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To investigate the occurrence of an entomopathogen microsporidia in cultured colony of fruit flies (*Drosophila melanogaster*) using PCR

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Abstract

Drosophila melanogaster is a genetically, economically and a scientifically chosen insect which serves as a model organism in various biomedical studies. From the last 100 years, the low cost, rapid multiplication and less maintenance have made the fly indispensable for scientific as well as genetic researches. Present study was designed to characterize the role of fruit fly as a 'host' in host parasitic relationship. Since microsporidia has shown devastating effects on wide range of invertebrates as well as vertebrates, *Nosema ceranae* belonging to genus *Nosema* was chosen to study the infestation status in *Drosophila melanogaster*. The presence of very low microsporidian spores counts in the light microscopy urged to characterize the 16sr RNA gene sequence (small subunit) of *Nosema ceranae* as to be sure with no prevalence of the *Nosema ceranae* in *Drosophila melanogaster* in spite of knowing that fruit flies belong to phylum arthropoda- a choice phylum of *Nosema ceranae* infection. Molecular characterization was performed using gradient PCR. Results suggests that there was no infection of *Nosema ceranae* in fruit flies but provides insights for future studies to work on all the possible aspects and reasons which enable flies to not to get infected with *Nosema ceranae* spores.

Parasitism is a type of relationship between two organisms or different species, where one organism is a parasite that lives on or inside another organism (host) causing it harm, adapted structurally to this way of life which is a close relationship in nature[14]. Wilson[22] had characterized parasites as the predators that eat prey in units of less than one and

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contemplated that parasite belongs to diverse categories like protozoans, hookworms, lice, mosquito, fungi etc.

Parasites when present inside the host reduce the fitness of the organisms which could be due to parasitic castration to behavior modification of the host; the main function of the parasite is to survive within or on the host and increase its own fitness. Parasites interact with many species and act as vectors[18]. Classification strategies vary with interaction between host and parasite. The types may include ectoparasites that lives outside the host surface and endoparasite that live inside the host; meso-parasites enter the opening of hosts but remain partially embedded[19], also microparasites complete life cycle inside the host whereas macroparasites completes life cycle outside the host[15]. Multiple ways exist for parasitic invasions. Out of many parasites, one important parasitic group is microsporidia[4]. The group belongs to sister fungus group and earlier it was regarded as a protozoan[16]. After examining the gene expressions and protein analysis a separate phylum is formed in which 1200 species are kept divided into 144 genera[21]. Out of 1200 species one microsporidian species belonging to the genus *Nosema* shows effects on a huge range of organisms belonging to invertebrates and vertebrates. *Nosema ceranae* has caused devastating effects on honey bees in past 15 years since it was discovered[5]. *N.ceranae* is an obligatory intracellular parasite of *Apis mellifera*[8] and a main cause of colony decline of benefitting pollinators *i.e.* honey bees. A major disease colony collapse disorder shook the whole world when the whole colony of honey bees died within few days[13]. *Drosophila melanogaster* (commonly known as fruit fly) belonging to the phylum arthropoda is an economically and scientifically important insect (animal model) to study human diseases[9] and has contributed a lot in the discovery of many different principles. It has played a significant role in the study of genetics. The chromosomal theory of inheritance was proposed by T.H. Morgan in 1902, was a result of his continuous efforts on experimenting on *Drosophila melanogaster*. Later the research continued on the same animal model and a concept of genetic map came into light by Sturtevant. There are various characters due to which the insect is considered best suited for researches in genetics and molecular biology, some of which are: -

- It multiplies very quickly in captivity
- It has a short life span
- And also, very easy to culture as seeks no or minimal care. These traits make the fly an ideal organism to be used in scientific research.

Study area :

Drosophila melanogaster (fruit flies) were collected from fruit market of Rajnikhand, Lucknow (UP). The collection was done in a very specified time interval between 23rd February 2022 to 23 rd April 2022 (two months). Georgette cloth was also used to trap fruit flies. Later on, the flies were brought into a separate chamber having rotten bananas and seasonal fruits. *Drosophila melanogaster* were cultured in the Parasitology laboratory, Departmentry of Zoology at BBAU, Lucknow using some rotten and fresh fruits. Fresh bananas, grapes, papaya and rotten strawberries were used to

culture the fruit flies properly. For study purpose fruit flies were captured and kept in 70% ethanol. Box was kept in a shady place till culturing of flies.

Light Microscopy :

One gm *Drosophila melanogaster* (Fruit fly) were weighed with weighing balance (Model - CB220) and were crushed using mortar and pestle. Homogenized sample was transferred to 1 ml eppendorf tube and centrifuged (Model R24) at 8000g for 5 minutes. Process was repeated 5 times to purify the pellet. Tubes were vortexed and left undisturbed. A drop of homogenized sample was kept on a glass slide and presence of spores was examined under the Imaging microscope (Model EVOS XL).

Molecular characterization :

1000 µL of lysis buffer was added to homogenate pellet infected with microsporidia in 1 ml eppendorf tube. Mixture vortexed and kept aside for 15 minutes. Then 50 µL of proteinase K and 25µL of RNAase were added to the solution and left undisturbed for 5 minutes. The tube was heated at 65ºC for 4 hours and left undisturbed overnight. On next day, eppendorf tubes were slightly vortexed and centrifuged at 14000 rpm for 2 minutes. Supernatant was transferred to a fresh eppendorf tube (1 ml in 5 tubes).After this equal volume of PCI was added to the tube. Tubes were inverted 20 times and centrifuged 14000 rpm for 15 minutes. Tubes were kept on ice for 5 minutes. The upper layer formed was transferred to a new eppendorf tube. Equal volume of 100% chilled ethanol and 50µL of 3M NaOAc was added. Tubes were vortexed to mix. Tubes were incubated at -20ºC for 4 hours. After that it was centrifuged at 14000 rpm for 30 minutes. DNA pellet settled and supernatant was carefully decanted. Pellet was washed with 1 mL 70% ethanol and centrifuged at 10000 rpm for 5 minutes. The supernatant was again removed and pellet was air dried for 2 hours until the smell of ethanol goes away. 50 µL of storage TE buffer was added to the pellet and preserved at -20ºC till further use.

Agarose gel electrophoresis :

To confirm the presence of extracted genomic DNA, 0.8% agarose Gel Electrophoresis was performed. 0.8% of agarose powder was weighed using weighing balance at Department of Zoology (DOZ), BBAU. Agarose powder was then mixed with 1X TAE (100 ml) buffer, beaker was heated at 70ºC till the solution turns transparent and was then allowed to cool. When the gel reached 60-65ºC, 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 65ºC for 1 hour water bath to loosen the DNA threads. 1 µL Bromophenolblue dye + 4 µL genomic DNA were mixed using a pipette were loaded into well of gel in the electro-phoretic unit. Unit was run at 75 V for 30 minutes. 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 = 50µg/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 by amplifying Small Subunits 16srRNA gene.

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Forward sequence: 5' CGGCGACGATGTGATATGAAAATATTAA 3'
Backward sequence: 5' CCCGGTCATTCTCAAACAAAAAACCG 3'

Table-1. PCR setup

Constituents of PCR	Volume
Reaction Mixture	in µL
DNA(10 ng/µl)	2.0 µL
DD Water	9.5µL
Forward Primer(10µM)	0.5µL
Reverse Primer(µM)	0.5µL
PCR Mastermix (G Bioscience)	12.5µL
Total reaction mixture	25µL

Table-2. PCR programme carried out in PCR thermo cycler (Model AB Biosystems.)

Procvess stage	Temperature (℃)	Time (in seconds)
Initial denaturation	94℃	120s
Final denaturation	94℃	30 s
Annealing	56,58,60℃	30 s
Extension	72℃	40 s
Final extension	72℃	600 s
	4	∞

Number of cycles = 30

The DNA samples that were amplified by given primers set were allowed to run on 1.8% agarose gel along with 6µL of 50 base pairs DNA ladder (G Bioscience 50bp ladder) and product band size was checked under UV transluminator (Model VL-6.LC). Images were captured.

Results

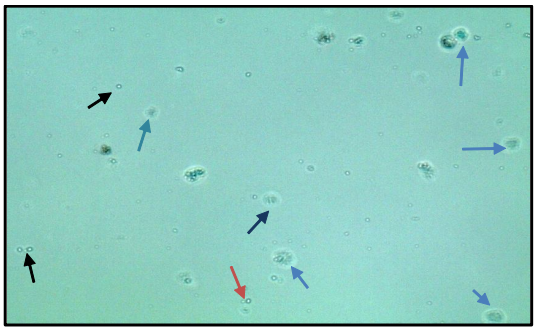

FIG 1: Light micrograph of homogenized fruit flies showing microsporidian like organisms in gut wall. Black arrows showing matured spores, red arrow showing dividing spores and blue arrows showing amoeboid cyst.

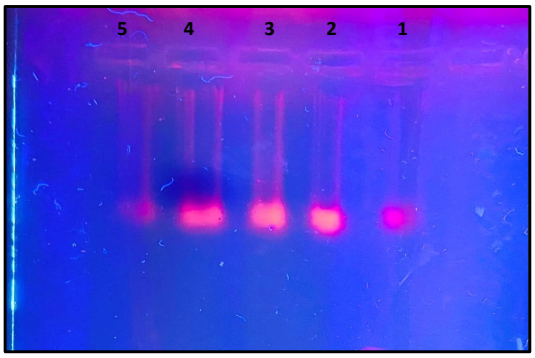

FIG 2: Right to left : Lane 1-5 showing the genomic DNA isolated from fruit fly using the CTAB based method of genomic DNA extraction protocols. Bands were observed as dark and intensified under UV transluminator.

Table-3. DNA quantification by spectrophotometer, purity check by O.D 260/280

Sample	Absorbance at 260 nm	Absorbance at 280 nm	Ratio 260/280	DNA concentration
Drosophila melanogaster	0.15nm	0.09nm	1.66	7500µg/ml

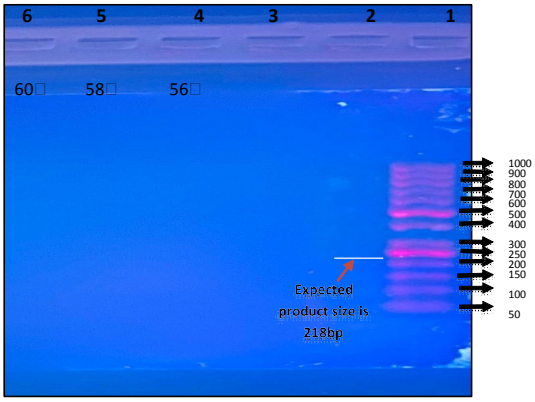

FIG. 3: Right to left : 1.8 % agarose gel image having Lane – 1 showing 50 base pair ladder ; Lane 2 showing expected PCR product size of 218 bp and Lane 3-6 showing no amplified DNA. Lane 4 showing PCR product run with 56ºC annealing temperature, lane 5 with 58ºC and lane 6 with 60ºC.

Microsporidian spores show infection in variety of insects belonging to various orders. Their parasitic behavior establishes a strong host-parasite relationship. As these parasites are obligatory and intracellular in nature, they always require host to complete the life cycle and thus can never be cultured in any medium in a laboratory. Present study was designed to verify if there is any host parasitic inter relation between Drosophila and microsporidia. Following observations have been made while perform the research work.

It was observed that there were spores like organisms present in the fruit flies but the number was very less and spores were present in scattered manner (Fig. 1). Clustered microorganisms signified presence of less infection which could be from bacteria, fungi or some protozoan. At few places some of the amoeboid structures were seen too which could also show some form of infection.

Genomic DNA was isolated from the body of fruit fly using CTAB buffer method and DNA was run on 0.8 % agarose gel. Very bright intensified bands of DNA were observed as in figure- 2. with slight contamination of RNA and proteins. Table-3 shows the absorbance of DNA at 260 and 280 nm. The optical density of the sample came out to be 1.66 which is not exactly 1.8 (pure DNA). After preparing the reaction mixture, gradient PCR was run at 56ºC, 58ºC ,60ºC annealing temperatures to exactly predict the annealing temperature of primers having 218 bp expected amplicon size (Fig- 3). **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 1.8% agarose gel (Fig-3)**. 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 now confirms the presence of some other parasite or protozoa which should be further

examined by new researchers.

A lot of researchers have found with a lot of supporting evidence that *Nosema* infects variety of arthropods like honey bees, bugs, crustaceans etc. Nosemosis is a disease caused by *N.apis* and *N.ceranae* which affects honey bees at their adult stages where it starts to affect adversely by increasing death rate or reducing life span of the host; and also puts negative effects on colony strength. Botias *et al.,*[1] found that *N.ceranae* is highly infectious in nature causing significant reductions in honey production or population size. This causes changes in pollination patterns. Hatcher *et al.,*[6] studied *Nosema granulosis* infecting crustaceans which were vertically transmitted. This transmission was seen in multiple generations. *Nosema* infections are devastating to many members of arthropoda and it was irrespective of the fact that every organism may have different immunity and response generated against the infection caused by *Nosema*. Infections have shown their effects and strength of infection varies with the hosts. *Nosema algerae* also known as *A.algerae*[12] mostly infects mosquitoes and also acts as a human pathogen which causes myositis. Another strain of *Nosema* is *Nosema locustae*[20] that affects grasshoppers, crickets, caterpillars and kills them after the parasitic invasion.

It was also found that parasite successfully established inside the host irrespective of the location. Many other scientists who worked on Nosema and hosts (belonging to arthropods) like Carton and Nappi[2], Lindquist[11], Sorrentio[17], Keebaugh and Schlenke[10] suggested that research work related to *N.ceranae* can be performed using other host like *Drosophila melanogaster* since it also belongs to the phylum arthropoda and bears unique characters for scientific studies. Keeping in the view, the current work was designed and performed on fruit flies. Though *Drosophila* is a member of phylum arthropoda where *Nosema* infection is very common, no infection of *Nosema ceranae* was reported which points to the possible developed immunity present in *Drosophila* playing an important role in protection from microsporidian infection but this requires work to be performed at a higher level with proper molecular diagnostic techniques. Hoffman[7] found with the help of molecular diagnostic techniques that *Drosophila* can sense the infection and can also differentiate between the type of infection as well as the causal microorganism behind it. It was observed that genes which were taking part in the immune system of fruit flies were much similar to the genes concerned with innate immune defences of the mammals. The fact that mammals have developed immune system than other lower organisms and the result showing similarity of *Drosophila* immune response similar to mammals can illustrate the reason to why *Nosema* infection was not found in *Drosophila* and could be a topic for research in future. Although *Drosophila melanogaster* does not have acquired immunity rather it shows cell mediated innate immunity. This can pave a new way for upcoming researchers to find out about the host parasite relations concerned with *Drosophila* and *Nosema.* Not only in arthropoda but microsporidia has also shown its effects on mammalian group (humans). Didier and

Weiss[3] found out about the significance of microsporidians which can cause infections in AIDS patients, immuno-compromised patients or non-HIV populations. This may be a pioneer research work in future relating microsporidia and human interaction and possible diagnostic techniques which may help humans to counter the worse effects of *Nosema* pathogen. However, the parasite was not prevalent in *Drosophila melanogaster* but new researchers may investigate on other *Nosema* strains. There are still possibilities of *Nosema* infection in fruit flies but species (strain of Nosema) may be different which could infect them.

References :

1. Botías e*t al*., (2008). *Environmental microbiology, 10*(10): 2659-2669.
2. Carton; A.J. Nappi (1997). Drosophila cellular immunity against parasitoids., *13*(6): 218–227.
3. Didier, Elizabeth S., and Louis M. Weiss (2011). *Current opinion in infectious diseases 24*(5): 490.
4. Franzen, Caspar (2004). *Trends in parasitology 20*(6) : 275-279.
5. Fries *et.al*., (2010) *Veterinary parasitology* 170.3-4 : 212-217.
6. Hatcher, Melanie J., Jon C. Hogg, and Alison M. Dunn (2005). *International Journal for Parasitology 35*(3): 265-274.
7. Hoffmann, Jules A (2003). Nature 426(6962): 33-38.
8. Huang, Wei-Fone, *et al*. (2007) *Apidologie 38*(1): 30-37.
9. Jennings, Barbara H. (May 2011). *Materials Today. 14* (5): 190–195.
10. Keebaugh, Erin S., Schlenke and A. Todd (2014). *42*(1): 111–123.
11. Lindquist, K., O. Terenius and I. Faye (2004). *EMBO Reports, 5*(2): 207–212.
12. Monaghan SR, *et al*., (Feb 2011). *In vitro Cell Dev Biol Anim. 47* (2): 104–13.
13. Oldroyd, Benjamin P (2007). *PLoS biology 5*(6) : e168.
14. Poulin, Robert (2007). Evolutionary Ecology of Parasites. Princeton University Press. ISBN 978-0-691-12085-0.
15. Poulin, Robert (2011). D. Rollinson and S. I. Hay (eds.). The Many Roads to Parasitism: A Tale of Convergence. Advances in Parasitology. Vol. 74. Academic Press. pp. 27–28.
16. Silar, Philippe (2016). Protistes Eucaryotes: Origine, Evolution et Biologie des Microbes Eucaryotes. HAL. p. 462. ISBN 978-2-9555841-0-1.
17. Sorrentino; Yves Carton and Shubha Govind (2002). Cellular Immune Response to Parasite Infection in the Drosophila Lymph Gland Is Developmentally Regulated., *243*(1): 0–80.
18. Suzuki, Sayaki U., and Sasaki, Akira (2019). *The American Naturalist. 194*(1): 90–103.
19. Vecchione, Anna; Aznar, and Francisco Javier (2008). *Journal of Marine Animals and Their Ecology. 7* (1): 4–11. Archived from the original (PDF) on 10 April 2018. Retrieved 11 April 2018.
20. Vega, Fernando E., Kaya, Harry K., eds. (2012). Insect Pathology (2nd ed.). Academic Press. p. 5.
21. Weiss *et al*., (1999), The microsporidia

and microsporidiosis : 196-224.

22. Wilson, Edward O. (2014). The Meaning of Human Existence. W.W.Norton & Company.. Parasites, in a phrase, are predators that eat prey in units of less than one. Tolerable parasites are those that have evolved to ensure their own survival and reproduction but at the same time with minimum pain and cost to the host.