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Colloidal Textile Dye-Based Dipstick Immunoassay for the Detection of Infectious Flacherie of Silkworm, *Bombyx mori* L.

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Infectious flacherie of silkworm *Bombyx mori* is caused by *B. mori* infectious flacherie virus (BmIFV) and causes severe crop loss to sericulturists. In the present study, a colloidal textile dye-based dipstick immunoassay is developed for the detection of infectious flacherie in silkworms. Colloidal textile dye (blue D2R) with $A\lambda_{max}$ at 620 nm was sensitised with 500 µg/ml of purified anti-BmIFV IgG. The dye-antibody reagent detects purified antigen up to 10 ng/ml and BmIFV infection in diseased larval extracts (up to a dilution of 10^{-5}) and faecal matter extracts (up to a dilution of 10^{-2}) by forming clear blue dot within 30 min. It was observed to be stable for three months period at 4°C. The efficacy of textile dye-based dipstick immunoassay was on par with HRP-based dipstick immunoassay and fluorescent antibody test, and better than latex agglutination and ouchterlony tests in the detection of BmIFV. The dye-based dipstick immunoassay method provides a simple, sensitive and less expensive test for the detection of BmIFV infection in silkworms.

Key words: Infectious flacherie virus, *Bombyx mori*, Colloidal textile disperse dye, Dipstick immunoassay

Introduction

Infectious flacherie of silkworm, *Bombyx mori* is caused by *B. mori* infectious flacherie virus (BmIFV), a picornavirus (Francki *et al.*, 1991). This disease prevails in all sericultural countries and is reported to cause severe damage to sericulture. BmIFV produces atypical morphological symptoms in diseased larvae, spreads in silkworm populations to dangerous proportion and causes severe crop loss as the larvae fail to spin cocoons. Yamazaki *et al.* (1960) and Aizawa *et al.* (1964) established the etiology of infectious flacherie in silkworms. The prevalence of BmIFV infection was reported in sericultural areas in India (Sato, 1992; Sivaprasad *et al.*, 1993).

Several immunoassays have been used to identify/detect the diseases of silkworms. For the detection of BmIFV in silkworms fluorescent antibody test (Inoue and Ayuzawa, 1972; Sato *et al.*, 1978), ouchterlony test (Sekijima, 1971), single radial immunodiffusion test (Seki and Sekijima, 1976), electrosynersis (Sekijima and Ohtsu, 1976), precipitin tests (Sekijima, 1971), neutralisation test (Kurisu *et al.*, 1975), haemagglutination (Sato and Kawase, 1971), enzyme-linked immunosorbent assay (Shimizu, 1982) were reported. Shimizu *et al.* (1983) reported latex agglutination test as the most sensitive assay for the detection of BmIFV infections in silkworms. Routinely the BmIFV infections are detected either by fluorescent antibody test (Inoue and Aizawa, 1972) or pyronin-Y and methyl green stating (Iwashita and Kanke, 1969).

Ever since Engvall and Perlmann (1971) first pioneered their use, enzyme-linked immunosorbent assay (ELISA) systems are being used extensively to detect/identify pathogens due to its high sensitivity and specificity. However, ELISA system has distinct drawbacks, which make it unsuitable in the field studies and not-so-well equipped laboratories. The practical utility of the ELISA-based systems had been improved with the usage of nitrocellulose membrane as test matrix instead of microtitre polystyrene plate (Scott, 1989) and colloidal gold (Hsu, 1984) or textile disperse dyes (Gribanu *et al.*, 1983; Nataraju *et al.*, 1994). The colloidal dye based dipstick immunoassay employs nitrocellulose membrane as test matrix and disperse textile dye antibody reagent as reporter for the antigen-antibody complex. This assay combines specificity

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and sensitivity at lesser cost for diagnosing microbial diseases and utilised for the early detection of nuclear polyhedrosis caused by BmNPV in silkworms. In the present study, a colloidal dye-based dipstick immunoassay is described for the detection of BmIFV in silkworms.

Materials and Methods

Purification of BmIFV

BmIFV was purified from silkworm larvae inoculated with BmIFV infected larval extract. The larvae were homogenised in 0.05 M Tris-HCl buffer, pH 7.6 containing 0.005 M EDTA and 0.05% β-mercaptoethanol. The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was clarified with 10% chloroform (v/v) by stirring for 20 min at 4°C and centrifuged at 10,000 rpm for 10 min at 4°C. Polyethylene glycol (MW 6,000) and NaCl were added to the aqueous phase to a final concentration of 8% (w/v) and 0.5 M (w/v) and incubated at 4°C for 2 hrs to precipitate the virus. The precipitate was collected by centrifugation at 10,000 rpm for 10 min at 4°C. The precipitate was suspended in 1/10 volume 0.05 M Tris-HCl buffer, pH 7.6 and centrifuged at 6,000 rpm for 10 min at 4°C. The supernatant was layered onto 20% sucrose cushion and centrifuged at 25,000 rpm for 2 hrs at 4°C in a Hitachi RPS-55 rotor. The virus pellet was suspended in 0.05 M Tris-HCl buffer, pH 7.6 and layered onto linear sucrose density gradient (10 – 40%) and centrifuged at 25,000 rpm for 2 hrs at 4°C. The light scattering virus band was collected and sedimented by high speed pelleting at 40,000 rpm for 2 hrs at 4°C. The purified virus pellet was suspended in 50 mM Tris-HCl buffer, pH 7.6 and protein concentration was estimated following the procedure described by Lowry *et al.* (1951).

Production of polyclonal antibodies

Purified BmIFV (100 µg/ml) was emulsified in Freund's complete adjuvant and injected simultaneously into two New Zealand white rabbits for immunisation. The booster doses were given 4 times at weekly intervals, intramuscularly, with the similar amount of antigen emulsified in Freunds incomplete adjuvant. The rabbits were bled 2 weeks after the final booster and antisera were collected by low speed centrifugation and stored at –20°C for further use. The titre was estimated direct antigen coating-ELISA (DAC-ELISA) as described by Mowat and Dawson (1987).

Purification of immunoglobulin

Anti-BmIFV immunoglobulins were purified by $(NH_4)_2SO_4$ precipitation (to a final concentration of 33%; v/v) followed by gel filtration (Sephadex G25, Pharmacia, Sweden) and affinity chromatography (HiTrap Protein G, Pharmacia, Sweden). The purified IgG was quantified and stored at 20°C. The immunoglobulins were quantitated in Shimadzu UV-VIS spectrophotometer at 280 nm (1.4 O. D. = 1 mg/ml).

Preparation of colloidal disperse dye-antibody reagent

The dye-antibody reagent was prepared by following the procedure described by Snowden and Hommel (1991) and Nataraju *et al.* (1994). Disperse textile dye D2R ($A\lambda_{max}$ = 620 nm), with blue hue (Indian Dye Stuff Industries Limited, Ranoli, India) was used for the preparation of colloidal disperse dye antibody reagent. Dye particles at a conc. of $A\lambda_{max} = 10$ in 10 mM potassium phosphate buffer, pH 7.4 containing 2.7 mM NaCl were mixed with 500 µg/ml of anti-BmIFV IgG and incubated for 2 hrs at 37°C and overnight at 4°C with gentle end to end mixing. Following incubation, the dye-antibody complex is spiked with 1/5 volume of 10 mM potassium phosphate buffer, pH 7.4 containing 5 mM NaCl and 30% BSA and incubated for 1 hr at 37°C to stabilize the dye particle surface. The antibody-dye reagent was centrifuged at 10,000 rpm for 20 min and the pellet was re-suspended in 33 mM potassium phosphate buffer, pH 7.4 containing 125 mM NaCl, 5% BSA and 0.02% NaN_3 to the final concentration of $A\lambda_{max} = 10$.

Preparation of dipsticks

Different concentrations of anti-BmIFV IgG (1 – 4 µg/3 µl dot) were captured onto nitrocellulose membrane attached to dipsticks (7 × 75 mm nitrocellulose membrane; 0.45 µ; DCN-II; Advanced Microdevices, Ambala, India). The dipsticks were washed in 0.15 M potassium phosphate buffer, pH 7.4 containing 0.85% NaCl (PBS) thrice at 15 second intervals and incubated in PBS containing 3% BSA (PBS-BSA) for 30 min at 37°C and washed thrice with PBS at 15 second intervals, air-dried and stored at 4°C or used immediately.

Dipstick assay for BmIFV detection

Anti-BmIFV antibody dipsticks prepared with concentrations (1 – 4 µg/3 µl dot) and blocked with PBS-BSA, as described above were used. Purified BmIFV was used as antigen (1000 to 1 ng/ml). The dipsticks were incubated in various antigen dilutions for 1 h and washed gently for 15 seconds under running tap water. The dipstick sets were then placed in dye-antibody detection reagent at 37°C for 5 min to 1 hr, briefly rinsed in tap water and observed for the coloured dots. The reactions were graded as negative (–), faint (F), clear (+), strong (++) and very strong (+++). To compare the textile dye-based dipstick immunoassay

with HRP (horseradish peroxidase) based assay, one set of dipsticks was incubated in HRP-labelled anti-BmIFV antibody for 1 hr. After washing for 15 seconds in tap water, blue-black coloured dots were detected using 0.03% diaminobenzidine be in PBS containing 0.06% H_2O_2.

Detection of BmIFV in larval and faecal matter extracts

Silkworm larvae out of I moult were individually inoculated with BmIFV diseased larval extract (10% homogenate) and reared singly in petridishes. The antigen samples were collected from each petridish and prepared for dipstick immunoassay by homogenisation in 0.1 M Tris-HCl buffer, pH 8.0 (silkworm larvae infected with BmIFV at 9 ml/g; faecal matter from diseased larvae at 9 ml/100 mg). The sensitivity of HRP- and dye-based tests was determined by using purified BmIFV and diluted antigen extracts to detect BmIFV infection in silkworms and faecal matter from diseased larvae.

Specificity of the assay

The specificity of dye- and HRP-based dipstick immunoassays to detect BmIFV infections was checked by using extracts of larvae infected with BmIFV, BmDNV1, BmNPV, *Bacillus thuringiensis*, *Serratia marcescens*, *Streptococcus* sp. and *Nosema bombycis* and faecal matter from larvae infected with BmIFV and BmDNV1 as antigens; purified BmIFV as positive control; healthy larval extract, faecal matter and buffer as negative controls. Normal rabbit serum control was maintained in both the tests. These tests were conducted thrice with 6 samples in each case to confirm the reproducibility of the results.

Practical application of the assay

The practical applicability of the dye-based dipstick immunoassay was checked with 50 healthy and 98 BmIFV inoculated silkworm larvae (III instar) as antigens. The conc. of capture antibody used was 4 µg/dot. These samples were also subjected to BmIFV detection by DAC-ELISA (Mowat and Dawson, 1987), ouchterlony and fluorescent antibody tests.

Results

At an antigen (purified BmIFV) concentration of 5 ng/ml, the titre of polyclonal antibodies raised against BmIFV was 1:10000 in DAC-ELISA. The results of present study indicate that colloidal dye-based dipstick immunoassay could detect BmIFV antigen (Table 1 and Fig. 1). A clear blue coloured dot has developed within 30 min in the presence of a minimum of 10 ng/ml antigen. Four µg/3 µl

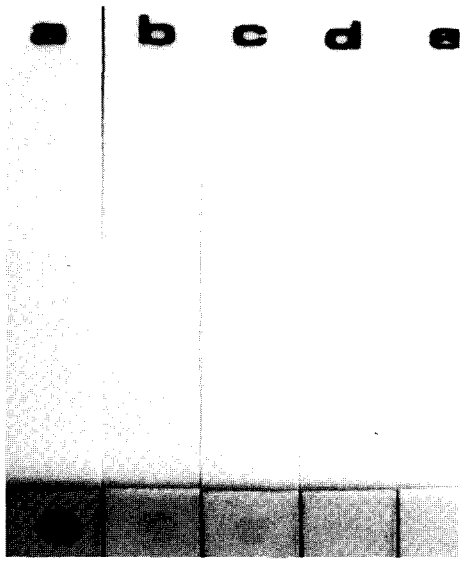

Fig. 1. Colloidal textile dye-based dipstick immunoassay for the detection of BmIFV. Dipsticks with 4 µg/dot affinity purified anti-BmIFV IgG as capture antibody developed blue coloured dot after incubation in antigen sample followed with dye-antibody reagent. (a) +++: very strong, (b) ++: strong, (c) +: clear; (d) F: faint and (e) −: negative reaction at 100, 10 5 and 1 ng/ml antigen (purified BmIFV) conc. and buffer control (PBS, pH 7.4).

Table1. Sensitivity of enzyme- and dye-based dipstick immunoassay for the detection of purified BmIFV

Anti-BmIFV IgG conc. (µg/dot)	Purified BmIFV conc. (ng/ml)									
	HRP-based					Dye-based				
	1000	100	10	5	1	1000	100	10	5	1
1	++	++	++	+	F	++	+	+	F	F
2	+++	+++	++	+	F	+++	++	++	F	F
3	+++	+++	++	++	F	+++	++	++	+	F
4	+++	+++	++	++	+	+++	+++	++	+	F

+: clear; ++: strong; +++: very strong; F: faint; -: negative reaction.

anti-BmIFV IgG/dot as capture antibody was comparatively better than lower concentrations. However, development of faint coloured dot was observed with 1 μg/dot capture antibody at an antigen conc. of 10 ng/ml. Different concentrations of capture antibody also developed faint coloured dot with ng/ml of antigen. Five-hundred μg/ml anti-BmIFV IgG was observed to be optimum to sensitise the colloidal textile dye. The sensitivity was similar in detecting BmIFV in each of six replicates in three separate experiments conducted.

A comparison of HRP- and dye-based dipstick immunoassays indicated the higher sensitivity of HRP system. The HRP system with 4 μg/dot of capture antibody could detect a minimum of 1 ng/ml of antigen through a clear brown coloured dot within 5 min. Even 1 μg/dot capture antibody could detect 10 ng/ml of antigen. The periodical testing (once in a week up to six months) of the dye-antibody reagent to determine its shelf life revealed that it could detect 10 ng/ml BmIFV up to three months with consistent results (six replicates in each experiment).

The colloidal dye-based assay could detect BmIFV infection in diseased larval extract (up to a dilution of 10^{-5}) and faecal matter extract (up to a dilution of 10^{-2}). The results were almost comparable to that of HRP-based dipstick immunoassay (Table 2). The assay was carried out with similar results in three separate experiments.

Table 2. Sensitivity of enzyme- and dye-based dipstick immunoassay for the detection of BmIFV infections in the extracts of silkworm larvae and faecal matter of BmIFV diseased larvae

Antigen dilution	Dipstick immunoassay	
	HRP-based	Dye-based
Larval extracts		
10^{-1}[a]	+++	+++
10^{-2}	+++	++
10^{-3}	++	+
10^{-4}	+	+
10^{-5}	+	+
10^{-6}	F	F
10^{-7}	–	–
Faecal matter		
10^{-1}[b]	+++	++
10^{-2}	++	++
10^{-3}	F	F
10^{-4}	–	–
10^{-5}	–	–

[a]Silkworm larvae were homogenised in 0.1 M Tris-HCl, pH 8.0 (9 ml/g).

[b]Faecal matter was homogenised in 0.1 M Tris-HCl, pH 8.0 (9 ml/100 mg).

+: clear; ++: strong; +++: very strong; F: faint; –: negative reaction.

The colloidal textile dye-based dipstick immunoassay was highly specific in the detection of BmIFV infections. No coloured dots were observed with extracts of larvae infected with BmNPV, BmDNV1, *B. thuringiensis*, *S. marcescens*, *Streptococcus* sp. and *N. bombycis* and faecal matter of larvae infected with BmDNV1, healthy larvae and faecal matter, and buffer. Normal rabbit serum control maintained also showed negative reaction in both dye- and HRP-based dipstick immunoassays. On the other hand, clear coloured dot was observed in case of purified BmIFV and extracts of BmIFV infected silkworm larvae and faecal matter from the infected larvae in both the assays. This clearly demonstrates the fact that occurrence of BmIFV could be specifically identified in the field when the silkworms are affected by various viral and bacterial diseases. The specificity tests were carried out six replications in two separate experiments.

The dye-based dipstick immunoassay could detect BmIFV infection in all the 98 BmIFV inoculated larvae tested. However, the assay was observed to be positive in two out of 50 healthy larvae tested. In DAC-ELISA and fluorescent antibody test, it was observed that all the positive and negative samples gave expected results. The A492 values ranged from 0.368 – 0.766 and 0.089 – 0.124 in the BmIFV inoculated and healthy larval extracts, respectively. In the ouchterlony test, no precipitin line was observed in case of only six BmIFV inoculated larval extracts.

Discussion

The atypical symptoms produced by the BmIFV in silkworm larvae make it difficult to diagnose the infectious flacherie in silkworms even at late stages of infection. Even though the ELISA reported by Shimizu (1982) for the detection of BmIFV is highly sensitive (detects 3 ng/ml antigen) and specific, it is not a ready-to-use diagnostic method. The colloidal textile disperse dye-based dipstick immunoassay developed in the present study is a useful diagnostic method for the detection of BmIFV infections in silkworm larvae as well as the extracts of faecal matter from diseased larvae.

The textile dye-based dipstick immunoassay requires a period of one hour involving two simple steps, I: incubation in antigen sample and II: incubation in dye-antibody reagent. This assay combines previously described techniques such as sandwich-ELISA and dot binding

immunoassay to form a simple, sensitive and economical test. It compares favourably with enzyme based dipstick assay in terms of storage and cost efficiency. The dye-antibody reagent in the present study was observed to be functional even after three months storage at 4°C as liquid suspension. Similar observations was made by Nataraju *et al.* (1994), but earlier Snowden and Hommel (1991) reported that dye-antibody reagent could be stored for a period of one week at 4°C as a liquid suspension or kept frozen at 20°C or lyophilised for long-term storage. The assay is specific in detecting BmIFV, as it did not show any reaction with extracts of larvae diseased with other common silkworm pathogens and faecal matter from larvae diseased with BmDNV1.

The 100% detection of infectious flacherie by colloidal textile dye-based dipstick immunoassay in silkworm larval extracts proves that this assay is a feasible technique for the detection of BmIFV. However, a small discrepancy, such as non-specific or false positives (up to 4%) was observed in healthy larval samples and this may be attributed to non-specific adherence of host proteins to the capture antibody. The results of ouchterlony (six false negatives out of 98 samples) clearly demonstrate the limitations of the test, as higher antigen conc. is required to detect the pathogen. The colloidal textile dye-based dipstick immunoassay forms a simple and less expensive technique for the detection of BmIFV infections in silkworms. The diagnosis of the disease is impossible with the light microscopy (pyronin Y and methyl green staining), and fluorescent antibody method has limitations in its filed applicability. The disease cannot be detected even at late infection stage due to atypical symptoms. An early detection by an efficient diagnostic method, such as dye-based dipstick immunoassay could aid in proper management of the disease, rejection of infected crop, thorough disinfection of rearing house and appliances followed by raising of next crop under ideal conditions, thereby limiting the loss due to BmIFV infection below economic injury levels.

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