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Purification, Serological and Molecular Studies on an Egyptian Isolate of Faba Bean Necrotic Yellows Virus (FBNYV) Infecting Faba Bean Plants

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Faba bean necrotic yellows virus (FBNYV) was isolated from naturally infected faba bean plants in Egypt expressing severe chlorosis, necrotic, leaf rolling and stunting. The identification was based on tests by indirect ELISA, symptomatology, serological properties and polymerase chain reaction (PCR). Serological testing of 150 samples from faba bean plants showing virus- like symptoms and collected randomly from 3 governorate in Egypt showed that percentage of FBNYV natural infection was 17-23.6% from the growing seasons 2007-2009. The virus was purified by PEG and sucrose density –gradient centrifugation. The purified virus preparation had an ultraviolet absorption spectrum typical of the nucleoprotein with A_{260}/A_{280} being 1.42. Yield of purified virus was 0.13mg/100g infected leaf tissue. Results of using polymerase chain reaction showed that, the simultaneous amplification of a 272 bp fragment of the FBNYV genome by using specific primer. The size of the PCR fragments was in agreement with expected for such nucleotide sequence. Serological and molecular methods, including enzyme-linked immunosorbent assay (ELISA), dot-blot immunoassay (DBIA), and immunocapture polymerase chain reaction (IC-PCR) were compared to evaluate their usefulness for diagnosis of this virus. Each method was tested with partially purified virus preparations and tissue samples from infected faba plants. Indirect ELISA was more sensitive than DBIA with all sample tested. Results showed that FBNYV was detectable in faba bean leaves up to $1:10^{-5}$ dilutions, by indirect ELISA and up to $1:10^{-3}$ dilutions by DBIA. End-point dilutions of partially purified virus preparations from indirect and DBIA were 50 and 250 ng/ml, respectively. The most sensitive method was IC- PCR, in which the virus was detected in sap of infected leaves diluted to $1:10^{-9}$ and 2ng/ml in partially purified virus preparation IC-PCR showed 16-fold and 32-fold greater sensitivity than indirect ELIAS and DBIA for detection of the FBNYV in partially purified FBNYV. Electron microscopy revealed virus particles were found scattered within the cytoplasm and occasionally in chloroplast and mitochondria which were highly deformed losing their structure.

Keywords: Faba bean necrotic yellows virus, ELISA, IC-PCR.

Legume crops are important protein source for human diets in many parts of the world and are widely cultivated for their value as food, forage and green manure. Viruses infecting legume crops have been reviewed and their importance was

indicated (Mazyed *et al.*, 1975; Hampton *et al.*, 1978; Allam *et al.*, 1979; Bos, 1982; Makkouk *et al.*; 1988 and Vega *et al.*, 2007).

Faba bean necrotic yellows virus (FBNYV, family *Nanoviridae*) causes an economically important disease of crops and pasture legumes in West Asia, North Africa, Sudan and Ethiopia (Makkouk *et al.*, 1992 and 1994; Franz *et al.*, 1996 and Shamloul *et al.*, 1999). The virus has a wide host rang as 58 host of legume species have been identified (Katul *et al.*, 1993; Franz *et al.*, 1998 and Shamloul *et al.*, 1999) Since the beginning of the 1990, this virus has been caused serious economic losses to faba bean in Egypt (Abdel-Salam and El-Sharkawy, 1996; Abdel-Salam, *et al.*, 1997). The main host is faba bean (*Vicia faba*. L). Early – infected plants remain stunted, showing leaf yellowing followed by necrosis and plant death (Ortiz *et al.*, 2006).

Isometric virus– like particles are closely associated with the disease and have been regarded as the putative causal agent although their infectivity has not yet been shown. The putative causal agent is not transmitted mechanically but by aphids in persistent manner. The virus– like particles measure 18 nm in diameter, contain a capsid protein of about 22 KDa and circular ssDNA of about 1Kb are called (FBNYV) (Katul *et al.*, 1993). Based on these criteria (FBNYV) is very similar to banana bunchy top virus (BBTV) (Thomas and Diretzyen, 1991).

In Egypt, the cultivated faba bean area is around 170.106 feddans, with an average productivity of about 244.109 tons (Anonymous, 2010).

A survey of faba bean viruses was conducted in Egypt during two growing seasons of 1993 and 1994 by Makkouk *et al.* (1994) using DAS- ELISA. They found that, FBNYV was the most frequent being (50.6%).

FBNYV is persistently transmitted by various aphid species (Franz *et al.* 1998), and with no report of mechanical transmission. Seed-transmission of an Egyptian isolate of FBNYV has been reported (Abdel-Salam and El-Sharkawy, 1996).

The aim of the present research conducted with development and application of ELISA, DBIA, and IC-PCR procedures for detection of FBNYV. A direct comparison of the sensitivity of these methods was made to evaluate their usefulness for diagnosis of this virus. The rapidity, easily, and sensitivity of these methods for the diagnosis of infected plants.

Materials and Methods

Isolation and identification:

Samples from faba bean plants showing severe chlorosis, necrosis, leaf rolling and stunting were collected from faba bean fields and used in this study. All samples were used as the source of inoculua for aphid specie *(Aphis craccivora)* inoculation of healthy faba bean. Test plants were kept under greenhouse conditions for assessment of symptoms expression. After symptoms appearance, FBNYV infection was identified by indirect ELISA, symptomatology and PCR.

2- Field survey:

Samples from faba bean plants with symptoms suggestive of virus infection were collected from 8 faba bean fields from 6 locations in 3 governorates, *i.e.* Alexandria, Behera and Kafr El-Shiekh, in Egypt during 2007- 2009. Samples were checked by indirect ELISA, for virus presence.

3- Virus Purification:

Two- week old faba bean (cv. Giza 3) were inoculated with FBNYV by aphid transmission. Three weeks later stems and leaves of symptomatic plants were harvested and stored at -80°C until use. About 100 g leaves and stem tissue was processed essentially as described by D'Arcy *et al.* (1989). The frozen tissues were further macerated in Warring blender using 2.5 vol. 0.1M sodium citrate buffer, pH 6, containing 5 µl/ml 2-mercaptoethanol, 1 µg/ ml Na N_3 and 20mg/ ml pectinase, and stirred overnight at room temperature. Following clarification of the extracts with 0.25 vol. chloroform / butanol (1:1) and low- speed centrifugation. Virions were concentrated and purified further by precipitation in polyethylene glycol 600 (8g in 100 ml), differential centrifugation and sucrose density – gradient centrifugation. Virus yield were estimated spectrophotometrically using the extinction coefficient of 3.6 for SCSV (Chu and Helms, 1988).

Serological assay:

a- Indirect ELISA:

Indirect ELISA was performed using antigen-coated plates as described by Koenig (1981) and modified by Fegla *et al.* (1997). Plant samples were prepared in 0.05 M sodium carbonate buffer (pH 9.6) and incubated at 4°C overnight. Wells were blocked by 0.2% bovine serum albumin (BSA) and 1.0% gelatin in PBST and incubated for one hour, followed by 100 µl of virus-specific IgG (1 µg/ml) in PBS containing 0.5% (v/v) Tween-20 (PBST) with incubation for 2 h at 37°C. Alkaline phosphatase –conjugated goat anti-rabbit IgG (100 µl of 1:1,000 dilution in PBST; (whole molecule, enzymatic activity 475 units /ml) was added and the plates were incubated for 2 h at 37°C. The ELISA values, measured by Multi Scan Ex ELISA reader, were expressed as absorbance at 405 nm. Absorbance values of at least double that of healthy control, were considered positive.

b- Dot-blot immunoassay (DBIA):

Nitrocellulose membrane was pre-wet in 100% methanol for 10 s and then washed in distilled water for 1 min. The membrane was marked into 1-cm squares and a 2-µl sample was spotted in the centre of each square. Membranes were blocked with 5% (w/v) non fat dry milk (NFDM) in PBS buffer for 1 h with gentle shaking at room temperature. After a brief rinse in PBST, the membrane was incubated with virus-specific IgG at 2 µg/ml (1:500) in PBST with gentle agitation at room temperature for 1h. Membrane was washed in three changes of PBS, including 0.5% (w/v) NFDM for 10 min, then incubated with alkaline phosphates-conjugated goat anti-rabbit IgG (AP-GAR; Bio-Rad), 1:1,000 dilutions in PBST for 1 h at room temperature. Finally, the membrane was removed from the second antibody dilution, dipped in distilled water, washed twice in TBST, 10 min each. And dried for a short time between filter paper. Prepared substrate solution containing 5- bromo-4-chloro-3-indolyl phosphate (BCIP) at 0.15 mg/ml and nitro

blue tetrazolium (NBT; Sigma-Aldrich, St. Louis, MO) at 0.30 mg/ml were applied for colour development. After colour development, the reaction was stopped by washing the nitrocellulose membrane in 0.01 M Tris-HCl containing 0.05 M EDTA, pH 7.5. The positive reaction of DBIA was indicated by the development of purple colour on the blots. The negative reaction developed no colour or green colour (Kamenova and Adkins, 2004).

Molecular biology studies:

a- Nucleic acid extraction:

Nucleic acids were extracted from FBNYV purified virions as described by the technical bulletin of High pure PCR template preparation kit (Roche). 200 µl of purified virus was mixed with 200 µl of Binding buffer and 40 µl of 20 mg/ml proteinase K was added. The mixture was incubated at 72°C for 10 minutes and 100 µl of isopropanol were added. The samples were pipetted into the upper reservoir of a combined High Pure filter tube-collection tube assembly and centrifuged in a standard table top centrifuge for 1 min at 10000 g. The flow through was discarded and the filter tube was combined with a new collection tube. 500 µl of Inhibitor removal buffer was added to the upper reservoir and centrifuged for 1 min at 10000 g. The flow through was discarded again and 500 µl of wash buffer was added twice to the upper reservoir and centrifuged for 1 min at 1000 g. The filter tube was inserted into a clean 1.5 ml reaction tube and 200 µl of a prewarmed elution buffer was added to the filter tube and centrifuged for 1 minute at 10000 g. so, the microfuge tube now contains the eluted DNA.

b- PCR amplification of FBNYV:

The coat protein gene of FBNYV was generated by a PCR-based strategy using specific primers P19 (5¯TTATTGTTAAATG TAATTCACCTAT-3) and P6 (5¯CA CTTCAACATAAACTCTG-3), located in the C5 (Ortiz *et al*, 2006). PCR mixes comprised 1 µl of each primer, 2.5 µl 10 mM dNTPs, 0.3 µl *Taq* DNA polymerase, 2.5 µl *Taq* DNA polymerase buffer, 2 µl MgCl and 1 µl of total nucleic acid extract. The reaction mixes were denatured at 94°C for 4 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min followed by 1 cycle of 72°C for 10 min. Following amplification, PCR products were analyzed by electrophoresis on native 2% agarose gel and detected by ethidium bromide staining

c- Immunocapture polymerase chain reaction (IC-PCR):

Sterile 0.5-ml polypropylene microcentrifuge tubes were coated with 100 µl of FBNYV specific IgG (1 mg/ml diluted 1:500 in 0.05M sodium carbonate buffer, pH 9.6), incubated for 3 h at 37°C and washed there times with PBST at 200 µl/tube. 100ng of purified FBNYV was mixed with PBST pH 7.4. The tubes pretreated with the antibody were washed three times with washing buffer (PBST buffer) at 200 µl/ tube and 100 µl of the purified virus was added. The mixture was vortexed and centrifuged at 4000 g for 2 min. The tubes were incubated at 4°C overnight and washed three times with washing buffer; 20 µl of above the mentioned P19/ P6 primer mix was added. PCR was performed using the above cycle conditions. The amplification products from PCR were analysed on a 2% agarose gel stained in ethidium bromide and the bands were photographed under UV illumination.

Electron Microscopy:

For electron microscopy leaves of faba bean (cv. Giza 3) were inoculated with FBNYV by aphid transmission Two- week old leaf tissues Leaf tissues were 1x3 mm) and fixed in a mixture 2% glutaraldehyde and 2% par formaldehyde in 0.05 M cacodylate buffer pH 7.0, for 2 hr at room temperature under vacum, rinsed with the same buffer, then postifixed in 1% osmium tetroxide (O_sO_4) for 2 hr. Sample were prestained in 0.5% uranyle acetat at 4°C, overnight. Tissue dehydration was achieved by running through an ethanol series, then imbedded in Spurr's epoxy-resin. Ultra thin sections of 10 nm thickness were cut by glass knife on a LKB ultramicrotome. Sections were double stained with 2% uranyl acetate for 10 min then lead citrate for 5 min and examined in a Jeol JEM-100 ex electron microscope, Faculty of Science, Alexandria University, Alexandria, Egypt.

Results

Isolation and identification:

Faba bean necrotic virus (FBNYV) was isolated from naturally infected faba bean plants, and identified basing mainly on serological reaction, symptomatology and PCR.

Serological reaction by indirect ELISA:

FBNYV was serologically detected with the corresponding antiserum by indirect ELISA in tested faba bean leaf showing severe chlorosis, necrotic, leaf rolling and stunting symptoms either alone or in combination with other viruses(Fig 1). FBNYV antiserum positively reacted with the isolated virus when used at a dilution of 1: 500 in indirect ELISA.

Field survey:

Results obtained from testing faba bean samples collect from 6 areas (locations) in Egypt are summarized in Table (1). High percentage of natural infections by FBNYV were detected on faba bean plants in Abis, EL-Banger, EL-Nahta, Hosh-Esa, Nobaria and Sagha.

Fig. 1. Natural infection of FBNYV on faba bean plant. A) Natural infected on faba bean leaves. B) Natural infected on pods and seeds of infected plant.

Table 1. Percentage of natural infection with FBNYV tested by ELISA

Location	Season			Total
	2007	2008	2009	
Alexandria				
Abis	3/14	0/7	3/10	6/31
Behera				
EL-Banger	--	0/8	1/9	1/17
EL-Nahta	4/18	6/15	4/15	15/48
Hosh-Esa	1/6	2/9	-	3/15
Nobaria	2/6	0/8	0/5	2/19
Kafr El-Shiekh				
Sagha	--	--	6/20	6/20
Total	10/44	8/47	14/59	32/150
Average *	22.7	17	23.6	21.3

* Number of plants infected with FBNYV /total number of plants tested X 100.
-- No samples collected.

Virus purification:

The virus was purified from naturally infected faba bean leaf tissues. The U.V. absorption spectra of the purified virus preparation revealed typical spectra of nucleoprotein (Fig. 2). The yield of the purified preparation of virus was 0.13mg/100g fresh tissue. Purified Particles had a A_{260}/A_{280} ratio 1.42.

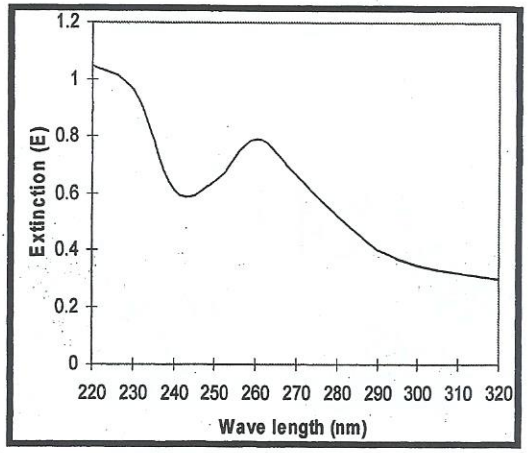


Fig. 2. Ultraviolet absorption of spectrum of purified FBNYV.

ELISA:

Data in Table (2) show that ELISA was sensitive to an end point dilution of $1:10^{-5}$ with sap extracted from infected faba bean leaves. Meanwhile, the level of detection by DBIA was 50 ng/ml of purified virus preparation (Table 3).

DBIA:

The sensitivity of DBIA was $1:10^{-3}$ with sap extracted from infected faba bean leaves (Fig. 3A) and 250 ng/ml of purified virus preparation (Fig. 3B).

Table 2. Sensitivity of indirect ELISA for detection of FBNYV in different dilution of infected faba bean plant sap

Dilution	Absorbance value (E405nm)	
	Healthy	Infected
1:10	0.123	0.319
$1:10^2$	0.127	0.305
$1:10^3$	0.128	0.294
$1:10^4$	0.125	0.270
$1:10^5$	0.121	0.253
$1:10^6$	0.125	0.179
$1:10^7$	0.096	0.131
$1:10^8$	0.095	0.115
$1:10^9$	0.085	0.101
$1:10^{10}$	0.080	0.097
$1:10^{11}$	0.069	0.072

Table 3. Indirect ELISA Absorbance values (A 405nm) of different dilutions of purified FBNYV

Purified FBNYV dilution (ng)	Absorbance values (E405nm)	
	Control	Purified FBNYV
1000	0.156	1.232
500	0.143	1.214
250	0.154	1.070
50	0.135	0.338
10	0.128	0.214
5	0.125	0.193
2	0.121	0.164
0.5	0.118	0.148

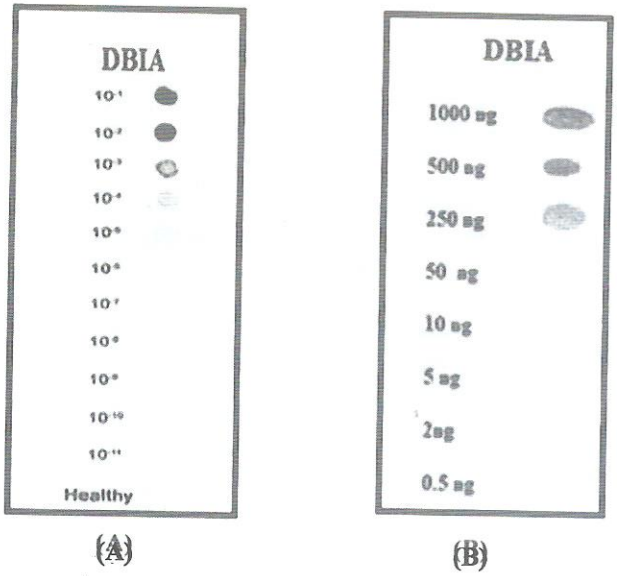


Fig. 3. Sensitivity of DBIA for detection of FBNYV antigen in serial dilutions of: (A) sap expressed from infected leaves, (B) purified preparation of the virus.

Detection of FBNYV by PCR:

Polymerase chain reaction was performed on total DNA extracted from infected leaf tissues. The obtained results confirmed the specificity of the primers which used in this study. The size of the amplification obtained product was approximately 272 bp for the isolate of FBNYV (Fig. 4).

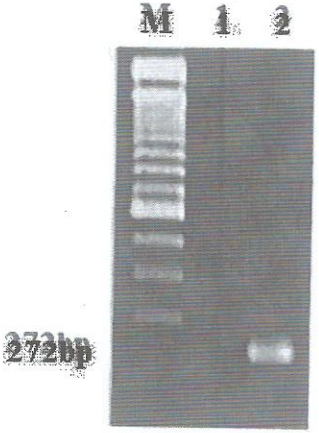


Fig. 4. Agarose gel electrophoresis showing the PCR amplification products of FBNYV coat protein gene. Lane (M): DNA marker, (1) Control (healthy) and (2) ZYMV.

IC-PCR:

IC-PCR detection using partially purified virus preparation or crude extracts of infected faba bean plants, products of the expected size of 272bp observed in agarose gels following IC-PCR amplification.

The Sensitivity of IC-PCR for virus detection was determined from serial dilutions of virus- infected plant tissue homogenate and partially purified virus preparation. Amplification products with purified virus preparations were obtained as little as 5ng /ml (5000pg /ml). The detected limit of infected faba bean extracts was at a dilution of $1:10^9$ (Fig. 5 A & B).

The detection limits of IC-PCR, indirect ELISA and DBIA were compared (Table 4). Using partially purified virus preparations, IC-PCR was 25-fold and 50-fold more sensitive than indirect ELISA and DBIA. IC-PCR showed 32-fold and 16-fold greater sensitivity than DBIA and indirect ELIAS for detection of the FBNYV in leaf extracts from faba bean.

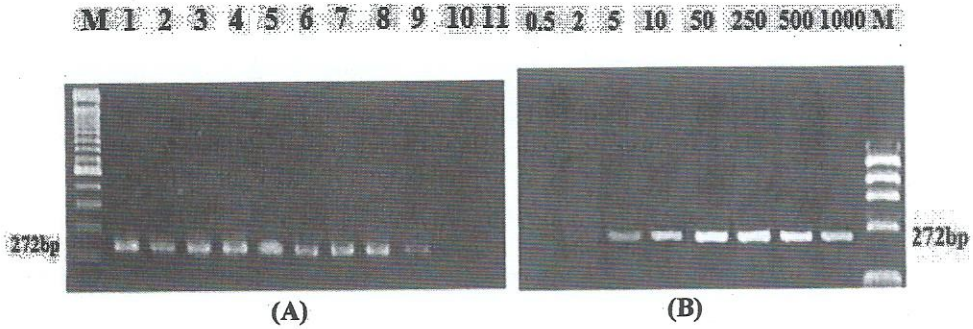


Fig. 5. Detection of FBNYV by immunocapture polymerase chain reaction antigen in serial dilutions of (A) infected faba bean plants, (B) purified preparation of virus.

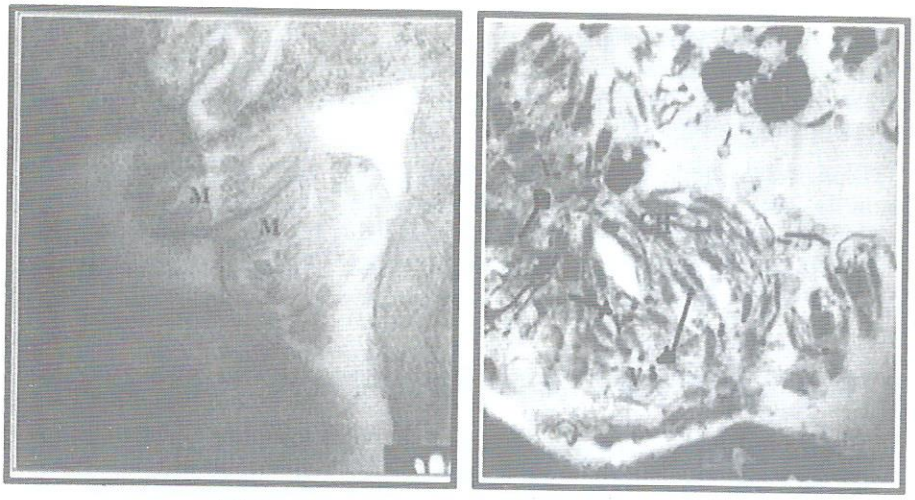
Table 4. Detection limits of IC-PCR, indirect ELISA and DBIA for FBNYV

Method	Virions	Sap Dilutions
IC-PCR	5 ng	10^9
ELISA	50ng	10^5
DBIA	250ng	10^3

Electron Microscopy:

Electron microscopy of ultrathin section prepared from faba bean infected leaves revealed that mitochondria had reduced size, but occasionally became abnormally larger (up 5-6 folds). Mitochondria were highly deformed losing their structure. Virus particles were found scattered within the cytoplasm and

occasionally in the stroma of chloroplast (Fig. 6 A). It could not be decided whether the particles had indeed been located in intact mitochondria or whether out membranes of these organelles which were already broken in the process of mitochondria disintegration (Fig. 6 B).



(A) (B)

Fig. 6. Cytological alterations observed in faba bean infected with faba bean necrotic yellows virus. A. portion of cytoplasm in mesophil cell containing deformed mitochondria (M). B. part of a chloroplast that contains virus particles in the stroma (arrow).

Discussion

FBNYV was recorded in many Arab countries of West Asia and North Africa as Syria, Jordan, Iraq, Iran, Ethiopia, Egypt, Algeria and Morocco (Katul *et al.*, 1993; Franz *et al.*, 1996; Al-Nsour *et al.*, 1998; Abraham *et al.*, 2000 and Makkouk *et al.*, 2002).

The presented survey, carried out during seasons of 2007 to 2009 in six areas located in three governorates in North Egypt, showed that percentage of the virus incidence ranged from 21.7%, 17% and 23.7%. On the same line, where in the 1991-1992 and 1998-1999 growing seasons the virus occurred at an epidemic scale on faba beans in Middle Egypt, leading to significant losses (Makkouk *et al.*, 1994). Abdel-Salam and El-Sharkawy (1996) found that FBNYV caused serious economic losses to faba bean in Egypt. Abraham *et al.* (2000) confirmed our results that found virus incidence based on visual estimation ranged from 0-80%. However, there was a wide variation in virus occurrence and incidence in different areas across the country.

The application of polymerase chain reaction (PCR) resulted in the simultaneous amplification of a 272 bp fragment of the FBNYV genome by using specific primer for FBNYV. The size of the PCR fragments was in agreement with expected for such nucleotide sequence. Ortiz *et al.* (2006) found that the nucleotide sequence was deposited in the Gen Bank Nucleotide Sequence Database under the accession number DQ830990. FASTA analysis showed that the sequence of 272 within the C5 component was 93.75% identical to the two previously sequenced isolates of FBNYV of Syrian and Egyptian origin.

The purification procedure applied in the present investigation is similar to that previously reported for FBNYV (Katul *et al.*, 1993 and Abdel-Salam *et al.*, 2003). The ultraviolet absorption spectra for the purified viral preparations were typical for nucleoproteins. The yield of viruses as well as their spectrophotometrical data i.e. A260/A280, Amax/A min fall in the range reported for the corresponding virus (Katul *et al.*, 1993 and Abdel-Salam *et al.*, 2003).

Relative sensitivities of certain serological methods and IC-PCR for detecting FBNYV in extracted sap of infected plants and purified preparation were studied. Using dilution end point as criterion for sensitivity, indirect ELISA was more sensitive than DBIA. By indirect ELISA FBNYV was detected in extracted plant sap dilution up to $1:10^5$ and 50 ng /ml in prepared from purified virus, while with DBIA the virus could be detected in sap extracted of infected plant at dilution up to $1:10^3$ and 250ng/ml of purified virus preparation.

DBIA and indirect ELISA procedure were compared. It was found that ELISA was more sensitive than DBIA likely due to a greater affinity of the antigen for the polystyrene ELISA plates than the nitrocellulose membrane (Kamenova and Adkins, 2004). Regarding serological methods, the fact that indirect ELISA was found to be the more sensitive method among serological tests is in a confirmation to finding of others (Makkouk *et al.*, 1993; Fegla *et al.*, 2001 and Fath-Allah, 2006b).

IC-PCR was the most sensitive of the techniques studied for detection of the FBNYV, as previously observed for other viruses (Wetzel *et al.*, 1992; Nolasco *et al.*, 1993; Rowhami, 1998 and Kamenova and Adkins, 2004). IC-PCR allowed detection of purified FBNYV as at 2ng/ml. Readily detected amplification products were observed at such high dilution as $1:10^9$ infected leaf faba beans. Our results generally are in line with those reported by (Wetzel *et al.*, 1992 and Maria *et al.*, 2001).

Kamenova and Adkins (2004) reported that IC-RT-PCR was 62-fold more sensitive than DAS-ELISA when using partially purified *Florida hibiscus virus* preparation and 16-fold greater sensitivity than DAS-ELISA for detection of the virus in leaf extracts from infected hibiscus.

The cytopathic changes induced by FBNYV were all important details typical for alterations induced by spherical viruses. Mitochondria or chloroplasts were deformed losing their structure such similar consideration already has been discussed by (Russo and Matell, 1972 and Fath-Allah, 2006a).

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تنقية ودراسات سيرولوجية وبيولوجيه على عزلة مصرية لفيروس اصفرار وموت الفول التى تصيب الفول

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تم عزل فيروس اصفرار وموت الفولFBNYV من نباتات فول مصابة طبيعيا فى مصر عليها اعراض اصفرار، نيكروزس، التفاف للاوراق وتقزم . ولقد تم تعريف الفيروس على اساس نتائج دراسات السيرولوجى باستخدام تقنية الاليزا غير المباشرة indirect ELISA ، الاعراض و تفاعل البلمرة المتسلسل PCR. تم عمل حصرللفيروس خلال الفترة من ٢٠٠٧- ٢٠٠٩ فى٦مناطق تابعة لثلاثة محافظات وجمعت ١٥٠ عينة عشوائيا واختبرت بتقنية الاليزا غير المباشرة وكانت النسبة المئوية لإنتشار الفيروس تتراوح من ١٧- ٢٣,٦% . تم تنقية الفيروس باستخدام البولى اثيلين جليكول ثم سكروز متدرج الكثافة وكانت كمية الفيروس المتحصل عليها ١٣ملجم/ ١٠٠جم اوراق مصابة ، اظهرت قراءة طيف الامتصاص للاشعة فوق البنفسجية للتحضير الفيروسى النقى انها مطابقة للنيكلوبروتين فكانت قيمة ٢٦٠/٢٨٠ تساوى١,٤٣. تم استخدام تقنية تفاعل البلمرة المتسلسل PCR أوضحت النتائج أن الجزء الذى تم تضخيمه من الجينوم بواسطة PCR بالنسبة لفيروس FBNYV كان حولى 272 زوج من القواعد وذلك بإستخدام نوعين من البادئات المتخصصة. حجم ناتج تفاعل PCR كان متوافقا مع ما هو متوقع من الاختبار لهذا الجزء من التتابعات النيوكليوتيدية. تم مقارنة الطرق السيرولوجية والجزيئية وهى تتضمن تقنية الاليزا غير المباشرة والارتباط المناعى النقطى DBIA و تفاعل البلمرة المتسلسل IC-PCR وذلك لتقيم مدى حساسيتها لتشخيص الفيروس، وتم اختبار كل طريقة مع انسجة النبات المصابة ومع الفيروس النقى . اوضحت النتائج ان تقنية الاليزا غير المباشرة كانت اكثر حساسية من الارتباط المناعى النقطى حيث امكن من خلال التقنية الكشف عن الفيروس فى العصارة المستخلصة من النبات المصاب، فى تخفيفات الفيروسية النقية الى ١: $١٠^{٩}$ ، ٥٠ نانوجرام/ مل على التوالى، بينما من خلال تقنية الارتباط المناعى النقطى فقد تم الكشف عن الفيروس حتى تخفيفات ١ : $١٠^{٣}$، ٢٥٠ نانوجرام/ مل على التوالى. اما تفاعل البلمرة المتسلسل كان اكثر الطرق حساسية حيث تم الكشف عن الفيروس فى تخفيفات عصارة النبات المصاب حتى تخفيف ١: $١٠^{٩}$، وامكنه الكشف عن الفيروس فى كمية صغيرة وصلت الى ٢ نانوجرام/ مل. قطاعات الميكروسكوب الالكترونى اظهرت الجسيمات الفيروسية فى البلاستيدة التالفة، الميتوكوندريا شديدة التشوهة.