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SHORT COMMUNICATION

Development of slide agglutination method for detection of *Nosema mylitta* in tasar silkworm using polyclonal antibody produced against proteins of pebrine spores.

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Abstract: Production and marketing of quality tasar cocoons is the source of livelihood for the majority of poor tribal peoples of central and north eastern states of India. Due to outdoor rearing of the tasar silkworms, the worms are amenable to different diseases including transovarially transmitted protozoan disease called pebrine caused by *Nosema mylitta*. The present study was aimed to develop polyclonal antibody based detection method for pebrine spores during mother moth examination which will be sensitive in nature in comparison with microscopic detection. In the present, the pebrine spores were purified from the pebrinized larvae. The spore proteins were purified and used for the production of polyclonal antibody in rabbit. The antiserum was used for the detection of pebrine spores both on glass slides and microscope. The visual and microscopic observation revealed that, the antibody in the dilution of 1:2000 showed agglutination of pebrine spores. Further, the different concentration of pebrine spores was subjected to agglutination. The results revealed that, the concentration up to 10^{-4} showed the agglutination of pebrine spores (single spore in the microscopic field). The results also confirms that, due to its low range of spore detection, antibody based pebrine detection method can be easily employed in breeding stations of the tasar industry which helps to produce disease fee layings.

Keywords: *Antheraea mylitta*, *Nosema mylitta*, Spore proteins, Polyclonal antibody, Agglutination

Introduction:

Rearing of tropical tasar silkworm (*Antheraea mylitta* D.) is practiced in outdoor condition in forest regions of central and north eastern parts of India which exposes larvae to different pathogens. Among the different pathogens infecting tropical tasar silkworm, Pebrine is a dreaded disease caused by protozoa, *Nosema mylitta* which causes yield loss up to 25-40% in tropical tasar silkworm farmers field (Sahay *et al.,* 2000). Pebrine disease is transmitted through mother moth to offspring transovarially along with secondary source of infections (Madhusudhan *et al.,* 2015).

Detection of the pathogens is an important measure to control the diseases. Presently the pebrine disease infecting tasar silkworm is detected by using microscopic method. This method requires highest manpower and most importantly time consuming. In case of mass emergence of moths, the individual mother moth examination is impossible to carry out in basic seed multiplication centers which ultimately lead to spread of disease in tasar silkworm farmer's field.

Serological methods are being employed in the detection of pathogens. Some insights about the spore wall proteins of *Nosema bombycis* has been provided by Wu *et al.* (2008). Some efforts have been done to detection of pebrine by using *N. bombycis* by using latex agglutination test (LAT) (Hayasaka and Ayuzawa, 1987; Shamim *et al.,* 1997). In the similar way, the easy and sensitive serological detection method needs to be developed for the pebrine disease infecting tropical tasar silkworm (*Antherea mylitta* D). Hence, the present study was aimed to produce polyclonal antibodies against pebrine proteins and utilization of antibodies for the detection of pebrine spores during mother moth examination which will facilitates easy eradication of pebrine menace in tasar culture.

Materials and Methods

Collection of pebrinized tasar silkworm and purification of pebrine spores

Nosema mylitta infected tasar silkworms were collected from the rearing plots of Central Tasar Research and Training institute during the period 1st crop rearing (July, 2014 to August, 2014) and were maintained in the indoor condition.

The infected fifth instar tropical tasar silkworm larvae were homogenized and centrifuged at 10,000 rpm for 10 minutes by utilizing the discontinuous percoll gradient (25, 50, 75 and 100%) solution. The pellets were rinsed in sterile distilled water and stored at 4^0C.

Production of antigen and antiserum

Purified spores were heated at 65oC for 10 min in 0.2M Phosphate buffer, pH 7.2 and mixed with equal volume of Fruend's complete adjuvant and emulsified. Antiserum was prepared in adult white rabbits. Pre-immune serum was collected as the control serum before the rabbits received their first injection. Subcutaneous injections were given at 7-day intervals for 4 weeks using 1 ml ($1x10^9$ spores/ml) of heated treated spore suspensions per injection. They were bled 7 days after the final injection. The specificity and titre were checked by immunodiffusion (Sironmani, 1997).

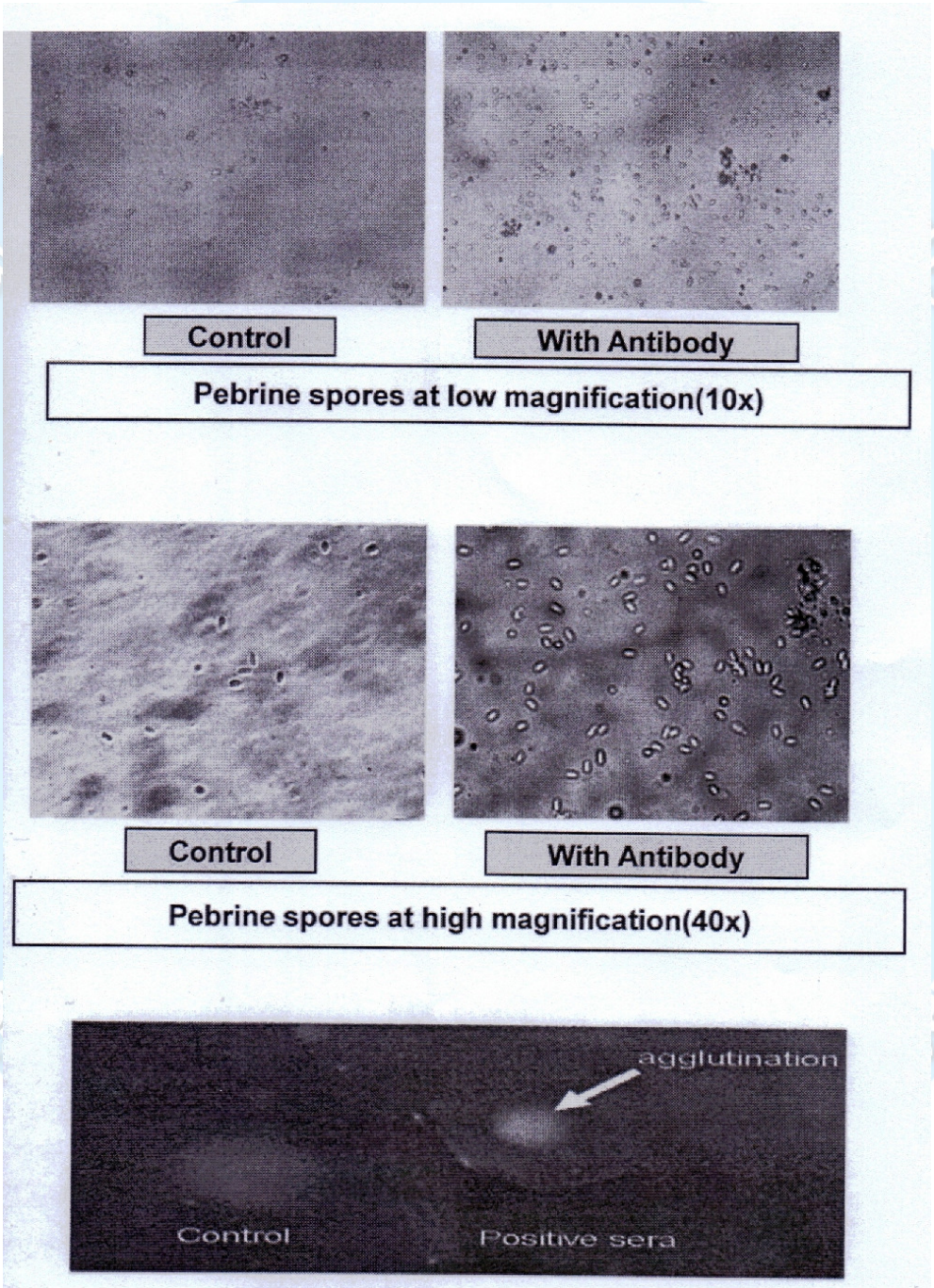

Fig. 1. Photographs showing Agglutination of pebrine spores by using polyclonal antibody on glass slides and phase contrast microscope.

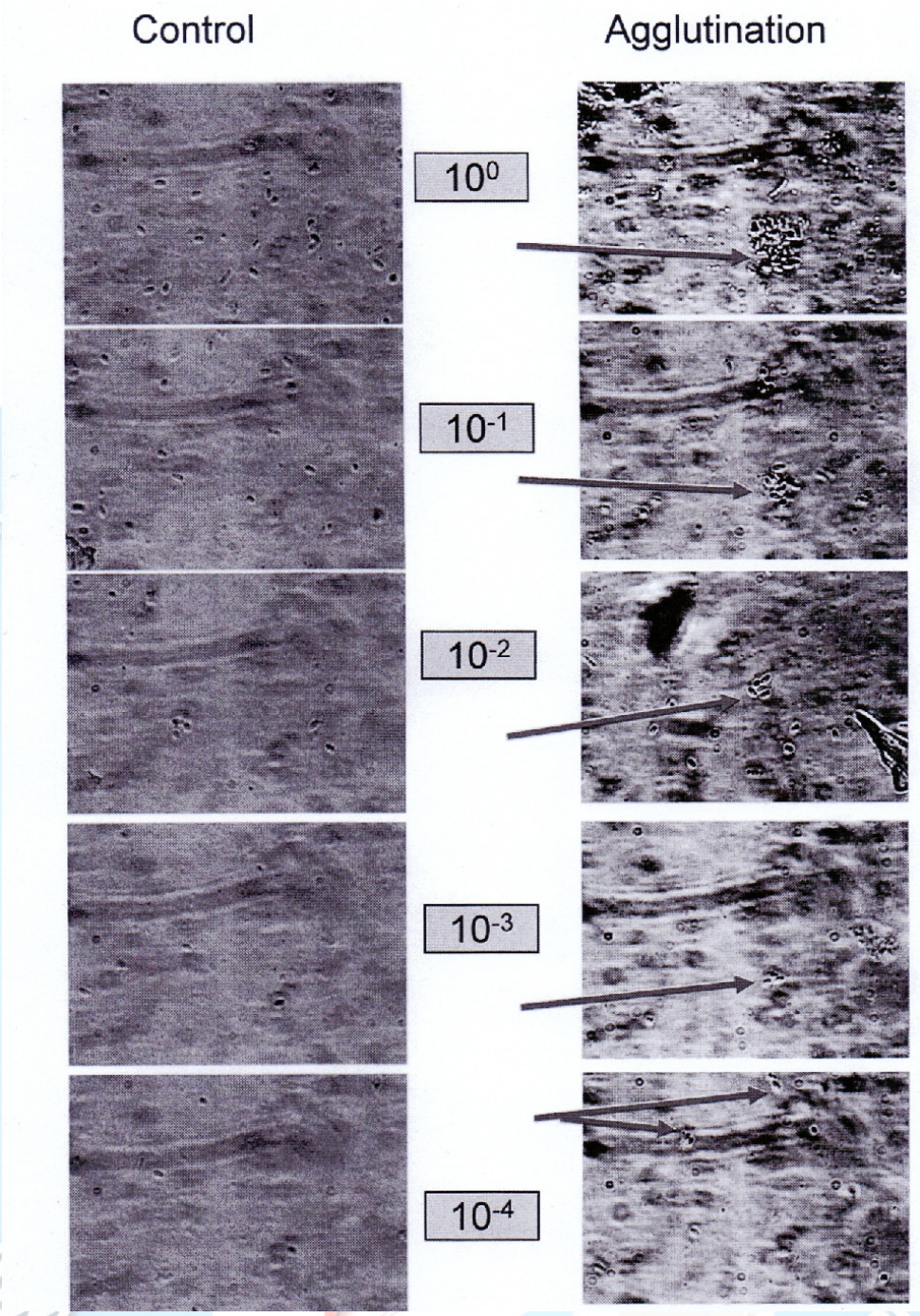

Fig. 2. Photographs showing agglutination of pebrine spores using polyclonal antibody in different concentration.

Slide Agglutination test:

A drop of pebrine infected sample was mixed with an equal amount of polyclonal antibody on clean slides with glass rod at room temperature and observed under phase-contrast microscope for spore- antibody agglutination. Five samples were observed in each category. The observation was recorded in as agglutination positive (+) for positive affinity and agglutination negative (-) indicating negative affinity (Bashir *et al.*, 2011).

Sensitivity of polyclonal antibody for the detection of pebrine spores.

The specificity and sensitivity of the polyclonal antibody different concentration of pebrine were used for agglutination (10^0, 10^{-1}, $10^{-2}$and 10^{-3} and single spore). After agglutination, the slides were observed for spore's agglutination.

Results

Slide agglutination test:

The antibody showed positive agglutination against pebrine spores (purified and positive sample observed during sample observed during mother moth examination). The white coloured agglutination of pebrine spores were noticed on slide. Whereas phase contrast microscopic observation in both low and high magnification revealed the complete agglutination of pebrine spores (Fig. 1).

Sensitivity of polyclonal antibody

The sensitivity evaluation of raised polyclonal antibody showed agglutination at different dilution 10^{0}, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} (single spore) in phase contrast microscopic observation in high magnification (600x magnification). The results revealed that, the polyclonal antibody raised was sensitive enough to agglutinate even single spore present in the microscopic field (Fig. 2).

Discussion:

The yield of tasar silk is lower than expected primarily due to infection of several diseases. In India, the extant of cocoon crop loss due to the silkworm diseases is nearly 40% (Sahay *et al*, 2000). Detection of the pathogens is prime importance in controlling of diseases in field. Typical symptoms of pebrine disease in the tasar silkworm are appearance of black spots on the larval skin and reduced growth of larvae in comparison with healthy ones. This is one method of identifying the pebrine disease in field. The other method practiced for the detection of the pebrine infection in mother moth by using microscopic method. This method requires manpower and time consuming. It has got some constraints i.e., impossible to screen more number of samples and physical strain to examiner (especially causing strain to eyes of examiner), which in turn helps in spread of pebrine disease in the field.

Serological methods such as Enzyme-linked immunosorbent assay, (ELISA), indirect fluorescent antibody techniques, latex bead agglutination, fluorescent antibody technique, slide agglutination test, and monoclonal antibody detection (Zhaoxi *et al*., 1990) were being used for the detection of pebrine infection in different stages of life cycle of Mulberry silkworm. According to their observations, all these methods are accurate in detecting pebrine infection. Shamim *et al.* (1997) produced monoclonal antibodies against pebrine spores of mulberry silkworm. They have used Latex agglutination test for the detection of pebrine.

In the present study, antibody generated against proteins of pebrine showed the good agglutination against pebrine spores during mother moth examination. The antibody showed agglutination up to lowest number of spores which were noticed in the single microscopic field. Similar work was carried out by Aronstein *et al.* (2011) to analyse the range of spore detection by the antibody in bees. The result of their work showed that, Antibody at 1:5000 dilutions can detect spores at $1x10^{3}$ concentrations. They also opined that, single infected bee can produce over $50x10^{6}$ spores ; this level of sensitivity will allow detection of a very low level of *Nosema* infection in bee colonies (Aronstein *et al*., 2011).

The results of present study confirms that, antibody based detection kit can be developed and utilized for the identification of pebrine infection in tasar silkworm at any stage of the life cycle. The results also confirms that, due to its higher level of sensitivity in pebrine spore detection, antibody based pebrine detection method can be easily employed in breeding stations of the tasar industry which helps to produce disease fee layings. The results confirms antibody based technology can be developed for easy identification of pebrine spores which will benefit the tasar industry immensely.

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