# Electron microscopic observations on the anatomy of microsporidians isolated from insect pests of mulberry and other agricultural crops

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***Abstract***

*A number of insect pests of mulberry and other agricultural crops were collected using insect collection net and crushed individually in mortar and pestle. The homogenate of each individual insect was examined under phase-contrast microscope at 600 X magnification for presence / absence of microsporidian infection. The spores were noticed in the samples of Catopsilia crocale, Catopsilia pyranthe, Diaphania pulverulentalis, Pieris rapae and Spilosoma oblique. The isolated spore were tentatively designated as NIK-1Cc, NIK-1Cpy, NIK-1Dp, NIK-1Pr and NIK-1So respectively. The spores were purified using percoll and processed for anatomical observations at National Institute of Mental Health and Neuro Sciences (NIMHANS), Bangalore, Karnataka and compared with the spores of the standard microsporidian strain Nosema bombycis.*

*The results showed variation in exospore construction which was slightly crumpled in NIK-1Pr, NIK-1So, wavy in NIK-1Cc, corrugated in NIK-1Cpy, smooth in NIK-1Dp and Nosema bombycis. Anatomical observations also revealed 9, 10, 11 and 12 polar filament coils in NIK-1Dp, NIK-1Cc, NIK-1Pr and in NIK-1Cpy and N. bombycis respectively. However, 15 coils of polar filament were recorded in case of NIK-1So. The number of the coils of the polar filament is reported to be one of the important criterion for the characterization and as per observations under report, these microsporidia seem to be different from each other and also from the standard strain N. bombycis.*

**Keywords:** Agricultural, Insect, Mulberry, Microscopy, Microsporidian and Pests.

## Introduction

Microsporidians are the unique pathogens of insects. Over thousand species belonging to different genera are reported to be found infecting nearly all insects orders. Over half of the susceptible insect hosts occur in two orders i.e. Lepidoptera and Diptera.

Most of the entomopathogenic microsporidia occur in genus *Nosema* and more than 150 described species are found in 12 orders of insects[1]. The perpetual incidence of microsporidian infection in silkworms may be due to various sources including alternate hosts.

Review of literature shows that the different microsporidians have been isolated from various sericultural practising countries of the world and their descriptions were mainly based on spore morphology but due to lack of ultra structural details, it caused unnecessarily lot of duplication.

In recent reports regarding the identification of microsporidians, it was felt necessary to study at least a minimum of ultrastructural details like number of coils of the polar filament, nucleus number angle of tilt of polar filament etc.[7] for which transmission electron microscopic studies is an essential for tool[19]. Hence in the present report, the spore anatomy and serological affinity of five microsporidians isolated from insect pests of mulberry and agricultural crops are compared with that of the standard strain *N. bombycis*.

## Material and Methods

Microsporidians isolated from insect pests of mulberry and agricultural crops and also the standard strain *Nosema bombycis* were cultured and purified by following the standard procedure of Sato and Watanabe. The purified spores of each microsporidian were processed by Transmission Electron Microscopy at National Institute of Mental Health and Neuro Sciences (NIMHANS), Bangalore, India. The spores were fixed in 2.5% (w/v) glutaraldehyde ($C_5H_8O_2$) in phosphate buffer saline (PBS, pH 7.2) kept at 4ºC for 24h, washed several times with buffer (pH 7.2) till the odour of the fixative was completely removed.

The samples were post fixed in 1% (w/v) Osmium tetraoxide ($OsO_4$) for 2hrs, washed with phosphate buffer saline, dehydrated in an ascending series of alcohol of 70, 80 and 90% (1hr in each change), stained with Enbloc stain (2% uranyl-acetate in 95% ethanol) and dehydrated in absolute alcohol for 1h (Two changes each for 30 minutes). The samples were then passed through propylene oxide (2-changes of 15 min each for clearing) and were infiltrated with araldite and propylene oxide in the ratio of 1:1 for 12h. The samples were centrifuged and sediments were infiltrated again with fresh araldite (3-changes of 4h each) embedded in araldite and kept at 60ºC for 48h. Semi-thin sections (1µ) were cut with glass knives of microtome (Leica EMUC-6).

The sections were stained with 1% toludine blue, dried on hot plate, washed under running tap water, dried and

observed under light microscope (Motic). Ultra thin sections 70-80 nm (700-800°A) were double stained with uranyl acetate and lead citrate, observed and photographed under 60KVA (JEM-100CX-II) electron microscope at different magnifications to study the internal structure and coiling pattern of the polar filament.

## Results

The results with regard to the Transmission Electron Microscopy of the isolated microsporidians and *N. bombycis* are presented in table 1 and plate 1. The transmission electron micrograph of the spore of the microsporidian NIK-1Pr isolated from *P. rapae* indicated that the mature spore consists of a spore wall comprising an inner endospore and an outer exospore. The exospore is slightly crumpled in outline. The polar filament is arranged in a single layer close to the spore wall and consists of 11 isofilar coils (Table 1 and plate 1a). The polar filament is connected to an anchoring apparatus- the "anchoring disc" at the anterior pole. The anchoring disc is enclosed in a polar sac called as polaroplast.

In the posterior end of the spore, a distinct posterior vacuole is present. The spore is uninucleate and the nucleus occupies a central portion of the spore between the polaroplast and the posterior vacuole. The average single coil length and width are 0.068 and 0.059 µm respectively and the coil length-width ratio is 1.15: 1.

The ultrastructural studies of the spore of the microsporidian NIK-1Cc isolated from *C. crocale* revealed that the spore consists of a spore wall comprising a clear inner endospore and an outer exospore which is wavy in outline. The polar filament has 10 isofilar coils and lies in the spore as a single layer close to the spore wall (Table 1 and plate 1b). At the anterior part of the spore, the anchoring disc or polar cap is present to which the basal end of polar filament is attached. The anchoring disc is enclosed in the polaroplast.

Towards the posterior end of the spore, an oval posterior vacuole is present. The spore has two closely associated nuclei (diplokaryon) which lie between the polaroplast and the posterior vacuole. The average single coil length and width are 0.045 and 0.043 µm respectively and the coil length-width ratio is 1.04: 1.

Similarly, the results of the transmission electron microscopy of the spore of the microsporidian NIK-1Cpy isolated from *C. pyranthe* show that the spore has an endospore and an exospore. The endospore is smooth and the exospore is corrugated consisting of distinct folds or ridges. The coiled region of the polar filament is arranged close to the spore wall in a single layer and consists of 12 isofilar coils (Table 1 and plate 1c).

The anchoring disc of the polar filament is present as a deep dome at the anterior extremity of the spore and is embedded in the polaroplast.

Towards the posterior extremity of the spore, the posterior vacuole is present. The spore consists of a single nucleus that is found between the polaroplast and the posterior vacuole. The average single coil length and width are 0.064 and 0.056 µm respectively and the coil length-width ratio is 1.14: 1.

The transmission electron micrograph of the spore of the microsporidian NIK-1So isolated from *S. obliqua* shows that the mature spore exhibited cytology similar to most of the microsporidia and consists of a spore wall comprising a smooth inner endospore and a slightly crumpled exospore. The polar filament is arranged in a single layer close to the spore wall and consists of 15 anisofilar coils (Table 1 and plate 1d). In the anterior portion of the spore, a bell-shaped anchoring disc is present which is enclosed in the polaroplast.

Towards the posterior extremity of the spore, an oval posterior vacuole is present. The spore is diplokaryotic consisting of two nuclei lying in close vicinity to each other found in the central part of the spore between the polaroplast and the posterior vacuole. The average single coil length and width are 0.056 and 0.046 µm respectively and the coil length-width ratio is 1.21: 1.

The ultrastructural studies of the spore of the microsporidian NIK-1Dp isolated from *D. pulverulentalis* revealed that the spore consists of a spore wall comprising a clear inner endospore and an outer exospore which is smooth in outline. The polar filament has 9 isofilar coils and lies in the spore as a single layer close to the spore wall (Table 1 and plate 1e).

At the anterior part of the spore, the anchoring disc or polar cap is present to which the basal end of polar filament is attached. The anchoring disc is enclosed in the polaroplast. Towards the posterior end of the spore, a spherical posterior vacuole is present.

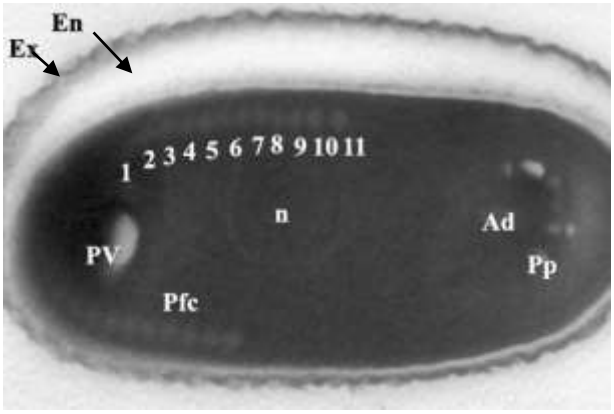
The spore is uninucleate consisting of a single nucleus which lies between the polaroplast and the posterior vacuole. The average single coil length and width are 0.052 and 0.042 µm respectively and the coil length-width ratio is 1.23: 1.

The internal ultra structure of the spore of the standard microsporidian strain *N. bombycis* shows that the spore consists of a bilayered spore wall comprised of smooth inner endospore and outer exospore. The polar filament has 12 isofilar coils and lies in a single layer close to the spore wall (Table 1 and plate 1f).

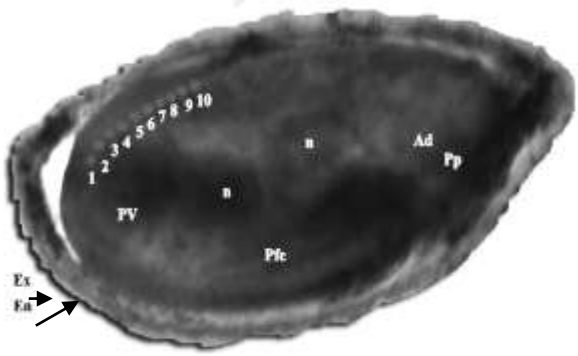
The spore is binucleate and the nuclei occupy a central portion between the polaroplast and the posterior vacuole. Other features include an anchoring disc positioned in the anterior portion of the spore enclosed in the polaroplast and a posterior vacuole positioned in the posterior portion of the spore. The average single coil length and width are 0.078 and 0.073 µm respectively and the coil length-width ratio is 1.06: 1.

**Table 1**
**Ultrastructural features of microsporidia spores**

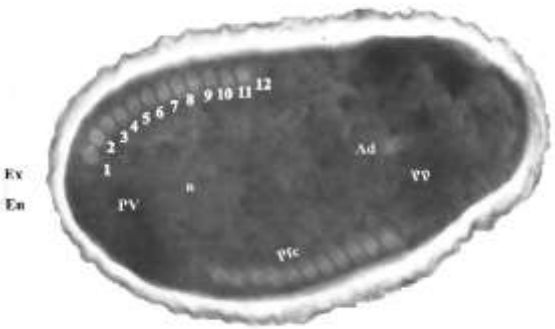
| Microsporidian isolates | No. of coils of polar filament | Coil size (µm) | | Coil Length-Width ratio | No. of nuclei | Exospore construction |
|---|---|---|---|---|---|---|
| | | Length | Width | | | |
| NIK-1Pr | 11 | 0.068 | 0.059 | 1.15:1 | 1 | Slightly crumpled |
| NIK-1Cc | 10 | 0.045 | 0.043 | 1.04:1 | 2 | Wavy |
| NIK-1Cpy | 12 | 0.064 | 0.056 | 1.14:1 | 1 | Corrugated |
| NIK-1So | 15 | 0.056 | 0.046 | 1.21:1 | 2 | Slightly crumpled |
| NIK-1Dp | 09 | 0.052 | 0.042 | 1.23:1 | 1 | Smooth |
| *N. bombycis* | 12 | 0.078 | 0.073 | 1.06:1 | 2 | Smooth |



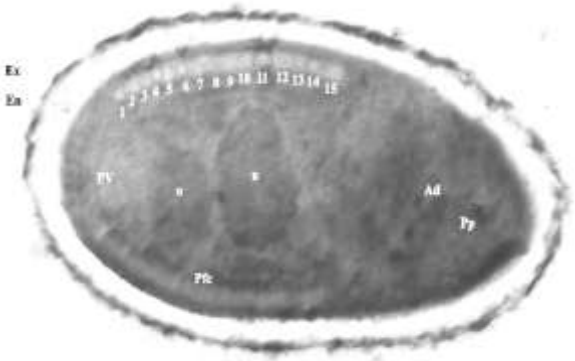

**a). NIK-1Pr having 11 coils of polar filament**



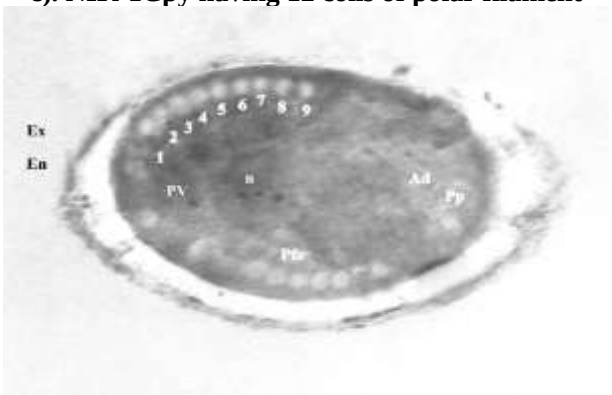

**b). NIK-1Cc having 10 coils of polar filament**



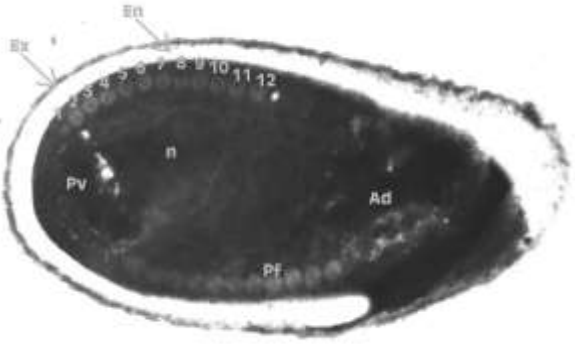

**c). NIK-1Cpy having 12 coils of polar filament**




**d). NIK-1So having 15 coils of polar filament**




**e). NIK-1Dp having 9 coils of polar filament**




**f). *N.bombycis* having 12 coils of polar filament**

**Plate 1: Longitudinal section of different microsporidians showing internal structure (Ex-Exospore; En-Endospore; Ad-Anchoring disc; Pfc-Polar filament coils; Pp-Polaroplast; PV-Posterior vacuole; n-Nucleus)**

## Discussion

Transmission Electron Microscopy is essential tool for studying the internal ultrastructure of cells, tissues, organs or unicellular organisms like microsporidians and to characterize them from unusual or new locations without causing any confusion.[19] Microsporidia are characterized by the presence of infectious unicellular spore stage, containing uninucleate or binucleate sporoplasm and an extrusion apparatus always with a polar filament ending in an anchoring disc at the apical part of the spore.[18]

The ultrastructural studies of the isolated microsporidian spores were carried out through TEM showing that the internal structure of the isolated microsporidia and *N. bombycis* have few similarities. The spores of the isolated microsporidia as well as *N. bombycis* indicate the presence of extrusion apparatus which occupies most of the spore and consists of the polar filament with its anchoring apparatus and the polaroplast. The other characteristic features include the posterior vacuole, nucleus, endospore and exospore. The spores of the microsporidia under study viz. NIK-1Cc and NIK-1So were binucleate consisting of two nuclei.

On the other hand, the spores of the microsporidia viz. NIK-1Pr, NIK-1Cpy and NIK-1Dp were found to be uninucleate consisting of a single nucleus. The spores of the standard microsporidian strain, *N. bombycis* are binucleate. The ultrastructure of many *Nosema* spp. has been described.[15-17] (The number of coils of polar filament, their arrangement related to one another and the angle of tilt provide a useful taxonomic criterion for differentiating microsporidian species.[6]

The number of coils of the polar filament within the spores may vary in a single species which is typically observed in case of *Nosema bombycis* – one with a few coils and a second type with many coils.[5] In present study also, a variation in the number of coils of the polar filament of the microsporidia under study was observed. The microsporidian NIK-1Pr was found to possess a polar filament consisting of 11 coils whereas the same in case of NIK-1Cc was only 10.

In other microsporidia viz. NIK-1Cpy, NIK-1So and NIK-1Dp, the polar filament consisted of 12, 15 and 9 coils respectively. In the standard strain, *N. bombycis*, the number of coils of the polar filament were recorded as 12. The number of coils of the polar filament of microsporidian spores varies from 44 in *Nosema apis*, 3 to 5 in *Encephalatozoon cuniculi*[11], 15 to 17 in *Nosema artemiae*, 12 in *Nosema bombycis*, 11 in *Nosema* sp. M12 and 8 in *Pleistophora*, 8 to 10 in *Nosema couilloudi*[15], 12 to 14 in *Nosema birgii*[17], 7 to 9 in *Nosema galerucellu*[16], 15 to 18 in *Nosema nisotrae*[15], 13 in *Nosema chaetocnemae* sp., 12 in NIK-1s, 10 in NIAP-6p, 13 in NIAP-7g, 14 in NIK-5d, 15 in NIK-2r, 16 in NIK-4m, 9 in NIK-8b and 8 in NITN-9n[10] and 11 in $Lb_{ms}$.[1]

The exospore construction is an important taxonomic attribute for determination of the generic position of microsporidia.[7] The isolated microsporidia showed a variation in their exospore construction and was observed to be slightly crumpled in NIK-1Pr, wavy in NIK-1Cc, corrugated in NIK-1Cpy, slightly crumpled in NIK-1So and smooth in NIK-1Dp microsporidia. The spores of the species of genus *Nosema* usually possess uniformly structured exospore.[12]

## Conclusion

The spores of *Nosema* sp. C01 isolated from *Pieris rapae* in Korea have an exospore with a wavy outline.[4] From these observations, it is clear that these spore have features of typical microsporidia but differ from each other and also from *N. bombycis* in anatomy, however, further more studies are needed to characterize them at species level.

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