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Testing the Presence of an Opportunistic Pathogen *Nosema ceranae* (Microsporidia) in Housefly (*Musca domestica)* using Polymerase Chain Reaction

Jaiswal Kamal, Mishra Suman, Kumari Prachi, Singh Amit and Sharma Saumya*

Department of Zoology, Babasaheb Bhimrao Ambedkar University, Lucknow, Uttar Pradesh, India

**Corresponding Author*

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Abstract: Houseflies, being vector of serious human disease, have been extensively used in the laboratory to research on aging and sex determination. It also plays an important role in breaking down the organic matter and thus makes its contribution in food cycle. Houseflies belong to the phylum Arthropoda and serves as vector for many pathogens transmiting disease from lower to higher vertebrates. Microsporidiosis (also known as nosemosis) is a disorder of honeybee and various other pollinators belonging to the class Insecta that are affecting nation's economy up to a great extent. Microsporidian spores have reportedly infected insects, fishes, crustaceous, human and thus causing huge threat to society. In order to establish a clear understanding for its role in the transmission of microsporidiosis, the present study was designed to detect and identify a microsporidian parasite- *Nosema ceranae* by amplifying 16sRNA (Small Subunit rRNA) gene using molecules technique Polymerase Chain Reaction (PCR). Results of the present study suggested no existence of *Nosema ceranae* spores as, in spite of performing gradient PCR, no amplification of gene occurred. The research work paves a way for other researchers to investigate the possible reasons behind the absence of *Nosema ceranae* infection in housefly and also on why the infection is not prevalent in this model organism.

Keywords: *Musca domestica*, Microsporidia, *Nosema ceranae,* Arthropoda, Vectors, Pathogen, Polymerase Chain Reaction, Molecular characterization

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Introduction

Parasitism is an obligate non-mutual symbiotic relationship between two organisms in which one organism, the parasite utilizes the other, the host as habitat, diverting the resources for their development, multiplication and existence, getting benefits at the expense with no harms to the host (Roberts *et al*., 2000).There are two categories of parasites affecting living organisms: ectoparasites (living on external surface of host) and endoparasites (living inside the body of host) (NAMRC, 2010). The characterization between the host and parasite relationship can be featured as

to express in the quantitative terms which can be manipulated to give a huge comprehension of this relationship (Crofton *et al*., 1971). There are cycles of host–parasite, adaptations–counter adaptations that define the so-called co-evolutionary process, and are one of the most prominent characteristics of host–parasite interactions (Sorci and Garnier, 2019). All the pathogens have a mechanism to infect their host and parasitism may make hosts more susceptible to predation (Odell and Godwin, 1984). Out of many pathogens, the most pervasive pathogen are microsporidia, the important agricultural parasites that infect the species of arthropoda like honeybee and silkworm (Fadhilah *et al*., 2021). Microsporidia refers to obligate intracellular parasites that are partially eukaryotic, characterized by acute reduction or even absence of typical type of eukaryotic cellular components and possess (Corradi *et al*.,2009). Nosemosis is a serious microsporidian disease which infects many species of honey bees and are caused by the spore formation from a unicellular fungi *Nosema ceranae* and *Nosema apis* (Formato *et al.,* 2022). *Nosema ceranae* and *Nosema apis*, are the intracellular microsporidium parasites that infect the honeybee mainly in the epithelial cells of midgut (Forsgren, 2010). Some reports suggest that *Nosema apis* and *Nosema ceranae* are the two species which heavily cause infection to various houseflies (Milbrath *et al*., 2015). Evidences showed that in some specific regions, *Nosema ceranae* is the most deadlier species infecting some species of houseflies (Gisder *et al*., 2013). Houseflies are always found in relation with the activities of human. These are considered as the most serious pests at animal exhibitions worldwide and are referred as a model organism for the studies of insect's olfaction. (Scott *et al*., 2014). The body color of housefly varies from light to grey in color and has chitinous exoskeleton with bilaterally symmetrical body. An adult housefly measure 6-9 mm long. Fly has one pair of membranous true wings and the second pair of wings are modified into a drum appendage having stick-like structure. They have reddish and large compound eyes. Female houseflies are moderately larger than male houseflies having 9 abdominal segments in males (Ahmadu *et al*., 2016). To develop a host parasite relationship, present study was designed to check whether *Nosema* infects housefly *Musca domestica* which tends to be prone to microsporidiosis. All researches that have been conducted earlier suggested to choose *Musca domestica a*s a potent host for *Nosema ceranae.*

Materials and Methods

Musca domestica (housefly) were collected from fish market of Telibagh, Lucknow (UP), India. The collection was done in a specified time interval between 23rd February 2022 to 23rd April 2022 (two months). Insect collecting net was used to trap housefly.

Light Microscopy:

2 g *Musca domestica* (housefly) were crushed by using mortar and pestle. The homogenized sample was obtained and transferred to 2 ml eppendorf tube and centrifuged (R24) for 10 min at 8000g. This process was repeated 10 times for the purification of pellet. The eppendorf tubes were vortexed and left undisturbed. A drop of homogenized sample was kept on a glass slide and presence of spores were observed under the Imaging microscope (Model EVOS XL).

Molecular characterization:

In 1 ml eppendorf tube, 800 µl of lysis buffer was added to homogenate pellet infected with microsporidia and the mixture was vortexed and kept aside for 20 min. After that 50 µl of proteinase K and 30µl of RNAase were added to the solution and left undisturbed for 5 min and the tube was heated at 65 C for 4 h and left undisturbed overnight. On next day, eppendorf tubes were slightly vortexed and centrifuged at 15000 rpm for 3 min to facilitate separation of the different phase of the extraction. In fresh eppendorf tube (1 ml in 5 tubes) supernatant and equal volume of PCI was added and inverted 30 times and centrifuged at 15000 rpm for 10 min. Tubes were kept on ice for 10 min to obtain three layers. The upper layer formed was transferred to a new eppendorf tube. Equal volume of 100%

chilled ethanol and 50 µl of 3M NaOAc was added. Tubes were vortexed to mix. Tubes were incubated at -20 C for 4 h and then centrifuged at 15000 rpm for 30 min. After centrifugation, DNA pellet settled and supernatant was carefully decanted. The obtained pellet was washed with 1 ml 70% ethanol and centrifuged for 10 min at 10000 rpm. The supernatant was again removed and pellet was air dried for 2 h until the smell of ethanol goes away. 50 µl of storage TE buffer was added to the pellet and preserved at -20 C till further use.

Agarose Gel Electrophoresis:

To confirm the presence of extracted genomic DNA, 0.8% agarose powder was weighed, then mixed with 1X TAE (100 ml) buffer and heated at 70 C till the solution turns transparent and then allowed to cool. When the gel reached 60-65 C, 0.5 µl EtBr was added to it. At optimum temperature gel was casted in a tray with a comb and allowed to solidify. Genomic DNA was heated at 65 C for 1 h to loosen the DNA threads.1 µl Bromophenol blue dye + 4 µl genomic DNA were mixed and were loaded into well of electrophoretic unit and was run at 80 V for 20 min. DNA bands were observed by visualizing gel under UV transluminator (Model VL-6.LC).

DNA quantification :

DNA was quantified using spectrophotometer (Model LT 2201) by using the following protocol:

- 1 OD of dsDNA = 50 µg/µl
- 3 µl of DNA was mixed with 2997 µl of distilled water = 3000/3=1000 dilution factor
- Purity was checked by taking ratio of OD by 260/280.
- DNA concentration was calculated by using following formula: standard OD at 260 nm × dilution factor
- DNA concentration = OD at 260nm×50×1000

Polymerase chain reaction:

To characterize the microsporidian spores, the following reported primers (Martin, 2007) were used.

Gradient PCR was carried out (Tables 1, 2) by amplifying Small Subunits 16srRNA gene.

218 MITOC:

FORWARD SEQUENCE: 5' CGGCGACGATGTGATATGAAAATATTAA 3'

BACKWARD SEQUENCE: 5' CCCGGTCATTCTCAAACAAAAAACCG 3'

The DNA samples obtained were allowed to run on 2% agarose gel along with 6 µl of 50 base pairs DNA ladder (G Bioscience 50bp ladder) and product band size was checked under UV transluminator (Model VL-6.LC).

Table 1: PCR setup

Constituents of PCR reaction mixture	Volume in µl
DNA (10 ng/µl)	4.5
DD Water	10.2
Forward Primer (10µM)	1.5
Reverse Primer (µM)	1.5
PCR Mastermix (G Bioscience)	7.3
Total reaction mixture	**25**

Table 2: PCR programme carried out in PCR thermocycler (AB Biosystems)

PROCESS STAGE	**TEMPERATURE (C)**	**TIME (in seconds)**
Initial denaturation	94 C	120s
Final denaturation	94 C	30 s
Annealing	56, 58, 60 C	30 s
Extension	72 C	40 s
Final extension	72º C	600 s
	4	∞

Results and Discussion

The microsporidia, *Nosema* belonging to family-Nosmatidae, is a serious disease causing organism that infects arthropods. The infection results into a heavy damage to the respective host and decreases the overall productivity of an organism by changing its physiological behavior. Many studies have shown the adverse effects of *Nosema* infections on its host like honey bees, silkworms, etc. Present study was designed to verify if there is

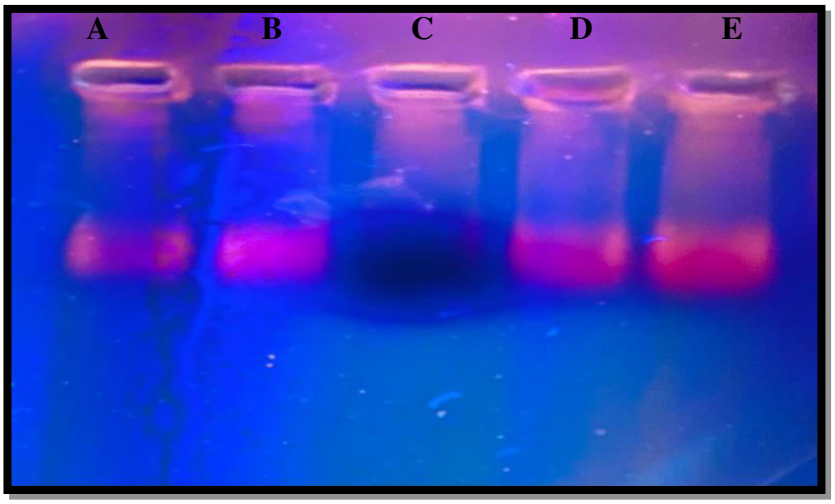

Fig. 1: Lane A-E showing the genomic DNA isolated from housefly using the CTAB based method of genomic DNA extraction protocols. Bands were observed as dark and intensified under UV transilluminators.

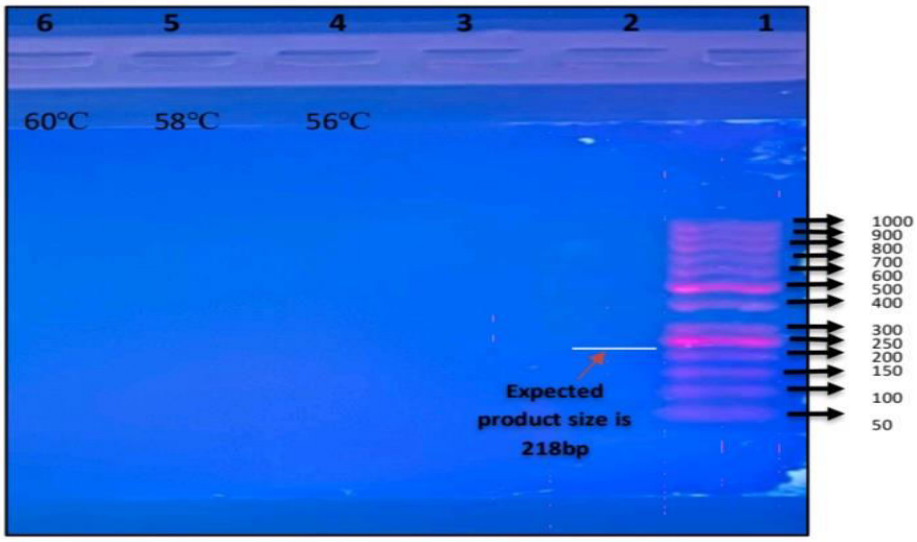

Fig. 2: Right to left: 2 % agarose gel image having Lane – 1 showing 50 base pair ladder ; Lane 2 showing expected PCR product size of 218 bp and Lane 3-6 showing no amplified DNA. Lane 4 showing PCR product run with 56 C annealing temperature, lane 5 with 58 C and lane 6 with 60 C.

any host parasitic inter-relation between housefly and microsporidia. Genomic DNA was extracted from the body of housefly (using CTAB buffer method). Intensified bands were observed while performing the 0.8 % gel electrophoresis (Fig. 1). Extracted DNA was quantified and the optical density was 1.66 which is not exactly 1.8. PCR was run at 56 C, 58 C and 60 C to exactly predict the annealing temperature of primers having 218 bp expected amplicon size. The band of 218 base pairs could have been observed but no such band was observed in PCR product run using PCR set up program depicted by Martin (2007); though ladder was separated very nicely on 2% agarose gel (Fig. 2). A continuous effort to examine the presence of *Nosema* spores was made but no such prevalence was observed in the sample. However, slight infection of spores observed in light micrograph (Fig. 3) now confirms the presence of some other parasite or protozoa

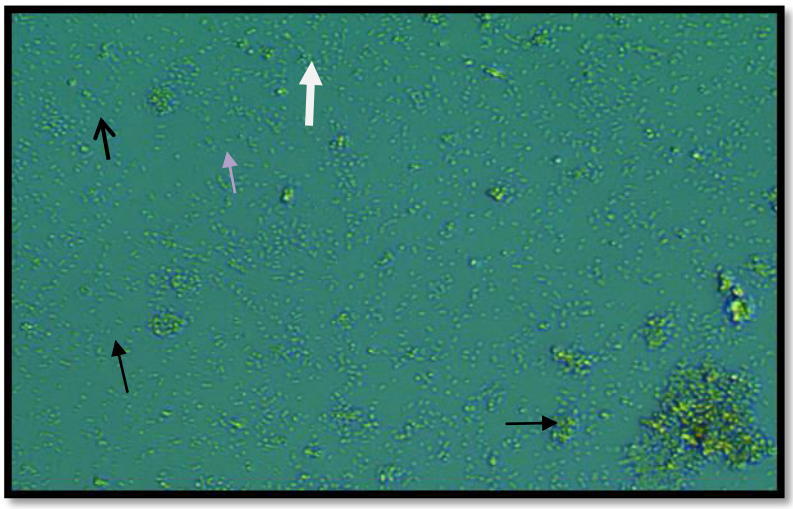

Fig. 3: Light microscopy of homogenized housefly showing very less infection in gut wall spores. Orange arrow showing matured spores. White arrow is showing dividing spores and Black arrow showing amoeboid cysts.

which should be explored.

All organisms are exposed to innumerable parasites either inside or outside the host body. The housefly, *Musca domestica* regarded as vector and are most common insect which carries microorganisms from unhygienic places like garbages, decaying matter, feces and human food. They infect organisms by contaminating their food and eventually transmit several types of diseases. Current studies have found the parasites associated with the group of microsporidia, which have multiple host species that include bumblebees, *Apis mellifera*, *Apis cerana,* grasshopper, silkworm, fishes, crustaceans and many more. Nosemosis is a serious microsporidian disease which infects many species of honeybees and are caused by the spore formation from a unicellular fungi *Nosema ceranae* and *Nosema apis* (Formato *et al*., 2022). Out of many species of *Nosema*, *Nosema ceranae* is found to be deadlier microsporidia infecting the above mentioned species. For example, in case of species of bumblebee, *Nosema* infection not only endangers the population of bumblebee but also shows adverse effects to rest of the domestic pollinator circles and to honeybees (Arbula *et al*., 2015). Thenosemosis type C caused by microsporidium *Nosema ceranae* is considered as the global disease infecting the adult honeybees and have adverse effects on both strength and the productivity of the colonies of honeybees (Botlas *et al*., 2013). Pebrine disease caused by *Nosema bombycis* infects lepidoptera. In the course of infection, various types of secretory proteins are synthesized by *Nosema bombycis* to control the host body (Huang *et al.,*2018). The species of silkworm mainly *Bombyx mori* suffers from microsporidiosis, *Nosema bombycis* causes infection that ranges from persisting to death resulting in great loss to the sericulture industries as well as decreasing the overall production of silkworms. The intensity of pathogen where the host organisms have high or low risk of infection, generally shows significant symptoms. Likewise, microsporidial parasite have different rate of exponential growth on their suitable host and have different forms of pathogenicity (Bhat *et al.,* 2009). From the recent studies, it has been reported that the presence of *Nosema* in HIV-AIDS patients have been found to suppress the pathogenicity of HIV. Keeping in the view, the current work was designed and performed on housefly. However, the parasite was not prevalent but new researchers may investigate on other *Nosema* strains. There are still possibilities of *Nosema* infection in housefly but species may be different which could be infecting them. The broad and explanatory study on the prevalence of *N.*

ceranae in housefly, host parasite interaction and the insect epidemiology should be the prospective of research and study in future.

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