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Research article

IMPACT OF A NEW MICROSPORIDIAN INFECTION ON LARVAL AND COCOON PARAMETERS OF THE SILKWORM, *BOMBYX MORI* L.

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ABSTRACT: Microsporidiosis is an important disease of silkworm caused by pathogenic microbes belonging to Phylum microsporidia. Various workers have isolated a number of microsporidia from silkworm. Recently, a new microsporidian was isolated from the silkworm by the authors of the present study. The microsporidian spores were studied for their morphology, pathogenicity and the transmission. These characters of the new microsporidian were compared with that of *Nosema bombycis* spores, a standard strain causing Pebrine disease in silkworm. The purified spores isolated from the silkworm were ovo-cylindrical in shape and measured 4.03 ± 0.24 µm in length and 2.30 ± 0.14 µm in width with 1: 0.57 length width ratio. In contrast, the spores of *Nosema bombycis* were oval in shape and were 3.80 ± 0.01µm in length, 2.60 ± 0.01µm in width and 1: 0.68 length width ratio. At the inoculation doses of 1 $\times 10^3$ and 1×10^4 spore/ml, the infection rate in the moths was found to be 3.00 and 8.67 % with new microsporidian and *N. bombycis* respectively. At subsequent high inoculation doses 29.00 to 63.00 % infection at moth stage was recorded. The microsporidian has resulted in low larval and pupal mortality but remarkably high infection percentage in moth stage at 1×10^5 and 1×10^6 spore/ml inoculation doses. Studies also indicated that infection by the new microsporidian in mother moth did not impact the larval health and cocoon parameters in the next generation. In case of *Nosema bombycis*, cocoon parameters were significantly affected as the inoculum dose increased from 1 $\times 10^3$ to 1 $\times 10^6$ spores/ml. The larvae hatched from the eggs laid by *Nosema bombycis* infected moths (20 and above spores/field) did not survive up to cocooning. The results have been discussed in light of the studies carried out.

Key words: Cocoon parameters, new microsporidian, *Nosema bombycis*, Silkworm, *Bombyx mori* L.

INTRODUCTION

Pebrine is the deadliest of all silkworm diseases. The most common microsporidian routinely encountered to cause this disease is *Nosema bombycis* [1]. However, there are many other microsporidia, which are encountered from time to time in silkworm. While involving in the general monitoring of pebrine in one of the Basic Seed Farm in Karnataka, India, authors came across the presence of microsporidian spores, which were distinctly different from the known strain, *Nosema bombycis* in shape and size. This difference was clearly visible at a magnification of 600X. It was also noticed during the 3 years of observations, that *Nosema bombycis* was not seen in these crops. Earlier, a few other microsporidia were also reported to cause disease symptoms in varying degrees other than the *Nosema bombycis* [2]. Reports are also available on the cross infectivity of these microsporidia from insects to silkworm [3,4,5]. Different researchers have studied their morphological characters, pathogenicity, serological affinity and ultra structure. Recently, Central Sericultural Research and Training Institute (CSR&TI), Mysore has isolated few microsporidian spores, which are different from the standard *Nosema bombycis* from silkworm, and studied their morphology and carried out preliminary studies on pathogenicity and transmission rate. Keeping in view of the recurring occurrence of the same microsporidian in Basic Seed Farm, it was felt necessary to conduct systematic and comprehensive study of microsporidiosis caused by the isolated microsporidian.

The characterization is also crucial for determination of the identity of the microsporidian infecting the silkworm, *Bombyx mori* L. This will also add to the existing knowledge on the microsporidiosis in silkworm and will help in devising suitable strategies for the management of microsporidian diseases caused by specific microsporidia. In the present study, observations are recorded on the morphology, pathogenicity and the impact of new microsporidian infection on the larval and cocoon parameters of silkworm the results are discussed with a view to its possible management strategies.

MATERIAL AND METHODS

The popular bivoltine breed, CSR2 was selected to the study the impact of new microsporidian infection on silkworm and the cocoon characters. The layings of the selected breed were received from the Germplasm Bank of CSR&TI, Mysore. The larvae were reared following standard method of rearing under hygienic conditions till the beginning of the III instar.

Isolation and purification of New microsporidian spores from silkworm: After the detection of infection during routine moth/larval testing at P3 Basic Seed Farm, Mysore, the homogenate containing spores of new microsporidian was collected and filtered using wet layer of absorbent cotton and subsequently centrifuged at 3,000 rpm for 5 minutes. The centrifugation process was repeated 2-3 times by adding distilled water to the sediment. The sediment obtained was suspended in minimal volume of distilled water and subjected to percoll gradient centrifugation [6] and pure inoculum of microsporidian was obtained. The sediment was suspended in distilled water and thoroughly mixed on cyclomixer thus giving the stock suspension of microsporidian spores. To prepare the inoculum of required concentration, stock inoculum was suitably diluted and quantified to estimate the spore concentration following standard haemocytometer count method [7]. The desired concentration of suspension was obtained from the stock suspension of known spore concentration by serial dilution.

Morphological Characterization of New microsporidian spores: Purified spores of microsporidian were subjected to morphological characterization following the standard method [2] and compared with the spores of the known strain, *Nosema bombycis.* To determine the spore size, the spores were first immobilized using a drop of mineral oil. Fifty spores of each microsporidian were measured for their length and width following the standard micrometry method [2].

For Scanning electron microscopy, the samples of purified spores were air-dried at room temperature, transferred on to double-stick carbon tape mounted on copper stubs sputter-coated with gold at about 20 nm thickness and observed under a JEOL-100 CX-II electron microscope with an ASID-4D scanning attachment (Tokyo-Japan) at 20 kV. The spores were observed for their shape, size and texture and photographed and compared with the spores of *N. bombycis*.

Impact of microsporidian infection on silkworm and cocoon parameters

Different concentrations (1×10^3, 1×10^4, 1×10^5 and 1×10^6 spores/ml) were prepared from purified spores of microsporidian by serial dilution of the quantified stock inoculum. These concentrations were inoculated to the silkworm by smearing the spore suspension on to the mulberry leaves and fed to silkworms on day zero of III instar (After II moult) larvae. The larvae were allowed to feed on the treated leaves for six hours. Each inoculum concentration formed a treatment. For each treatment, three replications of 100 larvae were maintained. Different sets of larvae were inoculated separately with different concentrations. Another set of larvae inoculated with different concentrations of *N. bombycis* spores was maintained separately for comparison. After six hours, the larvae were fed with normal leaves and reared till cocooning. Observations on the mortality due to the concerned microsporidia were recorded daily. The dead larvae and pupae were homogenized and examined for the specific microsporidian infection under phase contrast microscope. The emerged moths were also individually homogenized and examined under the phase contrast microscope, Nikon (Type - 104) to calculate the percentage of infection at larval/pupal and moth stages. The data on the mature larval weight, single cocoon weight, single shell weight and silk ratio also was recorded and the data was statistically analyzed. The results were compared with control batch (reared without inoculation of any microsporidian pathogen).

Impact of transovarian transmission of new microsporidian infection on silkworm and cocoon parameters

To study the impact of infection through trans-ovarian transmission on silkworm and cocoon characters, the infected mother moths (collected from the earlier experiment from both new microsporidian and *N. bombycis* infection) were individually homogenized and the homogenates were filtered, centrifuged and the sediments were observed under phase contract microscope and recorded the number of spores/field.

Based on the number of spores *viz*., 2, 5, 10 20, 100, 200 and 300 spores/field the layings laid by the concerned female moths were collected and brushed for next generation. The larvae hatched from these trans ovarian transmitted layings were brushed separately and reared under optimum rearing conditions up to the spinning. During the rearing, Observations on the mortality due to the concerned microsporidia were recorded daily. The dead larvae and pupae were homogenized and examined for the specific microsporidian infection under phase contrast microscope. The emerged moths were also individually homogenized and examined under the phase contrast microscope. The data on the 10 mature larval weight, single cocoon weight, single shell weight and shell ratio (S. R%) was also recorded and statistically analyzed. The results were compared with control batch (larvae reared from disease free layings – without microsporidian infection).

RESULTS

The observations on the morphological characteristics of the isolated New microsporidian spores and *Nosema bombycis* spores are presented in Table 1 and Figure 1 and 2. The purified spores isolated from the silkworms reared at P3 Basic Seed Farm, Mysore were ovo-cylindrical in shape and measured 4.03 0.24 m in length and 2.30 0.14 in width with 1: 0.57 length width ratio. In contrast, the spores of *N. bombycis* were oval in shape and were 3.80 0.01 m in length, 2.60 0.01 m in width with 1: 0.68 length width ratio. The impact of the isolated new microsporidian and *N. bombycis* on the larval and cocoon parameters at different concentrations of pathogen inoculation is furnished in Table 2. Result shows that at different inoculation doses of new microsporidian, 10 mature larval weight ranged between 40.83 and 42.23g whereas in a normal control, the larval weight was 42.53 g. These results suggest that the larval weight was not affected by any of the inoculation doses of new microsporidian spores. In case of pupation rate also, there was no significant variation among inoculated batches as well as control batch. The mortality during larva and pupa stages ranged between 0.00 and 4.67 % due to infection with new microsporidia. The cocoon characters *viz*., single cocoon weight, single shell weight and S.R % did not show significant variation.

Table 1. Morphological characterization of new microsporidian and *Nosema bombycis* spores

S. No.	Microsporidian spore	Shape	Length (µm)	Width (µm)	Length width ratio
1	New microsporidian	Ovo-cylindrical	4.03 0.24	2.30 0.14	1: 0.57
2	*Nosema bombycis*	Oval	3.80 0.01	2.60 0.01	1: 0.68

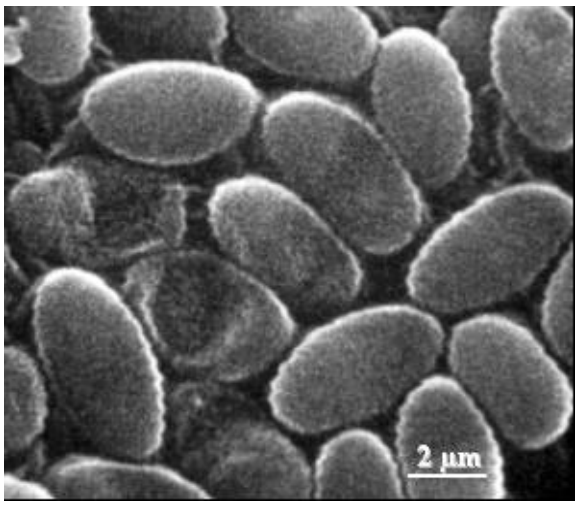

Figure 1.Scanning Electron Micrograph of New microsporidian spores

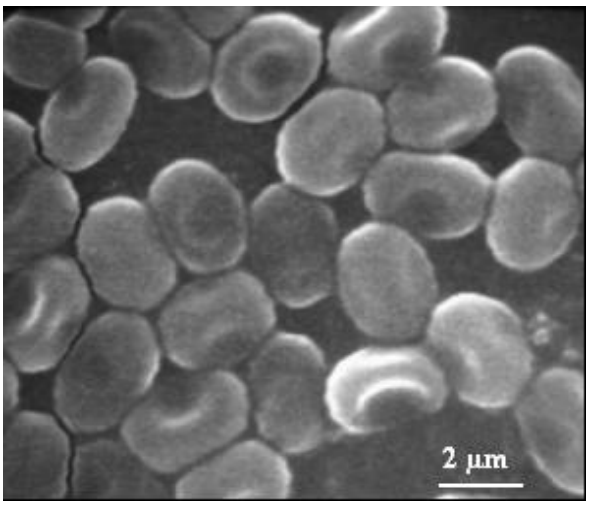

Figure 2.Scanning Electron Micrograph of *Nosema bombycis* spores

The infection levels in moth stage however, recorded significant increase with the increase in inoculation dose. At the inoculation doses of 1×10^3 and 1×10^4 spore/ml, the infection rate in the emerged moth was 3.00 and 8.67 % respectively. At the subsequent high doses (1×10^4 and 1×10^5 spores/ml) of inoculation, the infection at moth stage was as high as 29.00 and 63.00 % respectively. In case of *Nosema bombycis* there was a significant reduction in the larval weight (40.46 to 38.66 g), survival rate (84.33 to 36.60 %), cocoon weight (1.555 to 1.490 g), shell weight (0.326 to 0.288 g) and S.R% (20.97 to 19.75) with the increase in inoculum's dose from 1×10^3 to 1×10^6 spores/ml. The mortality in larval/pupal stages due to *N. bombycis* infection increased from 11.33 to 50.67 % as the inoculum's dose increased from 1×10^3 to 1×10^6 spores/ml. Comparatively very high infection rate (86.00 to 100.00 %) in moth stage was observed as inoculum dose increased from 1×10^3 to 1×10^6 spores/ml of *N. bombycis*.

Table 2. Impact of new microsporidian and *Nosema bombycis* infection on the larval and cocoon parameters of the silkworm, *Bombyx mori L.*

Pathogen dose inoculated (spores/ml)	10 mature larval weight (g)	Pupa-tion (%)	Single cocoon weight (g)	Single shell weight (g)	S.R %	% mortality during larval/pupal stages	% infection at moth stage
New Microsporidian							
1×10^3	41.46	90.33	1.626	0.334	20.456	0.00	3.00
1×10^4	42.23	91.00	1.656	0.337	20.415	0.00	8.67
1×10^5	40.83	91.33	1.647	0.335	20.351	3.67	29.00
1×10^6	41.50	88.66	1.634	0.326	19.969	4.67	63.00
Control	42.53	90.33	1.726	0.345	20.00	0.00	0.00
CD at 5%	NS	NS	0.018	NS	NS	0.66	5.38
Nosema bombycis							
1×10^3	40.46	84.33	1.555	0.326	20.97	11.33	86.00
1×10^4	40.30	71.00	1.545	0.318	20.45	22.33	92.00
1×10^5	39.46	57.00	1.495	0.295	19.75	3733	100.00
1×10^6	38.66	36.60	1.490	0.288	19.34	50.66	100.00
Control	42.13	91.00	1.635	0.329	20.17	0.00	0.00
CD at 5%	0.58	1.62	0.03	0.02	0.79	6.27	4.46

Larval and cocoon parameters of the larvae brushed from transovarialy infected layings with new microsporidian and *Nosema bombycis* with varying intensity of infection in mother moth are furnished in Table 3. In case of new microsporidian infection, no significant impact on the larval and cocoon parameters was noticed in batches reared from moths with spore load from 2 – 300 per field in mother moths. However, at a spore load of 100 and above/field in the moth stage, marginal reduction in ERR% was noticed with higher mortality during larval/pupal stages due to microsporidiosis. With low intensity infection up to 5 spore/field at moth stage, 12.00 % infection at moth stage was noticed in the next generation. At an intensity of 10, 20, 100 and 200 spores/field at moth stage, 14.33, 20.33, 70.33 and 89.00 % infection respectively was recorded in moth stages. At an intensity of 300 spores/field in moths, infection was as high as 92.00 % in the resultant moths of the next generation. In contrast, In case of *N. bombycis*, significant reduction in the larval weight, single cocoon weight, single shell weight and SR% was noticed due to transovarian infection at all intensities of mother moth infection. At 2, 5 and 10 spore/field infection intensity, the larval/pupal mortality in the resultant crop was 12.66, 22.66 and 45 % respectively. At infection intensity of 20 and more spore/field, 100 % mortality was recorded in larval/pupal stage and there was no survival in these batches.

Table 3. Impact of transovarian transmission of new microsporidian and *N. bombycis* infection on the larval and cocoon parameters of the silkworm, *Bombyx mori L.*

S. No.	Intensity of infection in mother moth (spores / field)	10 mature larval weight (g)	Pupa-tion (%)	Single cocoon weight (g)	Single shell weight (g)	SR %	% mortality during larval / pupal stages	% infection at moth stage
New Microsporidian								
1	2	44.53	92.33	1.643	0.362	22.03	0.00	3.67
2	5	44.55	91.66	1.635	0.363	21.86	0.00	12.00
3	10	44.96	91.00	1.631	0.358	21.92	3.67	14.33
4	20	44.38	91.00	1.616	0.356	22.04	4.67	20.33
5	100	43.91	86.00	1.656	0.358	21.63	5.67	70.33
6	200	43.17	88.00	1.640	0.353	21.55	4.67	89.00
7	300	43.40	86.33	1.655	0.356	21.45	6.33	92.00
8	Nil (Control)	44.73	92.00	1.760	0.389	22.10	0.00	0.00
	CD at 5%	NS	3.02	0.06	0.02	NS	1.39	5.88
Nosema bombycis								
1	2	38.46	73.33	1.398	0.283	20.26	12.66	87.33
2	5	38.81	67.33	1.428	0.277	19.42	22.66	100
3	10	37.75	45.66	1.434	0.284	19.78	45.00	100
4	20	NS	NS	NS	NS	NS	100	-
5	100	NS	NS	NS	NS	NS	100	-
6	200	NS	NS	NS	NS	NS	100	-
7	300	NS	NS	NS	NS	NS	100	-
8	Nil (Control)	45.12	91.66	1.619	0.329	20.33	0.00	0.00
	CD at 5%	0.62	3.58	0.024	0.02	0.29	1.69	4.59

NS - No survivor

DISCUSSION

The morphology of the isolated new microsporidian spore clearly indicates that New microsporidian is ovo-cylindrical in shape when compared to the near oval shape of *Nosema bombycis*. Besides, the size of the new microsporidian is also different in terms of its length, width and length-width ratio. It is therefore clear, that based on simple microspcopic observation, the new microsporidian can be easily identified from *N. bombycis*. The observations on the pathogenicity and the impact on larval and cocoon parameters by new microsporidian clearly suggest that it is comparatively less pathogenic to the silkworm, *Bombyx mori* L. when compared to the *N. bombycis*. At a dose of 1×10^6 spores/ml/100 larvae out of II moult, the new micropsorian resulted in only 4.67% mortality due to microsporidian infection during larval and pupal stages when compared to 50.67 % mortality caused by *N. bombycis* at the same inoculation dose. During moth stage, however, the infection with new microsporidian increased many fold and at lower doses of 10^4 and 10^5 spore/ml itself showing 8.67 and 29.00 % infection respectively. In *N. bombycis* infected batches at these doses, the recorded infection was comparatively very high (92.00 and 100 % respectively). This high infection rate at moth stage by both the microsporidian is probably due to longer time available to the pathogen for its multiplication in the host. These results are in conformity with earlier studies [8,9] wherein similar observations have been recorded. However, the susceptibility of insects to microsporidia may vary with different larval instars and occasionally with the different stages *viz.*, larval, pupal and adult stage [10]. Multivoltine silkworm races have been reported to be more tolerant to pebrine than bivoltines [11,12]. Similarly, the study carried out on the mortality during larval/pupal stages and moth level infection in the batches reared from transovarially infected eggs with varying degree of infection in mother moth (2, 5, 10, 20, 100, 200 and 300 spores/field) also indicated that the new microsporidian is less pathogenic even in these transovarial transmitted batches compared to *Nosema bombycis* microsporidian.

It is also clear from the results that the infected batches with new microsporidia through transovarial transmission can record a high survival rate (86.33 to 92.33%) whereas with *N. bombycis* infected batches, there was no survival in batches reared from layings laid by moths with infection intensity of more than 20 spores/field respectively in case of batches.

CONCLUSION AND RECOMMENDATION

The present study assume significance as authors have come across frequent incidence of new microsporidian infection in all stages of silkworm which are different from *N. bombycis.* Another significant observation during the study is that the infection by most virulent standard strain, *N. bombycis* was rarely encountered during the period of this study lasting 3 years. Accordingly, there is no crop loss reported from any sericulture area or Basic Seed Farms due to Pebrine, as the new microsporidian did not significantly affect the crop performance and cocoon characters. Our study also indicate that the constant elimination of the new microsporidian by thorough mother moth examination at each generation and using layings laid by spore-free moths at Basic Seed Farm levels will effectively prevent any economic loss at subsequent levels of seed/commercial rearings as the new microsporidian is less pathogenic and do not cause any adverse impact on the economic parameters of the crop.

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