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Characterization and Ultrastructural Development of *Nosema* sp. (Microsporidia) Infecting European Honey Bee, *Apis mellifera* by Scanning and Transmission Electron Microscopy

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Abstract
Nosemosis is a deadly disease of honey bee mainly caused by three types of Microsporidia *viz*. *Nosema apis*, *Nosema ceranae* and *Nosema neumanni*. This microsporidian infection affects the honey bee health, leading to honey bee colony collapse and therefore pose a is a serious threat for beekeepers in developing countries like India. The diversity and prevalence of microsporidia in host organisms vary according to different climatic condition. In the present investigation, the prevalence of *Nosema* sp. in European honey bee was investigated. Further, ultrastructure of different developmental stages of *Nosema* sp. were characterized by light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) that primarily infect gut tissue of European honey bee. A total of 150 *Apis mellifera* were examined, out of which 90 were found positive with *Nosema* sp. infection. Therefore, the prevalence percentage of *Nosema* sp. infection in European honey bee was calculated as 60%. The mean size (Length × Width) of *Nosema* sp. under scanning electron microscope was measured as 4.01±0.17 µm × 2.67±0.08 µm. The exospores of the microsporidia were found sculptured with deeper ornamentation of ridges and furrows which is the unique characteristics of *Nosema* spores previously reported in European honey bee. The thickness of the exospore and endospore were measured as 24 nm and 162 nm respectively under TEM. Ultrastructural study of *Nosema* sp. showed different developmental stages which will be helpful for the management of nosemosis disease in European honey bee.

Keywords: Nosemosis, *Nosema* sp, *Apis mellifera*, Colony collapse, Prevalence

Introduction
Microsporidia are a diverse group of obligate, eukaryotic endoparasites that infect a wide range of hosts from invertebrates and vertebrates including insects, fish, rodents, rabbits, primates, humans and have a worldwide distribution (Han *et al*., 2021) [11]. They are one of the most outstanding groups of organisms in many respects which were first identified 168 years ago with the description of *N. bombycis*, a pathogen of silkworm *Bombyx mori* L. (Naegeli, 1857) [18].

More than 1700 species and 220 genera of microsporidia have been reported till date (Liu, 2024) [22]. Of these, the genus *Nosema* is most common in invertebrate hosts, contains more than 200 species. Microsporidia have a complicated life cycles and spores are the only life cycle stages that can endure outside of the host cell. This is the infectious stage which normally reaches the host through the gastrointestinal tract by horizontal transmission, whereas there is also report available of vertical transmission. Most microsporidia reproduce in two sequences that follow each other: first, by vegetative reproduction (merogony), which produces daughter cells (meront) with the potential either to repeat merogony or to enter second reproductive phase (sporogony), which is the formation of spores (sporont, sporoblast). Both merogony and sporogony division occur by binary fission, producing two daughter cells from each mother cell. Sometimes the daughter cells attach themselves to one another to create chain of cells. Because of their ultrastructural uniqueness and differentiation from other spore-forming microorganisms, the life cycle phases of microsporidia are crucial for characterization (Larsson, 1986) [17]. In European honey bees, three types of *Nosema* sp. have been reported till date *viz*. *Nosema* apis, *Nosema* ceranae and *Nosema neumanni* (Huang *et al*., 2007 [15]; Higes *et al*., 2009 [14]; Traver *et al*., 2012 [21]; Charbonneau *et al*., 2016 [2]; Chemurot *et al*., 2017 [3]) and thus contributing economic losses to beekeeping industries worldwide. Hence, for the proper management of beekeeping, it is important to know the pathogens infecting honey bee and particularly the microsporidian parasites. Further, the ultrastructural details of microsporidia in European honey bee are an important tool for its characterization and identification. Keeping in view, the present study deals with the study of different developmental stages of *Nosema* sp. isolated from the midgut tissue (primary site of infection) of *A. mellifera*.

Materials and methods
Collection of European honey bee
European honey bee, *Apis mellifera* were collected from flower garden by standard insect collection techniques with the help of an insect collection net (Fenemore and Prakash, 1992) [5]. After collection, the honey bees were transferred immediately to plastic boxes with perforated lids and brought to the laboratory and screened for the presence of microsporidian infection.

Screening of European honey bee for Microsporidian infection
The abdomen of each European honey bee was separated and weight was measured. The abdomen was then homogenized individually in 0.6% K_2CO_3 (4ml/g) solution and investigated for the presence of microsporidian spores. A smear was prepared from the respective homogenate and observed under light microscope at 400X. The sample with negative microsporidian infection were discarded whereas

samples with positive infection were processed and stored at 4 °C for further studies

Prevalence of microsporidian infection in Insects

The prevalence percentage of microsporidian infection in insects was calculated by the following formula:

$$\text{Prevalence \% of Microsporidia} = \frac{\text{No. of infected honey bees} \times 100}{\text{Total no. of collected honey bees}}$$

Light microscopy

The microsporidian spores isolated from *Apis mellifera* were subjected to morphological characterization following standard method as described by Fujiwara (1980) [8]. The size of the fresh microsporidian spores were measured accurately by using Nikon NSBR software under Nikon Type-104 phase contrast microscope at 400X without shrinkage and drastic distortion, often caused by fixation and staining. The information about size (length and width), shape, movement and refractivity of spores was obtained during the study. Data were pooled to calculate the mean size of the spores.

Ultrastructural Study

Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) is a useful technique for the investigation of surface structure of the microsporidian spores. A small piece of the honey bee gut infected with microsporidian spores were fixed in primary fixative of 2.5% (v/v) glutaraldehyde ($C_5H_8O_2$) in 0.1 M phosphate buffer, pH 7.4 for 24 hours followed by Secondary fixation of the sample in 1% osmium tetroxide (OsO_4), pH 7.4 for 1 hour. The sample was then washed thrice in 0.1 M phosphate buffer pH 7.4 to remove the fixative and dehydrated by increasing gradation of acetone (30%, 50%, 70%, 90% and 100%). The dried sample was then mounted on copper stub using carbon tape and scanned under a JEOL JSM-6490LV scanning electron microscope. The scanning electron microscopy of the microsporidia was done at the University Science Instrumentation Centre (USIC), Babasaheb Bhimrao Ambedkar University, Lucknow.

Transmission Electron Microscopy (TEM)

A small piece (1×1 mm) of infected gut tissue of European honey bee was fixed in 2.5% glutaraldehyde and 2% paraformaldehyde (PF), in 0.1 M phosphate buffer (pH 7.4) for 24 hr at 4°C. The sample was then washed thrice in 0.1 M phosphate buffer, pH 7.4 to remove the fixative (Reynolds, 1963) [19] and submitted to Sophisticated Analytical Instrument Laboratory, AIIMS, New Delhi for further processing.

Results

In the present study, a total of 150 *Apis mellifera* were collected, out of which 90 were found to be positive with *Nosema* sp. infection. Therefore, the prevalence percentage of *Nosema* sp. infection in European honey bee was calculated as 60% (Table 1). Further, the *Nosema* sp. were characterized by light microscopy, scanning electron microscopy and transmission electron microscopy.

Characterization of microsporidia by Light Microscopy

The live microsporidian spores were easily detected by their characteristic Brownian movement. The spores were oval in shape with variable sizes. The live microsporidian spores showed translucent properties with high refractivity whereas the germinated spores were easily distinguished from the live spores and observed as black empty spores under phase contrast microscope. Besides this, the microsporidian spores exhibited clear and bright glare with a characteristic halo effect. The diplokaryotic nucleus of the microsporidia were clearly observed under the phase contrast microscope.

The mean size (Length × Width) of *Nosema* spores were measured as 4.35±0.10 µm × 2.80±0.07 µm, each possess a diplokaryotic nucleus. Further, few *Nosema* spores were observed with protruding polar filament which is the unique invasion organelle of microsporidia (Figure 1, Table 2).

Ultrastructural characterization

Characterization of *Nosema* sp. by Scanning Electron Microscopy (SEM)

The surfaces of the microsporidian spores were clearly visible under scanning electron microscope. The sizes of the microsporidia get reduced to some extent by the fixative and dehydrating agents used to fix the spores for SEM study. The mean size (Length × Width) of *Nosema* sp. under scanning electron microscope was measured as 4.01±0.17 µm × 2.67±0.08

µm (Table 3). The honey bee gut observed under SEM was fully covered with microsporidian spores with a high intensity of spore load. The developmental stages of microsporidian spores were clearly observed under SEM. The meront stages were rounded in shape whereas the spore stages were oval in shape. Further, the spores undergoing binary fission were also observed under SEM. The exospores of the microsporidia were sculptured with deeper ornamentation with ridges and furrows which is the unique characteristics of *Nosema* spores previously reported in European honey bee (Figure 2).

Characterization of *Nosema* sp. by Transmission Electron Microscopy (TEM)

Transmission electron microscopy of *Nosema* sp. in the midgut epithelial cells of European honey bee revealed the presence different developmental stages, including meront (M), sporont (ST), sporoblast (SB) and spore stages (S) of microsporidia. All stages of the spore were in direct contact with the host cell cytoplasm. Some empty spores were also observed indicating the intracellular germination of spores (Figure 3). Meronts, the earliest developmental stages were bound by a plasma membrane. Sporonts were elongated and oval in shape with dense cytoplasm and no visible internal structures. Sporoblasts were generally smaller than sporonts with a more clearly defined cell wall and two nuclei. The anchoring disc was present in the anterior pole of the spore. The lamellate polaroplast occupied the anterior part of the spore adjacent to the anchoring disc but was not prominent. Infected host cells were enlarged and the cytoplasm contained a larger number of mitochondria and free ribosomes. Several host mitochondria were close to and surrounded the plasmalemma of meronts (Figure 4). This is the strategy of microsporidia to obtain ATP from their host cell by maximizing contact between host mitochondria and

the developmental stages of microsporidia. From a previous study it was observed that, host mitochondria were found around the parasitophorous vacuole (a developmental stage of microsporidium) of *Encephalitozoon*-infected cells, which is probably critical for energy uptake from host mitochondria (Hacker *et al*, 2014 [9]; Han *et al*., 2019) [10].
The cells with sporogonial phases, the rough endoplasmic content was greater. The *Nosema* sp. were ovo-cylindrical in shape and contained nucleus in the central region of the spore and located between the polaroplast and the posterior vacuole. A small posterior vacuole was observed at the posterior end of the spore (Figure 5). The diameter of the nucleus was measured as 830 nm. The width of exospores of the microsporidia was thinner than the endospore. The thickness of the exospore and endospore were measured as 24 nm and 162 nm respectively. The polar filament coils measured 41 nm in diameter (Table 4). The ultrastructural features of microsporidia infecting European honey bee confirmed that it belonged to the genus *Nosema* sp. because of the presence of all characteristic features of a typical microsporidium belong to genus *Nosema*.

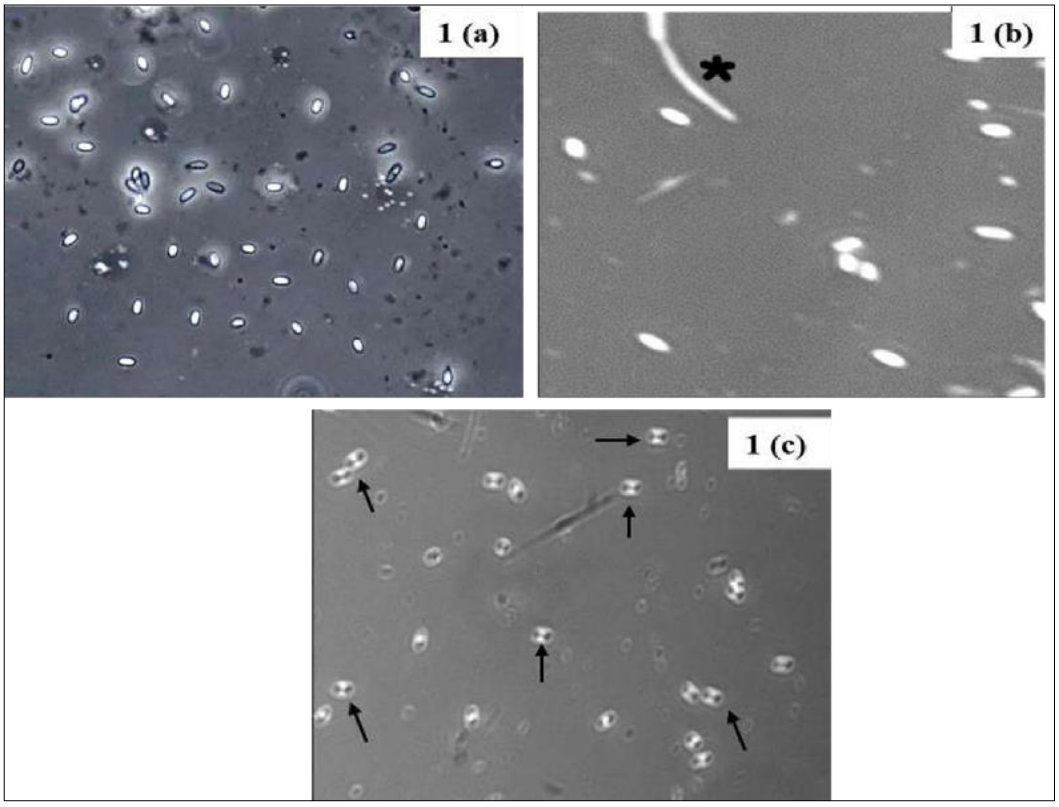
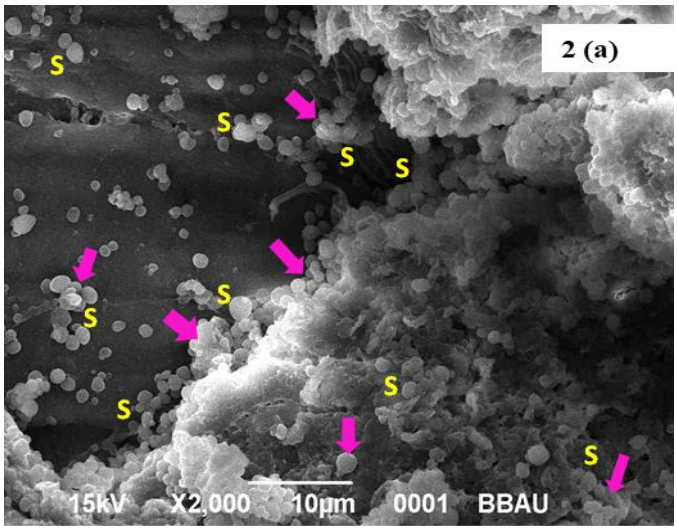


Fig 1: Phase contrast micrographs of (a) *Nosema* sp. suspension (b) A *Nosema* Spore (*) with protruding polar filament and (c) Diplokarotic *Nosema* spores (Arrow mark) isolated from infected European honey bee, *A. mellifera* viewed at 400X



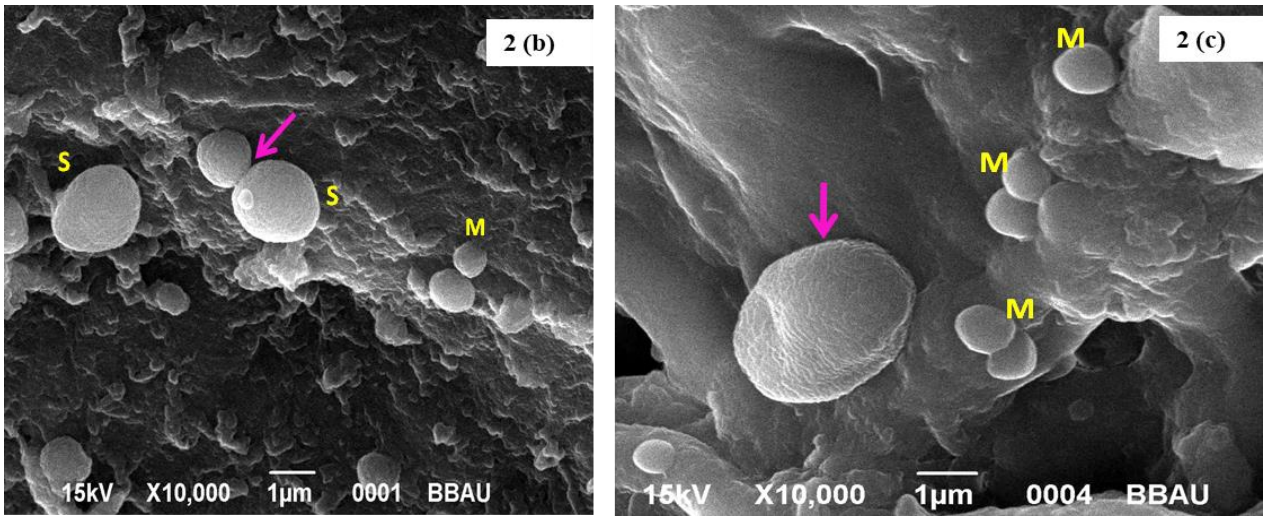


Fig 2: (a): Scanning electron micrograph of *Nosema* sp. with different developmental spore (S) stages, (b): *Nosema* sp. undergoing binary fission (Arrowhead) (c): A mature *Nosema* sp. (Arrowhead) showing ornamentation of ridges and furrows. Meronts (M) are immature developmental stages dividing by binary fission in the gut epithelial cell of infected European honey bee, *A. mellifera*.

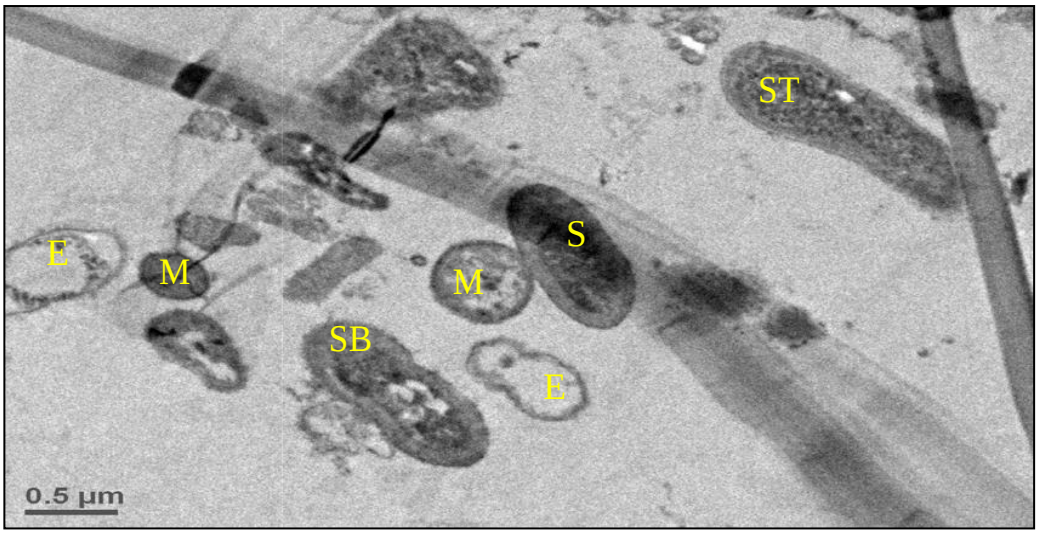


Fig 3: Transmission electron micrograph of midgut Epithelial cells of infected *A. mellifera* showing different developmental stages of *Nosema* sp., M: Meront, ST: Sporont, SB: Sporoblast, S: Spore, ES: Empty spore

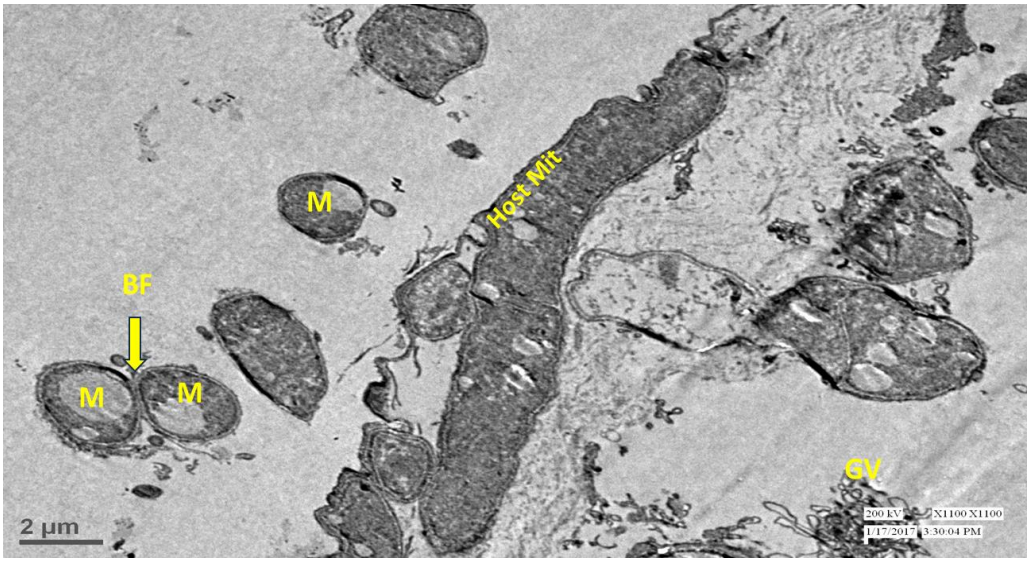


Fig 4: Transmission electron micrograph of midgut Epithelial cells of *A. mellifera* showing *Nosema* sp. surrounded by showing Golgi vesicle (GV) and Host mitochondria (Host Mit) in the early merogonial stages. Meronts (M) are dividing by Binary fission (BF) division. Developing spores derive energy from host mitochondria as spore do not possess mitochondria. However instead of typical mitochondria, the microsporidia possess mitosomes, which are remnants of mitochondria.

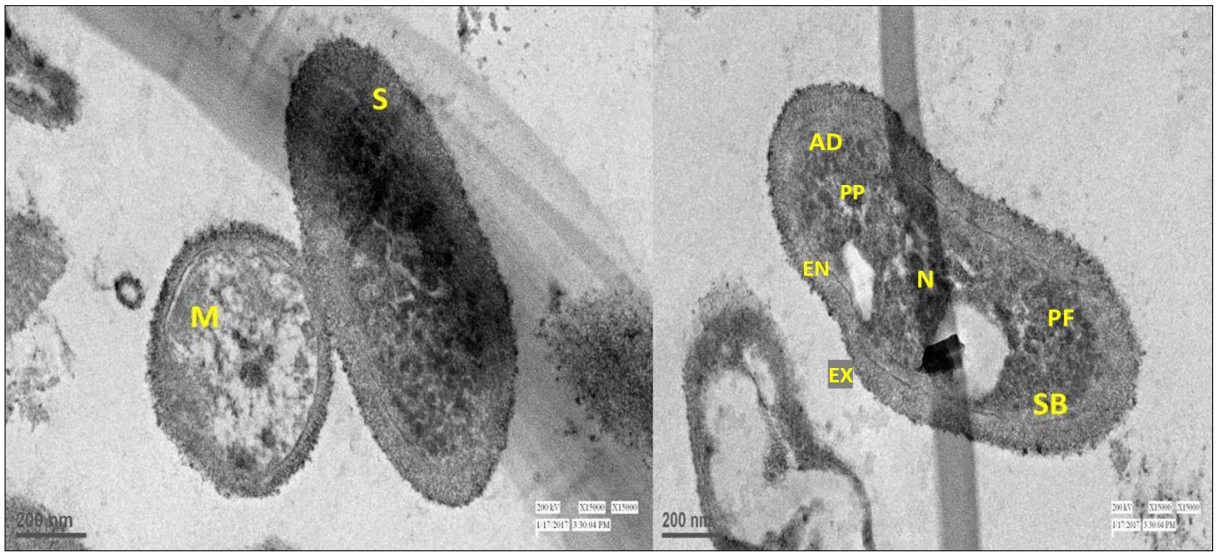


Fig 5: Transmission electron micrograph of Meront (M) which is round in shape and a developing spore (S) which is oval in shape, found in the gut of infected *A. mellifera*. A developed Sporoblast (SB) showing thickness of the spore wall Ex: Exospore, EN: Endospore, AD: Adhesive disk, PP: Polaroplast, PF: Polar Filament, N: Nucleus, PV: Posterior Vacuole

Table 1: Prevalence % of *Nosema* sp. infection in European honey bee, *A. mellifera*

Insect species	No. of honey bee collected	No. of honey bee infected with *Nosema* sp.	Prevalence % of *Nosema* sp. infection in honey bee
A. mellifera	150	90	60

Table 2: Morphometric details of fresh *Nosema* sp. in European honey bee, *A. mellifera* viewed under Phase Contrast Microscope at 400X

Insect pests	Isolated Microsporidia	Shape	Size (µm) N=10	
			Length±SD	Width±SD
A. mellifera	*Nosema* sp.	Oval	4.35±0.10	2.80±0.07

Table 3: Morphometric details of fixed *Nosema* sp. in European honey bee viewed under Scanning Electron Microscopy

Insect pests	Isolated Microsporidia	Size (µm) N=10		Texture of spore surface
		Length±SD	Width±SD	
A. mellifera	*Nosema* sp.	4.01±0.17	2.67±0.08	With Ridges and Furrows

Table 4: Ultrastructural details of *Nosema* sp. in European honey bee viewed under Transmission Electron Microscopy

Microsporidian Isolates from *A. mellifera*	Width of Polar Tubule (nm)	No. of nuclei	Diameter of Exospore (nm)	Diameter of Endospore (nm)
Nosema sp.	41	2	24	162

Discussion

The prevalence of *Nosema* sp. infection in European honey bees is documented in this study. Further, light and electron microscopic studies on the spore structure (Shape, Size), spore surface texture, different developmental stages and internal structure of the spore confirmed the *Nosema* sp. infection in European honey bee, *A. mellifera*. The study of spore ultrastructure elucidated the typical characteristics such as the presence of polar filament coils, an anchoring disc, polaroplast posterior vacuole and lack of mitochondria (Canning and Vavra, 2000) [1]. The formation of spores with walls made up of an electron-dense exospore, an electron-lucent endospore, 10–30 polar filament coils, and diplokaryotic nuclei are the characteristics of the microsporidia in the genus *Nosema* (Franzen and Müller, 1999 [6]; Huang *et al*., 2007) [15]. Earlier, the microsporidia known to infect the European honey bee have been identified as *Nosema apis* or *Nosema ceranae*, *Nosema neumanni* (Higes *et al*., 2006 [13]; Higes *et al*., 2007 [12]; Huang *et al*., 2007 [15]; Chen *et al*., 2009 [4]; Chemurot *et al*., 2017 [3]). In the present investigation, *Nosema* sp. infecting the European honey bee, *A. mellifera* were measured as 4.35±0.10 µm × 2.80±0.07 µm. Previous studies on the microsporidian infection in *A. mellifera* by some researchers suggested that, the microsporidium *N. ceranae* contained 20-23 polar filament coils whereas the microsporidium *N. apis* contained more than 30 polar filament coils (Fries *et al*., 1996) [7]. Recently, a new microsporidium *N. neumanni* have been reported from *A. mellifera* in Uganda which is completely different from both *N. ceranae* and *N. apis* and possess 10-12 polar filament coils (Chemurot *et al*., 2017) [3]. The size of *N. neumanni* was relatively smaller than the spore size of *N. ceranae* and *N. apis* which measured 2.36±0.14 µm in length and 1.78±0.06 µm in width. In contrast to this, the size of *N. apis* measured as 6.0×3.0 µm (Fries *et al*., 1996) [7] whereas the size of *N. ceranae* measured 4.4±0.41 µm in length and 2.2±0.09 µm in width (Chen *et al*., 2009) [4].

The average spore size of the *Nosema* sp. in this investigation was quite similar to the previously reported average spore size of *N. ceranae*. The *N. ceranae* infection in *A. mellifera* cause a death rate of 81.06% on the 16th days of post-infection, demonstrating the virulence of the nosemosis (Katna *et al*., 2018) [16]. It has been established by another study that the nosemosis spreads when infected bees feed the uninfected bees. Besides this, the younger uninfected bees that were fed by older infected bees developed the *Nosema* infections at a level 13-times higher than young uninfected bees unable to feed from older infected bees (Smith, 2012) [20]. So, it can be attributed that a single infected honey bee can be responsible for the rapid transmission of disease in the colony.

Conclusion

Microsporidian infections in insects are generally chronic, causing pathogenic effect on host and reduce their fecundity and life spans. The nosemosis disease in honey bee led to Colony Collapse Disorder globally and causes heavy economic losses. As honey bee having both economic and ecological significance, it is important to save them. From the present investigation, it can be concluded that, microsporidian *Nosema Sp.* are common in European honey bee population and the ultrastructural developmental stages of microsporidia will surely provide an insight to develop control strategies against nosemosis. Further, in future strategies can be developed to prevent the *Nosema* infection in the honey bee colony by giving antifungal treatment to the honeybee. This way the microsporidian infection in honey bee hives can be prevented and will ultimately boost the economy of bee keeping farmers in developing countries like India.

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