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Full-text article

INFECTION AND LONG-TERM CULTIVATION OF THE MICROSPORIDIUM *NOSEMA BOMBYCIS* IN SF9 INSECT CELL CULTURE

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The microsporidium *Nosema bombycis* is economically important as a causative agent of silkworm pebrine, and also as a potential biological control agent for lepidopteran pests such as *Dendrolimus sibiricus*. In this study, *N. bombycis* spores were isolated from diseased *Bombyx mori* larvae. After artificial stimulation in 0.1 M KOH, they were inoculated into the Sf9 cell line to initiate continuous proliferation of the parasite. After 7 days of initial infection, fresh Sf9 cells were added, establishing a long-term persistently infected culture. We quantified the percentage of infected cells and the dynamics of spore accumulation during primary infection and long-term cultivation, and determined the limits of cell survival and infectivity in the infected culture. Our studies enabled us to propose key parameters for utilizing this host-parasite system as a model for studying nosematosis and resistance mechanisms, as well as for the mass-production of spores for plant protection.

Keywords: microsporidia, *in vitro*, insect cell line, spore accumulation, infection rate

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Introduction

Microsporidia are a large group of fungi-related spore-forming obligate intracellular parasites (Becnel, Andreadis, 2014). They are widespread in the environment, infecting representatives of almost all large groups of animals. The highest diversity of parasitic species is found among arthropods, most notably insects (Solter et al., 2012; Bjørnson, Oi, 2014). The entire life cycle of microsporidia takes place inside the host cell, except for the spore, through which the infection spreads from one host to another with zoonotic, foodborne and water-borne ways of transmission (Cali, Takvorian, 1999).

Since microsporidia cannot be cultivated outside of the host cell and working with a whole host organism is not always feasible, the field of microsporidia research relies heavily on cell culture techniques. In vitro cultivation is generally focused on species of economic and clinical importance and historically started with *Nosema bombycis* infection of *Bombyx mori* primary ovarian tissue culture (Trager, 1937). With the development of continuous insect cell lines, it has become possible to cultivate many species of microsporidia for extended periods. Among them there is a large group of biological control agents of agricultural pests such as *Paranosema locustae* (Khurad et al., 1991), *Vairimorpha necatrix (*Kurtti et al., 1990), *Nosema furnacalis* (Kurtti et al., 1994) and *Nosema mesnili* (Gupta, 1964). Microsporidia of clinical interest as pathogens of human (e.g. *Encephalitozoon cuniculi, E. hellem, E. intestinalis*, *Vittaforma corneae, Anncaliia algerae,* and *Trachipleistophora hominis)* are succesfully maintained in vitro in various mammalian cell lines (Visvesvara, 2002; Molestina et al., 2014).

Some microsporidia from insects can be grown in mammalian cells including *Tubulinosema ratisbonensis* (Lowman, 2000) and *Anncaliia algerae* (Undeen, 1975). The latter was propagated also in insect and fish cell cultures (Monaghan, 2011; Visvesvara, 2002; Undeen, 1975). In general, heterologous cell lines, differing in origin from the natural hosts, are widely used for *in vitro* cultivation of insect microsporidia. For example, *Paranosema locustae*, a parasite of grasshoppers has been successfully propagated in heterologous fat body cell line from a moth *Mythimna convecta* (Khurad et al., 1991) and honeybee-pathogenic microsporidia *Vairimorpha ceranae* cell culture model was developed using heterologous lepidopteran cell line IPL-LD-65Y, established from the ovaries of the gypsy moth *Lymantria dispar* (Gisder et al., 2011).

The microsporidium *N. bombycis* is a highly destructive pathogen in the sericulture industry, known for causing a fatal disease called pébrine (Hukuhara, 2018). In fact, the history of in vitro studies of microsporidian biology started with this pathogen (Trager, 1937). After the first experiments involving cultivation in primary tissue explants of *Bombyx mori*, it was subsequently maintained in different cell lines. Spodoptera frugiperda Sf21 and Antheraea eucalypti cell lines were first used to study basic biology and life cycle of *N. bombycis* (Kawarabata, Ishihara, 1984; Kawarabata, 2003). Recently, preference has been given to cell lines derived from *Bombyx mori* (like BmE-SWU1 and BmN-SWU1), a natural host of the microsporidium. *Bombyx mori* cell lines provide the most biologically relevant context for studying molecular infection mechanisms and host immune responses (Dong et al., 2021; Yu et al., 2021; Dong et al., 2022).

In this study we established a Sf9 cell line consistently infected with *N. bombycis.* We characterized the main parameters of infection process and long-term cultivation. Sf9 cell line, derived from the fall armyworm *S. frugiperda*, was

chosen due to its availability, ease of cultivating and ability to support propagation of *N. bombycis and other* pathogens (Huang et al., 2018a; Zheng et al., 2021). We quantified the percentage of infected Sf9 cells and the dynamics of parasite spore accumulation during the initial acute phase and long-term cultivation. Furthermore, we assessed the time necessary for the parasite to achieve complete colonization of the host cell culture and subsequent developing of its infectivity. Characterizing these infectious properties of *N. bombycis* within a long-term culture system provides a foundation for establishing microsporidia-resistant cell lines. Such resistance could be achieved through genetic modification using RNA interference, single-chain antibodies, or CRISPR/Cas9 technologies—methods that are currently under investigation for the development of resistant silkworm strains (Huang et al., 2018a; Huang et al., 2018b; Dong et al., 2019).

Also, a persistent cellular infection of this parasite in an insect cell culture can provide mass spore production under sterile conditions for research purposes. Since *N. bombycis* exhibits high virulence against such a dangerous forest defoliator as Siberian moth, *Dendrolimus sibiricus* and infects crop pests *Spodoptera litura* and *Helicoverpa armigera* (Pei et al., 2021; Rumiantseva et al., 2024), the development of a technological method for generation of spore biomass of this parasite may be in demand in plant protection systems.

Materials and methods

Infection of Sf9 cells with Nosema bombycis spores

Sf9 cell line was obtained from ECACC General Collection (ECACC 89070101). Cells were cultured in Sf-900 III serum-free medium (SFM) (Thermo Fisher Scientific, MA, USA) in the adhesive culture at 27 °C and routinely maintained according to the manufacturer's instructions. For infecting with microsporidia spores, we used cells in the mid-log phase growth with viability of over 90 %. Viability of insect cells was estimated in the presence of an equal volume of 0.4 % Trypan Blue solution with Luna II automated cell counter (Logos Biosystems, South Korea).

Nosema bombycis spores were obtained from the Uzbek Research Institute of Sericulture (UzNIISh) in Tashkent, Uzbekistan. Small subunit ribosomal RNA gene sequencing showed 100 % identity to the type isolate of *N. bombycis* (Genbank accession # D85503) (Tokarev et al., 2019). Spores were isolated from infected *B. mori* larvae, purified and treated with antiseptic Multicide (Sante Pharm, Russia) as it was described previously (Dolgikh et al., 2022). To initiate primary cell infection spores were activated with 50 µl of 0.1M KOH water solution for 30 min and added to Sf9 cell suspension in SF-900 III SFM. Polar tube extrusion was observed within 5 min in 90 % of spores. Infection efficiency was ensured by mixing insect cells with parasite spores on an orbital shaker at 50 rpm for 30 min at 27 °C till no more extrusions of polar tubes were observed in a light microscope.

In all experiments, except for the primary infection, the infectious material was not the spores themselves, but a certain volume of the infected cell culture containing the calculated number of *N. bombycis* spores. Spores for most experiments were taken at a ratio of 10 spores per living cell, except for long-term infections and survival experiments, in which additional ratios of 1, 2.5, 5, and 7.5 spores per cell were taken.

For primary and long-term infections 6-well tissue culture treated plates were used with 1 ml suspension of 5×10^5 viable Sf9 cells in SF-900 III SFM in a well. For the survival and infectivity experiments, we used T25 tissue culture-treated flasks seeded with 5×10^6 viable cells, in addition to 6-well plates. Total volume of SFM was 4 ml. For primary infections, cells were detached from the wells of a 6-well plate every day for a week (7 time points) after infection, in other cases only after one week.

The following parameters of the infected culture were used: a number of viable cells, percentage of infected cells and spore accumulation. Volumes of cell suspensions were measured to calculate actual numbers of cells and spores. Concentrations of viable cells were measured using a Luna II automated cell counter (Logos Biosystems, South Korea). To determine the infection rate, smears of infected cultures on glass slides were fixed with absolute methanol and stained with Giemsa's solution. Infected cells were counted under Carl Zeiss AxioImager M1 microscope.

Total number of spores accumulated in the culture (spore accumulation) was quantified, as following: Sf9 cells were detached from the culture surface by pipetting, disrupted by sonication at 50 kHz for 15 sec, and released spores were counted using a hemocytometer. Identification of the spore type and measurement of the size of spores was carried out using phase-contrast and DIC optics of Carl Zeiss AxioImager M1 microscope with an Axiocam 712 mono camera and Zen 3.2 blue edition software.

Immunoblotting and immunofluorescence microscopy

The process of heterologous expression of *N. bombycis* β-tubulin in bacteria, production and purification of polyclonal antibodies against *N. bombycis* β-tubulin, as well as methodology of immunofluorescent and immunoblotting assays were previously described (Senderskiy et al., 2021; Dolgikh et al., 2022).

Statistical analysis

Primary infection experiments were repeated seven times. Long-term infections were performed in 5 replicates and the infectivity experiment in triplicates. Results were provided as mean ± standard deviation.

ANOVA, followed by the Tukey Honestly Significant Difference post hoc test for pairwise comparisons, was used to detect changes between different variants. Statistical significance was set at $p < 0.05$. The number of viable cells in the control and infected cultures was compared using t-test ($p < 0.05$). T-test ($p < 0.05$) was also used to compare spore sizes.

Results

Primary infection of Sf9 cell culture

Sf9 cell culture in 6-well plates with 5×10^5 viable Sf9 cells in each well was initially infected with *N. bombycis* spores that have been artificially activated in a solution of 0.1 M KOH according to Ohshima (Ohshima, 1964). The spore-to-cell ratio was 10:1, as it was found to be optimal for the establishment of microsporidia in cell culture (Trager, 1937). The spread of

infection was studied every day for a week with a reference to control (uninfected) cells.

In the control culture, cell density increased slowly during the first 3 days after seeding. Next 3 days cells proliferated rapidly each day significantly increasing cell density until they reached 100% confluence. Cell density on day 6 did not statistically differ from day 7, which indicated that the cell culture has entered the stationary phase. On day 8 the decreasing of viable cell quantity occurred due to depletion of one or more nutrients (Fig. 1A).

The growth pattern of infected culture resembled the uninfected one. First 3 days there was a slow propagation of cells, then on days 4–5 it accelerated and on day 6 it reached its maximum. A statistically significant difference between the infected and uninfected cell cultures was observed only on day 1 and day 7. In the first case, this can be explained by increased cell mortality at the onset of infection with microsporidia. Subsequently, on day 2 post infection, this effect disappeared, and the number of living cells is restored to a level that was not statistically different from the control cell culture**.** Starting on day 7, a clear divergence between the infected and uninfected cultures was evident, reflecting the impact of parasitic growth on host cell viability (Fig. 1A).

The total spore accumulation remained relatively stable over a week (Fig. 1B). In the first 3 days, the spore count was approximately half of the amount used for infection ($5x10^6$). This is probably due to difficulty of counting empty spore shells. The majority of spores were initially located inside or attached to the surface of Sf9 cells. Only on day 6 post-infection the spore count exceeded the number of spores in initial inoculum. The total increase in spore number on day 7 was only 1.1-fold.

During first 3–4 days after initial infection, the number of infected cells increased, as intracellular stages actively developed, becoming more visible in a microscope (Fig. 1C). The percentage of infected cells (Fig. 1D) peaked on day 3 and

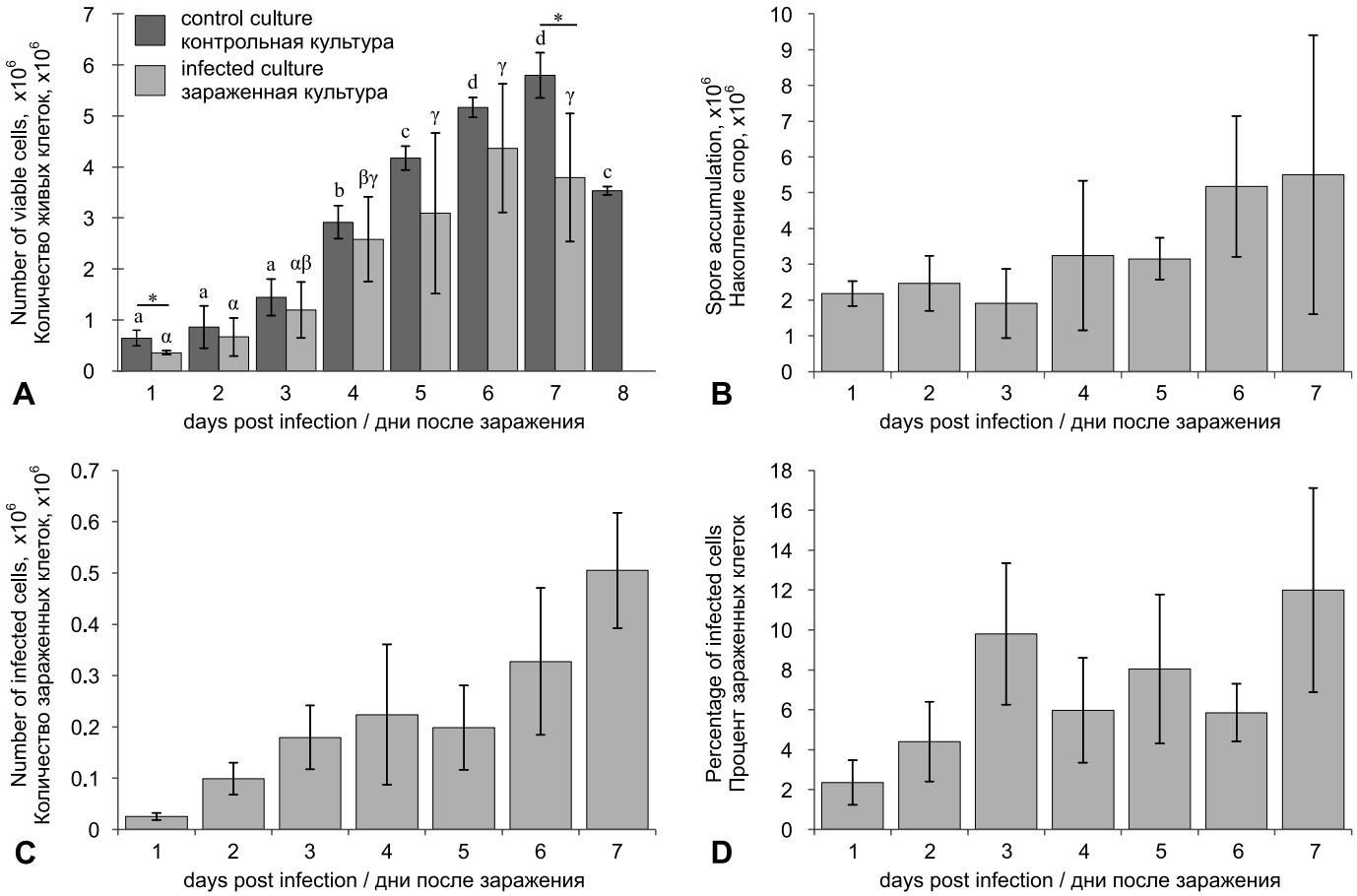

Figure 1. Sf9 cell culture over the first 7 days post-infection with *Nosema bombycis* artificially activated spores at a ratio 10 spores per cell: A – number of viable cells in the infected and uninfected cultures; B – total spore accumulation; C – number of infected cells; D – percentage of infected cells. Mean value and standard deviation are indicated. In histogram (A), different letters indicate significant differences between groups analyzed by one-way ANOVA, followed by the Tukey Honestly Significant Difference post hoc test ($p < 0.05$): Latin letters for the control culture and Greek letters for the infected culture. Asterisks denote statistically significant differences between control and infected cells compared using t-test ($p < 0.05$)

Рисунок 1. Sf9 клеточная культура первые 7 дней после заражения искусственно активированными спорами *Nosema bombycis* в соотношении 10 спор на клетку: A – количество живых клеток в зараженной и незараженной культуре; B – общее накопление спор; C – количество зараженных клеток; D – процент зараженных клеток. Указано среднее значение и стандартное отклонение. На гистограмме (A) разными буквами отмечены варианты, где различия между ними достоверно установлены с помощью однофакторного дисперсионного анализа (ANOVA) и апостериорного теста Тьюки для определения достоверно значимых различий ($p < 0.05$): латинские буквы для контрольной культуры, греческие – для зараженной. Звездочкой отмечены статистически значимые различия между контрольными и зараженными клетками, выявленные с помощью t-критерия ($p < 0.05$)

then decreased, remaining relatively stable from day 4 to day 6. This plateau may be due to the high rate of uninfected cell proliferation during this period. Subsequently, after completion of sporogony, the parasite was able to colonize neighboring cells, and therefore the infection began to spread rapidly.

Immunoblotting of Sf9 cultures infected with *N. bombycis* spores demonstrated that immunochemical methods with antibodies specific to parasite β-tubulins may be applied to assess microsporidia growth (Dolgikh et al., 2022). These antibodies were shown to have cross-reactivity with insect and microsporidia β-tubulins since these proteins have more than 70% identity. To visualize accumulation patterns of *N. bombycis* β-tubulin during 7 days of the culture growth, whole protein samples were prepared from infected cells, separated by electrophoresis and blotted against anti-β-tubulin antibodies (Dolgikh et al., 2022).

The host and parasite tubulins could be differentiated on Western blots. They produced two parallel bands corresponding to slightly heavier, the host, and lighter, the parasite tubulin molecules (Fig 2). Western blot analysis revealed gradual increase in host protein from the day 1 to the day 7, propotional to the cell culture growth (Fig. 2). *N. bombycis* β-tubulin became slightly noticeable on the day 3 and continuously accumulated until the end of the investigated period.

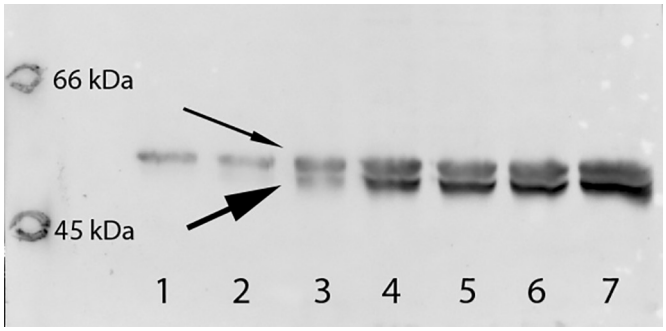

Figure 2. Western blot analysis of Sf9 cell culture over the first 7 days post-infection with *Nosema bombycis* spores using antibodies against the parasite β-tubulin. Specific staining of the microsporidian protein is indicated by thick arrows. Unspecific cross-reactivity of antibodies with *Spodoptera frugiperda* β-tubulin is indicated by thin arrows. Numbers correspond to days after infection

Рисунок 2. Вестерн-блот-анализ Sf9 культуры клеток, зараженной спорами *Nosema bombycis*, с антителами против паразитарного β-тубулина. Специфическое окрашивание белка микроспоридий указано толстыми стрелками. Неспецифическая перекрестная реакция антител с β-тубулином *Spodoptera frugiperda* указана тонкими стрелками. Цифры соответствуют дням после заражения

Long-term microsporidian infection

Nosema bombycis can persist in Sf9 insect cell culture following primary infection. In this study, the parasite was successfully subcultured for 6 months by weekly addition of healthy Sf9 cells to support its continuous propagation. Infected cell suspension, containing the counted number of spores was passaged to 5×10^5 fresh viable Sf9 cells in a well of 6-well plate at five different ratios: 1, 2.5, 5, 7.5 and 10 spores per cell.

At 7 days post-inoculation, higher infectious doses (5, 7.5 and 10 spores per cell) resulted in decrease in cell counts (Fig. 3A). Lower infection doses (1 and 2.5 spores per cell) had a minimal impact on viable cell counts, with no statistically significant differences from the control. After infection with the dose of 10 spore per cell, the number of viable cells did not differ between primary and long-term cultures. This suggests that activated spores and naturally fired spores from long-term culture had comparable effects on host cell viability. The total spore accumulation per well increased with increasing infectious dose from 1 to 7.5 spores per cell (Fig. 3B). At the dose of 10 spores per cell, the spore count was slightly lower. For infection dose 1 spore/cell, the number of accumulated spores increased 14.31-fold per week, whereas the higher dose (10 spores/cell) resulted in only a 6.47-fold increase (Fig. 3C). In contrast, during primary infection with 10 spores per cell, the spore count increased only 1.1-fold by day 7.

In long-term cultivation, the percentage of infected cells depended directly on the infectious dose (Fig. 3D). At the dose of 10 spores per cell, it was 2.591 times higher than in the primary infection. Thus, we can say that the parasite multiplied faster during long-term cultivation than during the primary infection.

Survival of infected cell culture

This experiment aimed to determine the fate of an infected culture in the absence of new host cells and to measure the time to complete host colonization by *N. bombycis*. For this purpose, $5x10^6$ viable Sf9 cells in a T25 flask were inoculated with long-term-infected culture, containing $50x10^6$ spores (spore-to-cell ratio 10:1). The suspension with $5x10^6$ viable Sf9 cells from infected culture was passaged weekly in a new culture flask until the number of viable cells fell below this threshold.

The parasite was shown to almost completely suppress the growth of insect cell culture in the third week after infection (Fig. 4A). From this moment until the end of the experiment, the number of living cells remained at approximately the same low level. The percentage of infected cells (Fig. 4B) and the total number of spores (Fig. 4C) showed rapid growth in the second week of the experiment, reaching maximum values by week 5. In general, by week 3 the parasite almost completely mastered the host cells under these conditions.

The effect of the infectious dose on the survival of the cell culture was studied in 6-well plates seeded with 5×10^5 viable Sf9 cells in a well at five spore-to-cell ratios: 1, 2.5, 5, 7.5 and 10 spores per cell. The infected cultures were passaged three times at weekly intervals using 5×10^5 viable Sf9 cells. The results showed that a lower infectious dose slowed the decrease in the host cell growth rate (Fig. 4D). Furthermore, it also reduced the rate of spore accumulation (Fig. 4E) and percentage of infected cells (Fig. 4F). However, even at a minimum dose of 1 spore per cell, *N. bombycis* did not allow the survival of the cell line.

The infectivity of the microsporidium in long-term culture

Infected cultures were routinely maintained by weekly subculture with fresh viable cells to ensure continuous parasite propagation. Here we assessed changes in parasite infectivity when cultures were deprived of fresh host cells. An infected culture was maintained for 4 weeks without addition of viable cells in T25 culture flasks. Each week, an aliquot from this

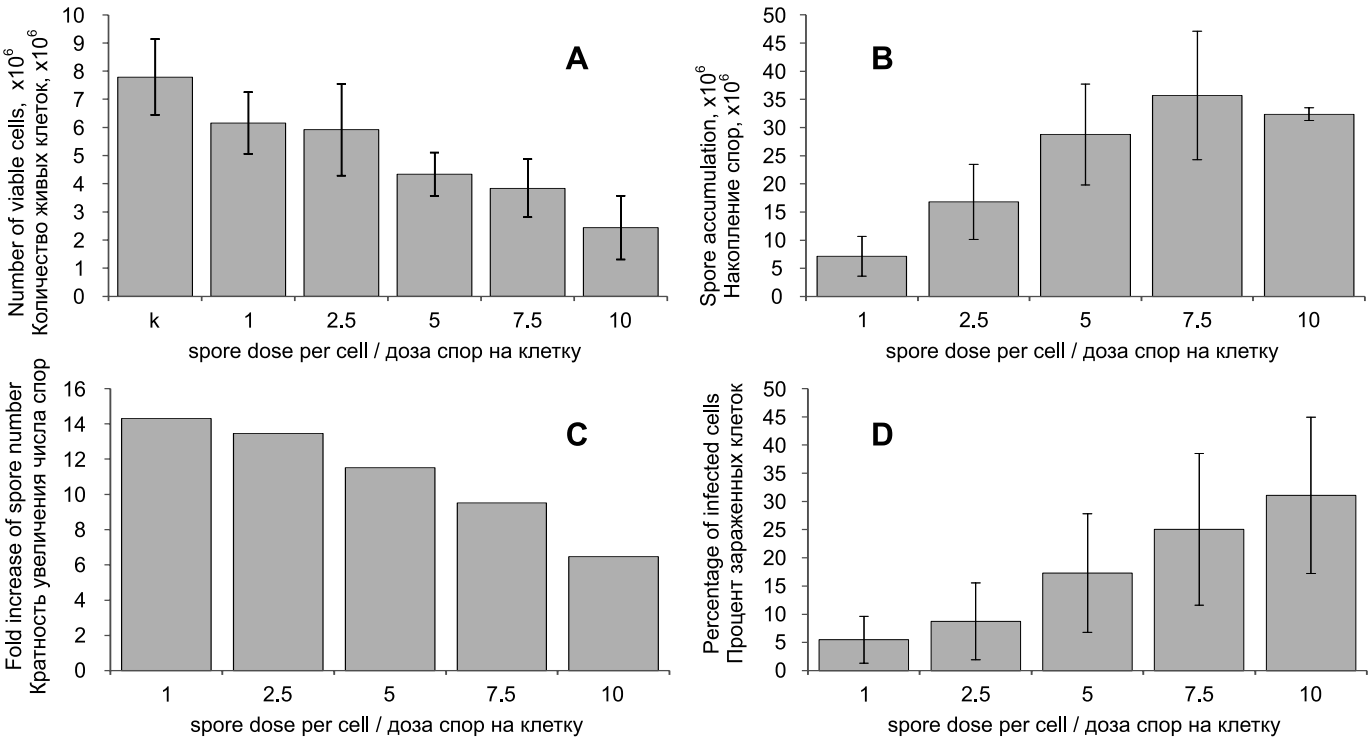

Figure 3. Effect of infectious dose on long-term *Nosema bombycis* culture parameters: A – number of viable cells; B – spore accumulation; C – fold increase of spore number; D – percentage of infected cells. Mean value and standard deviation are indicated

Рисунок 3. Эффект разных доз на параметры долговременной клеточной культуры *Nosema bombycis*: A – количество живых клеток; B – общее накопление спор; C – кратность увеличения количества спор; D – процент зараженных клеток. Указано среднее значение и стандартное отклонение

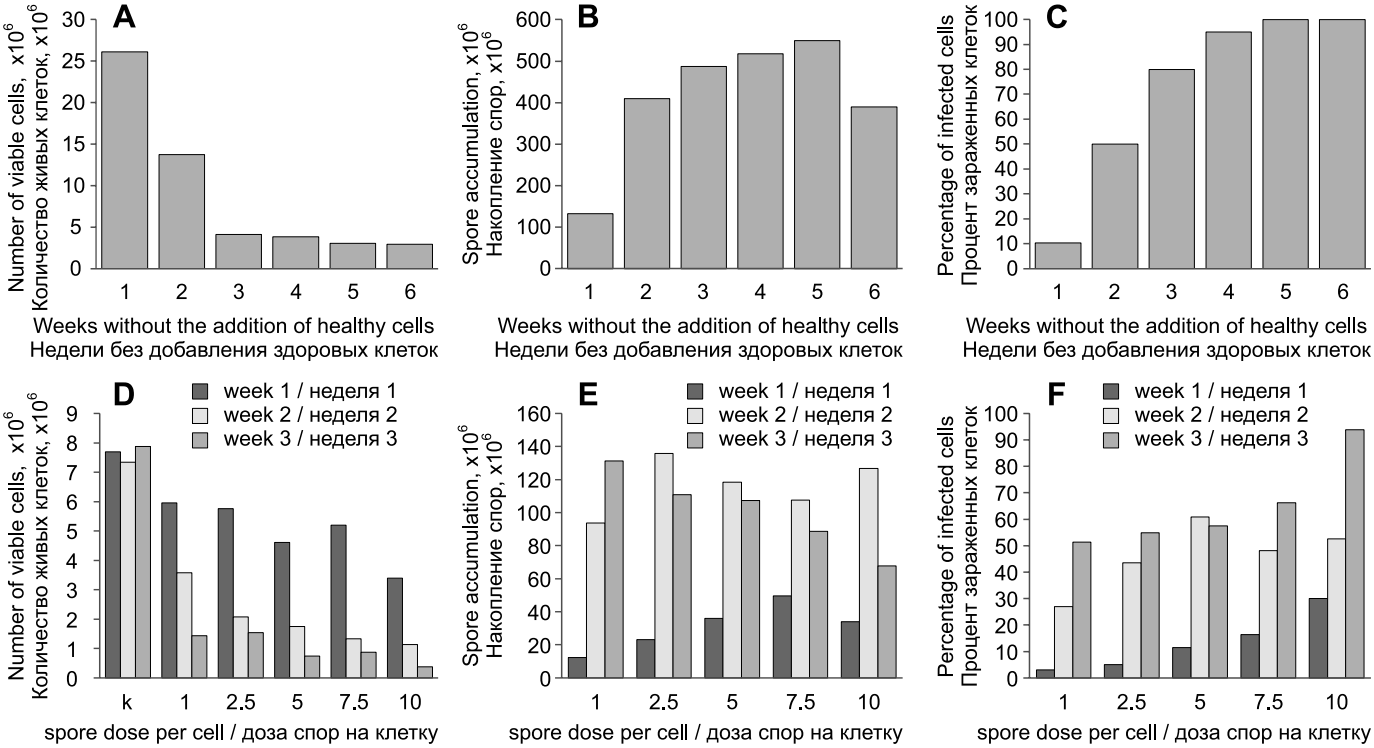

Figure 4. Sf9 cell culture infected with *Nosema bombycis* at a ratio 10 spores per cell over a 6-week cultivation without the addition of viable cells (A–C) and the effect of the initial infectious dose on culture survival (D–F). All cultures were maintained without addition of viable Sf9 cells: A, D – number of viable cells; B, E – spore accumulation; C, F – percentage of infected cells

Рисунок 4. Sf9 культура клеток, инфицированная *Nosema bombycis* в соотношении 10 спор на клетку в течение 6 недель без добавления жизнеспособных клеток (A–C), и влияние начальной инфекционной дозы на выживаемость культуры (D–F). Все культуры поддерживались без добавления жизнеспособных клеток Sf9: A, D – количество живых клеток; B, E – накопление спор; C, F – процент зараженных клеток

aging culture (source culture) was used to inoculate fresh Sf9 cells (acceptor culture) in 6-well plates at a standard multiplicity of infection (10 spores per cell).

Following infection, the number of viable cells remained approximately at the same low level, regardless of the age (1–4 weeks) of the original infectious inoculum (Fig. 5A). Statistically significant differences were observed only between the uninfected control and all infected variants. It was found that *N. bombycis* from a two-week source culture exhibited peak infectivity. When infected with this two-week culture, spore production and the percentage of infected cells in the acceptor culture approximately doubled, compared to infection with a younger one-week culture (Fig. 5B, C). In contrast, three- and four-week source cultures appeared to lose infectivity. The number of spores and the percentage of infected cells in the acceptor culture dropped sharply. This value never reached zero, probably because a significant number of pre-infected cells were carried over with the inoculum, as the source culture was nearly 100 % infected at the time of sampling.

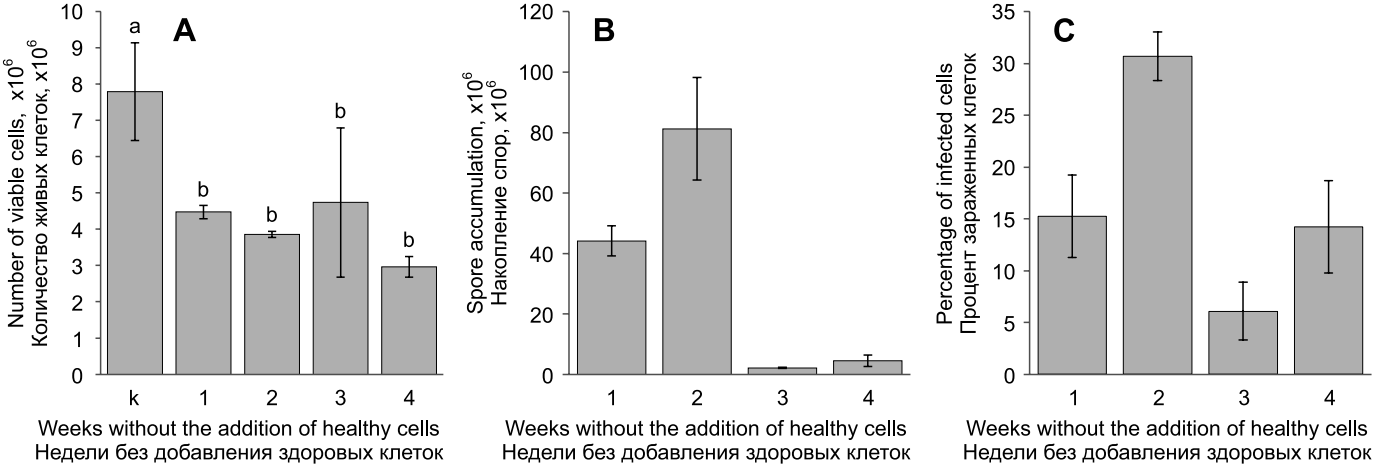

Figure 5. Sf9 cell culture parameters following infection with *Nosema bombycis* from cultures maintained without fresh viable cells for 1–4 weeks: A – number of viable cells; B – spore accumulation; C – percentage of infected cells. Mean value and standard deviation are indicated. In histogram (A), different letters indicate significant differences between groups analyzed by one-way ANOVA, followed by the Tukey Honestly Significant Difference post hoc test ($p < 0.05$)

Рисунок 5. Параметры Sf9 клеточной культуры после заражения *Nosema bombycis* из культур, содержавшихся без добавления свежих жизнеспособных клеток 1–4 недели: A – количество живых клеток; B – общее накопление спор; C – процент зараженных клеток количество живых клеток. Указано среднее значение и стандартное отклонение. На гистограмме (A) разными буквами отмечены варианты, где различия между ними достоверно установлены с помощью однофакторного дисперсионного анализа (ANOVA) и апостериорного теста Тьюки для определения достоверно значимых различий ($p < 0.05$)

Spores dimorphism

Sporogonial dimorphism is known to exist in the life cycle of *N. bombycis* (Iwano, Ishihara, 1991). In our study, we also observed the parallel formation of two types of spores, one with a thin wall and the other with a thicker one (Fig. 6). These spore types appear to correspond to the previously described primary and environmental spores, respectively (Kawarabata, 2003). Thin-walled spores were observed on the second day of primary infection, whereas thick-walled spores appeared only after the third day, which is consistent with published data. At later stages of primary infection, as well as during long-term infection, these spores occurred simultaneously in approximately equal quantities. It is difficult to accurately estimate the proportion of these spore types because they are very similar to each other. The differences become clearly visible only at high microscope magnifications using phase contrast optics, or DIC.

The sizes of the spores are given in the table (Table 1). A statistically significant difference is observed only in width. Primary spores are more pyriform, while environmental spores are more ellipsoidal. Under phase-contrast microscopy, primary spores appeared dark, which may indicate that they had already germinated (polar tube extruded), while environmental spores appeared bright, suggesting they were undischarged.

Spores from a long-term culture were isolated, purified, and successfully used to infect healthy Sf9 cells after activation.

Table 1. *Nosema bombycis* spore sizes

	Number of spores examined	length	width
Primary spores (thin-walled)	35	3.943 ± 0.522	2.284 ± 0.348
Environmental spores (thick-walled)	37	3.796 ± 0.279	2.138 ± 0.159

Таблица 1. Размер спор *Nosema bombycis*

	Количество измеренных спор	длина	ширина
Споры для передачи инфекции от клетки к клетке (тонкостенные)	35	3.943 ± 0.522	2.284 ± 0.348
Споры, передающие инфекцию через внешнюю среду (толстостенные)	37	3.796 ± 0.279	2.138 ± 0.159

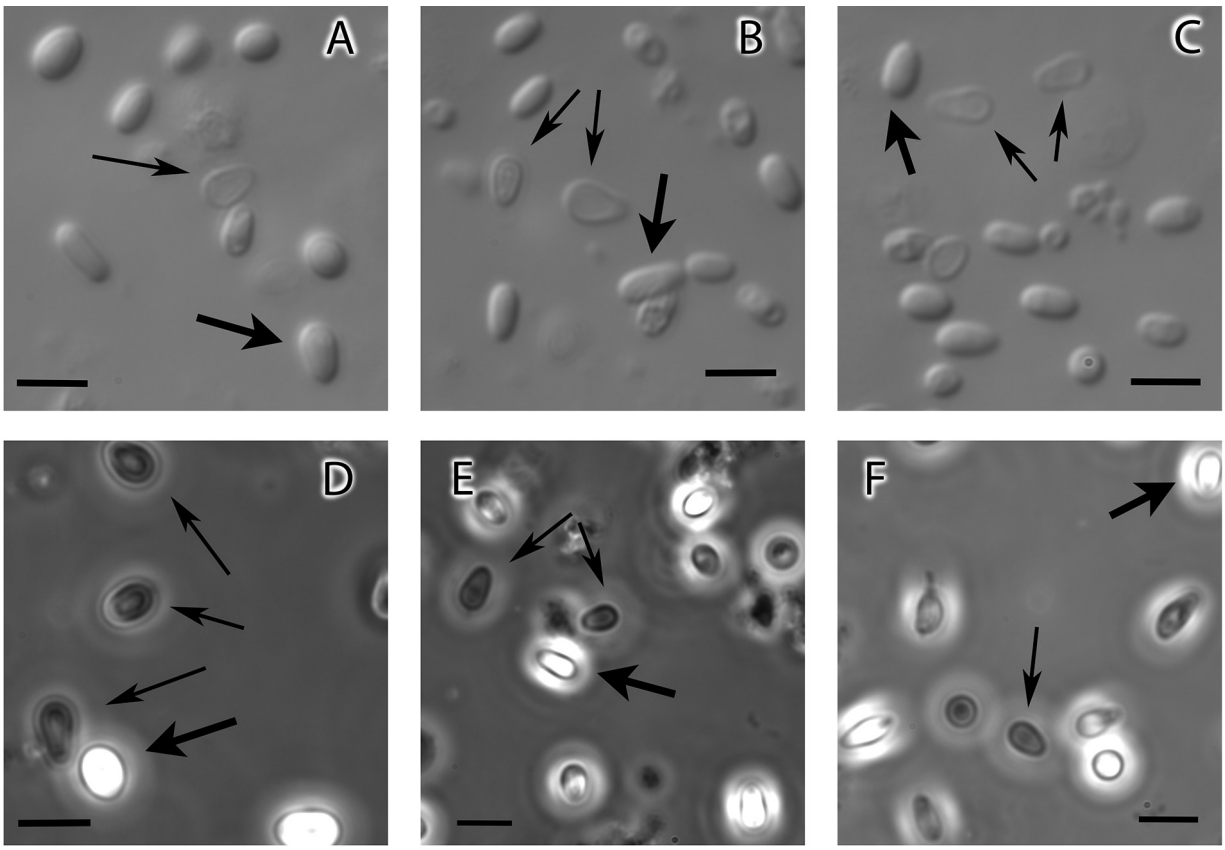

Figure 6. Spores of *Nosema bombycis*: environmental spores are indicated by thick arrows; primary spores are pointed by thin arrows. Spores are visualized in differential interference contrast (DIC) (A–C) and phase-contrast optics (D–F). Scale bars 5 μm

Рисунок 6. Споры *Nosema bombycis*: толстостенные споры обозначены толстыми стрелками, тонкостенные (первичные) — тонкими стрелками. Споры визуализированы в дифференциальном интерференциальном контрасте (ДИК) (A–C) и фазово-контрастной оптике (D–F). Масштабные линейки 5 мкм

Discussion

In this study it was demonstrated that the Sf9 cell line not only can be successfully infected with *N. bombycis* spores, as previously known, but is also suitable for continuous propagation of the parasite. This system can serve as a model for studying cellular mechanisms of resistance to nosematosis. It can also be promising for mass-production of spores of such insect pests as *Dendrolimus sibiricus*, *Spodoptera litura* and *Helicoverpa armiger*, to be used as biological control agents in plant protection.

As result of our studies, the dynamics of host cell reproduction was clarified, which is important for establishing parameters of culturing. Cell growth in the infected culture stopped on the day 6 after seeding, a day earlier than in the control, despite the fact that only 12 % of the cells were infected. We believe that this occurs due to accelerated depletion of the nutrients in infected cultures, and that one week would be an optimal period for subculture.

In long-term infection experiments, the use of small infectious doses (1 and 2.5 spores per cell) did not have a pronounced effect on the proliferation rate of host cells, which reached 100 % confluency 7 days after inoculation and also required subculture. Regardless of the infectious dose, the need to introduce healthy cells into the culture during passaging remains. Otherwise, the population of viable Sf9 cells decreased sharply over the next 2 weeks, and the parasite itself lost its infectivity after the third week. With weekly subculturing a long-term cell culture, the percentage of infected cells and spore accumulation remained approximately the same, corresponding to the dose of infectious material (a volume of infected culture containing a calculated titer of spores).

It was found out that the life cycle of this microsporidium includes production of 2 types of spores, environmental spores, and the ones, called “primary spores”, that are used for infection of neighboring cells (Iwano and Ishihara, 1991). We also recorded the presence of these two spore types. Environmental and primary spores differ slightly in shape and size, which makes the task of their separate counting in a hemocytometer very labor-intensive, so we did not differentiate these spore types. However, the question of whether cultured cells can be infected by environmental spores, requires further investigation.

As an alternative indicator of infection development, we assessed changes in *N. bombycis* β-tubulin concentration. Tubulin levels increased significantly during primary infection, while the total number of spores increased only a little bit, suggesting that this increase is associated with proliferation of intracellular stages, not spores.

While primary infection of Sf9 cell line with *N. bomb*ycis is convenient for testing susceptibility of genetically modified cells to this parasite over a short period, long-term cell culture

can provide a controlled model of microsporidian spread throughout the host body.

Basing on the data obtained, we can recommend the use of minimal infectious doses of 1 and 2.5 spores per cell, which provide relatively high rates of the parasite multiplication while having the least impact on the viability of host cells.

To maintain the infection, researchers often use a spore-to-cell ratio of 10:1 or greater to maximize infection rates (Jaronskiy, 1984). According to our data, the maximal accumulation of spores was observed at the ratio of 7.5 spores per cell. Furthermore, when infecting healthy Sf9 cells, it is preferable to use a two-week-old cell culture as the most infectious. An essential advantage of producing *N. bombycis* spores in cell cultures is sterility of the resulting infectious material.

In conclusion, our study has established the optimal conditions for the long-term maintenance of *N. bombycis* in Sf9 cells. By determining the baseline parameters for culture viability and infectivity, and by carefully optimizing the infectious spore dose, this model can serve as a versatile and reliable tool for both the mass production of spores and for molecular studies of host-parasite interactions.

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Полнотекстовая статья

ЗАРАЖЕНИЕ И ДОЛГОВРЕМЕННОЕ КУЛЬТИВИРОВАНИЕ МИКРОСПОРИДИИ *NOSEMA BOMBYCIS* В SF9 КУЛЬТУРЕ КЛЕТОК НАСЕКОМЫХ

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Микроспоридия *Nosema bombycis* имеет важное экономическое значение как возбудитель пебрины шелкопряда, а также как потенциальный агент биологической борьбы с чешуекрылыми вредителями, такими как *Dendrolimus sibiricus*. В данном исследовании споры *N. bombycis* были выделены из зараженных гусениц *Bombyx mori*. После искусственной стимуляции в 0.1 М растворе КОН их инокулировали в клеточную линию Sf9 для инициирования непрерывной пролиферации паразита. Через 7 дней после первичного заражения в инфицированную культуру были добавлены свежие клетки Sf9, что привело к созданию долговременной, постоянно инфицированной культуры. Мы количественно оценили процент зараженных клеток и динамику накопления спор как в течение первых семи дней после заражения активированными спорами, так и при длительном культивировании, а также определили пределы выживаемости клеток и инфекционности зараженной культуры. Проведенные исследования позволили предложить ключевые параметры для использования данной системы «хозяин-паразит» в качестве модели для изучения нозематоза и механизмов устойчивости, а также для массового получения спор в целях защиты растений.

Ключевые слова: микроспоридии, in vitro, клеточная линия насекомых, накопление спор, уровень заражения

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