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Molecular detection of microsporidia *Vairimorpha ceranae* and *Nosema bombycis* growth in the lepidopteran Sf9 cell line

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Summary

The search for new therapeutics and strategies to suppress microsporidiosis of domesticated insects requires cultivation of honeybee and silkworm parasites in available insect cell cultures as well as reliable methods of their detection. In this study, we infected the commonly used lepidopteran Sf9 cell line with the *Vairimorpha ceranae* and *Nosema bombycis* spores to evaluate molecular methods for microsporidia growth assay. The silkworm parasite *N. bombycis* effectively develops in lepidopteran cells and, according to literature data, its growth can be detected by qPCR analysis of β-tubulin gene copies in infected Sf9 cultures. Here, we used Western blotting with antibodies against *N. bombycis* β-tubulin to analyze Sf9 cultures infected with the parasite spores and demonstrated the prospects of immunochemical methods to assay its intracellular growth. Analysis of five genes of *N. bombycis* spore wall and polar tube proteins in infected cultures by reverse transcription (RT) PCR showed that expression of the polar tube protein PTP2 may serve as a specific marker of the parasite growth because only its transcripts were not detected in freshly inoculated Sf9 cells. The honeybee parasite *V. ceranae* infects Sf9 cells less efficiently. To find a sensitive and specific marker of the growth of this parasite, we analyzed the transcripts of its 13 genes in infected cultures using the same RT-PCR method. The spore wall protein SWP32 gene demonstrated the highest expression at the 4th-day post infection with *V. ceranae* spores, alongside with specificity of PCR-amplification, and the absence of transcripts in freshly inoculated cultures. Thus, quantitative PCR analysis of its expression may help to assay the *V. ceranae* intracellular growth in the Sf9 cell line.

Key words: domesticated insects, microsporidia, *Nosema bombycis, Vairimorpha ceranae*, Sf9 cell line, reverse transcription PCR, immunoblotting

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Introduction

Microsporidia *Vairimorpha* (*Nosema*) *ceranae* and *Nosema bombycis* are intracellular obligate parasites of honeybees *Apis mellifera* and silkworms *Bombyx mori*, respectively. They cause destructive epizootics of domesticated insects. The microsporidium *V. ceranae*, firstly described in the Asian honeybee *Apis cerana* (Fries et al., 1996), is widely spread around the world (Fries, 2010) and associated with honeybee colony losses (Martín-Hernández et al., 2018), at least in southern European countries (Gisder and Genersch, 2015). Fumagillin, an antibiotic found in the fungus *Aspergillus fumigatus*, had been used to control honeybee microsporidiosis for many years (Katznelson and Jamieson, 1952; Bailey, 1953; Williams et al., 2008; van den Heever et al., 2014). However, in 2018 Canadian company Medivet Pharmaceuticals Ltd. shut down production of this fungicide due to toxicity for mammals. The microsporidium *N. bombycis*, a highly virulent parasite of the silkworm *Bombyx mori* causes a devastating for the world sericulture disease, commonly known as pébrine. Since the first scientific record of pébrine in France in 1845, traditional methods of sanitation that include culling of the infected insects and eggs and using disease-free stocks, remain the primary strategy to control *N. bombycis* infection (Hukuhara, 2017). The control of these diseases has great economic value and demands a search for new effective therapeutics and strategies such as metal nanoparticles (Saleh et al., 2016; Dong et al., 2021), RNA interference (Paldi et al., 2010; Rodríguez-García et al., 2018; Holt and Grozinger, 2016; Huang et al., 2016; He et al., 2021) or heterologous expression of recombinant antibodies (Abs) (Huang et al., 2018a; Tsarev et al., 2019; Dolgikh et al., 2020). Inconvenience of working with live insects (seasonal availability, poor standardization of experiments, ethical problems) makes insect cell lines attractive for such studies. Cultivation of honeybee and silkworm parasites in insect cell lines requires reliable methods to evaluate their growth.

Fall armyworm *Spodoptera frugiperda*-derived Sf9 cell line and its progenitor line Sf21 are the most available and widely used in research, including heterologous protein expression (Arunkarthick et al., 2017). Lepidoptera parasites *N. bombycis* effectively develop in these lines and their growth in infected Sf9 cells have been assessed by quantitative PCR (qPCR) analysis of β-tubulin gene copies (Huang et al., 2018a; Huang et al., 2018b; Zheng et al., 2021). At the same time, there is little information on infection of *S. frugiperda*-derived cell lines with bee microsporidia, except for the reports on its ineffectiveness (Gisder et al., 2011; Senderskiy et al., 2020; Senderskiy et al., 2021).

In this study, we infected Sf9 cells with *N. bombycis* and *V. ceranae* spores to estimate the effectiveness of immunochemical, as well as reverse transcription PCR (RT-PCR)-based methods for detection of microsporidia growth. Besides, we tried to find *N. bombycis* and *V. ceranae* genes, which expression could be a specific and sensitive marker of the development of these parasites in Sf9 cells.

Material and methods

Infection of Sf9 cells with microsporidia spores

Sf9 cell line was obtained from ECACC General Collection (ECACC 89070101). Cells were maintained in Sf-900III serum-free medium (SFM) (Thermo Fisher Scientific, MA) in the adhesive culture at 27 °C without control of humidity and CO_2 concentration. For infecting with microsporidia spores, we used cells in the mid-log phase growth with the viability of over 90%. The 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).

N. bombycis spores were obtained from the Uzbek Research Institute of Sericulture (UzNIISh) in Tashkent, Uzbekistan. Spores were isolated from fat bodies of experimentally infected 5th instar *B. mori* caterpillars. Fat bodies were homogenized in distilled water and the homogenate was centrifuged at 600 g for 5 min. The spore pellets were washed three times in water, and additionally purified by centrifugation at the same mode in a density gradient of 50% Percoll (Merck, Germany), prepared with distilled water at 13000 g for 15 min. Before infection, 5×10^5, 10^7 or 2.5×10^7 spores were treated with 1 ml of the 0.1% antiseptic Multicide (Sante Pharm, Russia) (Tetz et al., 2017) for 30 min and washed with water as described above. To activate polar tube extrusion, washed *N. bombycis* spores were resuspended in 20 µl of the 10 mM KOH (Ohshima, 1937), incubated for 30 min and added to 500 µl of SF-900 III SFM with 5×10^5 Sf9 cells in a well of 12-well cell culture plate. We used a 10 mM KOH solution instead of 100 mM KOH (Huang, 2018a) to reduce the effect of alkali on the culture and avoid cell lysis. Polar tube extrusion was observed within 5 min in 90% of the microsporidia spores. Infection efficiency was ensured by mixing insect cells with parasite spores on an orbital shaker at 100 rpm for 1.5 h at 27 °C till no more extrusions of polar tubes were observed in a light microscope. Infected cell cultures were either pelleted at 600 g for 5 min and frozen at –80 °C, or maintained at 27 °C for 4 or 7 days without control of humidity and CO_2 concentration. In the latter case, the culture medium was supplemented with 5 µl of the Gibco® Antibiotic-Antimycotic solution (Thermo Fisher Scientific) per a plate well to prevent accidental bacterial contamination of the infected culture.

The spores of *V. ceranae* for cell culture infection were obtained from artificially infected honeybees. Infected midguts of living insects were dissected and homogenized in distilled water. Centrifugation of homogenates at 600 g for 5 min was followed by washing the spore pellet with distilled water and additional purification in 50% Percoll gradient, prepared with distilled water at 13000 g for 15 min. To infect insect cells, spores were sterilized for 30 min with antiseptic Multicide (Tetz et al., 2017), washed in sterile distilled water, and dried in a well of 24-well cell culture plate for 30 min under sterile conditions. Then 2×10^5 Sf9 cells in 500 µl of Sf-900 III SFM were added to 2.5×10^7 dried spores. Previously, a short-term drying followed by rehydration was used as an artificial trigger for extrusion of *V. ceranae* polar tubes (Gisder et al., 2011). In our case, we successfully stimulated their extrusion by rehydration of parasite spores in the culture medium (Senderskiy et al., 2021). After spore extrusion had been completed (1.5 h post infection), the cells were pelleted at 600 g for 5 min and frozen at –80 °C, or incubated at 27 °C for 4 days without control of humidity and CO_2 concentration, as described for *N. bombycis* spores.

Production of Abs against *N. bombycis* β-tubulin

The following procedures of (1) PCR-amplification of *N. bombycis* β-tubulin gene with forward (5'-CAAGGATCCATGAGAGAAATTATTCACTTACA-3') and reverse (5'-TCACTCGAGTTAATTTCCCATATAATCCCCTTC-3') primers using the parasite genomic DNA as a template (Tokarev et al. 2018), (2) its cloning in the vector pRSETa (Thermo Fisher Scientific) followed by heterologous expression in *E. coli*, and (3) production of immune serum to the recombinant protein and isolation of specific immunoglobulins (Igs) were performed according to the protocols identical to those described for production of Abs against *V. ceranae* β-tubulin (Senderskiy, 2021).

Western-blot analysis of *N. bombycis* β-tubulin in infected cell cultures

Five×10^5 Sf9 cells infected with *N. bombycis* spores in ratios of 1 and 20 spores per cell were cultivated for 1.5 h, 4 days and 7 days. After cultivation, the cells were pelleted by centrifugation at 600 g for 5 min, sonicated with 50 µl of 50 mM Tris-Cl (pH7.4) buffer solution, heated at 95 °C for 10 min with an equal volume of 2×sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol). Prepared samples (15 µl per lane) were loaded on 12% gel for SDS-PAGE and analyzed by immunoblotting with immune serum or isolated polyclonal Abs against *N. bombycis* β-tubulin diluted 1:1000 in TTBS (50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.05% Tween-20) and the second polyclonal Abs against rabbit Igs conjugated with horseradish peroxidase (HRP) (Bio-Rad, CA) also diluted 1:1000 in TTBS, as previously described (Dolgikh et al., 2009).

RT-PCR analysis of microsporidia transcripts in infected Sf9 cultures

For PCR with reverse transcription, 2×10^5 Sf9 cells were infected with *N. bombycis* in the ratio of 20 or 50 spores per cell or with *V. ceranae* in the ratio of 125 spores per cell. After 1.5 h, 4 days or 7 days post infection cells were pelleted by centrifugation at 600 g for 5 min and kept at – 80 °C before RNA isolation. Total RNA was isolated using Trizol reagent, DNase I, and RNA grade glycogen (all reagents were produced by Thermo Fisher Scientific) according to the manufacturer's instructions. To synthesize cDNA, 1 µg RNA in 12.5 µl of deionized water was mixed with 1 µl of 20 µM oligo-dT primers (Evrogen, Russia), denatured at 65 °C for 5 min and kept on ice before the addition of 1 µl of deionized water, 4 µl 5×reverse transcriptase (RT) buffer, 0.5 µl 25 mM dNTPs, 0.5 µl RevertAid M-MuLV-RT (100 U) and 0.5 µl RNAase inhibitor (2.5 U) (all reagents were produced by Thermo Fisher Scientific). In control samples, RT and RNAase inhibitor were replaced

Table 1. The list of primers used for PCR amplification of fragments or full-length copies of microsporidia *V. ceranae, N. bombycis*, and *S. frugiperda* genes.

Target gene	Sequence (5'-3')	NCBI ref., size (bp)
Vc beta-tubulin	TTGGGCTAAGGGTCACTACACG TCActcgagTTAATTAACGAATAAGTCTTCTGAATCT	XM_024475113.1 1030 (fragment)
Vc ubiquitin hydrolase	TGTggatccATGGTTGTAAGAGCTAAAAGAATAGAA** CACgaattcTTAAATTGTTTTAGAATAAAATACCAT	XM_024474448.1 894 (full gene)
Vc alpha, alpha- trehalase	TGTggatccATGCTACCCATTATTTTTATGACAGCA ACACgaattcCTATATATCAACCACACCTTCGTTATC	XM_024474995.1 1872 (full gene)
Vc disulfide isomerase	GTGTagatctATGTTGTTAATATTTCTATTACACTTT ACACgaattcTTAGACTCTTTTTTTGTTCTTACTTGA	XM_024476079.1 1482 (full gene)
Vc proteasome beta-subunit	TGTggatccATGTGTATTTCCTCCTTTTTATATCTT CACgaattcTTAATTAAGAATTTTTTCTCCAGAATA	XM_024476253.1 693 (full gene)
Vc alpha/beta hydrolase	TGTggatccATGTTTTTTAAAAATAAAAAAATAAGA CACgaattcTTAATGAAATATATTTTCTATCTCCTTCCA	XM_024475802.1 948 (full gene)
Vc hexokinase	TGTggatccATGAACTTTTTTCTGTTTTTTTATACC TCActgcagTTAATTCATTGTTTTTTTCGGTTTCACA	XM_024475587.1 1293 (full gene)
Vc proteasome alpha-subunit	GTGTggatccATGTTAGCTAGACTTTCAATACTATTC ACACgaattcTTATTTATCAGCTATAGACAAAAGTAA	ACOL01000014.1 2948..3706. 759 (full gene)
Vc ruvb-like 1 DNA helicase*	TCCTAAGGGATGTGGTAAAACCGCC CGATATGCACAACGTCTCCCACTT	XM_002995407.1 323 (fragment)
Vc checkpoint protein kinase*	TGGCTCCTGAGGTTGCAATGGAAA GTGTATCCCTGGCCACAGAACTTTCT	XM_002995655.1 303 (fragment)
Vc chitin synthase*	TGCGAAATGCATGGCGAGAGA GAGTTGCTTTCCAAAGGCTCGCT	XM_002996666.1 278 (fragment)
Vc spore wall protein 32*	AGGTAGACACTTTGGGATGCGGGA GCTGAAGGAATACCCGAGAAGCTGC	XM_002996303.1 531 (fragment)
Vc polar tube protein 2*	GCTTTTGTAGGATCTGTTCCCGGCA TGCTTTTTAGGTGGCAACTTGGCT	XM_002995446.1 269 (fragment)
Nb beta-tubulin	ACGTAGGGACATCAGATAACCAAT TCActcgagTTAATTTCCCATATAATCCCCTTC	KM207664.1 1229 (fragment)
Nb spore wall protein 25	CTTTCTGTATTCGGTGCCTTTTT AGTAACCCTCTTGATCTGTATTTC	EF683102.1 431 (fragment)
Nb spore wall protein 30	GGATACGTCTCAGGCGATGTT CTGCGGCTGAAAGATAGTAGAAG	EF683101.1 376 Fragment)
Nb spore wall protein 32	CCTTATATCGTCCACTTTACAACC AGCGGGGCATATTTTTCTG	EF683103.1 332 (fragment)
Nb polar tube protein 1	CCCGGAACAGCAGGAAATAAT GATGGCCACCGGTTGAAGATA	KY636450.1 445 (fragment)
Nb polar tube protein 2	CAATAATCCAGCCGAGTGTCAA AGTGGGGTACCTTCAGCAGTTT	HQ881498.1 462 (fragment)
Lepidoptera COXI subunit	ATTCAACCAATCATAAAGATATTGG TAAACTTCTGGATGTCCAAAAAATCA	universal primers 709 (fragment)

* - Primers were designed by Gisder and Genersch (2015).

** - Sites for restriction enzymes to clone PCR product into any plasmid are in small letters and underlined.

by 1 μL of deionized water. The cDNA synthesis was performed at 42 °C for 1 h using S1000 Thermal Cycler (Bio-Rad, CA). The reaction mixture was heated up to 95 °C for 5 min and diluted 2.5 times with deionized water. PCR was run with the primers specific to 13 *V. ceranae* and 6 *N. bombycis* genes (Table 1) according to the following protocol: 2 μl of diluted cDNA was mixed with 10 pmol of forward and reverse primers in 10 μl of deionized water (the final volume) and with 10 μl 2×DreamTaq Green PCR Master Mix (Thermo Fisher Scientific). After matrix denaturing for 3 min at 94 °C, followed by 30 cycles including denaturation (30s at 94 °C), annealing (30s at 55 °C), and synthesis (1 min at 72 °C), amplified fragments were analyzed in 1% agarose gel.

Results

Western-blot analysis of *N. bombycis* β-tubulin accumulation in infected Sf9 cells showed the prospect of immunochemical methods to assess the parasite growth

Immunoblotting of Sf9 cultures infected with 20 *N. bombycis* spores per insect cell demonstrated that immunochemical methods with Abs specific to

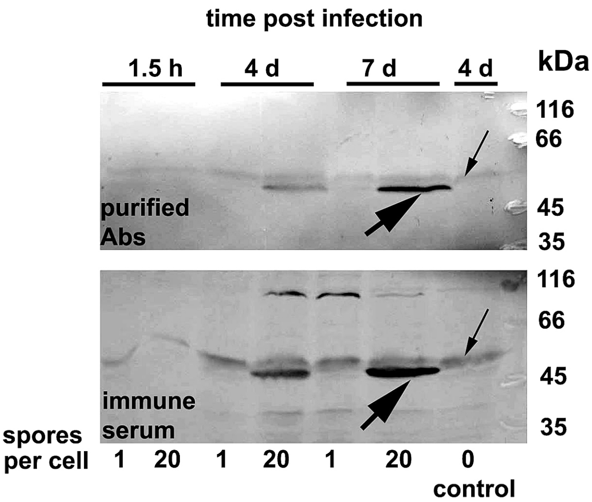

Fig. 1. Western blot analysis of *N. bombycis*-infected Sf9 cell cultures with Abs against the parasite β-tubulin. Specific staining of the microsporidian protein is indicated by thick *arrows*. Unspecific cross-reactivity of Abs with *S. frugiperda* β-tubulin is pointed by thin *arrows*.

parasite proteins like β-tubulins may be applied to assess microsporidia growth in this line. The protein band corresponding to *N. bombycis* β-tubulin became well detectable 4 days after the infection and was maximal at the 7th day of the parasite propagation (Fig. 1, indicated by a thick arrow). In the case of ratio of infection 1 spore per cell, *N. bombycis* β-tubulin band appeared only 7 days post infection being still hardly visible. In the control uninfected cells, the parasite protein was absent. Both immune serum and purified Abs also stained the protein band corresponding to the *S. frugiperda* β-tubulin (Fig. 1, indicated by a thin arrow). Since the insect and microsporidia proteins have more than 70% identity, such cross-reactivity was expected. The staining of this band was less intense compared to the parasite β-tubulin and did not depend on a ratio of infection. The staining of samples was more specific in the case of purified Abs than in the case of immune serum (Fig. 1).

RT-PCR analysis of transcripts encoding *N. bombycis* spore wall and polar tube proteins suggested a specific marker of the parasite growth in infected Sf9 cells

Infection of Sf9 cells by *N. bombycis* spores in ratios of 20 and 50 per insect cell followed by RT-PCR analysis of mRNA-transcripts of three spore wall proteins SWP25, SWP30, SWP32 at 1.5 h and on 4th and 7th days post infection demonstrated that significant amount of the transcripts was present in infected cultures already in the first hours of the parasite's development (1.5 h post inoculation) (Fig. 2). Expression of *N. bombycis* polar tube proteins PTP1 and PTP2 started later. Even without real-time qPCR, visualization of products of cDNA amplification in agarose gel suggests a lower content of PTP1 transcripts compared to the ones of *N. bombycis* SWPs. In the case of the PTP2 gene, RT-PCR analysis did not reveal visible products of cDNA amplification in the samples obtained from freshly inoculated cultures 1.5 h post infection even if a ratio of infection was maximal (50 spores per insect cell). Thus, (1) the presence of *N. bombycis* PTP2 transcripts in infected cell cultures may only be due to their synthesis in the course of the parasite sporogenesis but not due to their release from discharged spores and sporoplasms after inoculation; (2) the expression of *N. bombycis* PTP2 gene may serve as a specific marker of the microsporidia intracellular growth.

RT-PCR analysis of transcripts encoding 13 *V. ceranae* proteins revealed a sensitive and specific marker of the growth of this bee pathogen in Sf9 cells

To find a specific and particularly sensitive marker of inefficient *V. ceranae* growth in infected Sf9 cultures, we analyzed the expression of 13 parasite genes by RT-PCR at 1.5 h and on 4th days post spore inoculation. Even though all used primer pairs (Table 1) amplified the PCR products of the appropriate predicted sizes when the parasite genomic DNA was used as a template (Fig. 3), we did not find a detectable expression of six genes encoding the following *V. ceranae* proteins: ubiquitin hydrolase, α,α-trehalase, α- and β- proteasome subunits, α/β-hydrolase, and hexokinase, although these proteins could be potentially involved in microsporidia-host relationships. Among five genes suggested by Gisder and Genersch for *V. ceranae* growth detection in infected IPL-LD-65Y cells of the gypsy moth *Lymantria dispar* (Gisder and Genersch, 2015), the transcripts encoding spore wall protein SWP32 demonstrated the most effective and specific amplification (Fig. 3, indicated by a thick arrow). The expression of the PTP2 gene was also well detectable (Fig. 3, indicated by a thin arrow); however, it was accompanied by unspecific amplification of an additional 400 bp band in the samples prepared 1.5 h and 4 days post infection.

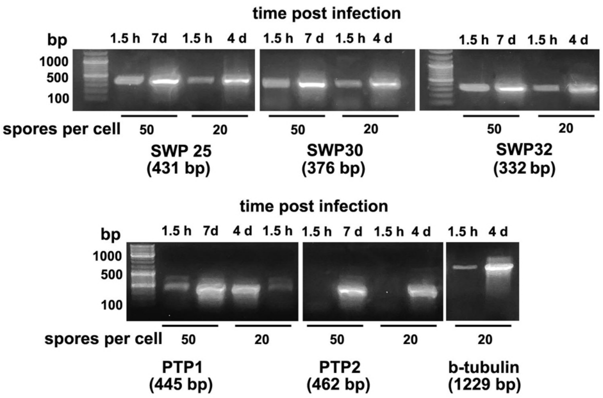

Fig. 2. Analysis of transcripts encoding *N. bombycis* spore wall, polar tube proteins, and β-tubulin in infected Sf9 cells by RT-PCR. The predicted sizes of amplified fragments are indicated in parentheses.

The expression of *V. ceranae* helicase, a checkpoint protein kinase, and chitin synthase genes was lower compared to the spore wall and polar tube proteins. Besides, both helicase and checkpoint protein kinase genes encoding "housekeeping" proteins showed the presence of mRNA transcripts in freshly infected cell cultures and could not serve as the specific markers of microsporidia growth. The same may be concluded about *V. ceranae* β-tubulin gene because its transcripts were also present in cell cultures 1.5 h post infection. As expected, the gene encoding subunit I of *S. frugiperda* mitochondrial cytochrome c oxidase, which we used to control the quality of RNA isolation and cDNA synthesis, showed similar expression at 1.5 h and 4 days post infection. Thus, the high content of the SWP32 mRNA-transcripts 4 days post infection, their absence in freshly inoculated cultures, and specificity of PCR-amplification suggested that expression of this gene may serve as a specific and sensitive marker of the *V. ceranae* growth in Sf9 cells.

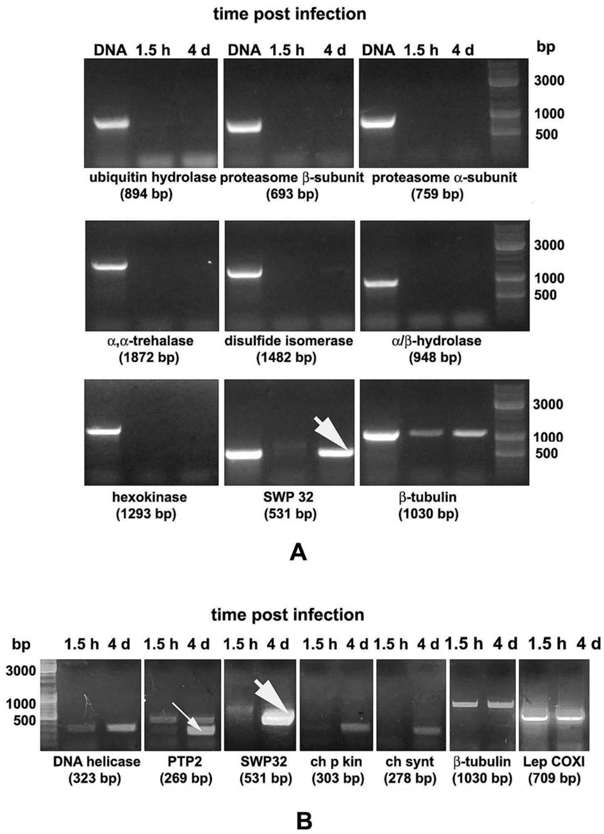

Fig. 3. RT-PCR analysis of transcripts of 13 *V. ceranae* genes in two independently infected Sf9 cell cultures (A, B). The predicted sizes of amplified full-size genes and fragments are indicated in parentheses. The most highly expressed genes SWP32 and PTP2 are marked with thick and thin *arrows*, respectively. SWP32 - spore wall protein 32; PTP2 - polar tube protein 2, ch p kin - checkpoint protein kinase; ch synt - chitin synthase; Lep COXI - subunit I of *S. frugiperda* mitochondrial cytochrome c oxidase. In the case of lanes DNA (A), genomic DNA was used as a template for PCR.

Discussion

The silkworm pathogen *N. bombycis* effectively infects *S. frugiperda*-derived cell lines, and at least three publications demonstrated that real-time qPCR analysis of the parasite β-tubulin gene copies in isolated genomic DNA may be used to assay its growth after inoculation of Sf9 cultures with 1 (Huang et al., 2018a) or 5 (Huang et al., 2018b; Zheng et al., 2021) spores per insect cell, although in two identical experiments this approach showed different results. In the first study of Sf9 cultures expressing the control eGFP-dsRNA and infected with 5 spores per insect cell, the number of copies of the β-tubulin gene increased on the 5th day after infection relative to the 3rd day by approximately 1.7 times (Huang et al., 2018b). In the recent study of the same research team, in the same Sf9 cells expressing the same control eGFP-dsRNA and infected at the same ratio of infection 5, the number of copies of the β-tubulin gene on the 3rd and 5th days post infection differed by 10 times (Zheng et al., 2021).

The drawback of PCR analysis of genomic copies of microsporidia genes in infected cultures is their contamination with DNA of discharged spores. For the correct estimation of microsporidia intracellular growth, such an approach requires the start point analysis followed by subtracting these values from the ones determined at the later points. The search for the genes expressed at the later stages of the parasite development whose mRNA transcripts are absent in the inoculum used to infect cell cultures is an alternative to the genomic DNA analysis. Infecting of the gypsy moth *Lymantria dispar* IPL-LD 65Y cell line (Goodwin et al., 1978) by *V. ceranae* spores demonstrated that, in contrast to housekeeping proteins and even chitin synthase, spore wall and polar tube components are necessary for the parasite spore formation at the late stages of the parasite intracellular development. Therefore, their genes start expression only 30 hours post infection (Gisder and Genersch, 2015).

The lepidopteran IPL-LD 65Y cells heterologous for the bee microsporidia were chosen as a model due to the lack of continuous hymenopteran cell lines (Gisder et al., 2011). Although the authors reported in the same paper that five other lepidopteran lines (including Sf9 cells) were susceptible to *V. ceranae* infection, they noted that only IPL-LD-65Y cells might be infected reproducibly with the microsporidia spores. This result and high ratio of infection (250 spores per cell), used in this study to inoculate the IPL-LD-65Y line (Gisder et al., 2011), demonstrated that bee pathogens infect lepidopteran cultures not very effectively. Previously, we raised Abs against *V. ceranae* β-tubulin over-expressed in bacteria *E. coli*, and used immunofluorescent microscopy to demonstrate a low rate (about 1%) of infection of Sf9 cultures with *V. ceranae* when 200 spores per 1 cell were added (Senderskiy et al., 2021). In the current work, we observed that transcripts of many *V. ceranae* genes were not detected in the infected Sf9 cultures using RT-PCR. However, as in the case of IPL-LD-65Y line infection (Gisder and Genersch, 2015), *V. ceranae* gene encoding spore wall protein SWP32 was specifically expressed in the late stages of the parasite intracellular development, and the presence of its transcripts can serve as a sensitive marker of microsporidia growth in Sf9 cells. Interestingly, in contrast to *V. ceranae* SWP32, all three investigated genes encoding *N. bombycis* spore wall proteins SWP25, SWP30, and SWP32, showed the presence of significant amounts of mRNA-transcripts in freshly inoculated Sf9 cultures.

Another approach to analyzing *N. bombycis* intracellular growth in Sf9 and other lepidopteran cell lines may be based on immunodetection of some parasite proteins in infected cultures using specific Abs. Since Abs against *N. bombycis* β-tubulin were previously applied for immunofluorescence microscopy of the parasite intracellular stages in infected silkworm *B. mori* BmE cells (Chen et al., 2017), we tried to verify whether polyclonal Abs against this protein may be used for Western-blot analysis of *N. bombycis* growth in infected Sf9 cultures. The successful detection of *N. bombycis* development in infected cultures by immunoblotting with anti-β-tubulin Abs and high intensity of the parasite protein staining, even with low sensitivity colorimetric (not chemiluminescent) HRP substrate, suggested that immunochemical approaches could be successfully used to analyze the microsporidia growth in Sf9 cells. Such quantitative or semiquantitative approaches for the parasite growth analysis may be based on ELISA (enzyme-linked immunosorbent assay) or Western blotting combined with digital image analysis (Janes, 2015). The problem of cross-reactivity of anti-β-tubulin Abs may be solved by constructing a chimeric protein, carrying only the parasite-specific peptides, or by searching for another *N. bombycis* protein suitable for production of diagnostic Abs.

Thus, a comparison of different methods to assay microsporidia growth in infected cell cultures suggests that the most sensitive one is the PCR-based analysis of transcripts highly and specifically expressed during intracellular development of parasites. It is sensitive enough to detect intracellular growth of *V. ceranae* that weekly infects Sf9 cells. In the case of the silkworm pathogen *N. bombycis*, which effectively develops in this cell line, intracellular development may be assayed by different methods based on the use of PCR, RT-PCR, or Abs specific to the parasite proteins. Further investigations should compare these methods and find an optimal approach that would allow tracking microsporidia development in cultured cell lines.

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