

Production of polyclonal anti- β -tubulin antibodies and immunodetection of *Vairimorpha (Nosema) ceranae* (Opisthosporidia: Microsporidia) proliferative stages in the midguts of *Apis mellifera* and in the Sf9 cell culture

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Summary

A number of methods to detect microsporidia infections are based on recovery of spores due to their thick refractive spore walls and presence of chitin that easily binds to fluorogenic and chromogenic stains. Visualization of intracellular stages in infected cells and organs has been always a challenge. In this report a new immunodetection method based on the use of β -tubulin antibodies, has been developed to mark the intracellular life cycle stages of *Vairimorpha (Nosema) ceranae*, an emergent pathogen of *Apis mellifera*. For this purpose, we carried out the heterologous expression of the *V. ceranae* β -tubulin gene in *E. coli* cells and obtained polyclonal antibodies to the recombinant protein. The antibodies were successfully applied for immunolabelling of microsporidian cells in insect midguts *in vivo* as well as in Sf9 cell culture *in vitro*.

Key words: honey bee nosemosis, immunofluorescence, Microsporidia, stages of intracellular development

Introduction

Microsporidia are a group of eukaryotic fungi-related intracellular parasites broadly distributed in the environment (Vavra and Larson, 2014). They infect almost all animal lineages and some unicellular hosts, such as amoebae and gregarines. Microsporidia infection is usually confirmed by microscopic examination of spores that have thick refractive envelope, so they can be easily detected

by light microscopy observations. Due to the presence of chitin spores are usually stained with chromotrope-based trichrome (Garcia, 2002) and chemofluorescent agents like Calcofluor White M2R dye (Chen et al., 2017).

The proliferative stages of microsporidia are more difficult to detect in light microscope due to their small size and optical transparency. At the same time, visualization of intracellular stages is of great importance for the study of parasite-

host relationships. Traditional Giemsa staining of infected cells and tissue samples provides information only about the development of life cycle stages and can help to find a focus of infection in histological preparations. Transmission electron microscopy is considered to be the best approach for the visualization of parasites within host cells and investigation of their fine cytological structures, but hardly can be used at low intensity of infection.

Fluorescence in situ hybridization (FISH) is considered to be one of the most promising methods for detecting intracellular stages of microsporidia. FISH probes against microsporidia rRNA sequences allow locating parasites in tissue samples and even determining the phylogenetic position of the parasites as rRNA targeted regions may be species-specific (Gisder et al., 2011; Dubuffet et al., 2012). Another promising method of microsporidia visualization is immunofluorescence assay (IFA). This method is based on specific interactions between immunoglobulins and microsporidia proteins, followed by treatment with secondary antibodies conjugated with a fluorescent label. In a series of works, devoted to the metabolism and secretory proteins of *Paranosema (Antonosporea) locustae*, antibodies against molecular chaperones Hsp70 were used to visualize infected cells and to separate parasites from host cytoplasm (Senderskiy et al., 2014a, 2014b). Recently, a novel method of labeling the proliferative stages of *Nosema bombycis* with β -tubulin antibodies was established (Chen et al., 2017). Beta-tubulin protein is widespread in the cytoskeleton as a major component of microtubules, which makes it a reliable marker of the parasite cell. The β -tubulin gene is conserved and yet variable enough to be used in molecular diagnostic protocols to detect microsporidia. For example, PCR detection methods of *Nosema* infection in silkworms (Esvaran et al., 2019) and *Enterocytozoon hepatopenaei* in shrimp (Han et al., 2019) basing on this gene, were developed. No cross reactivity of primers was observed when used with uninfected host DNA. Moreover this gene is applied in multi-gene phylogeny studies of crustacean parasites (Han et al., 2019) and multilocus genotyping of *Nosema bombycis* isolates (Tokarev et al., 2019).

In this study we proposed to exploit β -tubulin polyclonal antibodies for detection of intracellular stages of microsporidia *Vairimorpha (Nosema) ceranae*, an emergent pathogen of *Apis mellifera*. *V. ceranae* was first found in Asian honey bee *Apis cerana* (Fries et al., 1996). Then the parasite was discovered in western honey bee in Europe (Higes

et al., 2006) and all over the world becoming one of the most common pathogens of *A. mellifera* (Klee et al., 2007; Martin-Hernandez et al., 2007). Novel microsporidia-suppressing strategies, based on RNA interference and recombinant antibodies, are directed to interfere with crucial metabolic enzymes of these parasites during their intracellular development (Paldi et al., 2010; Rodríguez-García et al., 2018; Huang et al., 2016, 2018). The development of such approaches requires establishing of tissue and cell culture parasite-host model systems (Becnel and Andreadis, 2014). The goal of this work was to demonstrate immunolabeling in *V. ceranae* infected tissues *in vivo*, and in cultured insect cells. As there are no available permanent hymenopteran cell lines, we used the heterologous lepidopteran-derived Sf9 cell culture proved to be susceptible to *V. ceranae* (Genersch et al., 2013).

To visualize *V. ceranae* intracellular stages by IFA we amplified and overexpressed full-size parasite β -tubulin in *E. coli*, produced polyclonal antibodies against the recombinant protein and used them to detect microsporidian cells in infected Sf9 lepidopteran cell culture as well as in *A. mellifera* midguts.

Material and methods

PARASITE ISOLATION AND IDENTIFICATION

Honey bees *A. mellifera* naturally infected with microsporidia *V. ceranae* were collected from hives of private apiaries in the Leningrad region. Spores were isolated as previously described (Tokarev et al., 2018), and investigated with bright field optics of the microscope Axio Imager M1 (Carl Zeiss, Germany) to distinguish them from *Vairimorpha (Nosema) apis*. To confirm the absence of *V. apis* spores in preparations, DNA was extracted and analyzed by PCR with DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, MA). *V. ceranae*-specific primers 218MITOC F/R and *V. apis*-specific primers 312APIS F/R (Martín-Hernández et al., 2007) were applied. DNA extraction and PCR program parameters were the same as in the previous study (Tokarev et al., 2018). For further work, only *V. ceranae* positive *V. apis* free samples were used.

HETEROLOGOUS EXPRESSION OF *V. CERANA*E β -TUBULIN IN BACTERIA

The gene encoding *V. ceranae* β -tubulin (Gen Bank accession number KKO76433.1), was ampli-

fied by PCR using isolated genomic DNA, Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, MA), the forward primer CAAG GATCCATGAGAGAAATAATTCACCT ACAA and the reverse primer TCACTCGAGT TAATTAA CGAATAAGTCTTCTGAATCT (*Bam*HI and *Xho*I restriction sites are underlined). Amplification was performed using Tercyc PCR thermal cycler (DNA-Technology, Russia) with following parameters: initiation denaturation at 92 °C for 3 min, followed by 30 cycles of denaturation at 92 °C for 30 sec, annealing at 54 °C for 45 sec, elongation at 72 °C for 30 sec and a final extension at 72 °C for 10 min. The PCR product of about 1300 bp was purified from 2% agarose gel, inserted into the pRSETa expression vector (Thermo Fisher Scientific, MA) at *Bam*HI/*Xho*I sites and sequenced using T7 forward and reverse primers.

The constructed plasmid was transformed into *E. coli* BL21 (DE3)-derived C41 cells (Miroux and Walker, 1996) via electroporation at 1700 V using Electroporator 2510 (Eppendorf, Germany). Positive colonies selected on LB plates containing 0.15 mg/ml ampicillin were inoculated in 100 ml of the same liquid broth. The culture was grown in an orbital shaker at 220 rpm and 37 °C to OD₆₀₀ of 0.4, and expression was induced by addition of 0.7 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, final concentration) followed by cultivation for 15 h at 37 °C.

After expression, bacterial cells were pelleted by centrifugation at 3,000 g for 15 min, sonicated in 50 mM Tris-HCl (pH 7.4) buffer solution (TB) and centrifuged at 14,000 g for 15 min at 4 °C. The insoluble inclusion bodies were carefully washed in TB and the recombinant protein was dissolved by resuspending the pellet in 8 M urea followed by removal of insoluble debris at 14,000 g for 5 min. Samples for SDS-PAGE from the supernatant and the pellet of bacterial homogenate were prepared. Pellet has been resuspended in TB and urea in the supernatant volume. Sample preparation, SDS-PAGE and immunoblotting with monoclonal antibodies against polyhistidine sequence (Sigma-Aldrich, MO) diluted 1:2000 was carried out as it was described previously (Senderskiy et al., 2014b).

PRODUCTION AND PURIFICATION OF POLYCLONAL ANTIBODIES

Recombinant protein was dissolved in 8M urea to a concentration of 16 mg/ml, diluted twentyfold

with TB, mixed with an equal volume of Freund's adjuvant (complete for first injection, incomplete for the following ones) and used for immunization. Rabbit was immunized by four intramuscular injections (0.4 mg protein per injection) at 10-day intervals. Ten days after the last immunization, 15 ml of blood were collected and the serum was analyzed by immunoblotting. Specific antibodies were purified against recombinant protein and transferred to a nitrocellulose membrane as described previously (Dolgikh et al., 2009, 2011). The concentration of recombinant protein, extracted from inclusion bodies, was determined by Bradford's assay (Bradford, 1976).

HONEY BEE INFECTION

For experiments on artificial infection *A. mellifera carnica* brood was obtained from a private housekeeper in Belarus. The absence of natural microsporidia infection was confirmed by selective examination using microscopy and PCR. Foraging bees were planted in 0.5 l plastic bottles and then fed sugar solution supplemented with *V. ceranae* spores. A standard infection doze was one million spores per bee.

Ten days post-infection bees were dissected to isolate midguts for IFA and immunoblotting. For immunoblotting, midguts were homogenated in TB and heated at 95 °C for 10 min with an equal volume of 2× sample buffer (125 mM Tris-Cl (pH 6.8), 4% SDS, 10% 2-mercaptoethanol, 20% glycerol). For IFA the midguts were fixed in PBS (phosphate-buffered saline) with 4% paraformaldehyde (Serva, Germany) for 24 h, washed in PBS, incubated in 30% sucrose for cryoprotection, and frozen in liquid nitrogen. Frozen sections (10 μm thickness) were prepared with the Microm HM 520 cryotome and placed on microscope slides. Immunoblotting and IFA with newly raised antibodies were performed as described before (Senderskiy et al., 2014b; Timofeev et al., 2017) in three independent infection experiments.

CELL CULTURE INFECTION

Sf9 cell line derived from fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae) pupal ovarian tissue, was obtained from ECACC General collection (ECACC 89070101). Cells were cultured in Sf900III SFM (Thermo Fisher Scientific, MA) and maintained according to manufacturer's inst-

ructions. Cells for infections were in the mid-log phase growth and their viability was over 90%.

V. ceranae spores for cell culture infections were obtained from artificially infected living bees. After midgut dissection and homogenization in distilled water, spores were additionally purified on 50% Percoll gradient and sterilized with antiseptic Multicide for 30 min followed by washing in sterile water. Then 10^8 spores in water were dried in a well of 6-well plate for 30 min and cell suspension has been added at a ratio of 1 cell per 200 spores. In 5-day post-infection the cells were separated from spores using 20% Percoll gradient.

The cells were fixed for 24 h by the addition of 8% paraformaldehyde (Serva, Germany) prepared in PBS to an equal volume of cell suspension. IFA with polyclonal antibodies was carried out as previously described (Timofeev et al., 2017). The experiments on *in vitro* infection followed by fixation and IFA were independently repeated 3 times.

Results and discussion

The full-size gene encoding *V. ceranae* β -tubulin, was amplified by PCR using specific primers and genomic DNA as a template. Sequencing of the cloned DNA fragment confirmed its correct amplification and insertion into pRSETa vector at *Bam*HI/*Xho*I sites in the frame with an N-terminal 4 kDa peptide containing a polyHis tag.

After the transformation of *E. coli* C41 cells by this construct and subsequent heterologous expression, recombinant β -tubulin was accumulated in the inclusion bodies but not in the soluble fraction of bacteria broken by sonication (Fig. 1, lanes 1, 2). This finding simplified the procedure of protein isolation. Inclusion bodies were just pelleted from the disrupted cells by centrifugation, carefully washed and recombinant protein was easily dissolved in 8 M urea (Fig. 1, lane 3). Isolation of inclusion bodies from 100 mL of bacterial culture yielded approximately 16 mg of the recombinant product.

The solubilized β -tubulin was used to generate rabbit immune antiserum. Immunoblotting of purified polyclonal antibodies against nitrocellulose bands with immobilized recombinant protein, demonstrated that isolated antibodies specifically recognized a protein with a mass approximately 50 kDa in the samples of *A. mellifera* midgut infected with microsporidia (Fig. 1, lane 4). The size of this band corresponded to *V. ceranae* β -tubulin molecular weight (49.2 kDa) predicted according to its

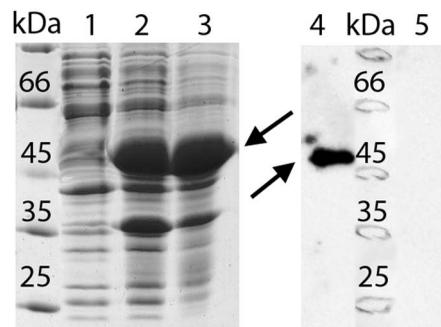


Fig. 1. SDS-PAGE of recombinant *Vairimorpha (Nosema) ceranae* β -tubulin protein (lanes 1-3) and immunoblotting with *Apis mellifera* midgut extract (lanes 4, 5). Lane 1 – soluble fraction of sonicated *E. coli* cells, 2 – insoluble fraction of sonicated *E. coli* cells, 3 – inclusion bodies dissolved in 8 M urea, 4 – infected midgut extract, 5 – uninfected (control). β -tubulin protein is indicated by arrows.

amino acid sequence. This protein band was not detected in midguts of uninfected (control) bees (Fig. 1, lane 5).

Immunofluorescence assay was performed on cryosections of infected midguts of *A. mellifera*. Antibodies to parasite β -tubulin specifically labeled stages of intracellular development (meronts and sporonts). The pattern of β -tubulin antibodies staining was not homogeneous (Fig. 2, A). Consistent clusters of filaments were observed in the cytoplasm around the nuclear area. The maximum density of fluorescent signal was concentrated at the periphery of the cell, closer to the plasma membrane, displaying the irregular ring structure. Likely, this rings corresponded to the elongated shape of merogonial stages. Spores and sporoblasts remained unstained as their envelope were impermeable for immunoglobulins.

Incubation of Sf9 cells with previously dried *V. ceranae* spores resulted in low rate of infection (about one percent). However, IFA provided an easy way to find even a single infected cell on entire preparation. Microsporidian proliferative stages developed in lepidopteran (Sf9) cell culture, demonstrated the same pattern of anti- β -tubulin staining, as naturally infected gastric epithelial cells of *A. mellifera* (Fig. 2, B).

The sequencing of some microsporidian genomes and genes enabled production of antibodies against parasite proteins expressed in heterologous systems. Due to a small size of microsporidia cells

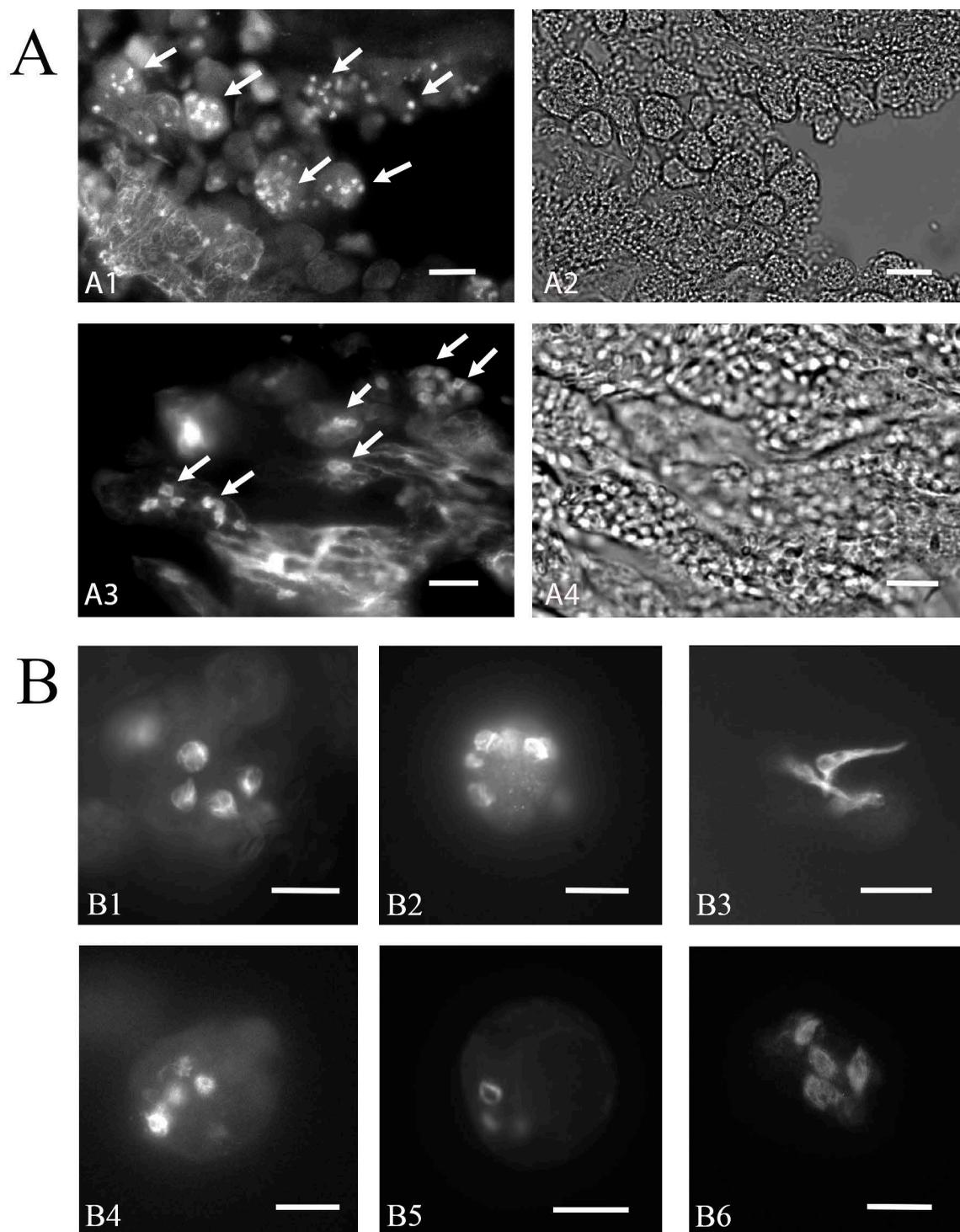


Fig. 2. Immunodetection of *Vairimorpha (Nosema) ceranae* intracellular stages. Cryosections of infected *Apis mellifera* midguts and Sf9 cells were stained with polyclonal β -tubulin antibodies, followed by treatment with secondary antibodies conjugated with Alexa Fluor 488 fluorescent label. A – Intracellular stages of *V. ceranae* (indicated by arrows) are seen in gastric epithelial cells; A1, A3 – fluorescence; A2, A4 – bright field microscopy. B – Infected Sf9 cells with intracellular stages of *V. ceranae*; immunolabeling with β -tubulin antibodies reveals clusters of filaments that mimic the shape of parasite's cell. Scale bars: A1-A4 – 20 μ m, B1-B6 – 10 μ m.

and spores, the most convenient method to precisely localize parasite proteins is immunoelectron microscopy (IEM). At the same time, IFA does not require such expensive equipment as IEM and is much more suitable for diagnostics. Besides, immunofluorescent microscopy is indispensable for co-localization of studied microsporidian proteins with other parasite or host cells molecules and/or organelles and compartments. In our previous study (Senderskiy et al., 2014b) we used antibodies against Hsp70 molecular chaperone to visualize microsporidia *Paranosema (Antonosporea) locustae* proliferative stages in host cell cytoplasm. To detect *Nosema bombycis* proliferative stages in infected silkworms and cell cultures, chinese authors used antibodies against microsporidian β -tubulins (Chen et al., 2017; Huang et al., 2018). In this study, we applied the same approach to detect *V. ceranae* intracellular stages in infected host tissues and cell culture. This method should help in testing new approaches to suppress *V. ceranae* intracellular development, in finding parasite secretome proteins, as well in immunodiagnosics of microsporidian early infection in honey bees and other hosts.

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