GENERATION OF BACMID-BASED RECOMBINANT BACULOVIRUS FOR BANANA BUNCHY TOP VIRUS COAT PROTEIN GENE EXPRESSION IN INSECT CELLS

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Banana Bunchy Top Virus (BBTV) is a plant virus belongs to the genus Babuvirus, family Nanoviridae, which infects banana plants in many countries all over the world and often resulting in large-scale crop losses (Dale *et al.*, 1992; Bhadra *et al.*, 2010; Kumar *et al.*, 2011; Abdel-Salam *et al.*, 2012; Stainton *et al.*, 2015).

BBTV is transmitted by an aphid vector *Pentalonia nigronervosa*, and in vegetative planting materials (Magee, 1940). Districted symptoms of banana bunchy top disease are dark green streaks in the veins of the leaf midrib and pseudostem, chlorosis of the leaf margins and narrowing as well as bunching of successive leaves.

Strongly infected Banana plants prevented from bearing fruit, where banana hands and fingers are normally distorted and twisted in cases of fruits production (Ferreira *et al*., 1997).

The BBTV is a multi-component single-stranded DNA genome. The six genome components of BBTV are approximately 1,000 nt long for each and called DNA-R, DNA-U3, DNA-S, DNA-M, DNA-C and DNA-N (King *et al*., 2012). DNA-R encodes a replication-associated protein (Rep), DNA-S is a capsid protein (CP), DNA-M a movement protein (MP), DNA-C a cell-cycle link protein (Clink) and DNA-N a nuclear shuttle protein (Nsp), whereas the function of DNA-U3 is unknown (Hafner *et al*., 1997; Aronson *et al*., 2000; Wanitchakorn *et al*., 2000a).

Sensitive, reliable and rapid early detection and identification system of plant viruses are essential to prevent spreading of the virus for effective disease control (Boonham *et al.*, 2014).

Traditionally, the polyclonal and monoclonal antibody production to be used for serological techniques require obtaining highly purified virus particles. However, virus purification is usually labor-intensive procedure with varying occasionally unsatisfactory results concerning the purity and concentration of the final preparation (Ling *et al*., 2007). One of the most promising methods to overcome these difficulties is the use of recombinant DNA technology to express the virus coat protein even in prokaryotic or eukaryotic cell systems. Hence, the purified protein can be successfully used for

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further antisera production (Alves-Junior *et al.*, 2008).

Baculovirus expression vector system (BEVS) is an efficient protein expression method that can be used for plant virus's diseases diagnosis. Baculovirus system can be used as an effective expression system for viral coat protein expression in insect cell culture with high protein expression yield to be subsequently used as an antigen for further generation of vial polyclonal antibodies. In this study, the expressed viral antigen coat protein of Egyptian BBTV will be produced using baculovirus expression system in insect cell culture and will be tested as a candidate antigen.

MATERIALS AND METHODS

Viruses and insect cell line

The Egyptian isolate of BBTV (Qalubia governorate) was used as a template for the amplification of BBTV *CP*. Sf9 cells, a clonal isolate of *Spodoptera frugiperda* Sf21 cells (IPLB-SF21-AE) (Thermo scientific), was used for recombinant protein production using baculovirus expression system. Cells were maintained in Excell-420 serum-free medium (Sigma) with an antibiotic-antimycotic mixture at 27°C as was described by O'Reilly *et al.* (1992).

Amplification of CP and GFP genes

Two oligonucleotide primers (Ha.004, F: 5′ GAC AAG AAG GAT CCA TGG CTA GGT ATC CGA AG ′3 and Ha.002, R: 5′ ATA AAG CTT TCA AAC ATG ATA TGT ′3) were used for PCR amplification of BBTV *CP*-ORF gene based on the published sequence of Wanitchakorn *et al.* (1997). Nucleotides in bold represent *Bam*H1 site in the forward primer and *Hin*dIII site in the reverse primer.

The PCR reactions were performed in a total reaction volume of 25 µl containing 1 µl of the appropriate forward-primer (10 pmol/µl), 1 µl of the appropriate reverse-primer (10 pmol/µl), 1 µl $MgCl_2$ (25 mM), 1.5 µl of dNTPs mixture solution (10 mM), 2 µl of DNA template (0.1-0.5 µg), 5 µl of 5X PCR reaction buffer and 0.5 µl of Goflexi Taq DNA polymerase (5 U/µl) (Promega). The reaction volume was completed to 25 µl using autoclaved bdH_2O. For a typical PCR reaction the following programme was used; an initial cycle at 95°C for 3 min; a total of 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min; final cycle at 72°C for 7 min to allow the completion of primer extension. The obtained PCR fragments were gel purified using the Qiaquick gele extraction kit (Qiagen), cloned into pGEM-T vector (promega) and sequenced to verify the correct sequences (data not shown). The pGEM-T-*CP* construct was digested using *Hin*dIII and *Bam*HI restriction digestion and the obtained *CP*-ORF *Hin*dIII/*Bam*HI fragment was cloned subsequently into pFastBac^{TM}Dual vector under Polyhedrin promoter previously digested by *Bam*HI and *Hin*dIII restriction enzymes. The generated construct was

denoted pFBD-CP_{polh}. The *GFP* gene was amplified based on Monster GFP vector phMGFP as a template using one pair of specific primers. The forward primer designed phMGFP-F (5'-CCG CTC GAG ATG GGC GTG ATC AAG CC-3'), and the reverse primer designed phMGFP-R (5'-CGG GGT ACC TTA GCC GGC CTG GC-3'), adding *Xho*I and *Kp*nI restriction sites to the 5'-end of phMGFP-F and phMGFP-R primers (under lined), respectively. The PCR reaction was performed using the same conditions as was described before for *CP* ORF amplification.

The amplified *GFP* PCR fragment was cloned into pGEM-T Easy vector and subsequently to pFBD-CP_{polh} construct, previously digested with *Xho*I and *Kp*nI, under the control of P10 promoter generating the bacmid cloning vector pFBD-CP_{polh}-$GFP_{p10.}$

Generation of the recombinant bacmid

In order to generate the recombinant bacmid cassette harbouring the BBTV *CP* and *GFP* genes, the recombinant pFBD-CP_{polh}-GFP_{p10} construct was transformed into DH10Bac competent *E. coli* using the manufacture instruction (Life technologies). One nanogram (1 ng) of the pFBD-CP_{polh}-GFP_{p10} DNA was transformed into 100 µl of the pre-chilled competent DH10Bac cells. The mixture was incubated on ice for 30 min and heat-shocked for 45 sec at 42°C. Immediately after cells heat-shocked, the mixture was chilled on ice for two minutes. About 900 µl of LB medium was added to the mixture and incubated at 37°C with shaking for four hours to facilitate transposition of the recombinant cassette into the bacmid mini-*att*Tn7 site. Five serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) of the obtained culture were performed and spread on LB-Agar plates contains (50 µg Kanamycin, 7 µg Gentamycin and 10 µg Tetracyclin). To facilitate Blue/white colonies screening, X-Gal (100 mg/ml) and IPTG (40 µg/ml) were added to each plate before spreading of the culture. All plates were incubated for 48 hour at 37°C. To verify successful transposition 10 clear white colonies were PCR analyzed using one pair of pUC/M13 primers designed M13 Forward 5′-(GTTTTCCCAGTCACGAC)-3' and M13 Reverse 5′-(CAGGAAACAGCTATGAC)-3′, flanking the insertion site of mini-*att*Tn7 in the recombinant bacmid. The recombinant bacmid DNA was isolated from 2 ml of overnight culture of single positive white colony following the manufacture instruction (Bac-to-Bac manual, Lifetechnologies).

Sf9 cells transfection and protein expression

The verified recombinant bacmid was transfected into Sf9 cells using the method as was described by O'Reilly *et al.* (1992). Briefly, Purified recombinant bacmid DNA (500 ng) and Cellfectin transfection reagent (Lifetechnologies) were mixed in a total volume of 210 µl of Excell-420 serum-free medium and kept for 30 min at room temperature. The mix-

ture was added drop wise to Sf9 cells (5x 10^9 Cells/plate) previously grown in 35-mm tissue culture 6 well-plate. The cells were incubated at 27°C for 5 hours then the medium containing Cellfectin/bacmid DNA mixture was removed and replaced with Excell-420 medium contains 10% Fetal bovine serum (FBS) and the appropriate antibiotics. Cells were incubated at 27°C for 72 h in a humidified incubator until appearance of signs of viral infection. The successful transfection was further confirmed by detection of GFP protein florescence using the dark filed of Ziss inverted fluorescent microscopy (Axiover 100, Ziss, Germany).

The supernatant contains Budded Virus (BVs) of the recombinant bacmid was separated from Sf9 cells as Stock1 and this was used to prepare Stock2 and Stock 3 *via* serial infection process of Sf9 cells for two week. The recombinant virus stock 3 was used to infect Sf9 cells (10 pfu/cell) using 25-Cm2 tissue culture flasks (Greiners), for 72 hour and the Sf9 cells were harvested and applied for total protein extraction.

Dot blot analysis

The Dot blot analysis was used to detect the specificity and sensitivity of the expressed BBTV coat protein according to the method described by Banttari and Goodwin (1995). Polyclonal antibodies generated against BBTV particles - Molecular Plant Pathogenesis Lab, AGERI, Giza, Egypt- were used to evaluate the expressed protein as a viral antigen. The cell lysate (infected and non-infected cells) was used as an antigen and extracts of banana BBTV infected tissue samples were used as positive control.

RESULTS AND DISCUSSION

Construction of the recombinant virus

The recombinant bacmid harboring both *CP* and *GFP* genes was generated in order to use the expressed recombinant protein as an antigen for further production of BBTV polyclonal antibodies as an alternative methodology instead of using viral particles. For this, a shuttle vector called vAc-CP_{polh}-GFP_{p10} was constructed (Fig. 1A). The BBTV *CP* gene was successfully amplified using DNA of infected BBTV tissues as a template and two specific primers (Ha.004, F/Ha.004, R) which gave a PCR fragment of 513 bp. This result was in agreement with results previously obtained by different reports (Nour El-Din *et al.*, 2005; Wanitchakorn *et al.*, 2000b; Karan *et al.*, 1994 & 1997).

The amplified 513 bp *CP* gene fragment was subsequently inserted into pFastBac-Dual vector (pFBD) downstream of the Polh promoter generating the modified plasmid pFBD-CP_{polh}. The improved synthetic version of *GFP* gene (hMGFP) was fully amplified using Monster GFP vector phMGFP as a template by one pair of specific primers designed phMGFP-F and phMGFP-R giving a fragment of 683 bp. The produced *GFP* fragment was inserted into the modified plasmid pFBD-CP_{polh} under the control of

P10 promoter to give the bacmid cloning vector pFBD-CP_{polh}-$GFP_{p10.}$ This construct was verified using PCR and restriction digestion analysis (data not shown). The obtained construct pFBD-CP_{polh}-GFP_{p10} was used to generate the recombinant bacmid vAc-CP_{polh}-GFP_{p10} using the Bac-to-Bac expression system (Fig. 1B). The recombinant virus harboring both BBTV *CP* and the *GFP* genes cassette was verified by PCR analysis (Fig. 2). As shown in Fig. (2), two primers of PUC/M13 designed M13-F and M13-R flanking the bacmid mini-*att*Tn7 site was used to amplify the whole cassette carrying both *CP* and *GFP* genes. About 10 clones were subjected to PCR analysis to verify the correct bacmid construct. PCR analysis resulted in 3758 bp fragment in one clone as expected from the correct recombinant construct. However the empty bacmid constructs gave the PCR fragment of about 300 bp. The recombinant bacmid harboring both *CP* and *GFP* genes was designed vAc-CP_{polh}-GFP_{p10}. The vAc-CP_{polh}-GFP_{p10} was transfected and amplified in Sf9 insect cells and used to prepare viral stocks to be used for recombinant protein expression.

The baculovirus expression vector system (BEVS) is a common eukaryotic cell expression system. This system has been widely used for high yield expression of heterologous proteins in insect cells and in insect larvae for research purposes and pharmaceutical applications (Luckow, 1991; Tani *et al.*, 2008; Kato *et al.*, 2010). BEVS has many advantages as a protein expression system such as; post-translational modifications, production of soluble recombinant proteins and antigenically, immunogenically, and has a similar function to their counterparts. The huge and flexible baculoviral genome of 130 kbp enables a high capacity for multiple genes or a large insert; express large amount of the foreign protein under the control of the very strong promoter of the NPV *polyhedrin (Polh)* or *p10* gene. Moreover, BEVS is safe since it doesn't infect mammals or other vertebrate animal cells (Luckow *et al.*, 1993). In order to further increase the protein production by the BEVS, a 128 bp simian virus 40 (SV40) polyadenylation signal sequence or SV40 polyA was added to the *polh* promoter-based transfer vectors Bac-to-Bac® pFastBac™ vectors. The SV40 polyA signal is recognized and used by the host RNA Polymerase II complex to process precursor mRNA and increase the stability of the mature mRNA as well as enhance the efficiency of mRNA translation in eukaryotic cells. However, in a study to investigate the influence of using SV40 polyA on enhanced green fluorescent protein (EGFP) expression, which is driven by the polyhedrin promoter, it was concluded that the mRNA levels increases in the presence of SV40 polyA but protein production was decreased in the BEVS when the polyhedrin promoter is used at different loci (Salem *et al.*, 2015). This work suggests that SV40 polyA in BEVSs should be replaced by an AcMNPV late gene polyA for optimal protein production.

GFP detection in infected Sf9 cells

The spreading of the recombinant virus vAc-CP_{polh}-GFP_{p10} in Sf9 insect cells was further analyzed by GFP protein florescence using the dark field of inverted fluorescent microscopy 72 hours post infection (hpi). The improved *GFP* full length gene was PCR amplified using Monster GFP vector phMGFP as template. The Monster Green Fluorescent Protein is encoded by an improved version of the green fluorescent protein gene originally cloned from *Montastrea cavernosa* (Great Star Coral). The synthetic gene (hMGFP) expresses 26 kDa protein that shows improved fluorescence intensity compared to the native gene. The amplified Monster GFP gene was cloned downstream of the very late promoter (*P10*) of the modified vector pFB-CP_{polh} to be inserted subsequently into the mutant vAc-CP_{polh}-GFP_{p10}. The green fluorescent protein was successfully detected in infected Sf9 cells (white arrows, Fig. 3) in comparison to non-infected cells, confirming the successful integration of the cassette carrying *GFP* and BBTV *CP* genes into the viral genome.

The importance of polyadenylation for *p10* gene expression in BEVS was analyzed using recombinant viruses with altered 3' untranslated regions (UTRs) using chloramphenicol acetyltransferase (CAT) as a reporter. Interestingly, after inactivation of the downstream AATAAA motif, CAT expression remained at the same level as obtained with a wild-type 3' UTR. Whereas, by replacing the *p10* 3' UTR with the SV40 PolyA terminator sequence, which is commonly, used in baculovirus expression vectors, this resulted in a reduction in reporter gene expression. These results concluded that in order to obtain high levels of protein expression under p10 promoters, baculovirus vectors that provide a wild-type *p10* 3' UTR are preferred over vectors containing the hsp70-lacZ-SV40 gene cassette (van Oers *et al*., 1999; Salem *et al*., 2015). However, the green florescence protein expressed under *P10* promoter was clearly observed in Sf9 cells infected with the recombinant virus vAc-CP_{polh}-GFP_{p10} and SV40 polyA.

Recombinant protein analysis

The sensitivity and specificity of the recombinant protein was evaluated by using polyclonal antibodies of BBTV via dot blot analysis. The results showed that the polyclonal antibodies generated against BBTV virus particles reacted positively with the produced recombinant BBTV-CP with strong signals as well as the BBTV infected banana plant extracts (as positive control). No colored signals have been detected with the extract of non-infected Sf9 cells (negative control). The dot blot analysis confirmed the sensitivity and specificity of the expressed BBTV CP to the polyclonal antibodies generated against BBTV particles (Fig. 4). Dot blot analysis is simple, relatively inexpensive and the result can scored visually, it is relatively more sensitive and economical compared to ELISA method (Rajasulochana *et al*., 2008; Sharma and

Misra 2011). Furthermore, the tested sample extracts can be blotted on the membrane at the field level and then send to the laboratory for further processing, accordingly a wide potential application of this technique can result in large scale detection of the virus infected plants using specific antibodies (Nour El-Din, 2013). These results suggested that the baculovirus expression system can be used effectively for BBTV CP expression to be used as a candidate antigen for further development of Polyclonal antibodies against BBTV infected plants.

The BEVS has been applied successfully for different viral coat protein expression in insect cells. Ardisson-Araújo *et al.* (2013) have used baculovirus expression system for coat protein gene expression of the Garlic Mite-borne Filamentous Virus (GarMbFV) under the control of *Polyhedrin* promoter (*Polh*). The generated construct was successfully produced large amount of the recombinant protein that was subsequently used for the generation of viral specific antibodies. In the same context, a recombinant baculovirus construct expressing glycoprotein E (gE) of the Egyptian BoHV-1.1 was performed. It was concluded that the expressed gE protein, as an antigen, is a promising specific and sensitive candidate for inexpensive detection of anti-gE antibody in both control and experimental bovine sera (El-Kholy *et al*., 2013). Furthermore, the production of recombinant baculoviruses expressing neurotoxin proteins such as scorpion toxin (Maeda *et al*., 1991), mite toxin (Tomalski and Miller, 1991) and cry1Ab of *Bacillus thuringiensis* (El-Menofy *et al.*, 2014), were successfully produced and were used as active toxins for biological control of insect pests.

The BEVS has the ability to produce high yields of authentically syntheses proteins (Luckow and Summers, 1988; Miller, 1988), due to the capability of insect cells for recognition and secretion of signal peptide (Davis *et al.*, 1992), post-translational modifications process such as glycosylation (James *et al.*, 1995), and phosphorylation (Hericourt *et al.*, 2000), the BEVS is now routinely used for the construction of recombinant proteins for a variety of purposes including; viral structural, gene function studies and the development of different diagnostic tools and vaccines production. Whereas, baculovirus harboring mammalian promoters (bacmams) was used for gene delivery (Hu, 2008) and high throughput screening in mammalian cells for drug discovery setting (Kost *et al.*, 2007).

This study concluded that using of baculovirus expression system vector (BEVS) for BBTV coat protein expression in insect cells can be used as an alternative promising candidate for the development of plant virus diagnostic kits of viruses that are in low titers, difficult to purify and present in mixed infections in their plant hosts.

SUMMARY

Bunchy top disease is one of the most destructive viral diseases caused by

the Banana Bunchy Top Virus (BBTV). Using of virus particles as an antigen for polyclonal and monoclonal antibodies production by common serological techniques has several disadvantages concerning the purity and concentration of the viral particles and by extension the produced antibodies. In this study, the coat protein gene (*CP*) of BBTV was expressed using baculovirus expression vector system (BEVS) under the control of the Polyhedrin promoter (*Polh*). Accordingly, the generated cassette consisting of the *CP* gene and the improved Green Florescent Protein (*GFP*) gene. The generated recombinant virus (vAc-CP_{polh}-GFP_{p10}) was proved using PCR analysis. Spreading of the recombinant virus in Sf9 insect cells was successfully detected using GFP protein florescence under inverted fluorescent microscopy. The generated recombinant virus was amplified in Sf9 cells and the expression of CP was tested using the infected cell lysate by Dot blot analysis. The anti-BBTV polyclonal antibodies serologically reacted with the recombinant CP which was expressed in Sf9 cells as well as to the BBTV infected plants. This result suggested the possibility of using the expressed CP of BBTV for further development of antisera that can be implemented for BBTV detection in infected plants.

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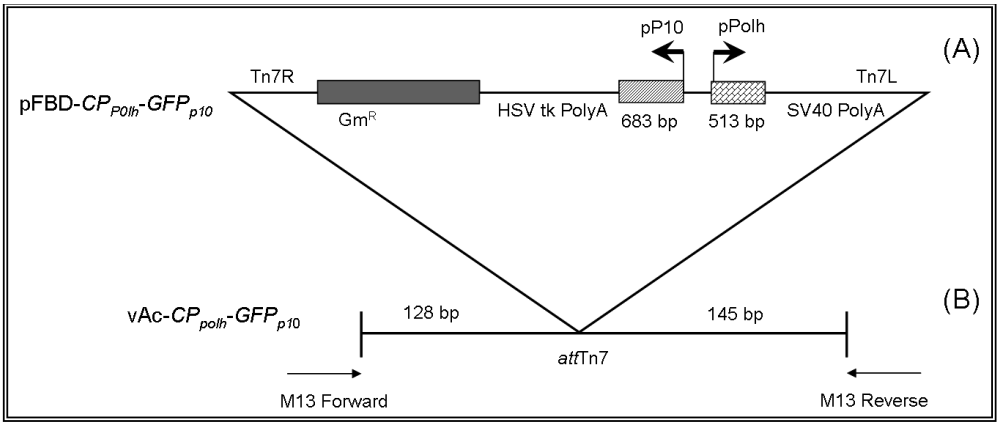


Fig. (1): Schematic representation of the construction of vAc-CP_{polh}-GFP_{p10} bacmid. (A) The bacmid cloning vector pFBD-CP_{polh}-GFP_{p10} harboring the BBTV *CP* under the control of Polh promoter, and the *GFP* gene under the control of P10 promoters. (B) The generated vAc-CP_{polh}-GFP_{p10} bacmid contains the *att*Tn7 transposition site. Black arrows show M13 Forward/M13 Reverse primers used for the PCR analysis of the recombinant bacmid.

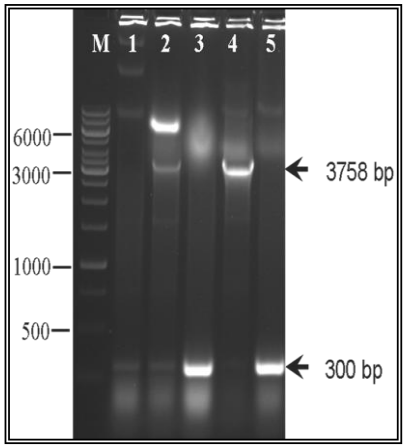
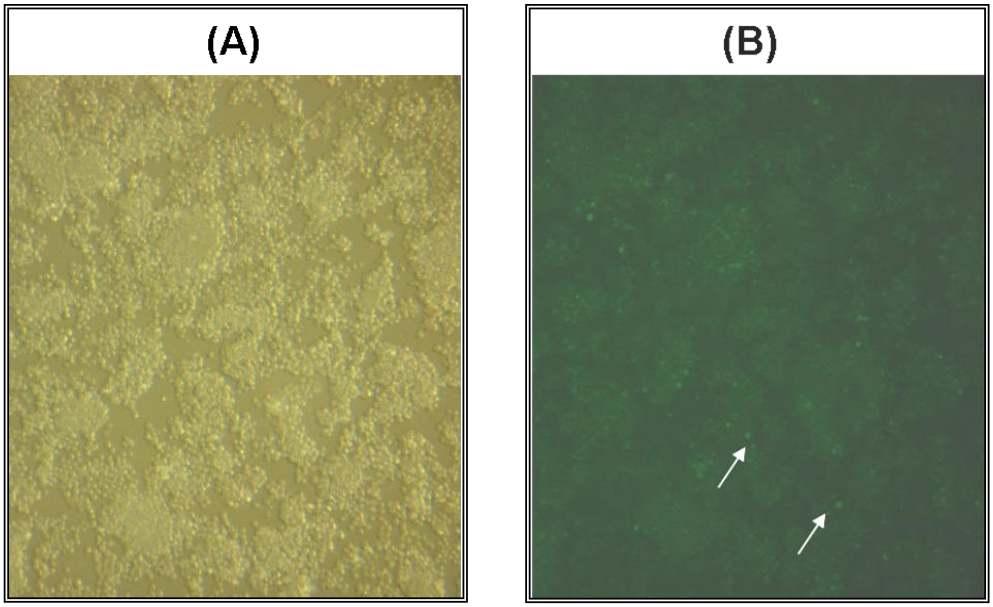


Fig. (2): Agarose gel (1 %) for PCR analysis of recombinant bacmid vAc-CP_{polh}-GFP_{p10} using M13 Reverse /M13 Forward primers flanking the *att*Tn7 transposition site. Lane 1: Negative control (blue colony). Lane 2: colony carrying both recombinant and empty bacmids. Lanes: 3 and 5: negative colonies carrying only empty bacmid (300 bp). Lane 4: Positive colony carrying BBTV *CP* and *GFP* gene cassette (3758 bp). M: 1 Kb DNA ladder marker.



Control Sf9 cells (mock infection) Infected Sf9 cells with vAc-CP_{polh}-GFP_{p10}

Fig. (3): Photograph of fluorescent microscopy analysis of Sf9 infected cells with vAc-Cp_{Polh}-GFP_{P10} using the green fluorescent protein. (A) Non-infected Sf9 cells (mock infection). (B) Infected Sf9 cells using vAc-CP_{Polh}-GFP_{P10} recombinant virus. White arrows show the spreading of green fluorescent protein inside the infected Sf9 cells nuclei.

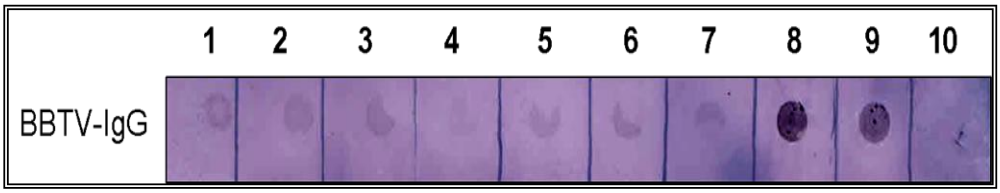


Fig. (4): Nitrocellulose membrane of dot blot analysis of the infected Sf9 cells using vAc-CP_{polh}-GFP_{p10} recombinant virus using BBTV polyclonal antibodies. Lanes 1-7: Banana infected tissues with BBTV (positive control). Lanes 8 & 9: Extracts of infected Sf9 cells using vAc-Cp_{polh}-GFP_{p10} recombinant virus. Lane 10: Non-infected Sf9 cells (mock infection).