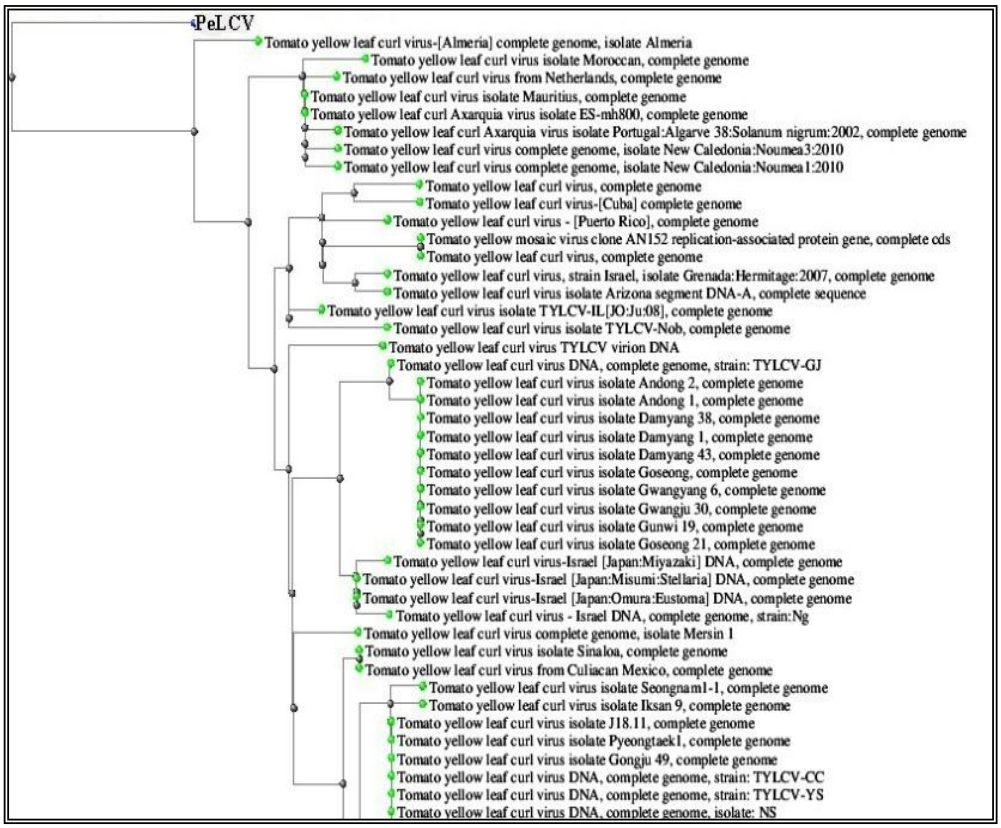

Fig. (4): Phylogenetic tree relationship produced by the alignment of the isolated nucleotide sequence with GenBank databases.

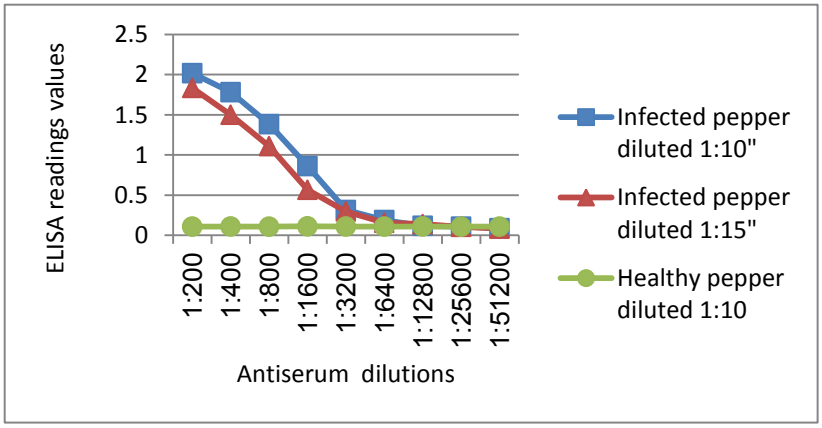

Fig. (5): Shows the ability of the raised polyclonal antibodies in the detection of specific antigen. Antibodies were used in double-fold dilutions from 200 to 51200 times as intermediate antibodies against two different concentrations of the antigen as well as the same two different concentrations from healthy pepper plants as a control.

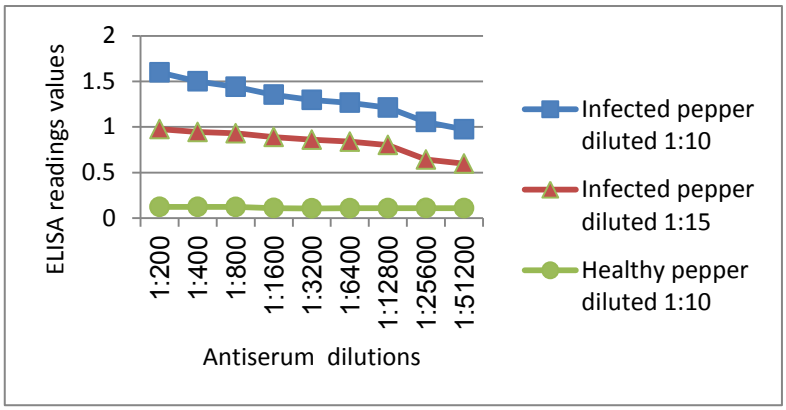

Fig. (6): Shows ability of the diluted PelCV raised polyclonal antibodies in detection. Antibodies were used in double-fold dilutions from 200 to 51200 times as trapped antibodies against two different concentrations of the antigen as well as the same two different from healthy pepper plants as a control.

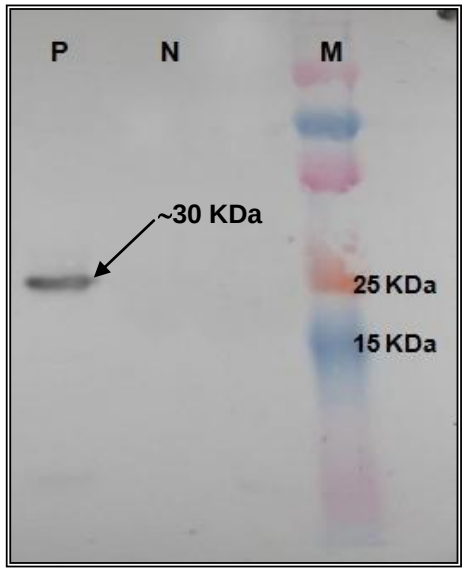

Fig. (7): Western blot analysis shows the positive signals approximately 30KDa developed from the raised antiserum reaction against the PeLCV infecting pepper plants. M: pre-stained protein marker. N: healthy pepper plant. P: pepper PeLCV infected.