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$F(ab)_2$-ELISA for the Detection of Nuclear Polyhedrosis Virus of Silkworm, *Bombyx mori* L.

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$F(ab')_2$-ELISA and direct antigen coating-ELISA (DAC-ELISA) were evaluated in the detection of purified *Bombyx mori* nuclear polyhedrosis virus (BmNPV) and nuclear polyhedrosis virus infection in silkworm larvae inoculated with BmNPV polyhedra. Although nanogram levels of BmNPV was detected in both DAC- and $F(ab')_2$-ELISA, similar concentrations of antigen was detected in case of $F(ab')_2$-ELISA even at higher dilution of antibody (up to 1 : 20 K). One hundred percent nuclear polyhedrosis infection was detected 6 hrs after inoculation in BmNPV infected silkworm larvae by $F(ab')_2$-ELISA. On the other hand, detection of 100% infection was observed only three days after inoculation in DAC-ELISA. In this study, it was observed $F(ab')_2$-ELISA was more sensitive than DAC-ELISA in the detection of purified BmNPV as well as nuclear polyhedrosis infection in silkworm larvae.

Key words: $F(ab')_2$-ELISA, Nuclear polyhedrosis virus detection, Silkworm, *Bombyx mori*, *Bombyx mori* Nuclear polyhedrosis virus

Introduction

Among the silkworm diseases, nuclear polyhedrosis virus is prominent and causes extensive crop loss through out the year in all the sericultural countries. *Bombyx mori* nuclear polyhedrosis virus (BmNPV) belongs to group I baculoviruses in the family Baculoviridae (Blissard *et al.*, 2000). BmNPV infections are usually detected at late stages and as a result the cocoon crop can not be harvested successfully. It would be extremely advantageous in the management of nuclear polyhedrosis, if early detection of BmNPV were ascertained in the silkworm rearings.

Enzyme-linked immunosorbent assays (ELISA) have been used extensively for their specificity and sensitivity to detect/identify diseases caused by microorganisms. Various forms of ELISA have been used to detect silkworm viruses in the past (Shimizu, 1982; Mike *et al.*, 1984; Arakawa and Shimizu, 1985; Ito *et al.*, 1985; Shi and Ding, 1989; Nagamine *et al.*, 1991). Fab fragment of immunoglobulin was first used in ELISA system [$F(ab')_2$-ELISA] to detect serological relationships among carlaviruses (Adams and Barbara, 1982). The Fab fragment, as a number of instances indicate proved to be superior to an intact antibody molecule in immunoassays. For example, when antibodies are used for diagnostic and imaging applications, they may show increased specificity possibly due to the removal of Fc portion of immunoglobulin, which is common to all antibodies regardless of their specificity (Glick, 1990). The diagnosis of any disease at appropriate or early stages plays an important role on the spread of particular disease, ultimately aiding in the management of disease itself. This is especially true in case of virus diseases. The present study describes the development of $F(ab')_2$-ELISA for the detection of BmNPV in silkworms during early stages of infection.

Materials and Methods

BmNPV was propagated by per oral inoculation of silkworm larvae immediately after IV moult with BmNPV polyhedra (1×10^6/ml) smeared onto mulberry leaf. The BmNPV was purified by following the procedure described by Sugimori *et al.* (1990). The BmNPV polyhedra were collected from diseased haemolymph and purified by linear sucrose density gradient (40 – 63%)

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centrifugation at 25,000 rpm for 30 min at 4°C. The virions were released from purified polyhedra by using 50 mM Na_2CO_3 and purified by linear sucrose density gradient (10 – 40%) centrifugation at 40,000 rpm for 2 hrs at 4°C.

Polyclonal antibodies were raised against BmNPV by injecting purified BmNPV (100 μg/ml) into New Zealand white rabbits. The booster doses were given 4 times at weekly intervals. Anti-BmNPV IgG was purified by $(NH_4)_2SO_4$ precipitation (to a final concentration of 33%; v/v) followed by gel filtration (Sephadex G25, Pharmacia, Sweden) and ion exchange chromatography (DEAE Cellulose, DE52, Whatman, UK). The IgG was subjected to substractive affinity chromatography using healthy silkworm haemolymph bound to CNBr activated Sepharose 4B (Pharmacia, Sweden). $F(ab')_2$ fragments were prepared from the IgG by pepsin hydrolysis (Clark and Bar-Joseph, 1984). The titre of antibodies was estimated by DAC-ELISA (direct antigen coating-ELISA) and $F(ab'_2)$-ELISA (Clark and Bar-Joseph, 1984; Barbara and Clark, 1982; Mowat and Dawson,1987) using purified BmNPV as antigen.

To detect BmNPV during progressive infection of nuclear polyhedrosis virus, the antigen samples were collected by homogenising the silkworm larvae in distilled water (9 ml/g tissue) containing few specks of phenylthiourea. The larval samples were collected at 6 hrs, 12 hrs and 1st, 2nd, 3rd, 4th, 5th and 6th days after inoculation. The antigen extract (100 μl) was mixed with 100 μl of carbonate buffer, pH 9.6 (DAC-ELISA) or phosphate buffered saline, pH 7.4 containing 0.05% Tween-20, 1% milk protein and 2% PVP [$F(ab')_2$-ELISA] in microtitre plate. Prior to the addition of antigen samples, the microtitre plate was filled with 200 μl of $F(ab')_2$ fragments in carbonate buffer, pH 9.6 in $F(ab)_2$-ELISA. Horseradish peroxidase labelled anti-Fc immunoglobulin was used to detect the antigen-antibody complexes and orthophenylenediamine was used as substrate. The reaction was stopped by the addition of 50 μl of 3N H_2SO_4. The absorbance values were read at 492 nm in Titertek ELISA Plate Reader. Healthy larval extracts, antigen and antibody dilution buffers were set in each microtitre plate as controls. Samples with absorbance values two-time greater than healthy larval extracts/buffer controls were considered as positive.

Results and Discussion

Anti-BmNPV IgG was able to detect ng/ml BmNPV at 1:5,000 dilution and mg/ml BmNPV at 1:20,000 dilution in DAC-ELISA, whereas ng/ml BmNPV at 1:20,000 dilution and μg/ml at 1:25,000 dilution was detected in $F(ab')_2$-ELISA. The A_{492} values at given antigen/antibody concentration/dilution were higher in case of $F(ab')_2$-ELISA as compared to DAC-ELISA (Fig. 1).

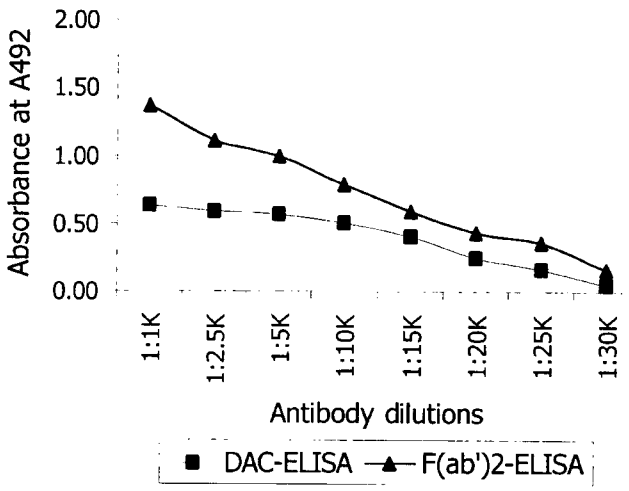

Fig. 1. Antibody dilution curve for anti-BmNPV IgG (μg/ml) at ng/ml purified BmNPV in DAC- and $F(ab')_2$-ELISA.

Table 1. Comparison between DAC-ELISA and $F(ab)_2$-ELISA systems in the detection of BmNPV during progressive nuclear polyhedrosis infection in silkworms

Duration After Inoculation	DAC-ELISA		$F(ab)_2$-ELISA	
	% BmNPV infected larvae	A_{492} Range	% BmNPV infected larvae	A_{492} Range
6 hrs	60	0.281 – 0.322	100	0.371 – 0.659
12 hrs	80	0.283 – 0.404	100	0.574 – 1.019
24 hrs	90	0.521 – 0.767	100	1.097 – 1.501
2nd day	100	0.528 – 0.830	100	1.016 – 1.522
3rd day	100	0.542 – 0.897	100	1.038 – 1.635
4th day	100	0.569 – 1.055	100	1.071 – 1.654
5th day	100	0.594 – 1.385	100	1.281 – >2.0
6th day	100	0.776 – 1.049	100	1.583 – >2.0

Both the systems, DAC-ELISA and F(ab')$_2$-ELISA detected BmNPV in the BmNPV inoculated larvae during progressive nuclear polyhedrosis virus infection (Table 1). Hundred percent infection was detected 6 hrs after inoculation in F(ab')$_2$-ELISA and on the other hand, only three days after inoculation only it was detected at similar levels in DAC-ELISA. The A_{492} values in the healthy larval extracts ranged from 0.043 – 0.054 and in the buffer controls from 0.021 – 0.028.

The sensitivity, specificity and economy of ELISA have made it preferred method for many filed and laboratory applications, where sensitivity and specificity of antigen detection together with economy and scale of operation are factors of prime importance. F(ab')$_2$-ELISA was more specific in detecting the viruses and in identifying the serological relationships (Adams and Barbara, 1982). Biotin-avidin system of ELISA (BAS-ELISA) and dot binding immunoassay (DBIA) could detect one ng and 10 ng of densonucleosis virus of silkworm, respectively (Arakawa and Shimizu, 1985; Shi and Ding, 1989). Double antibody sandwich-ELISA could detect 3 ng levels of infectious flacherie virus of silkworm (Shimizu, 1982). F(ab')$_2$-ELISA proved more sensitive than DAC-ELISA in the detection of BmNPV in both crude extracts of BmNPV infected silkworm larvae and purified virus (Table 1 and Fig. 1). Pulse labelling experiments conducted in BmNPV infected Bm-N cells showed that BmNPV induced polypeptides reacted with BmNPV antiserum and are believed to be structural polypeptides of BmNPV occluded virions (Sugimori *et al.*, 1991). The results obtained in the present study concur with the reports available in the literature about the capability of ELISA system and F(ab')$_2$ fragments in the detection of viral antigens at nanogram levels.

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