ISOLATION OF AN ENTOMOPATHOGENIC VIRUS IN THE GREEN STINK BUG *NEZARA VIRIDULA* (LINNAEUS) IN EGYPT

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ABSTRACT

The green stink bug *Nezaraviridula* is an important economic pest in Egypt and in many countries around the world and requires control management measures. During the establishment of a *N. viridula*colony in Egypt, a significant reduction in the insect productivity and high mortality rate were observed. Electron microscopy examination of dead insects homogenate revealed the presence of paraspherical virus like-particles with diameter of 25 nm. Virus purification on cesium chloride gradient showed one band at a buoyant density of 1.362. Extraction of nucleic acid from purified virus particles revealed a ~9 kb ssRNA linear genome and analysis of capsids showed two major proteins of 31 and 32 kDa and a minor 78 kDa protein. Infection of third instar nymphs by feeding on contaminated food induced similar disease symptoms and bioassay experiment confirmed virus concentration response mortality. Injection of a purified virus suspension to third instar larvae of *Galleria melonella* and *Spodopteralittoralis* failed to induce any mortality indicating that the virus is most likely host specific to *N. viridula.* This virus is similar to the previously reported NVV-1 virus in *N. viridula* from South Africa strongly suggesting that NVV-1 is widely distributed in Africa and might play an important role in regulating the insect population in field conditions or could be used to control this pest in heavily infested areas.

Key words: *Nezaraviridula*, Entomopathogenic viruses, Picornaviridae

INTRODUCTION

The green stink bug, *Nezaraviridula*(L.) is one of the most economically important pentatomid insect pests attacking various plant species in different parts of the world (Panizzi et al., 2000). The insect has a worldwide distribution occurring throughout the tropical and subtropical regions of the Americas, Africa, Asia, Australasia and Europe (Panizzi et al., 2000; Todd, 1989). In North America, the bug is distributed throughout the Southeast and in California it has been found increasingly since the mid-1980s (Hoffmann et al., 1991). In South America the insect is related to the increased acreage for soybean production and cause economic problem (Sosa-Gomez et al., 2005). In Europe, the growing number of reports in the North of France and Belgium during the 1990s indicates that this pentatomid is expanding its distribution northwards (Gallant, 1996). The expansion of this pest in Europe is presumably related in part to its association with a number of greenhouse crops. In Egypt, the insect is widely spread all over the country from the Mediterranean coast to Aswan and recently, its population density has tended to be high causing considerable damages to many field and horticultural crops (Khalafalla et al., 2014; Khattab , 2003)

This insect has a wide host range belonging to more than 30 families of plants, including a wide variety of agricultural crops and many weeds that can be used as intermediate host plants between cropping cycles (Todd, 1989). The insect attacks all parts of the plant and prefers growing shoots and developing fruits. Also, this insect causes indirect damage to plants by providing an entry slots for pathogenic and decaying organisms at the feeding punctures. Thus, even low population densities of the insect

are sufficient to produce major economic damages to the crop in question (Hoffmann et al., 1991). Important economic damages are caused worldwide to field crops including soybean, rice, corn, cotton, and tobacco and to a number of vegetable crops like tomato, sweet pepper and eggplant, both in the field and in greenhouses. In case of high density of the green bug, controlling the insect population is required. This control is largely based upon the use of conventional pesticides, including a number of carbamates, organophosphates and some pyrethroids(Jackal et al., 1990; Panizzi et al., 2000). Due to the negative impact of chemical insecticides to the environment, there is an increasing interest for alternative control agents. In several regions attempts to control outbreaks of the southern green stinkbug using parasitoïdes have been successful (De Clercq et al., 2002).

So far, no viral pathogen has been identified from *N. Viridula*in Egypt. A pathogenic virus was reported from this insect in South Africa (Williamson and von Wechmar, 1992) and a picorna-like from the stinkbug Brown-Winged Green Bug, *Plautiastali*(Nakashima et al., **1998).**During the establishment of a laboratory colony of this insect from adults collected from the field in Giza governorate in Egypt, we observed decrease in egg production and abnormal increase of nymph mortality. These symptoms prompted us to look for a putative causative agent responsible for these mortalities and which could be used in an integrated pest management program of this insect as an alternative for the chemical pesticides. In this paper we report for the first time the isolation and characterization of a Picorna-like virus similar to the NVV-1 virus isolated from the *N. viridula* in South Africa.

MATERIALS AND METHODS

Green stink bug *Nezaraviridula*

Adults were collected from the Faculty of Agriculture Experimental Station, Giza, and a colony was maintained on the potted broad bean *Viciafabae*L. supplemented with fresh green beans *Phaseolus vulgaris* L. This food was renewed at two days' intervals. Pots were covered with cylindrical glass 15 cm in diameter, 22 cm in length and covered with muslin. The deposited egg-masses were collected daily and placed in Petri dishes containing pieces of moistened cotton wool, supplemented with fresh green beans until hatching (Figure 1). After hatching, the 1st instar nymphs were collected and maintained in pots.

Virus isolation, purification and examining with Electron microscopy

The dead nymphs and adults were homogenized in Tris buffer (50 mMTris pH 7.8) and the homogenate was clarified by centrifugation for 10,000 g for 10 min to pellet the insect debris. The supernatent was then centrifuged at 140,000 g for 2 hrs to pellet the viruses. After resuspending in Tris buffer, the viruses were deposited at the top of a 15 to 45% sucrose gradient and centrifuged at 27,000 rpm, for 150 min in a Beckman SW28 rotor. The viral band was washed in Tris buffer by centrifugation at 140, 000 g for 2 hrs, the pellet was resuspended and examined under electron microscopy after negative staining with phosphotungstic acid.

Nucleic acid extraction and characterization

The nucleic acid was extracted from purified virions by phenol/chloroform method (Sambrook et al., 1989) and the nucleic acid precipitated by ethanol was resuspended in TE buffer (Tris 10 mM, EDTA 1 mM pH 7.8). The nucleic concentration was estimated by spectrophotometry and aliquots (1.3 µg) were treated with DNase or RNase following the supplier's instruction (Roche Biochmicals). After treatment, the nucleic

acid was analysed on 1% agarose gel along with non-treated samples as control.

Analysis of viral proteins by SDS-PAGE

Purified virus particles were solubilized in 4x concentrated loading buffer (125mM Tris pH 6.8, 2% SDS, 5% ß-mercaptoethanol, 50% glycerol, 0.01% bromophenol blue). After denaturation at 100ºC for 5 min proteins were separated by SDS-PAGE on 12% acrylamide gel (Laemmli, 1970). High-Range Rainbow™ Molecular Weight Marker proteins were used. The gel was stained with Coomassie blue and the protein size was estimated based on a comparison with molecular markers.

Virus transmission and bioassay

The virus pathogenicity was tested either by dipping nymphs in a suspension of heavily infected dead insects or by feeding them on fresh green beans leaves contaminated by deeping in this suspension. To correlate the mortality with the virus concentrations, a stock suspension of one dead insect/ml of distilled water was prepared. The insects were homogenized and the supernatant was clarified by centrifugation at 10,000 g for 10 min. The following dilutions of the supernatant were prepared 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. And third instar nymphs were contaminated either by dipping into the virus suspension or by feeding them on fresh green beans *Phaseolus* contaminated with the same virus suspension. The nymphs were starved for 6 hours before treatment. Contaminated food was cut into 9 cm pieces and placed with contaminated nymphs in plastic Petri dishes closed with muslin tissue for aeration (Figure 1). The food was placed on a layer of tissue soaked with water to maintain the humidity. Distilled water was used as mock control. Treated insects were maintained for 5 days and daily mortality was recorded.

Virus host range

The virus was injected into fourth instar larvae of *Galleria melonella* and *Spodopteralittoralis* to test its pathogenicity. Three replicates of 24 larvae of each species were injected with 1-2 µl of a 10^{-2} virus suspension prepared as above mentioned per larva. The injected larvae were fed on insect diet and maintained until larvae death or pupation.

RESULTS

Detection of Picorna-like virus in stink green bug

The observation of abnormal mortality and reduced fecundity in a green stink bug colony established from bugs collected in Guiza Governorate and maintained in the laboratory prompted us to look for a pathogenic agent in this colony. In the absence of external sign of fungal infection (Figure 2), we oriented the research for pathogenic bacteria or viruses. Examination of dead insect smears under light microscope did not show specific pathogenic bacteria (data not shown) or any viral occlusion bodies such as those from baculoviruses, cypoviruses or entomopoxviruses. We thus looked for small virus particles through virus purification and concentration as indicated in Materials and Methods. Examination by electron microscopy of clarified larval homogenates or pellets of high speed centrifugation revealed the presence of paraspherical virus particles 25 nm in diameter (Figure 3a). The close examination indicated also the presence of few larger paraspherical particles about 33 nm in diameter mixed with the small virus particles (Figure3b).

Determination of the viral nucleic acid

The viral nucleic acid was extracted from purified virus particles using phenol /chloroform methods and quantified using spectrophotometer. Migration of the nucleic acid by electrophoresis on a 1% agrose gel revealed a single band with an estimated size of 8.5 kb (Figure 5). To determine the nature of the viral genome aliquots of viral nucleic were treated with DNase or RNase and after treatment were analysed on agarose gel. As shown in

figure 5, treatment with DNase had no effect on viral genome whereas the viral nucleic acid was totally digested by RNase, clearly demonstrating its RNA nature (Figure 5).

Analysis of viral proteins by SDS-PAGE

SDS-PAGE analysis of a purified virus suspension revealed the presence of two major capsid proteins with sizes of 31 and 32 KDa and one less abundant band at 76 kDa (Figure 4B).

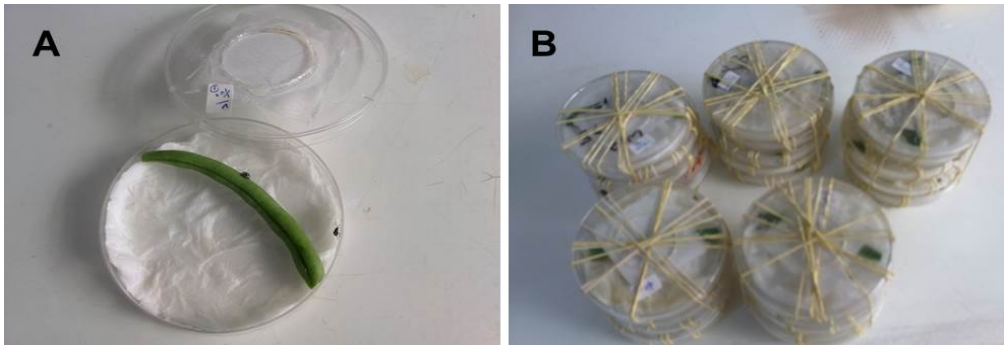

Figure 1. Experimental container to hold the green sting bug N. virudula nymph during bioassay tests. A: Open perti dish, B: closed warped perti dishes.

Figure 2. Viral disease symptoms in the green stink bug N. virudula insects.

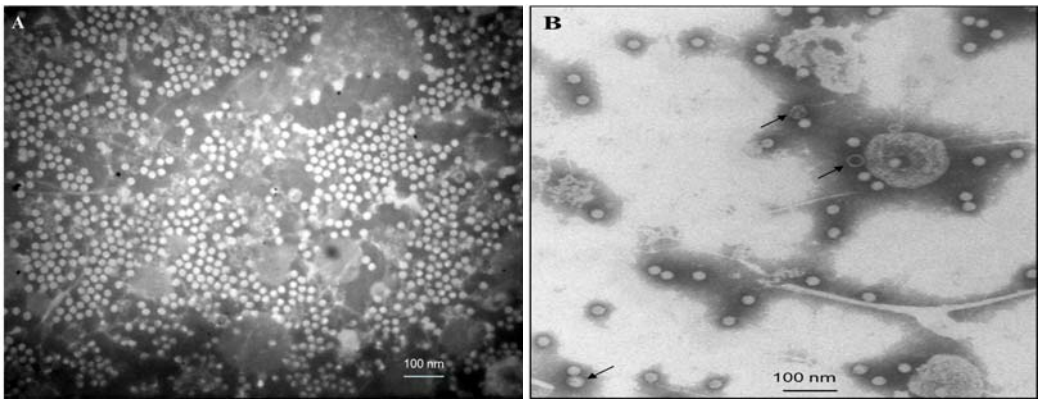

Figure 3. Electron microscope (EM) examination of purified Picorna-like virus particles isolated from the dead insects of green stink bug *N. virudula*. A: EM field show the small abundant virus particles, B: EM field show both virus particles size. Arrow indicates the large virus particles.

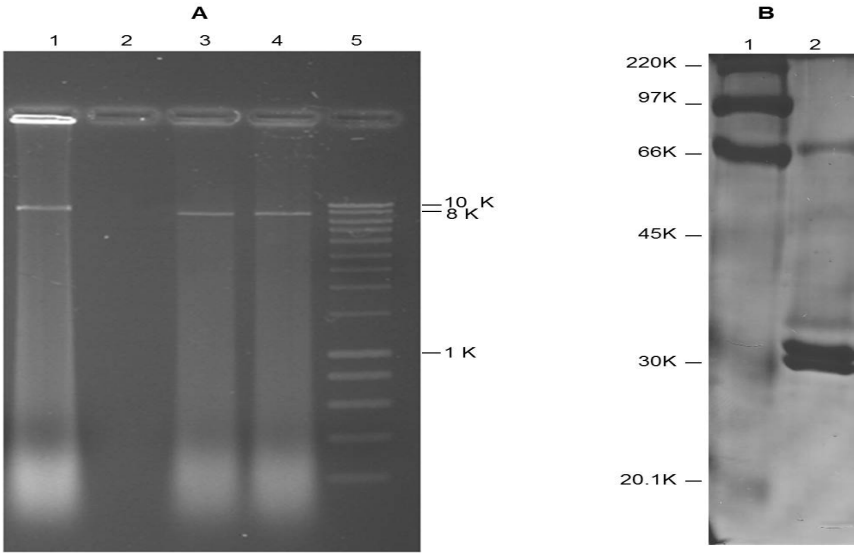

Figure 4.A: Nucleic acid of *N. viridula* isolated virus stained with ethidium bromide in a denaturing 1% agarose gel containing formaldehyde. Lane1and 4: extracted RNA of NVV-1; lane 2: extracted RNA of NVV-1treated with RNase, Lane 3: extracted RNA of NVV-1 treated with DNase, lane 5 size markers (DNA ladder; Smart ladder). (**B**) Structural proteins of PSIV in a SDS polyacrylamide gel (12%) stained with coomassie blue. Lane 1: protein ladder, Lane 2: Purified viral suspension.

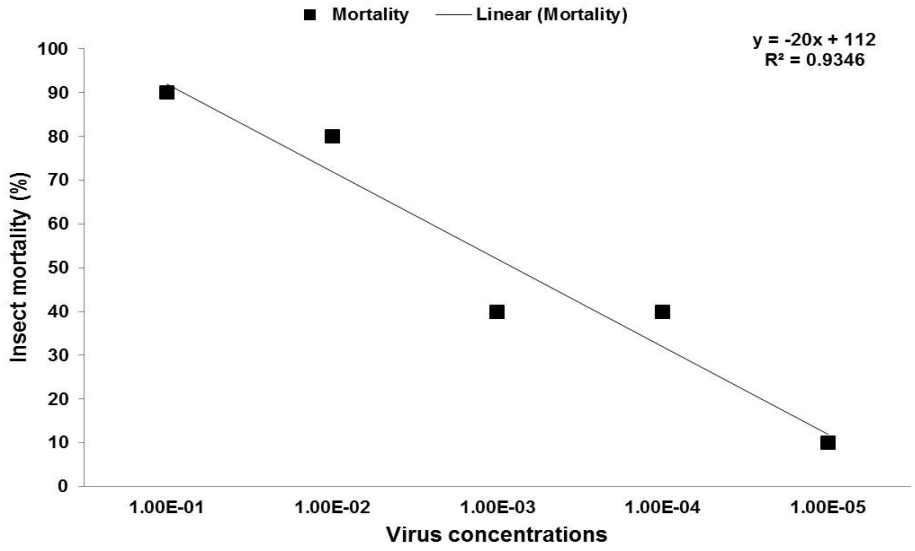

Figure 5. Bioassay test showing the relation between the virus concentration and insect mortality.

Virus pathogenicity and Bioassay

Contamination of third instar nymphs with the virus suspension prepared by homogenizing dead insects in distilled water induced the same pathology and cause high mortality at nymphal or adult stage. Contamination of the insect food with the virus suspension led to the same results, which indicates the pathogenic effect of this virus. These results were confirmed by feeding nymphs with food contaminated by different concentrations of a virus suspension and monitoring the daily mortality As shown in Fig 6, the highest concentration of the virus (10^{-1}) caused 90% mortality while the lowest concentration used (10^{-5}) caused only 10% mortality in the treated, insect thus clearly demonstrating a dose-dependent response.

Determination the host range of the virus

Injecting the virus suspension into the fourth instar larvae of *Galleria melonella* and *Spodopteralittoralis* did not cause any pathogenic effect and all injected larvae pupated normally indicating that the virus has a limited host range and is probably host specific to the stink green bug.

DISCUSSION

In this article we report for the first time the isolation and characterization of a small icoshedral RNA virus from *N. viridula*in Egypt. With a size of 25 nm, a genome consisting of a linear single-stranded RNA of about 9 kb and a capsid consisting of two major proteins of 32 and 34 kb this virus shares many similarities with the previously reported NVV-1 picorna-like virus isolated from the same species in South Africa (Williamson and von Wechmar (1992). These similarities support the idea that the virus we observed in our study is the same NVV-1 virus.

In addition to the 25 nm in diameter which corresponds to NVV-1 particles a few particles of 50 nm in diameter were also observed which probably corresponded to the NVV-2 particles previously reported by the same authors as Totiviridae(Williamson and von Wechmar 1992). However, the very low amount of the large virus particles did not allow detecting their genome by agarose gel. Furthermore, EM examination of virus suspensions after several passages by feeding the dead insect homogenate to healthy insects did not allow detecting the large virus particles anymore. The disappearance of large virus particles after several passages might result from its low pathogenicity compared to the small highly pathogenic virus particles.

A second picorna-like virus isolated from *Pentatomidae* insect is the PSIV isolated from the brown winged green bug*Plautiastali* (Nakashima et al., 1998; Sasaki et al., 1998). Although the similarity between this virus and the NVV-1 was reported, the protein profile of PSIV differed from the NVV-1 by having five capsid proteins of 35, 33, 30, 26, and 4.5 kDa proteins. The protein profile of the virus isolated in our study is more similar to that of NVV-1 virus than to that the PSIV virus.

Our results of *per os* contamination of healthy insect with the homogenate of dead nymphs clearly demonstrated its pathogenicity and bioassay results indicated that the mortality is dependant of the concentration of the virus.

Our unsuccessful attempts to infect Lepidopteran larvae, i.e. the wax worm *Galleria melonella* and the Egyptian cotton leaf worm *Spodopteralittoralis* indicates that contrary to several picorna-like viruses such as the cricket paralysis virus (CrPV) which have a wide host range (Plus et al., 1978), the *N. viridula* virus is host specific and might be a potential candidate for biological control for this pest.

In Egypt, during the breeding period, nymphal densities and oviposition are lowest in summer (Ali et al., 1979). The low summer densities were attributed to nymphal mortality from exposure to

high temperatures (25-28 "C). However, the low rate of reproduction could also result from delayed egg maturation, decreased egg production due to virus infection.

Taken together, our data demonstrate that a picorna-like virus similar to the NVV-1 previously isolated from South Africa was isolated from the *N. viridula* insect population in Egypt. This virus is mixed with low abundant and less virulent virus which may be similar to the NVV-2 as previously reported in South Africa too. This result indicates that although the different ecosystem in Egypt and South Africa the *N. viridula* maintain the infection with the same viruses. These results open the door to the possibility to use this virus as a bioinsecticides again this important insect.

ACKNOWLEDGMENT

We thank Dr XaviereJousset for her kind help in estimating the virus particles size.

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