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Scanning Electron Microscopy of Microsporidia Isolated from Gut Epithelium of Honey Bee (*Apis mellifera)*

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Abstract: Honey bees (Apoidea) are considered to be the best pollinators of various wild plants and crops that are not wind pollinated and have proved themselves as an essential part of worldwide biodiversity along with providing necessary products to biological community, who solely depend on bee products (honey, bee wax, propolis) for their livelihood. The fear of honey bees' decline has provoked an ultimate watchfulness to know more about the reason. The present study is designed to generate knowledge of the parasite- microsporidia isolated from the gut epithelium of honey bees' by performing scanning electron microscopy. Scanning electron microscopy (SEM) revealed the spore as oval and cylindrical with 7.04 x 4.83 µm size. Spore size observed is slightly larger than the standard values (4-6 x 2-4 µm). This might be due to climatic factors acting upon the microsporidian sp. SEM revealed the ultra-structural characteristics of the microsporidia, however, the exact species identification is only possible with the help of molecular techniques.

Keywords: Bee pollinators, Species, Infection, Parasite, Microsporidia, Scanning electron microscopy, Molecular techniques

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Introduction

Honey bees play an important role in maintaining life on earth as they pollinate nearly 2/3rd proportion of crops worldwide (Fontaine, 2005; Knight *et al*., 2005; Patel *et al*., 2021). It plays a crucial role in the conservation of ecological diversity and is considered to be the necessary part of the ecosystem. Much attention has been given on managing populations of bumble bees, social wasps, hoverflies and honey bees (Aizen and Harder, 2009). However, now-a-days, many pollinators population are decreasing day by day due to parasitic invasions (Aizen and Harder, 2009). Microsporidia are small, single celled, spore-forming microorganisms (extremely evolved fungus) and an obligate intracellular parasite of eukaryotes which chiefly parasitize on insects (e.g. silkworms, bees and mammals) (Keeling, 2009). The presence of polar filaments in spores, places the parasite under the phylum-Microsporidia (Issi, 1986). The disease nosemosis in honey bee populations is spread principally via the fecal oral way, that results in the Colony

Collapse Disorder (CCD) (Ptaszynska, 2014). The parasite establishes a considerable proportion of the noteworthy pathogens of insects and also produces huge impact on economically crucial insect species (Deepti *et al.,* 2018).

As reported by Keeling (2009) "*Nosema*" belongs to a sister group of fungi- microsporidia. The two most important species falling under genus *Nosema* are *Nosema apis* and *Nosema ceranae* (Fries, 1988). These species attack on the midgut epithelial cells (preferably) of honey bees (*Apis mellifera*) which includes worker bees, drones and queen. The way they infect honey bees and cause collapse of the whole honey bee colony, these are now believed to be the etiological agents of a "deadly honey bee disease" called- Nosemosis (Fries, 1988, 2010; Higes *et al.,* 2007, 2010). Nosemosis pessimistically affects efficiency and continued existence of honey bee populations, prolonged existence of adult bees, queen bees, bee biochemistry, brood rearing, collection of pollen grains and all the bee behaviors performed for communication amongst bees (Huang, 2011; Botias *et al.,* 2013). Formerly *N. ceranae* and *N. apis* were first time recorded in Asian honey bees (*Apis cerana*) and European honey bees (*Apis mellifera*), respectively and were believed to be species-specific in nature though *N. ceranae* was less reported in *A. mellifera* all over the world (Higes *et al.,* 2010; Fries, 2010; Paxton, 2010; Klee *et al.,* 2007). Few studies contemplated that geographic distribution of *N. apis* and *N. ceranae* may overlie with each other that results in co-existence and moreover co-infection in honey bees (Milbrath *et al*., 2015). It is also reported that *N. ceranae* has displaced *N. apis* extensively and has become ubiquitous in many countries (Klee *et al.,* 2007).

Present study is conducted to perform the molecular characterization of microsporidia isolated from gut epithelium of honey bee using light bright field and scanning electron microscopy. However, studies related to the behavior of infected honey bees (Nosema infected bee colonies) need more explorations and evidences.

Materials and Methods

Random collection of honey bees from different regions of Lucknow, India:

Honey bees (*Apis mellifera*) were collected from 5 different zones of Lucknow viz. Sitapur road (North zone), Barabanki road (East zone), Navabganj (West one), Raebareilly road (South zone) and Hazratganj (Central Zone) using insect collecting nets from July 2019 to March 2020.

Identification and Detection of Microsporidia:

Bees were brought to the parasitology laboratory of Babasaheb Bhimrao Ambedkar University and were kept at -20 C. After 40 min, midgut of bees was homogenized using potassium carbonate solution and temporary slide was prepared to visualize the presence of spores. After confirmation of the infected bee samples, the homogenised solution was centrifuged using Percoll (Sigma-Aldrich) by following the protocol mentioned by Tsai and Wang (2001) to get the crude spore pallet. Images were captured at 400X magnification in bright field microscope.

Scanning Electron Microscopy (SEM):

For scanning electron microscopy, samples were first air dried. Primary fixation was performed using 2.5 % glutaraldehyde for 3-4 h. Washed thrice with PBS (1%) 15 min each. Post fixed with 1% OsO_4. Left overnight. On the next day, spores were dehydrated with different grades of acetone (30%, 50%, 75%, 90%, 95% and 100%) and after dehydration sample was mounted on stubs, coated with gold and observed under Scanning Electron Microscope (Jeol, Japan; JSM 6490 LV) (Ptaszyńska, 2014) at 5K and 20K. Photographs were taken and examined.

Results

Our study suggested the same kind of ultrastructural properties studied so far. Figure 1 (A) showing the method of capturing bees from the fields of village Hindukheda (Lucknow). Figure

Fig. 1: (A) Collection of honey bees from fields of Lucknow region; (B) Matured spore observed under bright field microscope at 400 X magnification (white arrow).

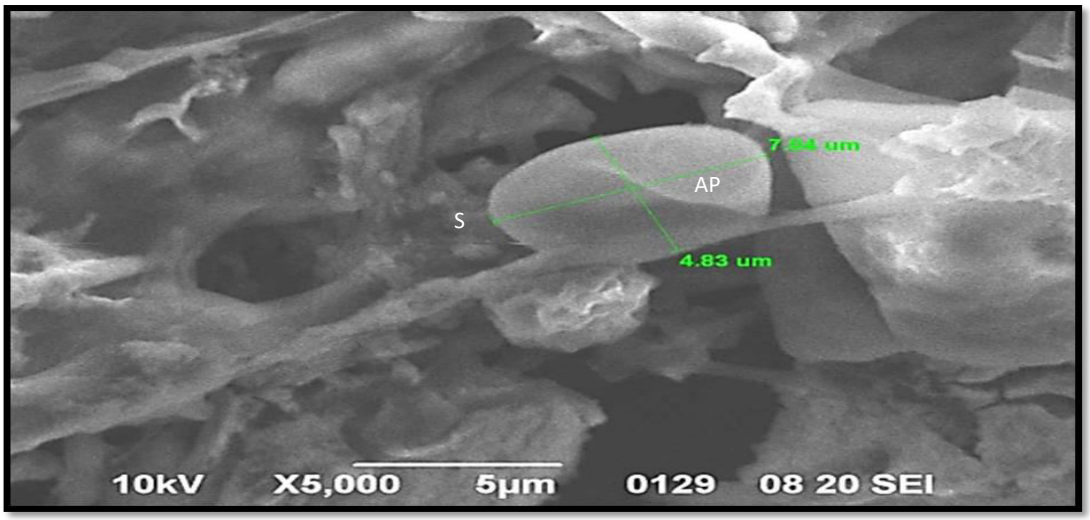

Fig. 2: Scanning Electron Micrograph of a microsporidian spore (S) with spore dimensions to depict the size of the spore. Polar tube is anchored in the epithelial wall of honey bee. AP: Anterior polaroplast.

1(B) showing a matured sporont (spore) visible under bright field microscope. Spores are oval and cylindrical in appearance with smooth surface and exhibited a vibratory movement at their particular axis which is considered as a characteristic feature of microsporidia (Brownian motion). The greenish luminescence imparted by the oval bodies confirmed that spores belong to phylum-microsporidia.

Scanning electron microscopy revealed various developmental processes performed by microsporidia for their existence. Figure 2 shows the anchoring behavior of microsporidia. Microsporidia use a polar filament that remains coiled inside the spore and emerges out at the time of infection. In the figure, spore is attached on the muscle of midgut of infected bee. The filament is not visible as it is put inside the gut epithelium. The ultrastructure specified the spore size at 5K magnification and 5 µm scale bar. Scanning electron micrograph showed spore dimensions as 7.04 x 4.83 µm however, spore size observed is slightly larger than the standard values (4-6 x 2-4 µm). This might be due to climatic factors acting upon the microsporidian sp. inhabiting in a particular area. A slight depression in the spore

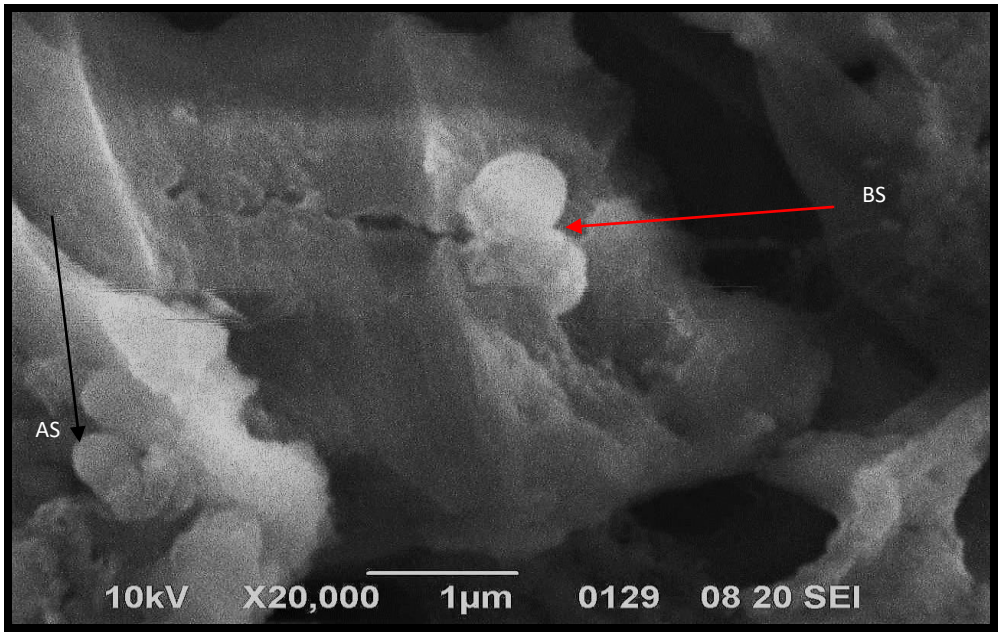

Fig. 3: Scanning Electron Micrograph of spore depicting the dividing meronts by red arrows (Binucleated spores, BS). Black arrow showing the anchored spores in bees' epithelial layer (AS).

wall confirmed it to be anterior polaroplast from where the polar tube emerges out.

Figure 3 denotes the mode of reproduction exhibited by the microsporidia. The life cycle involves the spherical binucleated meronts to undergo binary fission and produce four nuclei per celled condition i.e. tetra nucleated meront that further forms sporonts. These forms are achieved by repetitive karyokinesis and no cytokinesis. Red arrow denotes the binucleated meront undergoing binary fission. Black arrow depicts the matured anchored spores in the bee muscle.

N. apis and *N. bombi* are presently pertained synonyms, but they differ in their tissue specificities, spore sizes and also in the number of polar filament coils. *N. bombi* is considered to be the parasite of bumble bees at higher altitudes.

Discussion

The diversity of food accessible on earth is exclusively owed to bee pollinators as they pollinate a number of plants species (Fontaine, 2005). One major reason responsible for honey bee decline is a microscopic, unicellular, obligate parasite- Microsporidia that are spore forming parasites and infect vertebrates as well as invertebrates (Weiss and Keohane, 1999). The continuous declining of bees' populations has provoked a sense of working on reasons behind this menace. Our study is in support with similar findings with many researchers. Scanning Electron Microscopy was conducted to detect the chief morphology of spore. Ovo-cylindrical spores were detected in the homogenates that accurately exposed the structure of microsporidian spore. Ishihara (1969) isolated microsporidia that were multiplying in host cells in the form of meronts and sporonts and had a life cycle partaking only a single sporulation sequence in only one host individual.

Developmental stages revealed in this study, may get support from the results of Hylis *et al.* (2006) who gave an idea of life cycle of microsporidia explaining about the merogony and sporogonic stages that are typical of microsporidia of *Nosema.* Gray *et al.* (1969) proposed a similar finding in honey bee *A. mellifera* infected with *Nosema apis*. Long chains of schizonts or merogonial plasmodia were reported in this study.

Findings by Sulborska *et al.* (2019) showed the characteristic outline and sculpture of the external structure of the *Nosema* cell wall through scanning electron microscopy.

Conclusion

After examining the ultrastructural properties of microsporidia through scanning electron microscopy, the inter-relation between the species and genus *Nosema* could be established. Moreover, SEM has become an efficient technique to study the developmental stages and pathogenesis of microsporidia. It has been found that microsporidian are endoparasites of honey bees, but what essential is the implication of this character that has to be resolved. Adaptation is the ultimate goal of each and every organism but what influence a parasite is putting on the host is mandatory to be discovered. The spores were visualized under different magnifications for *Nosema* spp., however, the genus cannot be identified. Without DNA study, it can not be surely said and has to be explored more in future. Researches related to the behavior of bees infected with nosemosis with respect to pollen collection, flying, mating and honey production must be taken into consideration as no such knowledge exists till now.

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