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Isolation of a New Microsporidian sp. (NIK-5hm) forming Spores within the Haemocytes of Silkworm, *B. mori* L.

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While observing silkworm larval samples received from field, microsporidian spores formed within the haemocytes of silkworm haemolymph were observed. The spores of microsporidian sp. were purified and characterized for morphological characters *viz.*, size, shape as well as serological affinity with different *Nosema* spp. (M_{11} and M_{12}). The infectivity of the isolated spores to silkworm was also studied. The microsporidian sp. was found to be highly pathogenic to silkworm, *B. mori*. The isolated microsporidian sp. was designated as NIK-5hm, which formed ovocylindrical spore in the haemocytes of silkworm and differed in spore size (length, 4.55 µm & width, 2.10 µm) and shape from *Nosema bombycis* (NIK-1s), NIK-2r (*Nosema* sp. Mysore [3.6 & 2.8 µm]), NIK-3h (*Nosema* sp. M_{11} [3.8 & 1.8 µm]), NIK-4m (*Nosema* sp. M_{12} [5.0 & 2.1 µm]) and Lb_{ms} (*Nosema* sp. in Lamerine breed of silkworm [4.36 & 2.14]). In immonological test (Latex agglutination test), the isolated microsporidian spores did not react with antibody sensitized latex particles of *N. bombycis*, M_{11}, M_{12} and Lb_{ms} and thus are different type of microsporidian sp., parasitic to silkworm, *Bombyx mori* L.

Key words: Silkworm, *Bombyx mori*, Microsporidian sp., haemocytes, NIK-5hm

Introduction

Pebrine (microsporidiosis) is an insidious and chronic disease in silkworm, *Bombyx mori* L. caused by a highly virulent microsporidian parasite, *Nosema bombycis*. Approximately 600 among 1000 named species of microsporidia are observed in different insects (Sprague and Becnel, 1999). Most of the entomopathogenic microsporidia occur in genus *Nosema* and more than 150 described species have been reported in 12 orders of insects (Becnel and Andreadis, 1999). In India, four microsporidian sp. (NIK-1s, NIK-2r, NIK-3h and NIK-4m) were isolated from infected moths which differed in their shape, size, virulence and mode of transmission (Ananthalakshmi *et al.*, 1994). One microsporidian sp. associated with Lamerin breed of silkworm has also been characterized (Shabir Ahmad Bhat and Nataraju, 2004). Microsporidiosis in silkworm is also caused by different strains of *Nosema* spp., *Pleistophora* spp. (M_{24}, M_{25} and M_{27}), *Thelohania* spp. (M_{32}) and *Leptomonas* spp. (Abe, 1978; Jolly, 1986). These microsporidians not only infect the silkworm but also many other insect species especially the lepidopteran pests of mulberry. The microsporidian sp. gains entry into the silkworm rearing through contaminated mulberry leaves (Kishore *et al.*, 1994; Sharma *et al.*, 2003).

Most of the microsporidians normally infect silkwrom by *per os* method and spore formation is observed in different tissues like midgut epithelium, muscles, fat bodies, silk glands, malpighian tubules and gonads (Fujiwara, 1985; Sato and Watanabe, 1985; Han and Watanabe, 1988; Iwashita *et al.*, 1990; Kawarabata, 2003). Microsporidians penetrates in the Haemocoel and exist extracellularly in the haemolymph or intracellularly within the cells of various tissues and organs (Tanada and Kaya, 1993).

However, no reports are available on *Nosema* sp., forming the spores within the haemocytes of silkworm, *B.*

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mori. Recently, while microscopically examining the larval samples received from farmer's rearing (Kempynahundi, Karnataka), spores of a different type of microsporidian sp. were observed inside the haemocytes of silkworm, *B. mori*. The spore morphology is one of the most important characteristic features to distinguish them from other strains (Weiser, 1961; Sprague, 1977). Various biochemical and immonological techniques have recently been employed to further understanding of the relationships between species. Several PCR methods based on the amplification of rRNA gene fragments have been published for used in the diagnosis and species identification of insect microsporidia (Kawakami *et al.*, 2001). The present investigation covering the isolation, purification, morphological characteristics of the isolated spore, infectivity to silkworm and its immonological affinity using monoclonal antibody sensitized latex particles of different microsporidians (M11 and M12) and polyclonal antibodies of Lb_{ms} and NIK-1s strain of *N. bombycis* as control was taken up which clearly suggest that the microsporidian sp. isolated in the present study is different from the existing microsporidians.

Materials and Methods

The microsporidian sp. infected silkworms were homogenized, filtered and centrifuged three times at 3000 rpm for 15 min. After centrifugation, the sediment was suspended in 2 ml of distilled water and poured on the percoll in the ratio of 1 : 3 and centrifuged at 5000 rpm for 15 min. The sediment containing pure spores of microsporidian sp. was washed thrice in distilled water and stored in saline (0.85% NaCl) and kept at 4°C for further studies. The standard strain, *N. bombycis* kept in the silkworm pathology laboratory of this institute is also purified and kept for comparison.

Morphological characterization

The morphology is reported to be one of the important criteria for characterization of microsporidians. The purified spores of isolated microsporidia and *N. bombycis* were photographed under phase contrast microscope (Nikon, AFX-DX). The length and width of 100 spores was measured by ocular micrometer (Fujiwara, 1980) under phase contrast microscope.

Scanning electron microscopy

The purified microsporidian spores were dried at room temperature and transferred onto double stick cellophane tape pasted on copper stubs used for mounting specimen. Then the mounted stuffs were coated with 20 nm gold in a sputter coater (EMS-550) and viewed under a JEOL 100 CX-II electron microscope fitted with a scanning attachment (ASID-4D), scanning electron microscope (Tokyo, Japan). Micrograph was taken for determining the type. The observation was compared with standard strain, *N. bombycis*.

Serological affinity characterization

The purified spores were examined for their affinity to *N. bombycis* and different *Nosema* spp. by latex agglutination test using microsporidian spore-specific monoclonal antibodies sensitized latex kit of Yakult & Co., Japan (Mike *et al.*, 1988). The purified spores were used as antigens for the antibodies sensitized latex agglutination test. Two µl of purified isolated microsporidian sp. and 2 µl of *N. bombycis*, M_{11}, M_{12} (monoclonal) and Lb_{ms} (polyclonal) (strain of *Nosema* sp. which is commonly observed in Lamerin breed of silkworm) spore specific antibodies were mixed with a plastic rod and incubated for 5 min at room temperature. It was then observed under phase contrast microscope at 600 × magnifications for agglutination reaction. The agglutination reaction between isolated microsporidia / *N. bombycis* and the respective antibody was identified by the number of spores agglutinate.

Infectivity test

The stock of purified microsporidian spores was diluted to 1×10^6 spores/ml and smeared onto mulberry leaves. The smeared mulberry leaves were fed to second instar silkworm larvae of CSR_2, Bivoltine pure race that has been recently evolved for commercial exploitation in India. The silkworms fed with *N. bombycis* were served as control. A negative control was also kept without inoculation of isolated microsporidian sp. / *N. bombycis*. Three replications of 100 larvae each in treatment batches were maintained. The larvae were reared for 15 days and observed for mortality due to infection with microsporidian sp. The spores in the haemocytes were observed under the phase contrast microscope by taking a drop of haemolymph using a fine needle and puncturing the proleg of the infected silkworm. The spores inside the haemocytes were micro photographed at 600 × and 1500 × (oil immersion) magnifications.

Results

Morphological characterization of isolated microsporidia, NIK-5hm

The isolated microsporidian sp. has been designated as NIK-5hm on the basis of presence of microsporidian

Table 1. Characterization and infectivity of isolated microsporidian sp., NIK-5hm and standard strain, *N. bombycis*

Microsporidian isolate	Shape	Spore size (μm)		Monoclonal antibodies of different microsporidian spore				% Mortality to silkworm
		Length ± SD	Width ± SD	N. bombycis	M_{11}	M_{12}	L_{bms}	
NIK-5hm	Ovo cylindrical	4.55 ± 0.35	2.10 ± 0.30	-	-	-	-	65.00
N. bombycis	Oval	3.03 ± 0.15	2.40 ± 0.04	+	-	-	-	30.00

spores in side the haemocytes of silkworm, *B. mori* L. The spore size of NIK-5hm was 4.55 μm in length and 2.10 μm in width whereas, the spores of *N. bombycis* were 3.03 μm in length and 2.40 μm in width (Table 1). The variation in spore morphology between the NIK-5hm and *N. bombycis* is clearly discernible in electron micrograph at 20,000 magnifications. The NIK-5hm was ovocylindrical in shape when compared to *N. bombycis*, which was oval in shape (Table 1). The spores of NIK-5hm exhibited brownian movement like *N. bombycis*.

Serological affinity characterization

In the serological latex agglutination test, NIK-5hm did not agglutinate with monoclonal / polyclonal antibodies (*N. bombycis*, M_{11}, M_{12} and Lb_{ms}) sensitized latex particles and the spores were observed singly indicates negative, which shows that the new microsporidian sp. is serologically different from *N. bombycis* and other *Nosema* sp., M_{11}, M_{12} and Lb_{ms} (Table 1).

Infectivity of the isolated microsporidian sp. to silkworm, *B. mori*

When NIK-5hm was inoculated to silkworm, *Bombyx mori* at 1×10^6 spores/ml resulted in 65% mortality, whereas, *N. bombycis* at the same concentration caused only 30% of mortality (Table 1) indicating that NIK-5hm was highly pathogenic to silkworm and more virulent than *N. bombycis*. The haemolymph of infected silkworm larvae when examined under phase contrast microscope at 600 × (Fig. 1) and 1500 × (Fig. 2) magnification, revealed the presence of the spores of isolated microsporidian sp in haemocytes and in haemolymph.

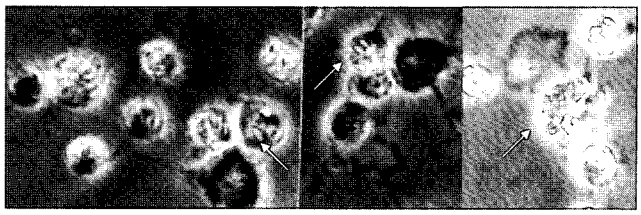

Fig. 1. Microphotograph of NIK-5hm in haemocytes of silkworm, *B. mori* at 600 × magnification.

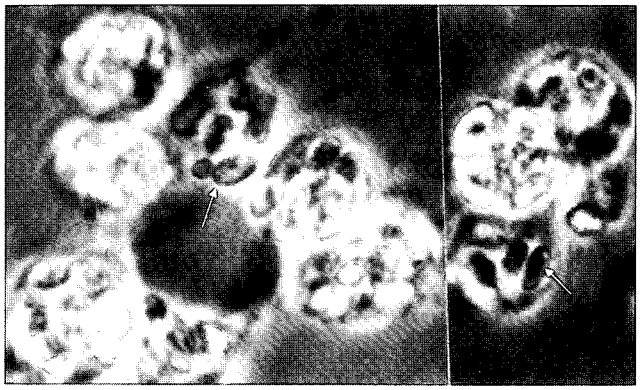

Fig. 2. Microphotograph of NIK-5hm in haemocytes of silkworm, *B. mori* at 1500 × magnification.

Discussion

The present study revealed that the microsporidian sp., NIK-5hm isolated from infected silkworm is different from *N. bombycis*. The serological affinity of NIK-5hm to monoclonal antibodies of *N. bombycis*, M_{11}, M_{12} and polyclonal antibodies of Lb_{ms} revealed that the spores of NIK-5hm does not react with any of the monoclonal antibodies sensitized latex particles of the microsporidians of *N. bombycis*, M_{11} and M_{12} and polyclonal antibodies of Lb_{ms}. This indicates that the NIK-5hm isolated from infected silkworm from Karnataka, India is different strain of the microsporidians isolated earlier in India.

The infectivity of NIK-5hm indicates, it is highly pathogenic and more virulent than *N. bombycis*. When the silkworms were inoculated with NIK-5hm, the spores of this microsporidium were formed inside the haemocytes. Later the haemocytes were disintegrated and spores released into the haemolymph of silkworm, *B. mori*. As per the available literature, the microsporidian spores have not been reported in the haemocytes of infected silkworm, *B. mori* in India and elsewhere. Several authors reported that the spore formation occurs in midgut epithelium, malphigian tubules, silk gland, fat bodies, adipose tissue, gonads, and trachea (Fujiwara, 1980; Fujiwara, 1985; Sato and Watanabe, 1985; Han and Watanabe, 1988; Iwashita *et al.*, 1990; Kawarabata, 2003). These observations also indicate that the microsporidian sp., NIK-5hm is

isolated for the first time from haemocytes of silkworm, *B. mori* in India and is different from other species of microsporidia.

From the present study, it is concluded that NIK-5hm isolated from silkworm, *B. mori* for the first time in India is different strain of microsporidian species parasitic to silkworm, *B. mori* on the basis of a) morphological variation when compared to *N. bombycis*, NIK-2r, NIK-3h, NIK-4m, b) negative reaction to the monoclonal/polyclonal antibodies of different microsporidian sp. and c) formation of spores within the haemocytes of silkworm, *Bombyx mori* L.

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