Investigation on the Presence of Bean Leaf Roll Virus (BLRV) Infecting Faba Bean (*Vicia fabae*) in Ismailia, Egypt

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Abstract: Virus isolate from faba bean (*Vicia fabae*) collected from eight locations in Ismailia governorate, Egypt. The virus as *luteoviruses* closely related to bean leaf roll virus (BLRV) was identified according to symptoms, mode of transmission, purification, RT-PCR. The main purpose of this study was identification of BLRV using direct ELISA and determined antiserum titer and antigen dilution end point using the microprecipitation test for imported and prepared antiserum with purified and clarified antigen. Samples were inspected for BLRV symptoms and direct ELISA was used to confirm the presence of BLRV infections. All tested plants collected from El- Tall El-Caber farms were gave positive reaction with specific BLRV antibodies by direct ELISA. Symptoms of BLRV stunting, leaf roll, mosaic, chlorosis and yellowing appeared on tested plant cultivars (Giza 714, Giza 429, Giza461, Giza717 and Giza3). The BLRV insect transmitted by six aphid species, all aphids species were obligatory aphid transmitted BLRV in a circulative persistent manner. The highest percentage of transmission was recorded (76.25 % and 70 %) by *A. pisum* and *Aphis craccivora* respectively followed by (33.75% and 28.75 %) by *Aphis fabae* and *Aphis gossypii* respectively while the lowest percentage was recorded (7.50%) by *Myzus persicae* persistent aphids (*Myzus persicae* and *Aphis craccivora*). The height percentage of plants infected by BLRV was observed with feeding period on the infected plants up till 2 and 3 days 100 % and 90 % respectively. On the other hand, the height percentage of infected plants (70 %) was infected when aphids had 10 and 15 minutes inoculation period. Reverse transcription-polymerase chain reaction (RT-PCR) assay was used for the detection and identification of the isolated virus from nucleic acid extracts of infected faba bean plants. Using specific oligonucleotide primer 5950C and 4F which amplified coat protein gene *cp* gene for detection of bean leaf roll virus (BLRV). A major product of approximately 400bp BLRV -*cp* gene was produced.

Keywords: Bean leaf roll virus, BLRV, Faba bean, *Vicia fabae*.

INTRODUCTION

Bean leafroll virus (BLRV) also known as pea leafroll virus, is distributed worldwide (Ashby, 1984). Faba bean, Vicia faba L, is considered as one of the most important food legumes in Egypt. The species is considered a major staple food crop that is important for human and animal nutrition in developing countries (Bond, 1987).

BLRV is distributed worldwide and has been found to be endemic in white clover in the southeastern United States (Damsteegt *et al.*, 1995). Like other members of the Luteoviridae, BLRV is transmitted obligately by aphids in a persistent manner (Ashby, 1984).

Cool-season food legumes (faba bean, lentil, chickpea and pea) and cereals (bread and durum wheat and barley) are the most important and widely cultivated crops in West Asia and North Africa (WANA), where they are the main source of carbohydrates and protein for the majority of the population. (Makkouk *et al.*, 2009)

Under certain environmental conditions, beans can improve soil fertility and reduce the incidence of weeds, diseases and pests when grown in rotation with other crops (Mwanamwenge *et al.*, 1998). Many viral diseases can affect faba bean plants, and this is considered a serious problem worldwide. Infection by certain viruses causes significant yield reduction and economic losses.

Broad bean plants showing symptoms suggestive of viral infection, such as stunting, leaf roll, mosaic, chlorosis, necrosis, and yellowing, were observed in the Andalucia, Baleares, Cataluna, and Murcia regions of Spain. A 4-year field survey showed the presence of five viruses: bean leaf roll luteovirus (BLRV), beet western yellows luteovirus (BWYV), bean yellow mosaic potyvirus (BYMV), tomato spotted wilt tospovirus (TSWV), and cucumber mosaic cucumovirus (CMV). Fresno *et al.* (1997).

Persistently transmitted aphid-borne viruses pose a significant limitation to legume and cereal production worldwide. Surveys conducted in many countries in WANA during the last three decades established that the most important of these viruses are: Faba bean necrotic yellows virus (FBNYV: genus Nanovirus; family Nanoviridae), Bean leafroll virus (BLRV: genus Luteovirus; family Luteoviridae), Beet western yellows virus (BWYV: genus Polerovirus; family Luteoviridae), Soybean dwarf virus (SbDV: genus Luteovirus; family Luteoviridae) and Chickpea chlorotic stunt virus (CpCSV: genus Polerovirus; family Luteoviridae) which affect legume crops, and Barley yellow dwarf virus-PAV (BYDV-PAV: genus Luteovirus; family Luteoviridae), Barley yellow dwarf virus-MAV (BYDV-MAV: genus Luteovirus; family Luteoviridae) and Cereal yellow dwarf virus-RPV (CYDV-RPV: genus Polerovirus; family Luteoviridae) which affect cereal crops. Loss in yield caused by these viruses is usually high when infection occurs early in the growing season. Leslie *et al.*, 2002.

The family Luteoviridae consists of eight viruses assigned to three different genera, Luteovirus, Polerovirus and Enamovirus. This family also consists of 12 unclassified and 20 tentative members Smith *et al.* (1999). Luteovirus virions are non-enveloped, icosahedral particles that are 25–30 nm in diameter and contain a single molecule of positive-sense ssRNA, 5.7–5.9 kb in size (Hull 2002). These viruses are transmitted in a persistent manner by aphid vectors and are mostly restricted to the phloem of infected hosts (Casper, 1998). Bean leaf roll virus (BLRV) belongs to the genus Luteovirus (family Luteoviridae). It occurs in Europe, the Middle East, India, and the USA and infects legumes, including French bean, faba bean (*Vicia faba*) and pea (*Pisum sativum*). BLRV is phloem-limited and present at very low concentrations, and it is transmitted by aphids in a persistent manner (Ashby, 1984).

The *Luteoviridae* family has been divided into three genera (*Enamovirus, Luteovirus* and *Polerovirus*) depending on genome organization, sequence similarity, and methods of gene expression (D'Arcy *et al.*, 2000). The single-stranded, positive-sense genomes of the *Luteoviridae* contain five to six open reading frames (ORFs) designated ORF 0 through ORF 6.

ORF 0 is unique to the *Enamovirus* and *Polerovirus* genera and encodes a protein of unknown function. ORFs 1 and 2 encode the replication-related proteins, which in luteoviruses are most similar to those of the *Tombusviridae*, while the replicases encoded by poleroviruses and enamoviruses are related to those of the *Sobemovirus* genus (D'Arcy *et al.*, 2000).

In all three genera, ORF 2 is expressed via a translational frameshift from ORF 1. ORF 1 overlaps ORF 2 by less than 20 nt in luteoviruses, but by more than 400 nt in enamo- and poleroviruses. The intergenic region between ORFs 2 and 3 is about 100 nt in luteoviruses and about 200 nt in polero- and enamoviruses. ORFs 3 and 5 encode the coat and readthrough proteins of the virus. Recently, parallels have been found between the coat proteins (CPs) of poleroviruses and members of the *Sobemovirus* genus (Terradot *et al.*, 2001), which suggests an even closer affiliation of the two genera. ORF 4, which is lacking in the enamoviruses, putatively encodes a movement protein.

The relationship of BLRV to other members of the *Luteoviridae* has been examined based on biological, immunological, and nucleotide sequence data. Serological (D'Arcy *et al.*, 1988; Smith *et al.*, 1996; van den Heuvel *et al.*, 1990) suggest that BLRV is related most closely to *Soybean dwarf virus* (SbDV). Sequences of the BLRV coat protein gene have been reported (Cavileer & Berger, 1994; Prill *et al.*, 1990) and predicted that BLRV was most closely related to poleroviruses. As mentioned above, SbDV has a genome organization similar to members of the genus *Luteovirus*, but may have arisen through recombination between a *Luteovirus* and a *Polerovirus* (Rathjen *et al.*, 1994 and Terauchi *et al.*, 2001).

The main purpose of this study was isolation and identification of BLRV using ELISA, symptomatology, aphid transmission, purification, antiserum production serological studies and distinguishable. DNA bands in diseased leaves for could be used as sign for the virus infection by RT-PCR technique. We describe for the first time, a RT-PCR in the detection of the RNA virus infecting faba bean (*Vicia faba*) plant.

MATERIAL AND METHODS

Detection and Biological Studies of BLRV:

Isolation, Propagation and Symptomatology of BLRV

Naturally infected broad bean samples were collected from, eight locations in Ismailia governorate, Egypt. Three farms per location and ten plants were collected from all farm. The bean leaf roll virus (BLRV) isolate was obtained from faba bean plants showing the identical symptoms suggestive of viral infection, such as stunting, leaf roll, mosaic, chlorosis, necrosis, and yellowing. Samples were inspected for BLRV symptoms and then marked for further detection assay by direct ELISA test, according to the method described by Clark and Adams (1977). Faba bean plants showing the identical symptoms of leaf roll grown and reacting positively BLRV antiserum as well as healthy faba bean plants were used for further experiments. The BLRV was isolated by aphid transmission using Pea aphid (*Acyrthosiphon pisum*) from those infected faba bean plants to healthy seedling. A virus isolate, was maintained by continuous aphid inoculation of faba bean cv. Giza3 seedlings during the experiments. Inoculated plants were kept in an insect proof green house (air–conditioned chamber at 22°C). Symptoms typical of those observed in the original field plants appeared 10 to 14 days after aphid transmissions in faba bean inoculated with pea aphids.

Aphid Transmission

The pea aphid (*Acyrthosiphon pisum*), cowpea aphid (*Aphis craccivora*), black bean aphid (*Aphis fabae*), cotton aphid (*Aphis gossypii*), potato aphid (*Macrosiphum euphorbiae*), and the green peach aphid (*Myzus persicae*) were collected from the faba bean, vetch and lentil field and identified in (plant protection department, faculty of agriculture, suez canal university, Ismailia – Egypt) and transferred to healthy Chinese cabbage seedlings in the green house. The off springs from the mature aphids were transferred to another healthy plant of Chinese cabbage and allowed to multiply on them. The upper leaves of faba bean plant with identical symptoms of BLRV were removed from the stem with petioles and placed in pitri dishes covered with wet filter paper. Non-viruliferous aphids of pea aphid, cowpea aphid, black bean aphid, cotton aphid, potato aphid, and the green peach aphid fasted for one hour and then transferred to diseased leaves. Non-viruliferous all types of aphids received a 48-h acquisition access period on symptomatic leaves. Aphids were then transferred to different cultivars from healthy faba bean plants (Giza3, Giza461, Giza717, Giza 429 and Giza 714) for a 72-h inoculation access period by 10 aphids per plant in the green house. Seedlings were sprayed by contact insecticide and transferred to air conditioned chamber at 20-22 °C and were kept for observation. Some plants were kept free

from insects and were subjected to healthy non-viruliferous insects from stock cultured as control. Four replicates were used for this test, and 20 plants per replicate. Ten insects were placed on each plant. The inoculated seedlings were kept in an insect proof cage till symptoms appearance. The same procedure was conducted to prepared the control except those insects were fed on virus free seedlings.

Acquisition Period

Non-viruliferous *Acyrthosiphon pisum* were allowed to feed on infected faba bean cv. Giza 3 plant for different periods.i.e.5, 10, 15, 30, 45 minutes 1, 4, 6 hours and 1, 2, 3, 4, 5, 6, 7 days. Aphids were then transferred to healthy seedlings of faba bean cv. Giza 3 by means of 1 aphid per plant and then they were left to feed for 3 days. Test plants were sprayed by contact insecticide and transferred to the air-conditioned chamber at 22°C, where they were held for observation. Ten plants were used in every trail.

Inoculation Period

Acyrthosiphon pisum were left to feed on the infected faba bean cv. Giza 3. Plant for 2 days, then transferred to feed on healthy seedlings of faba bean cv. Giza 3. for different intervals, 5, 10, 15, 30, 45 minutes 1, 4, 6 hours, and 1,2,3,4,5,6,7 days after which plants were sprayed by a 0.2 % Malathion spray. Plants were transferred to the air conditioned chamber, where they were held for observation. 10 plants each inoculated with one aphis were used in every trail.

Purification

The methodology of Ashby and Huttinga (1979) was used for Bean leaf roll virus (BLRV) purification from infected broad bean plants using polyethylene glycol precipitation of crude extracts followed by clarification with chloroform-butanol, differential centrifugation and density-gradient centrifugation. All centrifugation is therefore done at 15°C. Purified virus suspension was fractionated in vials and stored at -20°C for use Ashby and Kyriakou (1982).

Serological assays:

Preparation of antiserum from purified sBLRV:

Rabbit immunization:

New Zealand rabbit about 4.5 kg was used for antiserum production. A total of 6 mg purified virus was used for injection. Two routes of injection were applied. For intramuscular injections, virus was diluted with 0.85% NaCl in distilled water (W/V) and was mixed with an equal volume of freund's incomplete adjuvant. Injection was performed in the right and the left hind thighs, respectively using a 5ml-disposable syringe. For intravenous injection, virus was diluted with 0.85 % solution of NaCl in distilled water (W/V). Injection was made in the left ear at the marginal vein using 1 ml insulin disposable syringes.

Rabbit bleeding:

A week after last injection, the rabbit was bleed from the right ear. Blood was left to coagulate for 2-3 hrs at 37 °C then kept at 4 °C overnight. Antiserum was separated through centrifugation at 4000 rpm for 15 min. The obtained antiserum was used for titer determination and other serological tests.

Determination of Antiserum Titer and Antigen Dilution end Point Using the Microprecipitation Test

Antiserum titer was measured with the micropricipitation test (Van slogteren 1955). Clarified and purified sap of bean leaf roll virus were diluted with a 2-fold dilution series, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256 and 1/512 respectively, using phosphate buffer pH 7.6 containing 0.85 % Nacl. On the other hand, prepared BLRV antiserum and imported BLRV antiserum from (Institut National de la Recherche Agronomique de Tunisie (INRAT), Rue Hedi Karray, 2049 Ariana, Tunis, Tunisia) were diluted with the same buffer, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256,1/512, 1/1024 and 1/2048, respectively. Controls of healthy faba bean sap and normal serum were involved. The reaction was done between BLRV purified sap and its induced antiserum, BLRV purified sap and its imported antiserum, BLRV clarified extract and its imported antiserum and BLRV clarified extract and its induced antiserum. The reaction was performed in plastic Petri dishes (9 cm diameter) at room temperature by mixing equal aliquots (7 µl) of given dilution of antigen with another dilution of antiserum. The reaction mixture was covered with paraffin oil, then plates were incubated at 37°C for 2hr. before examination. Stereomicroscopy with descending light was used for detecting the reaction appearance. Plates were re-examined each 1/2hr. up to 6hr. virus dilution end point and antiserum titer were determined.

BLRV Identification by Direct ELISA

Eight samples were obtained from naturally infected broad bean plants which grown in Ismailia Governorate (Srapum, Abo-Soltan, Sabaa-Abar, El-Mahsama, Abo-Swair station, Riad, El-Blah and El-Tal El-kaber). At the First, these samples tested by direct ELISA test against three antibodies BLRV [imported from (Institut National de la Recherche Agronomique de Tunisie (INRAT), Rue Hedi Karray, 2049 Ariana, Tunis, Tunisia), prepared antiserum], BYMV (Bean yellow mosaic virus) and FBNYV (Faba bean necrotic yellows virus). One microtiter plate was used for only antibody; each plate contained 80 samples which were collected from eight different locations, two replicates for each sample. This procedure was carried out following the standard methods of Clark and Adams (1977).

Molecular Characterization

RNA Purification

Total RNA was extracted by SV total RNA isolation system by spin protocol as recommend by the manufacturer instructions of Promega fifty mg of BLRV-infected leaves of faba bean cv. (Giza3, Giza461, Giza717, Giza 429 and Giza 714) were ground in liquid nitrogen using a mortar and pestle. The purified RNA was stored at -20°C.

RT-PCR for Virus Identification

After total RNA extraction, detection was carried out using QIAGEN One Step RT-PCR Kit (Qiagene, Germany). To amplify the BLRV genome, a primer pair

designed based on the sequence available in GenBank (AF441393) was used. The antisense primer, BLRV 595°C (5'-CTTTCGCCACCTTAACAACA-3'), which started from base 5,950 at the 30 end of the BLRV genome, was used to synthesize cDNA from BLRV coat protein gene. The first-strand cDNA was then used in PCR, using BLRV5950C and the sense primer BLRV 4F (5'-AAAGTTGACACCTTTACAAGAG-3'), which started at the fourth base of the 50 end of the viral genome.

RT-PCR was performed for each primer pair in 20 µl mixture containing: 1 µg of total extracted RNA, 0.5 µM of each RT buffer (BRL), 1 µl RNasin (40 U; Promega) 0.3 M ß-mercaptoethanol. Synthesis of c-DNA was accomplished in water bath at 40°C for 1 h. Five microliters from the mixture were added to 45 µl of PCR mixture containing 10 X PCR buffer (Perkin Elmer), 0.2 µM unit AmpliTaq™ (Perkin Elmer) by primers pairs. Samples were placed in a Perkin Elmer GeneAmpli thermal cycler 2400. PCR thermocycling conditions were as follows: (i.e., RT at 45 °C for 30 min., Initial denaturation at 95 °C for 5 min., hot start at 95 ċ for 2 min., followed by 25 cycles of [denaturation at 92 °C for 1 min., annealing for 1 min. at special annealing primer 62 °C depending on primer pairs in the mixture and extension for 1 min. at 72°C.]. The final extension step was increased by 10 min at 72°C.

Agarose gel Electrophoresis

The PCR products were analyzed by gel electrophoresis on 1% agarose gel prepared in 1X TBE buffer (Sambrook *et al.*, 1989). After the electrophoresis was completed the gel tray was removed from the main until and placed in a glass tray containing a solution of 0.5 µg /ml ethidium. The gel was stained with ethidium bromide and examined using UV transilluminater and the PCR fragments of BLRV was confirmed as 400 bp.

RESULTS AND DISCUSSION

Detection and Biological Studies of BLRV

Isolation, Propagation and Symptomatology of BLRV

Two hundred and forty naturally infected faba bean plants were collected from, eight locations in Ismailia governorate.The original BLRV isolate was obtained from faba bean plants [Collected from El- Tall El-Caber location] which showing the identical BLRV virus symptoms. Samples were inspected for BLRV symptoms and direct ELISA was used to confirm the presence of BLRV infections. All tested plants collected from El- Tall El-Caber farms were gave positive reaction with specific BLRV antibodies by direct ELISA. This isolate was used for virus characterization and its effect of different cultivars of faba bean plants. According, symptomatology, insect, transmission, electron microscopy, serological reaction and RT-PCR.

Five plant cultivars belong to *Vicia faba* were inoculated with BLRV using infective pea aphid (*Acyrthosiphon pisum*), (Figer 1) Symptoms such as stunting, leaf roll, mosaic, chlorosis and yellowing appeared on tested plant cultivars (Giza 714, Giza 429, Giza461, Giza717 and Giza3) were appeared 10 to 14 days after aphid transmissions. The same result was obtained by Quantz and Völk (1954); Ashby and Huttinga (1979); Reddy *et al.* (1979) Ashby (1984) ; Schwinghamer *et al.*, 1999; Leslie *et al.*, 2002; Van Leur *et al.*, 2002 and Asma Najar *et al.* (2003). On the other hand, Larsen and Webster (1999) who mention that during the 1998 growing season, affected pea plants were observed that exhibited mild chlorosis and mottling, leaf rolling, and stunting symptoms in Italy regions.

Aphid Transmission

Six aphid species namely the pea aphid (*Acyrthosiphon pisum*), cowpea aphid (*Aphis craccivora*), black bean aphid (*Aphis fabae*), cotton aphid (*Aphis gossypii*), potato aphid (*Macrosiphum euphorbiae*), and the green peach aphid (*Myzus persicae*), were used to transmit BLRV. Results presented in table (2) show that all aphids species were obligatory aphid transmitted BLRV in a circulative persistent manner.The highest percentage of transmission was recorded (76.25 % and 70 %) by *A. pisum* and *Aphis craccivora* respectively followed by (33.75% and 28.75 %) by *Aphis fabae* and *Aphis gossypii* respectively while the lowest percentage was recorded (7.50%) by *Myzus persicae*. Similarly results had already been observed by Quantz and Völk 1954 ; Thottappilly, 1969; Ashby 1984 ; Damsteegt, *et al.*, 1990; Damsteegt, *et al.*, 1992; Damsteegt, *et al.*, 1995; Schwinghamer *et al.*, 1999 and Van Leur *et al.*, 2002.

Acquisition and Inoculation Period

Acyrthosiphon pisum were feeding on the infected plants cv. Giza 3 at different periods of i.e. 5,10,15,30,45 minutes, 1, 4, 6 hours and 1, 2, 3, 4, 5, 6, 7 days. Five aphid were used for each plant. Results recorded in table (3) showed that, *Acyrthosiphon pisum* adults became infective when they had 10 minutes feeding period on the infected plant. In addition, the heighest percentage of plants infected by BLRV was observed with feeding period on the infected plants up till 2 and 3 days 100 % and 90 % respectively, this percentage decreased 50 % to 60 % with feeding period of 4 and 5 days. On the other hand, the percentage became increased again to 80 % when 7 days feeding period were used.

Five individuals of viruliferous *Acyrthosiphon pisum* were allowed to feed on healthy cv. Giza 3 plants for different periods of 5,10,15,30, 45 minutes, 1, 4,6 hours, and 1, 2, 3, 4, 5, 6,7 days. Results recorded in table (3) indicated that the height percentage of infected plants (70 %) was infected when aphids had 10 and 15 minutes inoculation period. On the other hand, percentage of infected plants decreased from 70 % to 50 % when the feeding period was elongated to 6 hours. Also, the percentage of infected plants was increased again to100 % when the aphids feed on healthy plants for 2 and 3 days. In addition, results indicated that aphids remain infective for 7 days. Ashby 1984; Schwinghamer *et al.*, 1999 and Larsen and Webster (1999) who mention that the High aphid populations and disease levels of nearly 100% were observed in susceptible varieties. Samples from affected fields were analyzed for the presence of bean leaf roll virus (BLRV). Non viruliferous pea aphids (*Acyrthosiphon pisum* Harris) received a 48-h acquisition access period on

symptomatic leaves. Aphids were then transferred to Puget pea and Diana faba bean for a 72-h inoculation access period.

Purification of BLRV

The yield of the purified BLRV was about 1 mg/kg broad bean cv. Giza3 leaves. Infectivity on the tested broad bean cv. Giza3 plants with inoculated BLRV using *Acyrthosiphon pisum* and observed epical BLRV symptoms after 25 days from inoculation when saved under green house at 22 °C.

As a matter of fact it was the methods through which Ashby and Huttinga, 1979; D'Arcy *et al.*, 1990; Schwinghamer *et al.*, 1999 and Van Leur *et al.*, 2002. On the other hand, Elbeshehy (1999 and 2006), who found that the ratio of A 280/260 was 0.78 and the ratio of max. / min. was 1.23. The yield of the purified PLRV was about 0.7 mg/kg potato cv. Diamont leaves and 1 mg/kg Ph. Floridana leaves. Infectivity on the tested Ph. floridana plants and D. stramonium L. plants with inoculated PLRV using *M. persicae* (Suls) and observed epical PLRV symptoms after 25 days from inoculation.

Serological Studies

Determination of Antiserum Titer and Antigen Dilution End-point

Purified sap preparation of BLRV obtained from broad bean cv. Giza3 plants was used for rabbits immunization. Antiserum titer and antigen dilution end-point were determined using microprecipitation test under paraffin oil. Data in table (4) recorded that the titer of BLRV imported antiserum was 1 / 1024 and 1 / 8 at purified antigen dilution of 1/32 and 1/128, respectively. The dilution end-point of purified antigen was 1/128 when a dilution of 1/8 of imported antiserum was used. Results observed in table (5) showed that the titer of the prepared BLRV antiserum was 1/512 and 1/8, at purified antigen dilution of 1/8 and 1/32, respectively. The dilution end-point of the purified antigen was 1/32 when dilution 1/8 of prepared antiserum was used.

Data in table (6) reported that the titer of BLRV imported antiserum was 1/512 and 1/8 at clarified antigen dilutions 1/8 and 1/64, respectively. The dilution end-point of the clarified antigen was 1/64 when a dilution of 1/8 of imported antiserum was used. Data in table (7) showed that the titer of prepared BLRV antiserum was 1/256 and 1/8 at clarified antigen dilution of 1/8 and 1/16, respectively. The dilution end-point of the clarified antigen was 1/16 when a dilution of 1/8 prepared antiserum was used. On the other hand, the controls of normal serum, healthy plant sap and buffer NaCl 0.85 % gave negative reactions in all tests. Similarly results had already been observed by D'Arcy *et al.*, 1989; Hewings *et al.*, 1990; Damsteegt, *et al.*, 1999; Larsen and Webster 1999; Schwinghamer *et al.*, 1999; Van Leur *et al.*, 2002 and Kumari *et al.*, 2006.

BLRV Identification by Direct ELISA

The original BLRV isolate was obtained from naturally infected broad bean plants collected from El-Tal El-Kaber Farm of Ismailia governorate, the purified sap from BLRV isolate gave positive reaction with imported and prepared BLRV antibody and negative reaction with BYMV and FBNYV antibodies.

Table 1. The rabbit immunization scheme, used for antiserum production against BLRV.

Injection No.	Date of injection	Route of injection	Dosage mg/ml Purified virus
1	29-1-2004	Intramuscular	1
2	5-2- 2004	Intravenous	1
3	12-2- 2004	Intramuscular	1
4	19-2-2004	Intravenous	1
5	26-2-2004	Intramuscular	1
6	5-3-2004	Intravenous	1

Table 2. Transmission of BLRV by *Acyrthosiphon pisum, Aphis craccivora, Aphis fabae, Aphis gossypii, Macrosiphum euphorbiae, Myzus persicae*

Replicates	*Acyrthosiphon pisum*		*Aphis craccivora*		*Aphis fabae*		*Aphis gossypii*		*Macrosiphum euphorbiae*		*Myzus persicae*	
	Infectivity*	%of transmission	Infectivity*	%of transmission	Infectivity*	%of transmission	Infectivity*	%of transmission	Infectivity*	%of transmission	Infectivity*	%of transmission
R1	16/20	80	15/20	75	6/20	30	4/20	20	2/20	10	2/20	10
R2	15/20	75	13/20	65	8/20	40	6/20	30	3/20	15	2/20	10
R3	15/20	75	14/20	70	6/20	30	6/20	30	1/20	5	1/20	5
R4	15/20	75	14/20	70	7/20	35	7/20	35	2/20	10	1/20	5
Mean	15.25/2	76.25	14/20	70	6.75/20	33.75	5.75/20	28.75	2/20	10	1.50/20	7.50

* = Number of infected seedlings / Total number of inoculated seedlings.

Table 3. Effect of Acquisition and inoculation period on BLRV transmission by *Acyrthosiphon pisum* on *Vicia fabae* cv. Giza3.

Inoculation periods on Giza3	Acquisition periods on *Vicia fabae* cv. Giza3																													
	Time in minutes										Time in hours						Time in days													
	5		10		15		30		45		1		4		6		1		2		3		4		5		6		7	
	I/T*	%	*I/T	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%
Days3	0/10	0	1/10	10	2/10	20	3/10	30	3/10	30	3/10	30	4/10	40	4/10	40	8/10	80	10/10	100	9/10	90	5/10	50	6/10	60	8/10	80	8/10	80

Acquisition periods on Giza3	Inoculation periods on *Vicia fabae* cv. Giza3																													
	Time in minutes										Time in hours						Time in days													
	5		10		15		30		45		1		4		6		1		2		3		4		5		6		7	
	I/T*	%	*I/T	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%	I/T*	%
Days2	5/10	50	7/10	70	7/10	70	7/10	70	7/10	70	7/10	70	6/10	60	5/10	50	9/10	90	10/10	100	10/10	100	9/10	90	8/10	80	8/10	80	7/10	70

*Infectivity = Number of infected plant s(I) / Total number of inoculated plants (T) .

Table 4. Determination of imported antiserum titre and antigen (purified BLRV) dilution end point.

Dilution of antiserum / Dilution of purified sap	Antiserum dilution 1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048
1/4	+5	+5	+5	+5	+5	+5	+5	+5	+5	+5	-
1/8	+5	+5	+5	+5	+5	+5	+5	+5	+5	+5	-
1/16	+4	+4	+4	+4	+4	+4	+4	+4	+4	+4	-
1/32	+3	+3	+3	+3	+3	+3	+3	+3	+3	+3	-
1/64	+2	+2	+2	-	-	-	-	-	-	-	-
1/128	+1	+1	+1	-	-	-	-	-	-	-	-
1/256	-	-	-	-	-	-	-	-	-	-	-

- = No visible reaction +1 = Faint reaction +2 = Weak reaction +3 = Moderate reaction +4 = Strong reaction +5 = Very strong reaction

Table 5. Determination of induced antiserum titre and antigen (purified BLRV) dilution end point.

Dilution of antiserum	Antiserum dilution										
Dilution of purified sap	**1/2**	**1/4**	**1/8**	**1/16**	**1/32**	**1/64**	**1/128**	**1/256**	**1/512**	**1/1024**	**1/2048**
1/4	+3	+3	+3	+3	+3	+3	+3	+3	+3	-	-
1/8	+3	+3	+3	+3	+3	+3	+3	+3	+3	-	-
1/16	+2	+2	+2	-	-	-	-	-	-	-	-
1/32	+1	+1	+1	-	-	-	-	-	-	-	-
1/64	-	-	-	-	-	-	-	-	-	-	-
1/128	-	-	-	-	-	-	-	-	-	-	-
1/256	-	-	-	-	-	-	-	-	-	-	-

- = No visible reaction +1 = Faint reaction +2 = Weak reaction +3 = Moderate reaction +4 = Strong reaction +5 = Very strong reaction

Table 6. Determination of imported antiserum titre and antigen (infectious sap) dilution end point.

Dilution of antiserum	Antiserum dilution										
Dilution of purified sap	**1/2**	**1/4**	**1/8**	**1/16**	**1/32**	**1/64**	**1/128**	**1/256**	**1/512**	**1/1024**	**1/2048**
1/4	+3	+3	+3	+3	+3	+3	+3	+3	+3	-	-
1/8	+3	+3	+3	+3	+3	+3	+3	+3	+3	-	-
1/16	+3	+3	+3	-	-	-	-	-	-	-	-
1/32	+2	+2	+2	-	-	-	-	-	-	-	-
1/64	+1	+1	+1	-	-	-	-	-	-	-	-
1/128	-	-	-	-	-	-	-	-	-	-	-
1/256	-	-	-	-	-	-	-	-	-	-	-

- = No visible reaction +1 = Faint reaction +2 = Weak reaction +3 = Moderate reaction +4 = Strong reaction +5 = Very strong reaction

Table 7. Determination of induced antiserum titre and antigen (infectious sap) dilution end point.

Dilution of antiserum	Antiserum dilution										
Dilution of purified sap	**1/2**	**1/4**	**1/8**	**1/16**	**1/32**	**1/64**	**1/128**	**1/256**	**1/512**	**1/1024**	**1/2048**
1/4	+2	+2	+2	+2	+2	+2	+2	+2	-	-	-
1/8	+2	+2	+2	+2	+2	+2	+2	+2	-	-	-
1/16	+1	+1	+1	-	-	-	-	-	-	-	-
1/32	-	-	-	-	-	-	-	-	-	-	-
1/64	-	-	-	-	-	-	-	-	-	-	-
1/128	-	-	-	-	-	-	-	-	-	-	-
1/256	-	-	-	-	-	-	-	-	-	-	-

- = No visible reaction +1 = Faint reaction +2 = Weak reaction +3 = Moderate reaction +4 = Strong reaction +5 = Very strong reaction.

Molecular Characterization

RT-PCR for Virus Identification

In this work, we used a simple, specific and rapid method for the detection of BLRV coat protein gene in plants by RT-PCR. This technique was used as an effective method to confirm the validation of RT-PCR 400bp product.

The results shown in (Fig 2) demonstrated that the amplified sequence from total RNA was readily detected in all ELISA-positive samples. A major PCR product of about 400 bp *cp* was present in samples-bearing BLRV. Our results are in harmony with those reported for BLRV by Prill *et al.*, 1990 ; Cavileer and Berger, 1994 ; Larsen and Webster 1999 ; Smith *et al.*, 2000 ; Terauchi *et al.*, 2001; Leslie *et al.*, 2002 ; Shahraeen *et al.*, 2005 and Thekke Veetil *et al.*, 2009.

No specific product occurred for healthy material and no bands were found when the PCR assay was attempted without the initial RT step. Resulted in neither increased product yield nor enhance target-sequenee specificity. When cDNA was generated from infected plants. There are two bases less compared with obtained data by Pantaleo, *et al.*, 2001 using same primers whith BLRV isolate from Italy.

The procedure is quick, of diagnostic value able and can be used as a preliminary method to detect virus in infected plants

We fell that the serological and molecular procedures used in this study have potential merit for routine testing in diagnostic laboratories. The choices of the detected method are depend on the kind of experiment being carried out, the kind of property to be measured, the sensitivity and accuracy required.

Stunting, leaf roll, mosaic, chlorosis and yellowing symptoms were notice in two years ago from naturally infected broad bean plants from eight locations in Ismailia governorate in Egypt.

The disease still under recognition and investigation but needs more attention as a serious problem that could become uncontrollable and may be fast spreading via insect vectors circulative persistent manner and, also cloning, sequencing and phylogenies of BLRV compared between Egypt isolate and worldwide isolates.

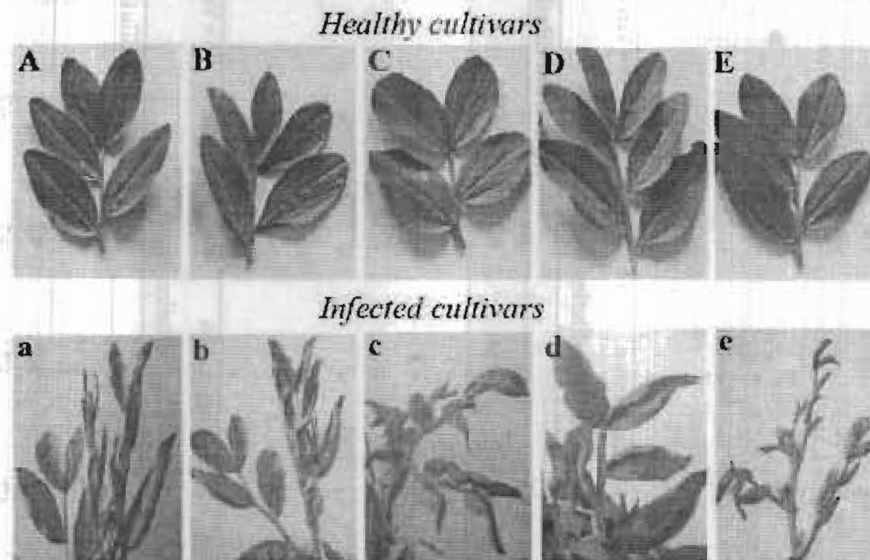

Figure 1. Symptoms caused by BLRV on faba bean plants.
Healthy leaves: A: cv. Giza 714, B: cv. Giza429, C : cv. Giza 461, D : cv. Giza 717, E : cv. Giza 3
Infected leaves: a, b, c, d and e Showed stunting, leaf roll, mosaic, chlorosis and yellowing

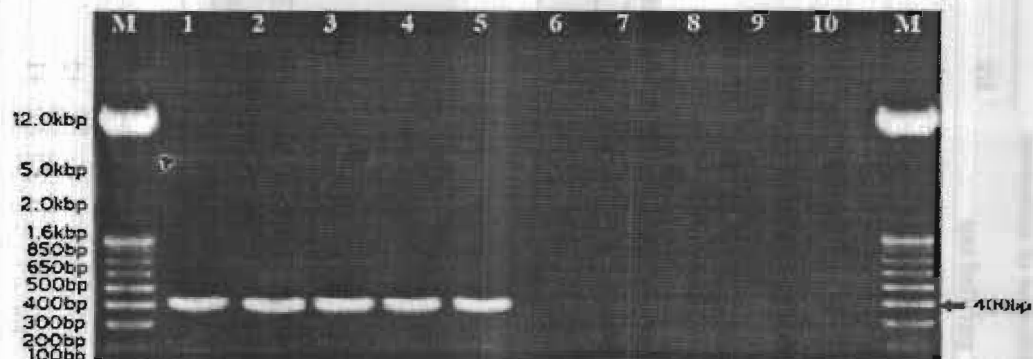

Figure 2. RT-PCR for BLRV-RNA extracted from infected tissues.
A product of 400 bp was produced from the amplification of BLRV-RNA.
- Lanes 1, 2, 3, 4 and 5: BLRV isolated from infected *Vicia fabae* CVs. Giza3, Giza461, Giza717, Giza 429 and Giza 714 leaves.
- Lanes 6, 7, 8, 9 and 10 *Vicia fabae* CVs. Giza3, Giza461, Giza717, Giza 429 and Giza 714
- healthy control leaves M : Marker.

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