Journal of Microbiological Methods 120 (2016) 72–78

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Quantitative PCR for detection of *Nosema bombycis* in single silkworm eggs and newly hatched larvae

Zhangwuke Fu, Xiangkang He, Shunfeng Cai, Han Liu, Xinyi He, Mingqian Li, Xingmeng Lu *

Laboratory of Invertebrate Pathology, College of Animal Sciences, Zhejiang University, Hangzhou 310058, China

ARTICLE INFO

Article history:
Received 6 September 2015
Received in revised form 25 November 2015
Accepted 2 December 2015
Available online 3 December 2015

Keywords:
Nosema bombycis
Pebrine inspection
Transovarial transmission
Real-time qPCR
Light microscopy

ABSTRACT

Pebrine disease is the only mandatory quarantine item in sericultural production due to its destructive consequences. So far, the mother moth microscopic examination method established by Pasteur (1870) remains the only detection method for screening for the causative agent *Nosema bombycis* (*N. bombycis*). Because pebrine is a horizontal and vertical transmission disease, it is better to inspect silkworm eggs and newly hatched larvae to investigate the infection rate, vertical transmission rate and spore load of the progenies. There is a rising demand for a more direct, effective and accurate detection approach in the sericultural industry. Here, we developed a molecular detection approach based on real-time quantitative PCR (qPCR) for pebrine inspection in single silkworm eggs and newly hatched larvae. Targeting the small-subunit rRNA gene of *N. bombycis*, this assay showed high sensitivity and reproducibility. Ten spores in a whole sample or 0.1 spore DNA (1 spore DNA represents the DNA content of one *N. bombycis* spore) in a reaction system was estimated as the detection limit of the isolation and real-time qPCR procedure. Silkworm egg tissues impact the detection sensitivity but are not significant in single silkworm egg detection. Of 400 samples produced by infected moths, 167 and 195 were scored positive by light microscopy and real-time qPCR analysis, respectively. With higher accuracy and the potential capability of high-throughput screening, this method is anticipated to be adaptable for pebrine inspection and surveillance in the sericultural industry. In addition, this method can be applied to ecology studies of *N. bombycis*–silkworm interactions due to its quantitative function.

1. Introduction

Microsporidia are obligate intracellular eukaryotic parasites that invade a variety of hosts ranging from protists to mammals (Hirt et al., 1999; Keeling et al., 2000; PJ K and GI M, 1998; Letunic, 2009). They produce spores containing an extrusion apparatus that consists of a coiled polar tube ending in an anchoring disc at the apical part of the spore. The polar tube is discharged under appropriate conditions through the thin anterior end of the spore, thereby penetrating a new host cell to inoculate the infective sporoplasm into the new host cells. Microsporidia gain access to host cells by phagocytosis as well. After phagocytosis, a special infection mechanism is used to escape from the maturing phagosomes to infect the cytoplasm of the cells (Franzen, 2005). The study of parasites depends on their detection, identification and quantification (Refardt and Ebert, 2006). Microsporidium *N. bombycis* is the first identified microsporidia; it is able to modulate the expression of several silkworm proteins belonging to different functional groups, such as molecular synthesis and transport, redox, regulatory proteins and enzymes for several metabolic pathways (He et al., 2014). Analysis of protein coding genes, especially α- and β-tubulins (Keeling et al., 2000; Keeling, 2003), and phylogeny of microsporidia based on LSUrRNA sequences (Van de Peer et al., 2000) now suggests that microsporidia are fungi. Pebrine disease, caused by *N. bombycis*, inflicted heavy losses on the sericultural industry in Europe and Asia during the mid-19th century (Becnel and Andreadis, 1999) and is still epidemic and causes tremendous economic losses in sericultural countries such as China. Pasteur (1870) discovered that the parasite could transmit from mother moths to progenies via transovarial transmission (Pasteur, 1870). Based on this observation, he developed the mother moth microscopic examination method to eliminate eggs laid by infected moths and produce *N. bombycis*-free eggs for the sericultural industry. After nearly 150 years, light microscopy examination remains the only method for pebrine detection in the sericultural industry. According to the mother moth microscopic examination method, all of the eggs from the infected mother moths are eliminated. However, most of the eggs from the infected mother moths are not infected with *N. bombycis* and it is a great waste in the sericultural industry. Moreover, this method requires skilled and experienced labourers to identify the unique structure of this pathogenic microorganism. There is an increasing demand for a direct, highly sensitive and labour-saving approach for pebrine inspection and surveillance in silk-producing countries. Compared to the conventional method, real-time qPCR is more accurate

* Corresponding author.
E-mail address: xmlu@zju.edu.cn (X. Lu).

http://dx.doi.org/10.1016/j.mimet.2015.12.003
0167-7012/

and saves labour. Furthermore, we are able to detect silkworm eggs and newly hatched larvae to investigate the infection rate in progenies. Therefore, a new standard of quarantine could be worked out on the basis of infection in progenies rather than elimination of all eggs from the infected mother moths.

Genetic assays have been developed such as conventional PCR and the loop-mediated isothermal amplification (LAMP) method for pebrine inspection of silkworm eggs (Hatakeyama and Hayasaka, 2003; JiPing et al., 2015), but they do not quantify spore levels in a single reaction. In recent years, real-time qPCR has been successfully utilized for detection and quantification of numerous pathogens including human pathogenic microsporidia (Menotti et al., 2003; Polley et al., 2011; Verweij et al., 2007; Wolk et al., 2002) and agricultural microsporidia (Bourgeois et al., 2010; Chaimanee et al., 2013; Erler et al., 2012). This technology is able to identify pathogens as well as to infer the initial copy number of the template DNA by measuring the amount of amplicons using fluorescent data at every cycle (Higuchi et al., 1993). The whole detection process is accomplished rapidly in a sealed-tube system, thus reducing the cross contamination from the environment or other samples. All of these factors indicate that real-time qPCR can offer accurate results for the diagnosis and surveillance of disease, as well as considerable advantages for inspection and quarantine in the sericultural industry. In this study, we designed a novel set of primers for a conserved region in the small-subunit rRNA gene to develop a real-time qPCR protocol for the detection of *N. bombycis* in single silkworm eggs and newly hatched larvae. Silkworm egg tissues impact the detection sensitivity but are not significant in single silkworm egg detection. Therefore, this method for only one silkworm egg or larva is reliable.

2. Material and methods

2.1. Silkworm and Nosema bombycis

Parent Baiyu and Qiufeng silkworm eggs were provided by the Quality Inspection and Quarantine Station, Agriculture Department, Zhejiang Province. Qiufeng × Baiyu is the most widely used breed in Zhejiang Province, China. The *N. bombycis* ZJ01 strain originally obtained from infected silkworms in Zhejiang, China (Mei and Jin, 1989), was stored at the Institute of Sericulture and Apiculture, Zhejiang University, and propagated in laboratory-reared silkworms as previously described (Cai et al., 2011).

2.2. N. bombycis-infected female moth preparation and egg production

Baiyu and Qiufeng were reared under identical conditions and divided equally into three groups (Baiyu: A, A′ and A″; Qiufeng: B, B′ and B″) after the fourth exuviation. Newly moulted silkworm larvae of groups A and B were fed mulberry leaves that were previously besmeared with *N. bombycis* spores (10^6 spores/mL, 10 μL/larva). The other groups of larvae were reared with fresh mulberry leaves. After eclosion, female moths in group A were mated with male moths in group B′ (Baiyu × Qiufeng); female moths in group B were mated with male moths in group A′ (Qiufeng × Baiyu). Four hours later, female moths were put onto cards to lay eggs. Female moths were examined by a phase contrast microscope after oviposition. Eggs laid by infected mother moths were collected and incubated in optimum hatching conditions (temperature at 25 °C, humidity between 70% and 80%). At the egg bluishing stage on day 9, which is just one day before hatching, half of the samples were aborted from incubation and stored at −80 °C. The remaining eggs continued incubation until hatching. Newly hatched larvae were also stored at −80 °C for further use. Groups A″ and B″ were used to produce healthy eggs and larvae (both Baiyu × Qiufeng and Qiufeng × Baiyu).

2.3. Real-time qPCR

2.3.1. DNA extraction

The single silkworm egg or newly hatched larvae or purified spore suspension was put into a homogenization tube (MP Biomedicals) and treated with 20 μL 5% NaOH for 10 min and then washed 3 times with ddH_2O. Before homogenization in a FastPrep-24 (MP BIO), 500 mg acid-washed glass beads (Sigma, diameter: 425 μm–600 μm) and 500 μL DNA extraction buffer (1 mM Tris, 1 mM EDTA, 0.5% SDS, pH 8.0) were added. The disruption programme is 6500 r/min, 20 s for 3 times. After the silkworm eggs or larvae were crushed, DNA was extracted by the phenol chloroform extraction method as described previously (Undeen and Cockburn, 1989). FDNA was dissolved in 100 μL TE (10 mM Tris, 1 mM EDTA, pH 8.0) and stored at −80 °C for further analysis.

2.4. The standard curve

For quantification of *N. bombycis* DNA and estimation of the pebrine infection rate and spore loads in silkworm eggs or larvae, a standard curve was constructed with recombinant plasmid as the standard substance. In brief, the primer pair Nb-ssu1092F (5′-GTCCCTGTTCTTTG TAC-3′) and Nb-ssu1227R (5′-ATCCTGCTAATGGTTCT-3′) was designed to amplify a 136-bp fragment located downstream of the *N. bombycis* small-subunit rRNA gene (GenBank accession No. EU864525.1), from site 1092 to site 1227. The PCR product was inserted into the pGEM-T Easy cloning vector (Promega, production no.: A1360) following the manufacturer's instruction, and the vector was then transformed into Trans5α competent cells (Transgen production no.: CD201) and incubated in a culture dish. Potential clones were picked, and positive recombinant plasmid was verified by sequencing and BLAST search. The standard curve was established from three replicates of 10-fold dilutions (5×10^8–5×10^0 copies/μL) of recombinant plasmid DNA with LightCycler® 480 System Software (Version 1.5.0.39).

2.5. Quantitative PCR procedure

Amplification was performed in 20 μL reaction volumes (10 μL 2 × SYBR Mix, 0.5 μL of each primer, 1 μL template DNA, 8 μL ddH_2O) in a LightCycler® 480 (Roche) using LightCycler® 480 SYBR Green I Master (Roche production no. 04,887,352,001). The optimum cycling conditions consisted of an initial denaturation (10 min at 95 °C) and 45 cycles (5 s at 95 °C, 20 s at 55 °C, and 15 s at 72 °C), followed by a melt-curve dissociation analysis to confirm the specificity of the PCR amplification. Experiments were set up in triplicate for each sample. Samples with a ≤40 amplification threshold cycle (Ct) and a consistent melting curve were scored positive.

2.6. Impact of the silkworm egg tissues on the sensitivity of qPCR

A total of 0, 1, 2, 4, 7, and 13 Qiufeng × Baiyu bluish eggs were mixed with 100 μL aliquots containing 1×10^7, 1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2, and 0 purified *N. bombycis* spores, respectively. A total of 63 samples were first mixed, and then DNA extraction and real-time qPCR detection were carried out as described above. Each concentration gradient was replicated three times. Analysis of mean values and variances was applied.

2.7. Evaluation of the sensitivity and reproducibility of real-time qPCR

A single Qiufeng × Baiyu bluish egg was mixed with 100 μL aliquots containing 1×10^7, 1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2, 1×10^1, and 0 purified *N. bombycis* spores. DNA extraction and real-time qPCR detection were carried out as described above. Each concentration gradient was replicated three times. Data were analysed with ANOVA.