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The Comparison of Antibodies Raised Against PLRV with Two Different Approaches - Viral Particles Purification and Recombinant Production of CP

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Abstract

Serology is one of the most important techniques which extensively used in different fields especially the agriculture one. Serological methods usually which are extensively used because of their specificity in disease diagnosis and relative ease of completion. The methods are widely used in plant virology include enzyme-linked immunosorbent assay, dot-blot immunoassay, immunospecific electron microscopy, and tissue-blot immunoassay. The main stone of the serology test is antiserum which is either mono or poly. Due to the high cost of the mono-antisera production and its low sensitivity of the ones immunized by the purified virus particles, the recombinant protein could be good substitution. In this study, we aimed to produce polysera using the viral particles and with the recombinant coat protein of the Potato Leaf Roll Virus as well and used both in rabbit immunization separately. The sensitivity, specificity, and reactivity of the two produced sera were tested in comparing with the real-time polymerase chain reaction. Results revealed that polysera produced by recombinant coat protein are more specific and sensitive than the sera produced by the purified particles. Moreover, the results obtained by dot and tissue blot confirmed the results obtained by enzyme-linked immunosorbent assay techniques. The real time-polymerase chain reaction results were similar to that obtained by serological methods except the time and the substrate. In conclusion, production of the polysera using recombinant protein is test easy, cheap, and sensitive test for on line vial detection.

Keywords: Potato Leafroll Virus (PLRV); Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR); rCP; Antiserum production; Dot Blot Immunoassay (DBIA); TBI

Introduction

Potato (*Solanum tuberosum* L.) is one of the most important vegetable crops in human nutrition having potential of vital food security. It is the fourth largest food crop cultivated in more than 100 countries throughout the world and has gained a status of globally traded commodity [1].

Potato is infected by at least 40 viruses and 2 viroids [2] and mixed viral infection is frequent [3]. The primary infection triggers the rolling of young leaves with upright growth pattern appearing pale yellow, tinged purple, pink or red with many cultivars. Whereas, secondary symptoms turned out to be severe with overall rolled leaves with leathery texture, stunted growth and tuber necrosis [4].

PLRV is a major menace for the potato production all over the world [5]. PLRV is the typical member of the genus *Polerovirus* of the Family *Luteoviridae* [6]. PLRV has a monopartite, single stranded RNA genome, transmitted by aphids in a circulative non-propagative manner and is mainly restricted to phloem tissues of infected plants [7]. PLRV forms 25 to 30 nm diameter isometric particles that encapsulate genomic RNA of about 5.9kb that contains six large open reading frames (ORF) [8]. In addition, the 3' end proximal ORFs are expressed via a sub genomic RNA synthesized in host cells during the infection process. The ORF3 encodes the major capsid protein (CP) of about 23 kDa and ORF4 encodes a 17 kDa putative movement protein. The ORF5 encodes the carboxy terminal region of the minor capsid component expressed by translational readthrough of the ORF 3 amber stop codon. The resulting full-length protein has a MW of about 74 kDa but, in preparations of purified virus, it is present in a C-terminally truncated form of about 54 kDa [9].

Recombinant coat protein (CP) was used as an immunogen to produce monoclonal antibodies (MAbs) and polyclonal antibodies (PAbs) against many plant viruses. It's an alternative approach to produce structural proteins of viruses, in particular CP, in *E. coli*, overcoming difficulties associated with the development of antibodies of good quality. It will be useful when purified viruses or virus proteins are not available especially with viruses are phloem-limited viruses, such as *Luteoviruses*, are present at very low concentrations in their hosts, resulting in low yield of purified virions. Also, the filamentous particles of *Potyviruses* are relatively low stable and they tend to aggregate with plant debris by Souiri et al. [10]. Therefore, polyclonal antibodies raised against purified virions contaminated with host tissue components, cross react with host antigens and often give variable background reactions, thus limiting their use in ELISA based diagnostic methods. Recombinant polyclonal antibodies (Pab) specific to *Tomato spotted wilt virus* (TSWV) [11], *Potato virus Y* [12], *Alfalfa mosaic virus* (AMV) Khatabi et al. [13] have been generated and used for serological detection of cited viruses [14].

Recombinant antisera for Egyptian isolates of both PVX [15] and PLRV [16] were induced using denatured CP technology. These antisera were reactive in I-ELISA but not in DAS-ELISA. Abdel-Salam et al. [8] described a modified technique involving the use of a mixture of native and denature CP for each virus in the antiserum production to enhance the binding capacity of the produced antibodies with their corresponding antigens in DAS-ELISA.

Serological methods widely used in plant virology include enzyme-linked immunosorbent assay (ELISA), dot-blot immunoassay (DBIA), immunospecific electron microscopy, and tissue-blot immunoassay (TBIA). Serologically based tests commonly are employed today because

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of their specificity in disease diagnosis and relative ease of completion [17-19].

We report in this study the sensitivity and specificity of the antisera produced by the recombinant protein in comparing with those raised by purified virus particles. Moreover, the advantages of the recombinant antisera are, rapidity, easy, and inexpensive for the diagnosis.

Material and Methods

qReal-Time PCR for detection PLRV-CP gene

The total RNA of *potato leafroll virus* was extracted from one gram of the infected leaf tissues by using RNeasy® Plant Mini Kit (QIAGEN, Germany) according to manufacturer's instructions. The total RNAs were reverse transcribed using reverse primer of PLRV-CP. In each reaction, 3 µL RNA (30ng) were added to 17 µL of reaction mixture (2.5 µL of 5x RT reaction buffer; 2.5 µL of 25 mM dNTPs; 1 µL of PLRV-CP R primer (100pmol); 0.2 µL of reverse transcriptase (200 u) and 10.8 µL of H_2O). The program was performed at 42°C for 1 h; enzyme stopped at 65°C for 20 min and final step at 4°C for 10 min. Consequently, qPCR SYBR®Green Kit (Thermo, USA) was used to quantify expression of coding region of coat protein gene in infected potato samples by leaf roll virus in assay after the normalization of certain concentrations. The specific primer DAF-CP sense 5`-AGTACGGTCGTGGTTAAAGG3-3 ` and DAR-CP antisense 5`-CTATTTGGGGTTTTGCAAAG3-3 ` were designed according to Presting et al. [20], were used to target the specific gene (CP for PLRV). A 18S rRNA- sense 5`-TACCTGGTTGATCCTGCCAGTAG-**3**` and 18S rRNA antisense 5`-CCAATCCCTAGTCTGCATCGT-**3**` were used as a housekeeping gene for normalizing RNA levels of the target gene. The qPCR SYBR Green based real-time PCR was performed in a total volume of 10µL followed by 1 µL cDNA 10 ng, 5 µL SYBER Green 1x, 0.7 µL DAF 10 ppm, 0.7 µL DAR 10 ppm up to 2.6 µL water. The reaction was performed with a pre-denaturation at 95°C for 15 min, and 40 cycles of denaturation at 95°C for 15 secs, at annealing at 60°C for 1 min and elongation at 72°C for 30 sec. Fluorescent signal measurements were carried out during the elongation step. The qRT-PCR reactions were carried out in thermo piko qRT-PCR apparatus. All qRT-PCRs were performed in duplicate. The qRT-PCR reactions were carried out in 10 µL into Thermo picko qRT-PCR plate 96 well.

Data analysis

Delta Delta Threshold cycle ($\Delta\Delta CT$) expression values were calculated for RNA samples of PLRV to determine gene expressions using 18S rRNA (reference gene) and the other PLRV-CP gene. $\Delta\Delta\ C_T$ expression = $2^{(-\Delta\Delta CT)}$, the equations show the mathematical model of the relative expression ration for the real time PCR. The ratio of the target gene is expressed in sample versus control in comparison to reference gene [21].

Virus purification

According to the method described by Gooding & Hebert [22] with some modification, the following processes were applied: Using blander for 5 min, 100g of infected leaves were homogenized in 0.5M Na_2HPO_4-KH_2PO_4 buffer (1:1 W/V) pH 7.2, containing 1% 2-β-mercaptoethanol. The homogenate was squeezed through cheesecloth. An 8-ml n-butanol/100 ml extract was added to the homogenate and stirred for 2 h at 4°C. Then, the emulsion was centrifuged at 12.000 rpm for 30 min. The supernatant was placed on the stirrer at 4°C and 4g polyethelen glycol (PEG, mo. Wt. 6000)/100 ml were added. The mixture was incubated on the stirrer for 2 hours at 4°C and centrifugation was performed for 15 min at 12.000 rpm. The small clear glassy pellet was resuspended in 20 ml of 0.01 M phosphate buffer, (pH 7.2)/100 ml of initial extract and centrifuged at 12.000 rpm for 15 min. Further purification was obtained by a second precipitation with PEG. A 0.4 g NaCl and 4 g PEG were added for each 10 ml virus suspension and incubated on the stirrer for 2 h at 4°C. Pellet was collected by ultracentrifugation for 2 h at 30.000 rpm. Finally, the pellet was immediately resuspended in 2 ml of 0.01M phosphate buffer, pH 7.2, mixed well; centrifuged at 12.000 rpm for 5 min and was stored at -20°C.

PLRV-CP, cloning and protein expression

This is done by using the protocol described by Dalia et al. [23].

Antisera production

The virus purified and the purified recombinant PLRV-CP fusion protein were emulsified with an equal volume of Freund's complete adjuvant for first injection and with Freund's incomplete adjuvant for subsequent three injections intramuscularly into a New Zealand white rabbit. The rabbit was first bled two weeks after the last immunization. The whole blood was kept for one hour at room temperature for clotting then the clot was released and the blood was heated at 37°C for 30 min then stored at 4°C overnight. The serum was decanted from the clot and centrifuged at 2,000 rpm to remove cell debris. The serum was filtered and brought to 0.025% sodium azide. Aliquots of serum, mixed with equal volumes of glycerol, were stored at -20°C. The serum fractions were collected and stored at -20°C until required according to El-Attar et al. [16].

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were separated by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) using the protocol described by Sambrooke and Russell [24]. Gel electrophoresis was performed using the Mini-PROTEIN II vertical gel electrophoresis system (BioRad); 5% staking gel and 12% resolving gel, The SDS gel electrophoresis was carried out at about 80 V in IX Tris/glycine-SDS running buffer. After electrophoresis, the gel was stained by shaking for 1 h in Coomassie brilliant blue R-250 stain and de-stained with de-staining solution overnight until the bands were clearly defined.

Serological detection of PLRV

The produced PLRV-antiserum was tested using I-ELISA, DAS-ELISA, DBIA and TBIA. Two antisera were used for comparison: Antiserum raised against virus particles (Viral antiserum) and Antiserum raised against PLRV coat protein (r CP antiserum).

I-ELISA and Double antibody sandwich-ELISA: The indirect-ELISA was performed according to Koenig [25] and modified by Fegla et al. [26]. Whenever, Double antibody sandwich-ELISA was applied according to Clark and Adams, [27]: The reactions were read visually (yellow color) by a Micro ELISA reader (Stat fax -2100), absorbance at 405 nm (ELISA value).

DBIA and TBIA: Both Dot Blot Immunoassay (DBIA) and Tissue Blot Immunoassay (TBIA) were approached according to Kamenova and Adkins [28] with some modifications. A nitrocellulose membranes (Amersham Biosciences Corp., Piscataway, NJ) was pre-wetted in 100% methanol for 10 secs and then washed in distilled water for 1 min. For DBIA, the membranes were marked into 1-cm squares and a 5 µL sample (homogenized in carbonate coating buffer used in ELISA) was spotted in the center of each square. For TBIA, leaves first were rolled into a tight bundle before being cut with a sterile razor blade into small pieces. Tuber and stem were cut transversely. Freshly cut edges of all tissues were pressed firmly against the membrane. Membranes for both assays were placed in petri dishes blocked with 5% (wt/vol) bovine

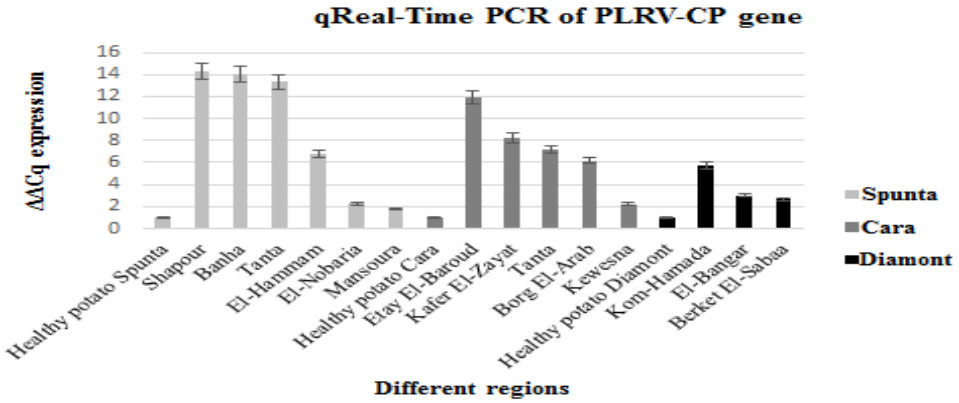

Figure 1: Histogram of the quantitative estimation for CP gene expression in leaves of three potato cultivars infected by PLRV from different regions.

serum albumin (BSA) in PBS buffer for 30 min with gentle shaking at 37°C. After a brief rinse in PBS-T for three times, the membranes were incubated with polyclonal antibodies in serum buffer with incubated at 37°C for 30 min. Membranes were washed in three changes of PBS-T, including 0.5% (wt/vol) Nitrocellulose membranes for 5 min each, then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma-Aldrich, Oakville, ON, Canada), at 1:7500 dilution in serum buffer at 37°C for 30 min then membrane was washed 3 times with PBST for 5 min. Membranes were then washed as before and incubated with freshly prepared substrate NBT/ BCIP sodium salt solution reagent with gently agitation for 1 min. The reaction was stopped by washing the membrane in deionized water for several minutes. The membrane was air dried on a filter paper and photographed.

Results and Discussion

qReal-Time PCR for detection PLRV-CP gene

PLRV is one of the most destructive viruses in potatoes [29] and in seed-production schemes, the absence or very low incidence of the virus is a prerequisite [30]. While most of the viruses infecting potatoes can be detected easily in leaf, stem and tuber tissues by Dot and Tissue blot. On the other hand, quantitative Real-Time PCR (qRT-PCR) is possibly the best method to analyze gene expression because of the large dynamic range; high sensitivity and reproducibility [31-35]. Thus, in order to estimate the relative gene expression of some viral genes understudy, the relative amounts of viral RNA for target PLRV-CP gene for three potato cultivars (Spunta, Cara and Diamont) were compared with the amount of viral RNA in healthy leaves samples. Results revealed that, gene expression in all examined infected samples was higher than that for the control samples. The highest expression values were; 14.32 (Spunta, komhamada), 11.93 (Cara, Etay El-Baroud) and 5.71 (Diamont, komhamada). But the lowest expressions of CP gene were observed with Spunta (Mansoura) is (1.75), with Cara (kewesna) 2.21 followed by 2.64 with Diamont (Berket El-Sabaa). Similarly, Arif et al. [36] tested 22 plants of 14 lines harbored both PVY-CP and PLRV-Replicase genes. They found sixteen plants of 11 double transgenic lines showed high level of the expressions for both genes. In addition, in the present study, the expression PLRV-CP gene varied from region to another for the same cultivar. We assume that expression variability of the viral genes in host cells due to time and place of sampling collection. Also, the relative environmental conditions; degree of infection; virus type and stage of viral life cycle; plant genotype and plant-virus interactions affect the expression level for specific genes Pallas and García, [37]. In some studies, Real Time PCR has been described for efficient detection of PLRV in dormant tubers but due to high costs of reagents and equipment involved in real time PCR, the method described herein is more applicable and cost-effective (Figure 1).

Purified of virus particles and r-coat protein

The purified virus protein was separated on SDS-PAGE and size of band 80kDa with RT about 53kDa respectively as shown in Figure 2A. This result are in agreement with Brault et al. [38]; Filichkin et al. [39]; Jolly and Mayo, [40]; Wang et al. [41] found within the read through protein (RTD) there is a highly conserved N-terminal region and a variable C-terminal region. The full length RTP can be detected readily in infected tissue, but in purified virus preparations a significant portion of the C-terminus of the RTD is proteolytically processed yielding a 51–58 kDa RTP. This phenomenon has been seen among other members of the family *Luteoviridae* and despite such truncations, the virus is still aphid transmissible [41]. Also, this result are similar with Bahner et al. [42] that reported the major protein component detected by staining with Coomassie blue was the 23 K protein but small amounts of a 53K protein were always present. A 53K polypeptide has been detected in protein from particles of PLRV in preparations purified from potato tissue and *Physalis floridana* using either Celluclast or Driselase Waterhouse, [43] to macerate the tissue prior to extracting the virus particles. Moreover, the purified *luteovirus* particles contain two types of proteins: a major capsid protein (CP) of ~22 kDa and a minor capsid component of 54 kDa, which is a truncated form of a translation read-through protein of the CP gene termination codon. The read-through domain (RTD) contains determinants responsible for virus transmission according to Gonçalves et al. [44].

The purified protein for rCP-PLRV was separated on SDS-PAGE analysis and an enriched expected size of band (approximately 23kDa) was observed (Figure 2). Also, Hossain et al. [45] amplified and cloned 346 bp amplicon of PLRV-CP gene. Our purified PLRV-CP fragments were then sub-cloned into expression vector and transformed into *E. coli* cells. The expressed proteins were purified and one band of ~23 KDa was detected on SDS-PAGE (Figure 2B). According to Mayo and Miller [46], PLRV virions are assembled mainly from the 23 kDa coat protein (CP), but contain minor amounts of readthrough protein (RTP) translated when the stop codon of the CP is suppressed.

Antiserum production for both viral particles and recombinant coat protein

The polyclonal antibodies were obtained from rabbit bleeding after two weeks from the last injection, as presented in Figure 3. The titers of antisera raised against PLRV were 1: 6400, 1/25600 and 1/51200 as determined by indirect ELISA. For experimental point of view, the

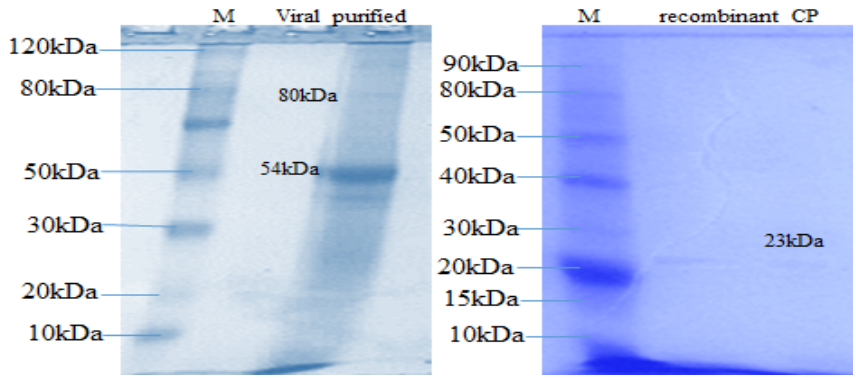

Figure 2: SDS-PAGE analysis showing, (A): virus purification of PLRV, (B): the purified PLRV-CP (23kDa) as a result of the recombinant vector (PLRV-CP gene) expression, where M, 200 kDa protein marker.

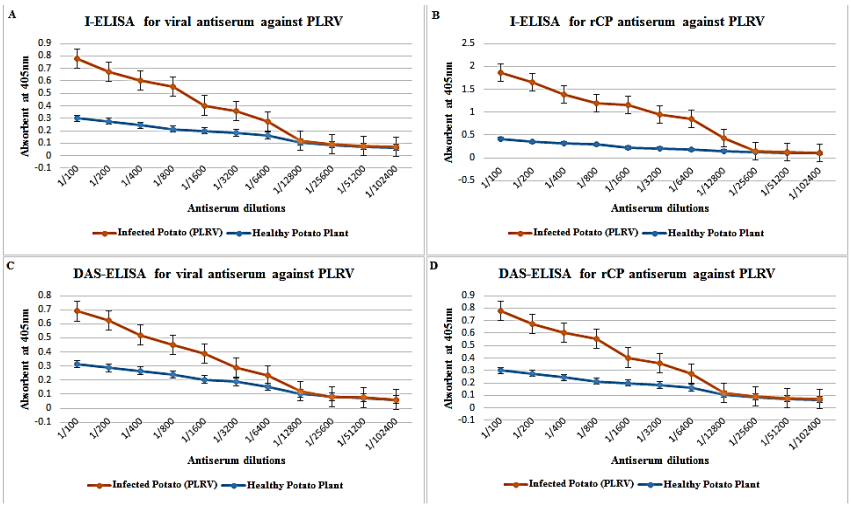

Figure 3: Indirect ELISA and DAS-ELISA using different dilutions of crude PLRV-CP rabbit polyclonal antibody. Healthy and PLRV (1:10, w/v) were tested. Goat-anti rabbit AP-conjugate was used as secondary antibody.

antiserum titer decreased gradually, as it reacts at 1:3200 and 1: 6400 for virus particles and rCP respectively (Figures 3A and 3B respectively). The obtained results were in agreement with that obtained by El-Sharkawy [47], who reported that the antiserum produced against *cow pea aphid borne virus* (CABMV) had titer of (1: 1024) by indirect ELISA. Antiserum obtained after first bleeding (1: 1024) was used in the subsequent experiments. The antiserum produced against PLRV-rCP was applied successfully for the sensitive detection of PLRV in potato plant more than that produced from viral particles. While DAS-ELISA found of virus purified and r CP results were 1/400 and 1/800 respectively (Figures 3C and 3D). Also, we observed sensitivity of Indirect than DAS-ELISA and absorbent value was highest in Indirect-ELISA than DAS-ELISA. In addition, Abdel-Salam et al. [8] noticed that the r-antisera for PVX and PLRV were induced through immunizing the animal with equal concentrations of virus CP prepared under denatured and native conditions. This would expose both epitopes (outer epitopes) and cryptopes (hidden epitopes) to the animal immune system and therefore enhance immunogeneictiy. The similar result detected by the Indirect ELISA had a greater sensitivity than either DAS- or cocktail-ELISA. The observed high sensitivity of indirect ELISA compared with DAS-ELISA could be due to the binding of more virus antigen on plates that were not percolated with specific antibodies, as suggested by Hsu and Aebig and cited by Hsu and Lawson [48]. Moreover, when evaluating the sensitivity of indirect and DAS-ELISA, their relative ability for accurate quantitation of viral antigens in crude extracts should be considered. For instance, those observed an increase in absorbance values by indirect ELISA with increasing dilutions of *C. quinoa* leaves and hibiscus roots. This likely reflects competition between viral and plant proteins for the finite number of binding sites on the microtiter plate, as previously observed by Lommel et al. [49]. Also, El-Attar et al. [16] addressed the possibility of using recombinant PLRV-CP to produce PLRV specific antisera and to test their suitability for use in serological diagnostic assays for surveys or in certification programs. This investigation suggested that the recombinant virus coat proteins expressed in bacterial cells have great potential as an alternative source of antigens for raising specific antibodies to plant viruses. Such recombinant virus coat proteins can be produced in large quantities and can be manipulated or modified as needed for specific uses. A similar suggestion was reported by Soliman et al. [15].

Dot blot immunoassay (DBIA) found to be sensitive enough for detection of PLRV in infected potato plants (leave, stem and tuber) as presented in Figure 4. A purple colour was obtained from tissues in positive reactions, whereas extracts from healthy plants remained green in representing negative reactions and colour less in blank. DBIA, the virus could be detected in sap extracted from leaves, stems and tubers of

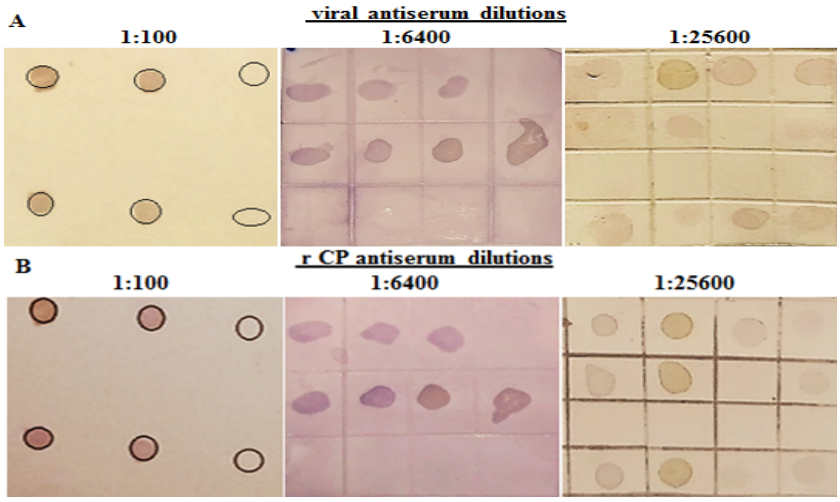

Figure 4: Detection of PLRV in potato leaf, stem and tuber samples using PLRV-antiserum produced for both (A) virus particles and (B) recombinant CP by Dot blot.

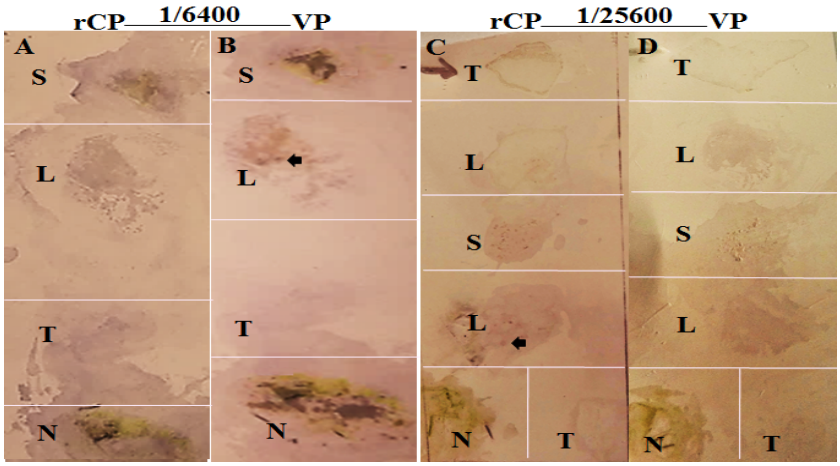

Figure 5: Tissue blotting immunoassay: (A and B); rCP and virus particles used 1/6400 dilution from antiserum, (C and D); rCP and virus particles used 1/25600 dilution from antiserum respectively.

Dilutions of polyclonal antibodies	DAS-ELISA		I-ELISA		DBIA		TBIA		RT-PCR	
	rCP	Vp	rCP	Vp	rCP	Vp	rCP	Vp	rCP	Vp
1/100	+	+	+	+	+	+	+	+	+	-
1/200	+	+	+	+	+	+	+	+	-	-
1/400	+	+	+	+	+	+	+	+	-	-
1/800	+	-	+	+	+	+	+	+	-	-
1/1600	-	-	+	+	+	+	+	+	-	-
1/3200	-	-	+	+	+	+	+	+	-	-
1/6400	-	-	+	-	+	+	+	+	-	-
1/12800	-	-	-	-	+	+	+	+	-	-
1/25600	-	-	-	-	+	+	+	+	-	-
1/51200	-	-	-	-	-	-	-	-	-	-
1/102400	-	-	-	-	-	-	-	-	-	-

Table 1: Comparison for both r CP and viral particles Polyclonal antibodies used detection of PLRV in potato samples by I-ELISA, DAS-ELISA, DBIA, TBIA and RT-PCR.

infected plants and used rCP antiserum and viral antiserum at dilutions up to 1×10^2, 1×10^3 and 1×10^5, respectively. Such results generally are in line with those reported by Chaicharoen et al. [50] and El-Sharkawy [47]. The rate of false DBIA positives was estimated. Since, DBIA has been found to be more sensitive than ELISA. The immunogenicity of the r-antisera for PLRV was also examined for detecting these viruses in commercial potato plants in the field. Similar results showed the high antigenicity of r CP of *Potato Virus Y* and PLRV immunogenicity of its r-antiserum upon evaluation with DBIA [8]. Also, Fulladolsa Palma et al. [51] investigated that the unlike ELISA, DBIA is not quantitative. The dot-blot immunoassay was successfully used to detect several genera of viruses in different hosts, including *Potyvirus* (PVY and PVA in potato, CYVV in clover), *Potexvirus* (PVX in potato, HVX in hosta), *Carlavirus* (PVS in potato), *Luteovirus* (PLRV in potato), *Cucumovirus* (CMV in pumpkin), *Tobamovirus* (TMVin Nicotiana benthamiana), and *Nepovirus* (TRSV in N. benthamiana). Signals from virus-infected samples were clearly visible for all of the samples tested, including those infected with PLRV, from which it is often difficult to detect a signal after ELISA due to low virus titer in the host plant.

Sensitivity of PLRV detection in leaves (L), stems(S) and tubers (T) extracts using TBIA was examined for the presence of PLRV in potato sap extracts. For PLRV was abundantly present in all tissues tested. Signals obtained from potatoes of PLRV infected plants are shown in Figure 5. Strongest blots detected in leaves and stems and sharper images were obtained from antiserum dilution 1/6400 produced from

rCP than Virus particles. While, the antiserum dilution 1/25600 high blot concentration was observed in leaves, stems than tubers, but, healthy potato plant was used as a negative control (N).

Results demonstrated in Table 1 revealed that, the dilution end point of the produced antiserum against PLRV was used at dilution 1/6400 and 1/3200 of rCP and Virus respectively by indirect ELISA and 1/25600 used in Dot-blot and Tissue blot for both antiserum but 1/800 and 1/400 of rCP and Virus particles respectively by DAS-ELISA.

Conclusion

We studied and compared the sensitivity, specificity and reactivity of antiserum produced from r CP more specific and sensitive than the antiserum produced by the virus purified particles. Serological methods such as DBIA and TBIA easy, rapidly, inexpensive, sensitivity and reactivity than I-ELISA, DAS-ELISA and RT-PCR.

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