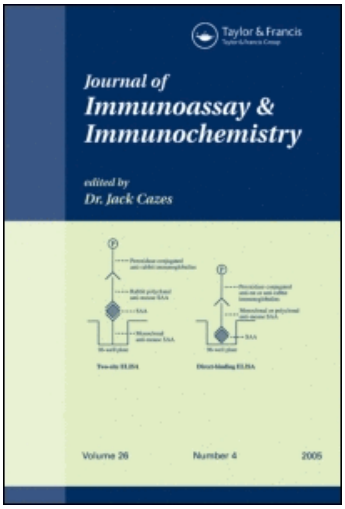


**Journal of Immunoassay and Immunochemistry**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

# Production of Monoclonal Antibodies Against *Nosema bombycis* and their Utility for Detection of Pebrine Infection in *Bombyx mori* L.

M. Shamim[a]; Debjani Ghosh[a]; M. Baig[b]; B. Nataraju[b]; R. K. Datta[b]; S. K. Gupta[a]
[a] Gamete Antigen Laboratory, National Institute of Immunology, New Delhi, India [b] Central Sericultural Research and Training Institute, Mysore, India

**To cite this Article** Shamim, M. , Ghosh, Debjani , Baig, M. , Nataraju, B. , Datta, R. K. and Gupta, S. K.(1997) 'Production of Monoclonal Antibodies Against *Nosema bombycis* and their Utility for Detection of Pebrine Infection in *Bombyx mori* L.', Journal of Immunoassay and Immunochemistry, 18: 4, 357 — 370
**To link to this Article: DOI:** 10.1080/01971529708005827
**URL:** http://dx.doi.org/10.1080/01971529708005827

JOURNAL OF IMMUNOASSAY, 18(4), 357-370 (1997)

# PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST *NOSEMA BOMBYCIS* AND THEIR UTILITY FOR DETECTION OF PEBRINE INFECTION IN *BOMBYX MORI* L.

M. Shamim[1], Debjani Ghosh[1], M. Baig[2], B. Nataraju[2], R.K. Datta[2] and S.K. Gupta[1]*

[1]Gamete Antigen Laboratory, National Institute of Immunology, New Delhi-110 067, India; and [2]Central Sericultural Research and Training Institute, Mysore-570 008, India.



## ABSTRACT

Latex agglutination assay based on monoclonal antibodies (MCAs) described in this communication may be useful for detection of Pebrine infection in silkworm. Four murine MCAs were produced against *Nosema bombycis* spore. In ELISA all 4 MCAs (IgM isotype) reacted with alkali treated Nosema spores and to variable extent with acetone precipitated surface protein. However, MA-310 and MA-542 showed a low degree of cross reactivity with BmNPV. In contrast, MA-503 and MA-515 were devoid of reactivity with BmNPV, *B. thuringiensis, S. marcescens,* Azotobactor, Rhizobium and normal hemolymph protein in ELISA. Latex beads sensitized with a combination of MA-503 and MA-515 (50 μg each per ml of 0.4% latex beads) could detect $1\times10^5$ Nosema spores per test. Sensitization of the latex beads with the cocktail of these two MCAs through protein-A bridge further led to a 10-fold increase in the sensitivity ($1\times10^4$ spores/test) of the assay. No agglutination was observed in presence of BmNPV, Rhizobium, Azotobactor, *E. coli, B. thuringiensis, S. marcescens* and normal hemolymph protein indicating the specificity of the test. The results obtained by latex agglutination assay on hemolymph samples of infected as well as normal larvae collected from field, II instar larvae infected in the laboratory and from infected mother moth revealed 100% correlation with results by microscopic examination.

**(KEY WORDS:** *Nosema bombycis* spores, Monoclonal antibody, Enzyme linked immunoassay, Latex agglutination test)

*Author for correspondence; FAX- 91-11-6162125; E-mail: GA@nii.ernet.in

## INTRODUCTION

Pebrine disease of silkworm, *Bombyx mori* L. caused by the microsporidian, *Nosema bombycis*, is widely prevalent and causes silkworm morbidity and mortality in sericulture industry. The pathogen, by its nature maintains in silkworm populations through transovarial transmission and secondary contamination by ingesting contaminated leaves. The infection is prevented by adopting prophylactory measures to avoid secondary contamination and systemic mother moth examination to reject the eggs produced by infected moths. Inspection by microscopy of mother moths for Pebrine infection after ovi-position is cumbersome due to the presence of other microsporidian spores which are similar in size and shape as that of *Nosema bombycis* [1]. However, serological techniques can provide an alternative for correct diagnosis of Pebrine infection. Various immunoassays such as fluorescent antibody tests [2, 3], indirect enzyme linked immunosorbent assay [4, 5] and inhibition ELISA [6] have been developed for specific detection of microsporidian spores. A simple and easy to perform latex agglutination test based on monoclonal antibody (MCA) with sensitivity of $1.3\times 10^8$ spores/ml have been reported [7]. In the present study, we report the development and characterization of 4 MCAs against *Nosema bombycis*. The utility of these MCAs in the development of a sensitive and specific latex agglutination test for the detection of Pebrine spores has been investigated.



## MATERIALS AND METHODS

### Isolation and Purification of *Nosema bombycis* Spores

Mature *Nosema bombycis* spores were collected by homogenization in physiological saline (0.85% NaCl) of the infected silkworm larvae and/or mother moths and centrifugation of the suspension at 1000×g for 10 min at room temperature. The pellet was washed five times with saline. The spores were further

purified by isodensity equilibrium centrifugation using Percoll (Sigma Chemical Co., St. Louis, USA) at 73,000×g for 30 min at 4°C according to the procedure described earlier [8]. Spore concentration was estimated with a hemocytometer and adjusted to $10^9$-$10^{10}$ spores/ml. Purified spores were stored at -20°C in physiological saline.

**Preparation of *Nosema bombycis* Surface Antigen**

*N. bombycis* spores ($10^9$ to $10^{10}$) were suspended in 0.5ml of 100 mM $K_2CO_3$ solution for 20 min at room temperature, and the suspension was gently mixed every 5 min. After incubation, pH of the suspension was neutralized by adding an equal volume of 1M Tris-HCl (pH 4.5). This suspension is termed as alkali treated Nosema spores. To prepare acetone precipitated surface antigen, the alkali treated Nosema spores suspension was centrifuged at 1000×g for 30 min at room temperature. To the supernatant an equal volume of cold acetone was added and incubated overnight at 4°C. The precipitated antigen was separated by centrifugation at 4000×g for 30 min at 4°C and dissolved in 50 mM phosphate buffer pH 7.4. Protein content of extract was determined by BCA protein estimation kit (Sigma Chemical Co., St. Louis, USA) and stored at -20°C until ready for use.

**Generation and Purification of Monoclonal Antibodies**

The inbred BALB/c mice reared at the Small Animal House Facility, National Institute of Immunology, New Delhi, were immunized subcutaneously with $1\times10^8$ *Nosema bombycis* spores per animal emulsified in complete Freund's adjuvant. After 4 weeks, animals were boosted intraperitoneally (i.p.) with the same dose of antigen in incomplete Freund's adjuvant. Antibody titers were checked by solid phase ELISA as described later. Prior to the fusion $1.5\times10^8$ spores/animal were

given in saline intravenously on three consecutive days. Hybrid cell clones were developed by fusing splenocytes obtained from immunized mice with SP2/0 Ag-1.4 mouse myeloma cells using 50% polyethylene glycol and HAT selection medium as described elsewhere [9]. Hybridoma cells secreting MCAs were identified by screening the culture supernatant in ELISA from the wells positive for the growth of hybrid cells. The positive hybrid cells were cloned twice by limiting dilution to obtain stable cell lines. Hybrid cell clones secreting anti-Nosema antibodies were grown as ascites in the peritoneal cavity of Pristane (Aldrich Chemical Co., St. Louis, USA) primed (0.5ml, i.p.) BALB/c mice. The immunoglobulins from ascites were precipitated by using 40% ammonium sulfate, dialyzed against distilled water and the precipitated IgM immunoglobulins were obtained by centrifugation at 4000×g for 20 min. The pellet was dissolved in 50 mM phosphate buffer (pH 7.4) containing 250 mM NaCl. Protein concentration of the antibodies were measured, aliquoted and kept at -20°C for further use.



**Enzyme Linked Immunosorbent Assay**

Murine MCAs were screened for the reactivity with Nosema spores by ELISA. Briefly, 96 well polyvinyl microtitration plates were coated with alkali treated Nosema spores ($10^6$/well) in 50 mM carbonate buffer (pH 9.6) by incubating at 37°C for 2 hr followed by over night at 4°C. Free binding sites were blocked by incubating the plates for 2 hr with 1% BSA in 50 mM PBS (pH 7.4). Culture supernatant (100 µl/well) obtained by growing hybrid cells or different concentrations of purified MCAs were added in duplicate to the plates and incubated for 1 hr at 37°C. The plates were washed with PBS containing 0.05% Tween-20 (PBST) and were incubated for 1 hr at 37°C with 100 µl/well of rabbit anti-mouse HRPO conjugate (1:5000 dilution, Pierce, Illinois, USA). After

washing four times with PBST, enzyme activity was revealed by adding 100 µl of 0.05% ortho phenylenediamine and 0.06% $H_2O_2$ in 50 mM citrate phosphate buffer (pH 5.0) in each well. The reaction was stopped by adding 50 µl/well of 5 N $H_2SO_4$ and absorbance read at 490 nm by ELISA reader (Anthos ht II, Anthos Labtec Instruments, Salzburg, Austria).

The specificity of the MCAs was checked by ELISA as described above. Microtitration plates were coated with alkali treated ($10^7$/well) BmNPV, *B. thuringiensis, S. marcescens,* Azotobactor, Rhizobium and *Streptococcus-A.* In addition plates were also coated with hemolymph protein (5 µg/well) obtained from healthy larvae. Isotyping of the heavy chain of the MCAs was determined by ELISA using anti-heavy chain antibodies from Sigma Chemical Co., St. Louis, USA.



**Agglutination Assay**

Polystyrene latex beads (0.81µm, Sigma Chemical Co., St. Louis, USA) were washed three times with GBS1 (0.1 M glycine buffer containing 0.15 M NaCl, pH 8.2) by centrifugation at 8000×g for 10 min at room temperature. The latex beads were sensitized with different concentrations (25, 50, 100, and 250 µg/ml) of MCAs (MA-503 and MA-515) individually or in combinations at 37°C for 2 hr followed by 4°C overnight with continuous gentle end-to-end mixing. MCAs sensitized latex beads were washed twice with GBS1 containing 0.2% BSA. The latex particles (0.4% final concentration) were suspended in GBS1-BSA containing 0.05% sodium azide and stored at 4°C.

Alternatively, using modified composition of GBS2 (1.5 M glycine, 3 M NaCl, pH 8.9), latex beads were initially sensitized with 6 µg/ml of recombinant protein-A (Sigma Chemical Co., St. Louis, USA) as described for MCAs. Subsequently, protein-A coated latex particles were incubated with a combination of either 25 or 50 µg/ml of MA- 503 and MA-515 each at 37°C for 2 hr and followed by 4°C

overnight with continuous end- to-end mixing. After washing twice with GBS2 containing 0.2% BSA the sensitized latex particles (0.4% final concentration) were finally suspended in GBS2-BSA containing 0.05% sodium azide. An additional batch of latex particles was directly coated with a combination of 50 µg/ml each of MA-503 and MA-515 using GBS2.

The test was put on a black glass agglutination plate, by mixing 25 µl of sensitized latex beads, 50 µl of GBS1 and 100 µl of different concentrations ($10^4$, $10^5$, $10^6$, $10^7$, $10^8$, and $10^9$/ml) of alkali treated *Nosema bombycis* spores. The slide was slowly rotated for 2-5 min and was kept on a flat surface to observe for visible agglutination. The test was graded as negative (-) in the absence of visible agglutination, and positive agglutination was graded strong positive (+++) and positive (++). In between the positive and negative agglutination it was regarded as indeterminate (±). The control set was run side by side where only buffer was used instead of Nosema antigen. The specificity of the test was checked using other antigens like BmNPV, Rhizobium, Azotobactor, *B. thuringiensis, S. marcescens*, *E. coli* and normal hemolymph of healthy larvae. In addition hemolymph samples were also collected from apparently healthy (n=10) and infected (n=15) *B. mori* larvae from the field (Farmer's House and Rearing Centers, Mysore, Karnataka). Furthermore, samples from 12 infected mother moths were also prepared. To evaluate the efficacy of the latex agglutination test, *B. mori* II instar larvae (3/group) were fed with mulberry leaves buttered with *N. bombycis* spores ($10^2$, $10^4$, $10^6$, $10^8$ spores/cm$^2$), sacrificed on day 4 and hemolymph samples collected and analyzed in latex agglutination assay.

## RESULTS

### Characterization of Monoclonal Antibodies

By fusing SP2/0 mouse myeloma cells with splenocytes from BALB/c mice

immunized with *Nosema bombycis* spores, four hybrid cell clones secreting MCAs reacting with Nosema spores were stabilized. The reactivity pattern of MCAs secreted by these clones in ELISA is shown in the Table I. All the MCAs are of IgM isotype. MCAs, MA-310, -503, -515, and -542 reacted to a variable extent with alkali treated *N. bombycis* spores, and acetone precipitated surface protein prepared from *N. bombycis* spores. MA-310 and MA-542 showed low degree of reactivity with acetone precipitated surface antigen as compared to MA-503 and MA-515. Moreover, these two antibodies also showed a low degree of cross reactivity with BmNPV. MA-310 in addition also reacted with *B. thuringiensis.* None of the MCAs reacted with *S. marcescens,* Azotobactor, Rhizobium and normal hemolymph protein obtained from the healthy larvae.



**Latex Agglutination Assay**

MCAs, MA-503 and MA-515 have been used individually or in combination at different concentrations (25, 50, 100, and 250 μg/ml of latex particles) for sensitization of latex beads. Combination of the MCAs (MA-503 & -515) at a concentration of 50 μg/ml each showed best agglutination with Nosema spores (Table II). The sensitivity and specificity of the agglutination test employing the latex beads sensitized with optimized concentration of MCAs, MA-503 and MA-515 is shown in Table III. Using this combination positive agglutination was observed at a concentration of $1\times10^5$ alkali treated spores per test. Agglutination with lesser clarity was observed with $1\times10^9$ spores/test. However, no agglutination was observed in presence of $1\times10^8$ or lower concentration of BmNPV, *Rhizobium, Azotobactor, E. coli, B. thuringiensis and S. marcescens.* Moreover, hemolymph protein from apparently healthy larva when tested at 50 μg per test failed to reveal any positive agglutination.

Latex particles coated with a combination of MCAs, MA-503 and MA-515 through a protein-A bridge, however, revealed a 10-fold higher sensitivity as

## TABLE 1

**Reactivity Pattern in ELISA of Murine Monoclonal Antibodies Against *N. bombycis***

| MCA No. | Isotype | Absorbance at 490 nm with *N. bombycis* Alkali treated | *N. bombycis* Acetone precipitated | BmNPV | *B. thuringiensis* | *S. marcescens* | Azotobactor Rhizobium Hemolymph[1] |
|---|---|---|---|---|---|---|---|
| 310 | IgM | 1.09 | 0.21 | 0.87 | 0.23 | 0.04 | 0 |
| 503 | IgM | 1.50 | 1.07 | 0 | 0.05 | 0.08 | 0 |
| 515 | IgM | 1.45 | 1.15 | 0 | 0 | 0.09 | 0 |
| 542 | IgM | 1.52 | 0.47 | 0.27 | 0.06 | 0 | 0 |

[1]Hemolymph was not alkali treated



## TABLE II

**Determination of Optimum Concentration of MCA Used Individually or in Combination for Sensitization of Latex Beads**

| Conc. (μg/ml) of MA-503 | Conc. (μg/ml) of MA-515 | Minimum number of *N. bombycis* spores required per test for positive agglutination |
|---|---|---|
| 25 | 0 | Nil* |
| 50 | 0 | $1X10^8$ |
| 100 | 0 | $1X10^7$ |
| 250 | 0 | $1X10^7$ |
| 0 | 25 | Nil* |
| 0 | 50 | $1X10^8$ |
| 0 | 100 | $1X10^7$ |
| 0 | 250 | $1X10^7$ |
| 12.5 | 12.5 | $1X10^8$ |
| 25 | 25 | $1X10^6$ |
| 50 | 50 | $1X10^5$ |
| 125 | 125 | $1X10^5$ |

Positive agglutination observed in < 3 min.

* No agglutination was observed upto $1\times10^9$ spores per test.

**TABLE III**

**Sensitivity and Specificity of MCA Based Latex Agglutination Test* for Detection of *N. bombycis***

| Antigen | Concentration of antigen/test | Agglutination |
|---|---|---|
| Pebrine | $1X10^4$ | – |
| | $1X10^5$ | ++ |
| | $1X10^6$ | +++ |
| | $1X10^7$ | +++ |
| | $1X10^8$ | +++ |
| | $1X10^9$ | ++ |
| BmNPV | $1X10^8$ | – |
| Rhizobium | $1X10^8$ | – |
| Azotobactor | $1X10^8$ | – |
| *E. coli* | $1X10^8$ | – |
| *B.thuringiensis* | $1X10^8$ | – |
| *S. marcescens* | $1X10^8$ | – |
| Hemolymph | 50 µg | – |

*Latex beads were sensitized with a combination of MA-503 and MA-515 at a concentration of 50 µg/ml each. Positive agglutination was observed in < 3 min.
+++ strong positive, ++positive, – no agglutination.

compared to the latex beads directly coupled with MCAs (Table IV). Positive agglutination was observed at a concentration of $1X\ 10^4$ alkali treated spores.

To determine the efficacy of the latex agglutination test, *B. mori* 2nd instar larvae were infected in the laboratory as described under Materials and Methods. Hemolymph samples were collected from 4 groups fed with varying amounts of

## TABLE IV

**Comparison of the Sensitivity of Protein-A Linked MCA Versus Direct MCA Based Latex Agglutination Test for the Detection of *N. bombycis***

| Latex coated with (μg/ml) | | | Concentration of antigen/test | Agglutination |
|---|---|---|---|---|
| Protein-A | MA-503 | MA-515 | | |
| 6 | 25 | 25 | $1X10^8$ | ++ |
| | | | $1X10^7$ | ++ |
| | | | $1X10^6$ | +++ |
| | | | $1X10^5$ | ++ |
| | | | $1X10^4$ | - |
| 6 | 50 | 50 | $1X10^8$ | ++ |
| | | | $1X10^7$ | +++ |
| | | | $1X10^6$ | +++ |
| | | | $1X10^5$ | +++ |
| | | | $1X10^4$ | ++ |
| 0 | 50 | 50* | $1X10^8$ | ++ |
| | | | $1X10^7$ | +++ |
| | | | $1X10^6$ | +++ |
| | | | $1X10^5$ | ++ |
| | | | $1X10^4$ | - |

*Latex beads were sensitized with same GBS (1.5 M glycine, 3.0 M NaCl, pH 8.9) as that for MCAs through protein-A bridge.

+++ strong positive; ++ positive; - no agglutination

Pebrine spores on day 4 following infection. All 12 samples were positive by latex agglutination test. However, the 10 hemolymph samples collected from apparently healthy larvae failed to give any positive agglutination. Out of 15 field samples collected from the infected larvae, 3 were positive by latex agglutination test. The microscopic analysis also revealed the presence of Pebrine infection in these samples. All 12 samples (negative) in the latex agglutination test revealed the presence of BmNPV. The 12 samples collected from the mother moth were found positive by latex agglutination test and microscopy for Pebrine infection.

## DISCUSSION

In the present study, generation of four MCAs against *Nosema bombycis* spores and their use in the development of a simple and sensitive latex agglutination test has been reported. We found a variable degree of reactivity of MCAs with whole spores in ELISA which was mainly due to loss of the spores from the ELISA plate during repeated washing. *N. bombycis* spores lacking mucous envelope may not stick properly to the well of polystyrene ELISA plate as has been reported previously [4]. However, alkali-soluble whole spore surface antigens are good for the diagnosis of microsporidiosis in insect feces and dried cadavers of infected silkworms [4]. Therefore, we have utilized two different antigenic preparations of Nosema spores (a) alkali treated spores and (b) acetone precipitated cell surface antigen. Both antigenic preparations are equally good for screening of MCAs in ELISA. All the four MCAs recognized alkali treated spores in indirect ELISA. However, two antibodies (i.e., MA-310 and -542) showed a low degree of reactivity with acetone precipitated surface antigens. One of the possibilities for low reactivity of MA-310 and MA-542 with acetone precipitated surface antigens may be due to non availability of antigenic determinants in the supernatant.

Serological techniques such as latex agglutination tests employing the latex beads sensitized with either polyclonal or monoclonal antibodies have been used extensively to detect various infections [7, 10]. Using a cocktail of two MCAs (MA-503 and -515) the sensitivity of the latex agglutination test was increased about 100-fold as compared with individual MCA coated latex agglutination test. The two MCAs may recognize different epitopes on the Nosema spore antigen and thus bring out better agglutination by cooperative interaction. Increase in the sensitivity (20-fold) of an enzyme immunoassay for human granulocyte - macrophage colony - stimulating factor has been reported by using a cocktail of three MCAs for coating as well as revealing the bound antigen [11]. Further 10-fold increase in the sensitivity ($1x10^4$ spores/test) of the latex agglutination assay was achieved by sensitizing the latex beads with the cocktail of these two MCAs through a protein-A bridge. It has been made feasible by the observations that mouse IgM antibodies can bind to protein-A in GBS2 having high pH (8.9) and ionic strength (1.5 M glycine, 3 M NaCl) [12]. The sensitivity of the latex agglutination assay described here ($1\times10^4$ spores per test or $1\times10^5$ spores/ml) was higher than the latex agglutination assay employing either polyclonal ($10^7$ spores/test) or monoclonal ($1.3\times10^8$ spores/ml) antibodies reported previously [7, 13]. The increased sensitivity of the latex agglutination assay employing latex beads sensitized with a cocktail of MCAs through protein-A may be due to higher flexibility and easy accessibility of antigen binding sites (Fab) of the immunoglobulins to react with antigen [14]. Furthermore, the results obtained on the hemolymph samples collected from the field as well as laboratory infected larvae have shown 100% specificity of the test. The presently described direct latex agglutination assay is less sensitive as compared to the previously described ELISA [4] and fluorescent MCA test [2, 3]. However, immunofluorescence assay and ELISA involve multiple steps, technical expertise and are dependent on

instrumentation. Latex agglutination tests on the other hand are simple to perform, do not require instrumentation and results can be obtained within 10 min. Currently, Pebrine is diagnosed by microscopic examination of smears from different metamorphic stages of silkworms for the presence of *Nosema bombycis*. The approach is an integral part of silkworm seed preparation as the pathogen is transmitted to the progeny through eggs.

The mother moth examination for Pebrine infection in seed production is laborious and time consuming. The spores are generally detected by microscopic examination, only if the smear contains greater than $10^6$ spores per ml. The analysis is further complicated by the presence of other microsporidians of the similar shape and size. The latex agglutination assay is 10-fold more sensitive compared to the microscopic examination and hence at the field level where a simple, one-step assay is preferred, the presently described latex agglutination will be a useful tool. It can be performed by a semi skilled worker and results obtained in a short time.



## ACKNOWLEDGMENTS

This work was supported by grant from the Department of Biotechnology and Silk Board, Government of India. The views expressed by the authors do not necessarily reflect the views of the funding agencies.

## REFERENCES

1. Han, M.S. and Watanabe, H. Transovarial transmission of two microsporidian in the silkworm, *Bombyx mori* and disease occurrence in the progeny population. J. Invertebr. Pathol. 1988; 51: 41-5.

2. Fujiwara, T., Ogimoto, K., Sudo, K. and Ueda, K. Immunofluorescence study of *Nosema bombycis* Nageli, a causative agent of the Pebrine. Acta. Sericol. 1966; 58: 14-19.

3. Sato, R., Kobayashi, M., Watabanabe, H. and Fujiwara, T. Serological discrimination of several kinds of microsporidian spores isolated from the silkworm, *Bombyx mori* by an indirect florescent antibody technique. J. Seri. Sci. Jpn. 1981; 50: 180-4.

4. Kawarabata, T. and Hayasaka, S. An enzyme linked immunosorbent assay to detect alkali soluble spore surface antigen of strains of *Nosema bombycis* microsporidian: Nosematidae. J. Invertebr. Pathol. 1987; 50: 118-23.

5. Zhaoxi, K., Weidong, X., Xunzhang, W., Qingxing, L. and Zhelong, P. A monoclonal antibody to *Nosema bombycis* and its use for the identification of microsporidian spores. J. Invertebr. Pathol. 1990; 56: 395-400.

6. Mike, A., Ohwaki, M. and Fukada, T. Preparation of monoclonal antibodies to the spores of *Nosema bombycis*, M11 and M12. J. Seri. Sci. Jpn. 1988; 56: 189-95.

7. Mike, A., Ohmura, H., Ohwaki, M. and Fukada, T. A practical technique of Pebrine inspection by microsporidian spore specific monoclonal antibody sensitized latex. J. Seri. Sci. Jpn. 1989; 58: 392-5.

8. Sato, R. and Watanabe, H. Purification of mature microsporidian spores by iso-density equilibrium centrifugation. J. Seri. Sci. Jpn. 1980; 49: 512-16.

9. Gupta, S.K., Bagavant, H., Koothan, P.T., Talwar, G.P., Yurewicz, E.C. and Sacco, A.G. Characteristics of monoclonal antibodies against porcine zona pellucida-3 and their relevance. Ind. J. Exp. Biol. 1992; 30: 1000-5.

10. Shimizu, S., Ohaba, M., Kanda, K. and Aizawa, K. Latex agglutination test for the detection of flacherie virus of the silkworm, *Bombyx mori* L. J. Invertebr. Pathol. 1983; 42: 151-5.

11. Zenke, G., Strittmatter, U., Tees, R., et al. A cocktail of three monoclonal antibodies significantly increases the sensitivity of an enzyme immunoassay for human granulocyte-macrophage colony-stimulating factor. J. Immunoassay 1991; 12: 185-206.

12. Mariani, M., Cianfriglia, M. and Cassone, A. Is mouse IgM purification on protein A possible? Immunol Today 1989; 10: 115-6.

13. Baig, M., Gupta, S.K., Nataraju, B., et al. Latex agglutination test for the detection of Pebrine in silkworm *Bombyx mori* L. Indian J. Seri. 1992; 31: 141-5.

14. Torrance, L. Use of protein-A to improve sensitization of latex particles with antibodies to plant virus. Ann. Appl. Biol. 1980; 96: 45-50.