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A comparative study on artificial germination of two microsporidia under the neutralization method

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ABSTRACT

Lamerin microspridia (Lb_{ms}) isolated from Lamerin breed of the silkworm, *Bombyx mori*, L. and the standard strain *Nosema bombycis*, were induced to germinate artificially by two-step procedure. Fresh percoll purified spores were activated by incubating them in potassium hydroxide solution (KOH) (pH 11.00) and germinated artificially by using phosphate buffer saline (PBS), (pH 7.00). Germinated spores appeared black under phase contrast microscope. Germination percentage amoung the microsporidia spores varied in different concentrations of KOH. The Lb_{ms} was found poor germinator as compared to *Nosema bombycis* in all the concentrations of the chemicals. The length of the extruded polar filament was also varied between the two microsporidia.

Keywords: Artificial germination, Lamerin, Microsporidia, Phosphate buffer, *Bombyx mori*.

INTRODUCTION

Microsporidia are a diverse group of spore forming obligate intracellular parasites infecting wide range of the host texa and have long been considered primitive eukaryotes, both on the basis of morphological features and on the basis of molecular, mainly ribosomal RNA- based phylogenies (Peer *et al.*, 2000). In 1857 these parasites were recognized as a pathogens in silkworms (Naegeli, 1857) and long before they were described as human pathogens, then were recognized as cause of disease in many nonhuman hosts including insects, mammals and fishes (Brayan, 1995; Canning and Lom, 1986; Canning and Hollister, 1987 and 1992). In addition to *Nosema bombycis* several other microsporidian species have been isolated from silkmoth (Govindan *et al.*, 1998). They differ in target tissue, virulence, mode of transmission and *in vitro* germination (Fujiwara, 1984a, b; Fujiwara, 1985; Fujiwara, 1993). Microsporidian spore (Lb_{ms}) was isolated from Lamerin breed of the silkworm, *Bombyx mori* L its morphology,

serology, pathogenicity to the host has been studied (Shabir Ahmad Bhat and Nataraju, 2004; 2005) In this paper our objective was to investigate its germination by artificial means under neutralization method.

MATERIALS AND METHODS

The microsporidia (Lb_{ms}) is associated with the Lamerin breed of the silkworm, *B. mori,* L for several generations without having been eliminating the breed. The microsporidia were isolated from the infected breed and purified by following the method of Sato and Wtanabe (1980). The spores were suspended in 0.85% solution of potassium hydroxide (pH 11.00) and preserved at 5°C until use. The purified spores of standard strain, *N. bombycis* were collected from Silkworm Pathology Laboratory of Central Sericulture Research and Training Institute Mysore, India for comparison. Fresh purified spores of Lb_{ms} or *N. bombycis* ($1 \square 10^7$) were incubated at different concentration (0.1, 0.3, 0.5, 0.7. 0.9, and 1%) of KOH solution (pH 11.00). The pH

of KOH solution was adjusted with either HCl or NaOH. The pH of KOH solution was measured by Lacom tester pH Scan Series pH meter. The spore samples treated with KOH were neutralized with phosphate buffer saline, (pH 7.00) following the method of Fujiwara (1993) and observed under phase contrast microscope at 600X magnifications for germination. The percent germination was calculated using the procedure involving haemocytometer count (Cantwell, 1974) for germinated and ungerminated spores followed by estimation. The spores, which appeared black under phase contrast microscopy and had an attached polar filament, were considered to be germinated whereas ungerminated spores were white and refringent.

RESULT

The results obtained from the present study are presented in table 1 and 2, Figure 1 and 2. The results indicated that the incubation of microsporidia spores in diff-

erent concentration of potassium hydroxide (0.1, 0.3, 0.5, 0.7, 0.9 and 1.00%) for different time durations ranging from 10-30 minutes and neutralized by adding phosphate buffer saline (pH. 7.00) resulted in the germination of microsporidia spores. The germination of activated spores occurred within 1-2 seconds. The spores of Lb_{ms} or *N. bombycis* lose their refringency and appeared black due to the extrusion of polar filament under phase contrast microscope (Figure 1a and b).

The spores, which did not germinate, remained bluish in color and exhibited refractive index.

The germination of microsporidia spores were observed for hatching and recorded under phase contrast microscope Nikon (Type-104) which was the usual way to identify the germinated spores from the un germinated ones (Fujiwara, 1993). The germination percentage was observed comparatively low in Lb_{ms} as compared to *N. bombycis* in all the treatments (Table 1 and figure 2).
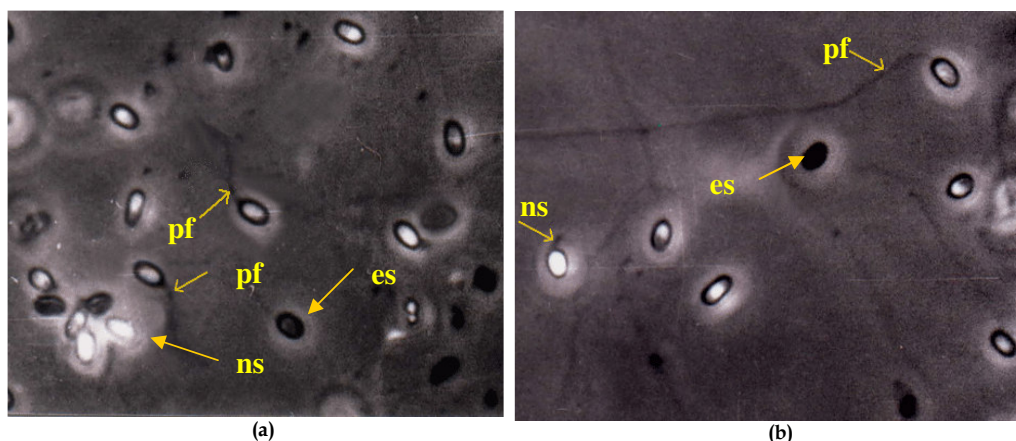

(a)   (b)

Fig 1. Germination and observation of extruded polar filament from the spores of Lb_{ms} (a) and *N. bombycis* (b): pf-polar filament, es-empty shell, ns-non germinated spores (scale bar = 0.05μm).

Table 1. Germination response of Lb_{ms} and *N. bombycis*.

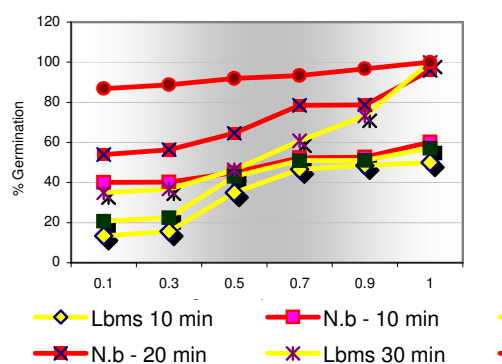| Microsporidian isolates	KOH solution (%)	Time duration of the treatments (Min)		
		10	20	30
Lb_{ms}	0.1	+	+	+
	0.3	+	+	+
	0.5	+	+	++
	0.7	++	++	+++
	0.9	++	++	+++
	1.00	++	++	+++
N.bombycis	0.1	+	++	+++
	0.3	+	++	+++
	0.5	+	+++	+++
	0.7	++	+++	+++
	0.9	++	+++	+++
	1.00	+++	+++	+++

+: ≤50%;   ++: 50 – 80%;   +++: ≥80%.

Fig 2. Germination percentage of Lb$_{ms}$ and *N. bombycis* after incubation in KOH for different time durations.

These observations showed that the Lb$_{ms}$ could germinate artificially but the germination percentage was comparatively low in all concentrations of potassium hydroxide. The polar filament could be seen as thread like structure attached to the germinated spores (Fig. 1a and b). The length of the extruded polar filament from germinated spores was 0.009 and 0.0296µm in Lb$_{ms}$ and *N. bombycis* respectively (Table 2).

Table 2. Measurement of the extruded polar filament of Lb$_{ms}$ and *N. bombycis*.

Microsporidian isolates	Length of extruded polar filament (µm)
Lb$_{ms}$	0.0099
N . bombycis	0.0296

TAXONOMIC SUMMARY
Spore: Found singly, ova cylindrical in shape,
Type host: Lamerin breed of the silkworm (Lepidoptera - Bombycidae)
Type Locality: Manipur a northeastern part of India
Germination response: Poor
Etymology: The name Lb$_{ms}$ refers to the host from which it has been isolated

DISCUSSION
Many reports have described the microsporidia from Lepidopteran hosts but the first description of microsporidia from silkworm was given by Naegelli (1857) and long before they were described as human pathogens.

Since that several other microsporidia belonging to the genus - *Nosema, Pleistophora, Thelohania, Vairimorpha,* and *Leptomonas* were reported to cause infection in silkworm

(Fujiwara, 1980, 1984a, b and 1985; Govindan *et al.*, 1998; Selvakumar *et al.*, 2005). Microsporidia, characterized by the production of resistant spores that vary in size depending upon the species. They possess a unique organelle, the polar filament that coiled inside the spores as demonstrated by its ultra structure. The polar filament, which serves as an inoculating needle, is a conspicuous and characteristic organelle of the microsporidian spore. Its presence in spores places an organism in the phylum Microspora (Issi, 1986).

The germination of microsporidia occurs in the gut of the host when spores are ingested and the sporoplasm is inoculated into the epithelial cells of the gut wall. Spore of vertebrate and invertebrate microsporidia can be induced to germinate artificially by various chemical and physical treatments (Keohane and Weiss, 1999).

Artificial germination of microsporidian spore is known to require two steps: the first is priming and the second is induction of sporoplasm extrusion. In suitable buffer or salt solution the spores germinate within a few seconds after priming by the action of certain chemicals. Since the priming effect of KOH is reliable to any silkworm microsporidia, this chemical has been used commonly.

Silkworm microsporidia other than *N. bombycis* isolates show peculiar reactions to germination media after KOH priming. The environmental spores of *Nosema* sp. NIS-M11 are poor germinators (Yasunaga *et al.*, 1991, 1992). The germination rate of spore depends on the preparation and storage conditions and the percentage of germinated spores varies from 3-5% (Sasidharan *et al.*, 2003). The observation on the polar filament is necessary in order to identify the microsporidia and to confirm that spores are microsporidia.

The viability of the microsporidian spores can be also assessed by their capacity to germinate. The polar filament is very elastic, stretching to 3-times the length of the coiled state when extruded from the spore. It is also elastic transversely so that its diameter increases 2-fold at the site of passage of sporoplasm (Lom and Vavra, 1963). The extruded polar filament is 0.1□m in diameter when seen under phase contrast microscope

and in some cases up to 400 m in length. The length measured with the light microscope has been used to characterize species, but such measurements have little value in classification because there is no way to check if complete extrusion has been occurred (Larson, 1988).

In the present study the microsporidian spores (Lb_{ms} or *N. bombycis*) after incubation in different concentration of KOH followed by rapid neutralization in phosphate buffer saline (pH 7.00) germination occurred within 1-2 seconds. Microscopic examination of germinated spores confirmed that the spores became dark required for polar filament formation and sporoplasm expulsion. It is necessary to use the highly purified spores for artificial germination that germinated spores will not be confused with immature spores that are black in color under phase contrast microscope.

Artificial germination test confirmed that the Lb_{ms} is a microspridian spore. The Lb_{ms} was poor germinator as compared to *N. bombycis* and the extruded polar filament was also found shorter as compared to *N. bombycis* polar filament. Before this study there was no information available on artificial germination of Lb_{ms} isolated from Lamerin breed of the silkworm *B. mori*. So present report gives the information on artificial germination of Lb_{ms} by the action of KOH. These observations clearly indicated that Lb_{ms} is comparatively poor germinator and the length of the polar filament is also shorter compared to that of the *N. bombycis*.

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