Detection of Entomopathogen Microsporidia in Declining population of Pollinators: Honey bees & Bumble bees

A Summary of the Thesis Submitted to the
Babasaheb Bhimrao Ambedkar University, Lucknow
in Fulfillment of Requirement for the Award of degree of

Doctor of Philosophy
In
Zoology

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
LUCKNOW
प्रज्ञा शील करूणा
ESTABLISHED 1996

Submitted By

Saumya Sharma

Enrolment No. - 955/16

Under The Supervision of

Prof. Kamal Jaiswal

DEPARTMENT OF ZOOLOGY
SCHOOL OF LIFE SCIENCES
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
(A CENTRAL UNIVERSITY)
VIDYA VIHAR, RAEBARELI ROAD, LUCKNOW- 226025 (U.P.), INDIA

2023

SUMMARY

There are several facets of the phenomena of parasitism in nature, out of which host-parasitic environmental relationship is incredibly changeable. For the purpose of investigating host-parasite interactions, microsporidian parasites serve as a suitable model. They contain 144 genera and 1200 species out of which 200 species fall to the genus Nosema. Microsporidia are said to be unique because of their "way of causing infection" in bees. The parasite is supposed to be causing infection in the patients of AIDS; however, it is a possible regulator in monitoring insect populations. Even though microsporidians have been explored for more than 165 years, there are still numerous elements of their life cycle, systematic classification and morphological traits that are obscure and call for more research.

Pollinators are critical to the survival of not just plants but also people, since we rely on bees for employment and a variety of other advantages. The activity of beekeeping not only contributes to the preservation of ideal living conditions for bees but also gives millions of people worldwide a means of support via income.

Microsporidia has been the subject of much research globally, however there are relatively few reports on it in India. To identify them visually and using molecular methods, it is necessary to research the variety of microsporidia in various important insects. The goal of the current thesis is to examine the morphology and molecular mechanisms of Nosema (microsporidia) in potential pollinators- honey bees and bumble bees.

First chapter of the thesis provides the description of microsporidia, together with its discovery, taxonomic classification, spore structure, life cycle and

its prevalence in bees. Together with the purpose and objective pertinent to the issue of the proposed topic, it also addressed the technique of diagnosis by microscopy and molecular techniques in context of the genus Nosema of microsporidia.

The second chapter provides a review of the literature that exemplifies the characterization of pathogens, their prevalence, morphological specifications, molecular characterization, the implications of microsporidian infestation on their own hosts, factors that influence microsporidian development and progression, and the reassignment of the genus Nosema to Vairimorpha based on certain phylogenetic characteristics. However, in the current work, the identified genus is referred to as Nosema.

The third chapter explains the incidence of microsporidia in bees recognized under light and scanning electron microscopy, that corresponds to the genus Nosema sp. Microsporidia have been observed severely infecting the honey bees, contributing to the fact of high prevalence of Nosema in honey bees of Lucknow and also being a potential reason for their decline in this region.

Out of the total 1212 honey bees collected, 1198 bees were found to be highly infected with the parasite providing the infection percentage to be 98.84%. Light microscopy revealed the external characters as well as various developmental stages of the spores. Mean spore count came out to be $8.25 \text{ X } 10^6$ spores/ml. The spores were found to be oval in shape. Various developmental stages like sporonts (s), sporoplasmic vesicles and meronts undergoing binary fission were observed. Honey bee homogenate observed at dark field mode showed the most versatile character of spores i.e bioluminescence which further confirmed the existence of infection. Numerous mature oval spores were found to be vibrating at their own axis (Brownian

motion), which is the second most vibrant feature of microsporidian spores. Furthermore, the spindle shaped meront developing into paired meronts were found undergoing several cycles of division to first separate and then mature into sporonts. In the Giemsa-stained smear, both the spore proliferation stage (merogony) and the spore maturation stage (sporogony) were simultaneously observed. The process of reproduction of microspores commonly known as binary fission was highly prevalent in the smears demonstrating the conversion of mature sporonts into sporoblasts. Oval young spores were found scattered in the homogenate. However, shape of spores does not confirm the species of the genus Nosema especially when there is a mixed infection and can also confuse between *N.apis* and *N.ceranae.* With the help of SEM, the morphology of the spores was principally disclosed. Due to heavy spore load the micrograph was able to show the reproductive phase of spores. The spore appeared sculptured with slight constriction in the mid, which denotes the presence of anterior polaroplast (AP) and release of sporoplasm inside the host cell. Spore was found properly anchored into the gut. The scanning electron micrograph revealed the size of the spore isolated from the homogenates of western honey bee as 7.04 µm X 4.83µm.

Further investigation of microsporidian infection was carried out bumble bees captured from various regions of Kashmir (India). Out of the total of 744 bumble bees collected 670 bees were found to be highly infected with the parasite providing the infection percentage to be 90.05%. Early detection of the spores was carried out by homogenisation, filtration and then purification using Percoll ®. Light microscopy revealed the spore shape to be ovo-elliptical. The light microscopy showed moderate infection rate in bumble bees' homogenates. Mean spore count came out to be 4.5 X 10^6 spores/ml thus moderate infection was seen. No stages of merogony, sporogony or post infection deformities were seen under bright field microscope. Giemsa smears

revealed many mature spores. The stages of merogony particularly the binucleated stage was observed. Matured sporonts post infecting the host tissue release sporoplasm were also seen enclosed in a thin and simple plasma membrane. Spores undergone injury due to mechanical crushing or improper development were also observed (deformed spores). Microsporidian Giemsa staining provides contrast coloration to show infection in tissue preparations; however, spore contents staining obscures the nucleus. Since the infection level came out to be less than that of honey bees, the process of filtration was also performed before the staining. With the help of SEM, a single mature spore was seen adhered to the epithelial lining of the bee. The size of the spore isolated from bumble bee homogenate is 3.86 µm X 2.21 µm.

LM and SEM revealed adequate morphological and structural characteristics of microsporidian spores in this study. The increased prevalence and variety of reports of these parasites call for additional investigation.

The fourth chapter of the thesis deals with the confirmation of the microsporidia infecting honey bees and bumble bees. Molecular techniques like PCR and Real-Time PCR were employed to test the occurrence of microsporidian pathogens. SDS based genomic DNA extraction was performed. DNA was quantified and purity was checked at OD $_{260/280}$ nm ratio using Nanodrop 2000 spectrophotometer.

Primer pair 321 APIS f/r was used in the case of HB (honey bee) isolated DNA and self-designed primer pair *N.bombycis* f/r was used in the case of BB (bumble bee) isolated DNA for species identification, by means of PCR and Real-Time PCR. PCR products obtained after conventional PCR method were allowed to run on 1.8 % agarose gel at 80 V with 50 base pair DNA ladder. Lustrous bands

depicting successful amplification were recorded on Gel doc system. The bands obtained in HB and BB PCR product samples corresponded in the ladder range between 250 bp to 300 bp. The sequence generated after HB PCR product analysis came out to be of 313 bp and BB PCR product of 295 bp. A Real-Time PCR was also conducted to confirm the infection in bees. Threshold cycles (Ct) value for *Nosema bombycis* (NB)= 22, and Ct value for *Nosema apis* (NA)=23 were recorded when calculated in Ariyamax software.

The amplified sequences were sequenced, cloned, and analysed. DOZNA has been identified as the microsporidian pathogen of western honey bee (*Apis mellifera)* prevailing in Lucknow region. Based on the arrangement and structure of the rRNA genes, it is determined that DOZNA belongs to the real Nosema group and has the greatest resemblance to *Nosema apis.* The obtained sequences were blasted with extremely comparable sequences in NCBI nucleotide BLAST. The amplified product sequence from a microsporidian-infected honey bee demonstrated 99.68% homology with *Nosema apis* strain with accession numbers X73894, KM242588, KM242587, and 95.18% similarity with *Nosema neumanii* accession number MF882996. *Nosema apis* was never reported earlier from honey bees of Lucknow region so this study confirms the first detection of *N.apis* spores in honey bees of Lucknow. Similarily, DOZNB has been identified as the microsporidian pathogen of bumble bee (*Bombus ternarius)* prevailing in Kashmir. Results throw light on the fact that DOZNB reveals 100 % homology with *Nosema bombycis* with an accession number JF443700. Moreover, it also showed 99.66% similarity with *Nosema fumifuranae* with accession numbers HQ457432, EU219083, and MN861969. *Nosema bombycis* has never been reported infecting bumble bees before and therefore the current report is the first report of bumble bees having *Nosema bombycis* as pathogen. The generated sequence

of amplified PCR product of microsporidian infected Honey bee, and Bumble bee were submitted to GenBank with accession numbers- OP586620 for *Nosema apis* (NA) and OP586756 for *Nosema bombycis* (NB).

The fifth chapter focuses on the relevance and prospects for the research on microsporidia that's been reported, as well as the necessity of light microscopic observations for deciphering the structure and life cycle of microsporidia of the genus Nosema into a three-dimensional, intelligible micro-structural depiction of subcellular host-pathogen interactions. It also explains about the future opportunities that can help in controlling this hazard by using plant-based products in apiaries and not chemicals.