# Establishment of Simplistic Moth Inspection System to Prevent *Nosema bombycis* Infection of the Silkworm, *Bombyx mori*

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## ABSTRACT

Present experiment designed for the review of theoretical basis for the inspection system of infected insects by *Nosema bombycis*. A microsporidian *N. bombycis*, known as the high virulence, produced at the average of $7 \times 10^8$ spores per female moth of the silkworm, Bombyx mori, enabled transovarial tranmission. Detectability of *N. bombycis* spores in the mass inspection was varied by dillution level, the higher limit of dillution with healthy moths was 1: 140 for 100% detection, 1:160 for 99.5%, 1:200 for 99.0%. For an efficient inspection under the microdcopic observation ($600 \times$), the lower limit of spore concentration was determined as 1,000,000 spores/m$l$, 60~80 moths could be applicable for a maximum sample unit of a lot. Following the present inspection unit conditioned 35 to 40 moths for a lot. *N. bombycis* spores were easily detectable from the preparation of crude homogenate with 2% KOH, even the step of centrifuge was omitted. The results suggested a new basis of rational mass inspection system of silkworm female moths to save the facilities, labor, and time

Key words : *Nosema bombycis*, Transovarial transmission, Microsporidia

## INTRODUCTION

The virulence of *Nosema bombycis* was greatly different from other microsporidia isolated from sericultural farms in Korea (Lim *et al.*, 1982) and in Japan (Tanaka *et al.*, 1972; Fujiwara, 1980). Transovarial transmission of *N. bombycis* to the silkworm progeny and the destructive effect on the sericultural industry were clarified, female moths of the silkworm supposed to be inspected after oviposition (Han and Watanabe, 1988). All the silkworm eggs must be discarded when the mother moths are found to contain *N. bombycis* spores. Traditional moth inspection method, considered as the only way to prevent pebrine, has been proposed for more than 100 years since Pasteur (1870).

However, the practice of current moth inspection system is insufficient, still prevalent pebrine in the developing countries lacking in facilities and methodology.

In recent years, previously undescribed microsporidia pathogenic to the silkworm have been studied their spore shapes with micromorphology, target tissue (Iwano and Ishihara, 1991), and identification (Han and Watanabe, 1987). Transovarial transmissibility of *N. bombycis* (Han and Watanabe, 1988), histopathology of gonad infection (Han, 1994) with the pathway of the vertical transmission (Han, 1996) was clarified. However, theoretical basis for the routine moth inspection system, the most important conditions to enforce or revise the present system by an establishment of rational methodology, has not been thoroughly studied.

The present study was conducted to assay the moth inspection unit by invstigation of *N. bombycis*

spores produced in the body of infected moth and transovarial transmissibility of those pathogens to their progeny populations. Subsequently, determined upper limit of moth numbers for a lot as the inspection unit, then clarified a proper number of moths per lot for the practice of efficient inspection system. Finally, established a basic theory to practice a rational moth inspection system.

## MATERIALS AND METHODS

### 1. Preparation of *Nosema bombycis* strains

The microsporidian *Nosema bombycis* of standard type was maintained in the laboratory condition, the wild type identified as *N. bombycis* was newly collected from sericultural farm. Those microsporidian spores preserved at 5°C were preinoculated, and reproduced before bioassay of transovarial transmissibility. Pathogenic spores from infected larvae or moths with *N. bombycis* were homogenized in 0.8% NaCl solution, and filtered through 3 to 4 layer of gauze. Suspention of microsporidian spores from the filtrate were roughly isolated by separatory funnel, simple purification by natural sedimentation of this method repeated for 3 to 4 times to earn the purified spore inoculum.

### 2. Bioassay of transovarial transmission

Original race of Jam107 and Jam126, commercial hybrid of Saseongjam and Daeseongjam were introduced, and reared the larvae by mulberry leaves or artificial diet after hatching of diapaused eggs by routine methods. Microsporidian inoculum of $10^7$ spores/ml was smeared on mulberry leaves or dropped on the artificial diet, and fed on the silkworm soon after molting at 5th instar larvae or exposured at around the end of larval stage before spinning. The progeny populations from infected moths were investigated transovarial infection in the dead eggs, dead larvae on the rearing, and the newly hatched larvae starved to death. The ratio of transovarial infection was calculated by the modified method of Han and Watanabe (1988) as the following formula : Transovarial transmission rate $(\%)=(a \times b+c \times d)/100$; a, % of dead eggs; b, % infection of a; c, % of hatched larvae; d, % infection of c.

### 3. Quantitative assay for the moth inspection

Infected moths collected after oviposition were homogenated individually, the homogenate measured its total volume was counted the spore concentration per ml of suspension. Each of the homogenate was adjusted the volume between 15 to 20 ml for convenience on spore counting. The number of spores produced in each moth was calculated from the total volume of individual preparation and its spore concentration per ml. The distribution of moth populations on their level of spore content was investigated, and the mean value of spores in crude homogenate was introduced as a original suspention. Samples for experimental inspection were prepared by dillution of the original suspention in the homogenate of microsporidian-free moths. The serial concentration of spore samples were prepared by twofold dillution from the first standard dillution of 1:40 suspention, 3 ml of 2% KOH solution per moth were added to homogenize the samples for mass inspection of microscopic observation by the magnification of 600 times.

## RESULTS AND DISCUSSION

### 1. Occurrence of infected silkworm moth with *Nosema bombycis*

Peroral inoculation at 5th instar larvae found their infectivity as high as 97 to 100% of the moths eclosed, those infectivity against commercial silkworm races appeared no significant difference between the sex nor the presented silkworm races of Saseongjam and Daeseongjam (Table 1). The infectivity of wild type of *N. bombycis* was as high as that of standard type in the male and female moths following inoculation at the final instar larvae, which showed the same tendancy as the infectivity of standard type of *N. bombycis* against the female moth of a commercial silkworm used in Japan (Han and Watanabe, 1988).

Common inoculation technique has been carried out for the early stage at any larval instar of the silkworm, however, infectivity at the later stage of the 5th instar larvae has been unknown. The incidence of infection at the later larval stage was confirmed by the larvae exposed at the time of 19 to 22

**Table 1.** Occurrence of infected moth of the commercial silkworm races, Saseongjam and Daeseongjam, following inoculation of standard type and wild type of N. bombycis at the 5th larval instar

| Pathogens | Silkworm Varieties | Sex | No. of the Moths | | % Moth Infected |
| --- | --- | --- | --- | --- | --- |
| | | | Tested | Infected | |
| Standard Type N. bombycis | Saseongjam | Female | 118 | 118 | 100.0 |
| | | Male | 34 | 33 | 97.1 |
| | Daeseongjam | Female | 24 | 24 | 100.0 |
| | | Male | 24 | 16 | 100.0 |
| Wild Type of N. bombycis | Saseongjam | Female | 166 | 164 | 96.8 |
| | | Male | 29 | 29 | 100.0 |

hours before spinning, and the inoculation time later than 10 hours before spinning was failed to earn infected moth. Silkworms reared by artificial diet preventive the contamination with microsporidia during the early larval stage, despite the worms reared by mulberry leaves are difficult to secure the microsporidian-free condition. Details on the peroral infection of the silkworm at the latest time of exposure, needed further study.

## 2. Spore population of infected moth in relation to Transovarial Transmission

Individual female moths infected with standard type N. bombycisz contained average number of $(7.7 \pm 0.60) \times 108$ spores in the silkworm of Daeseongjam, and $(6.2 \pm 0.23) \times 108$ spores in the Saseongjam. Spore amount per moth appeared from the lowest number of $1.7 \times 10^8$ to the highest of $1.6 \times 10^9$, distinguished the higher spore content of female moths, in the face of remarkably reduced body weight after oviposition. Represented the vigorous propagation of N. bombycis specifically in the female of the silkworm (Table 2). The number of N. bom-

bycis spores displays their virulence in the female moths, and spore concentration per host represented the advance of pathogenesis. However, the correlation of spore concentration per body weight with transovarial transmission appeared in the silkworm was not so clear, any level of presented spore cpopulation in the infected moths, therefore, considered as enough for their invadence to progeny eggs with the high rate of transmissibility (Fig. 1).

The high infectivity and transovarial transmissibility of standard and wild type pathogens still preserved. Although both type of N. bombycis strains repeated their generation for several decades in different environment of the field and laboratory condition. The similarity between wild type and standard type suggesting an ecologically unique phenomenon.

## 3. Evaluation of the sample size for moth inspection

Infected moths with N. bombycis were investigated the distribution of the populations by their spore content, which was varied from the highest of $1.7 \times 10^9$ to the lowest of $1.7 \times 10^8$ among 305 individuals. Av-

**Table 2.** Number of N. bombycis spores produced per infected moth of the silkworm, Bombyx mori ($\times 10^8$ spores)

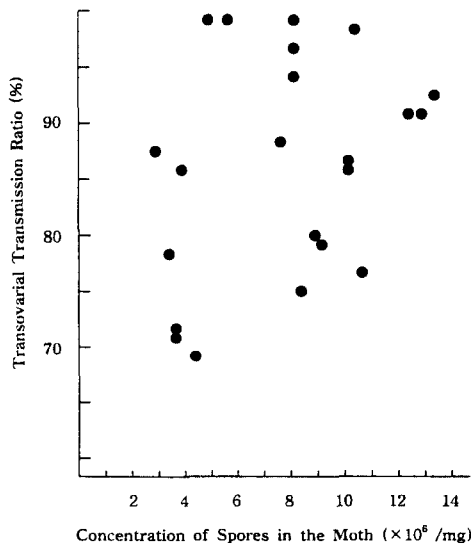| N. bombycis Strains | Silkworm Host | | No. Moth Inspected | Average No. Spores per Moth($\times 10^8$) | The Lowest to Highest levels($\times 10^8$) |
| --- | --- | --- | --- | --- | --- |
| | Varieties | Sex | | | |
| Standard Type N. bombycis | Daeseongjam | Female | 24 | $7.7 \pm 0.60$ | 2.04~16.64 |
| | | Male | 10 | $4.9 \pm 0.53$ | 1.52~10.70 |
| | Saseongjam | Female | 118 | $6.2 \pm 0.23$ | 1.74~11.25 |
| | | Male | 33 | $3.8 \pm 0.30$ | 1.68~6.64 |
| Wild Type N. bombycis | Saseongjam | Female | 164 | $7.1 \pm 0.17$ | 2.47~15.83 |
| | | Male | 29 | $4.6 \pm 0.32$ | 2.02~6.97 |

**Fig. 1.** *Distribution of infected moths of the silkworm in relation with their concentration of N. bombycis spores and those rate of transovarial transmission to their progeny : examined Saseongjam infected with standard type of the pathogen, the moths weighed after dry were measured spore concentration.*

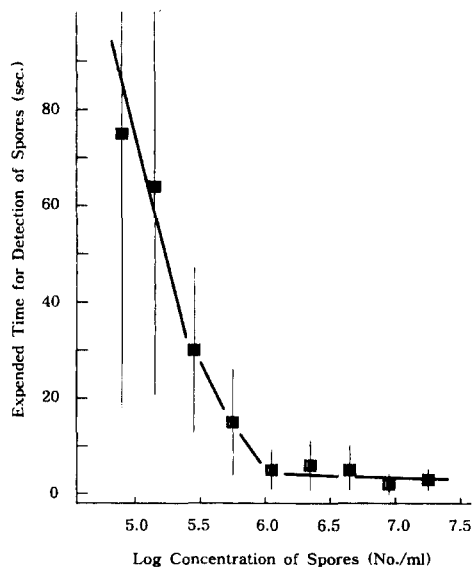with the lower spores shared 0.66% among the total moths, however, as many as 1.74 and 1.78×10⁸ spores.



**Fig. 2.** *Times (mean±SD) needed for the detection N. bombycis spores under the microscopic observation (magnification; 600×) in response to the concentration of spores included in the homogenate of moths with 2% KOH (w/v) as a sample suspention.*

erage spore content was $(6.8 \pm 0.14) \times 10^8$ per moth (Table 3). An experimental design for detection of infected moth, the spore content might be served as a limit of dillution for the sample size. Infected moths

**Table 3.** *Proportion of the female moths with various levels of spore content by infection of N. bombycis in the silkworm : the spores per moth was investigated for 305 individuals, and varied the range from the highest to the Lowest as 16.64 to 1.74 ($\times 10^8$) spores*

| Levels of Spore Content ($\times 10^8$/moth) | Number of Moths | Frequency (%) | % accumulation from the Highest |
|---|---|---|---|
| 16.0~16.9 | 1 | 0.33 | 0.33 |
| 15.0~15.9 | 1 | 0.33 | 0.66 |
| 14.0~14.9 | | 0.00 | 1.64 |
| 13.0~13.9 | 3 | 0.98 | 2.30 |
| 12.0~12.9 | 2 | 0.66 | 3.93 |
| 11.0~11.9 | 5 | 1.64 | 7.54 |
| 9.0~ 9.9 | 11 | 3.61 | 19.02 |
| 8.0~ 8.9 | 35 | 11.48 | 30.16 |
| 7.0~ 7.9 | 34 | 11.15 | 44.92 |
| 6.0~ 6.9 | 45 | 14.75 | 60.33 |
| 5.0~ 5.9 | 47 | 15.41 | 74.43 |
| 4.0~ 4.9 | 43 | 14.10 | 88.53 |
| 3.0~ 3.9 | 43 | 14.10 | 96.07 |
| 2.0~ 2.9 | 23 | 7.54 | 99.34 |
| 1.0~ 1.9 | 10 | 3.28 | 100.00 |
| Sum | 305 | \*Average=$(6.8 \pm 0.14) \times 10^8$/moth | |

Detectablity of the microsporidian spores was limited by those concentration of $3.8 \times 10^5$/ml as that observable at the least 1 spore per microscopic field by the magnification of $600 \times$. The more spores on the microscopic field needed for the rapid and easy detection, therefore, the spore concentration-time response was assayed (Fig. 2). Time needed for the detection of the spores under the microscope (magnification of $600 \times$) was about 2.57 to 2.26 sec. for the concentration of $1.8 \times 10^7$/ml or $9 \times 10^6$/ml. The time took about 4 to 6 sec. for the sample concentrations among $4.5 \times 10^6$ /ml, $2.3 \times 10^6$/ml, and $1.1 \times 10^6$/ml, none of the significant change was observed for those spore content. Apparent increase of time expence for detection from the concentration of $5.7 \times 10^5$/ml as 14.7 sec., and followed by 29.8 sec. for the concentration of $2.9 \times 10^5$/ml which relevent to the level below 1 spore per microscopic field (Table 4).

Theoretical value of a lot as proper sample size was determined on the bases of the experimental design, the scale of a lot for mass inspection in relation with the number of spores observable under the microscopic field (Table 5). The level of $3.9 \times 10^8$ spores per moth was occupied by 90% among the infected moth populations, and those become $128.67 \times 10^6$ spores/ml following homogenization with 3 ml of 2% KOH solution. The sample suspention was determined its limit of dillution as 340 times, which represent its detectability for a case included 1 of infected moth among in the 1,020 ml of suspention prepared by the homogenization of 340 moths. A level for the average spore content of $6.8 \times 10^8$, it could be

detectable at the dillution limit of 596 times. Sample suspension prepared by the moth contained $1.8 \times 10^8$ spores, $5.9 \times 10^7$ spores were exist per ml of homogenate, which could be checked efficiently. It was able to cover the 99.7% among the population of infected moths, and the level was also satisfied the present rule of 0.5% permission of infected moth. Dillution limit determined on the condition of 0% or 0.5 % permission, was 140 or 160 times. While the limit of dillution for practical use, counted for the time expense, up to the 60 times dillution was possible on the condition for 0% permission of infected moth. *N. bombycis* spores could be recognizable at the least 4 spores per microscopic field following the current rule conditioned 35 to 40 moths for a lot as a proper sample size.

· On the bases of the above results, moth inspection could be practiced without concentration of the sample suspension by centrifugal machine. The suggested idea needed additional consideration about the spore content of the moth infected at around the end of the 5th instar. The simple test by using 5 moths infected by inoculation at the time of 19 to 22 hours before spinning, provided a predictive data for the problem, and those spore content measured as 1.66 to $5.54 \times 10^8$ spores/moth. According to the customal moth inspection method, sample for a lot consist of 40 moths. Therefore, the lowest spore content appears the concentration of 1. $38 \times 10^6$ spores/ml, which was caused by homogenization in the 120 ml of sample suspension. It could be contained at least 3 spore per microscopic field, and relevant to the level of dillution for prac-

**Table 4.** *Experimental design for a detectablity of microsporidian spores from infected moths with various dillutions of homogenate*

| Dillution of Infected Moth by the Scale of a Lot | Number of Spores Observed per Microscopic Field | Number of Spores Included in the Moth Homogenate |
|---|---|---|
| 1/40 | $19.03 \pm 1.64$ | $6.00 \times 10^6$/ml |
| 1/80 | $10.64 \pm 0.76$ | $3.00 \times 10^6$/ml |
| 1/160 | $5.80 \pm 0.40$ | $1.50 \times 10^6$/ml |
| 1/320 | $2.80 \pm 0.34$ | $0.75 \times 10^6$/ml |
| 1/640 | $1.03 \pm 0.12$ | $0.38 \times 10^6$/ml |
| 1/1280 | $0.40 \pm 0.11$ | $0.19 \times 10^6$/ml |

*Observation of 3 to 4 microscopic fields for each of 5 replicates by the magnification of 600 times.
*Spore concentration of $2.4 \times 10^8$ spores/ml for dillution was based on the original homogenate of $7.2 \times 10^8$ spores/3 ml/moth, which was confirmed transovarial transmission in their progeny.

**Table 5.** *Theoretical value of dilution limit estimated for the detectablity of 90 to 100% of infected moth populations based on the previous data of spore numbers observable under microscope, detectability-time response, and distribution of infected moths by spore content (the volume for dilution by a lot of moth inspection was counted as 3 ml per moth)*

| Dilution of Infected Moth by a Lot | The Level of Spore Content for the Specimen by a lot on the Detection of Infected Moths at the Rate Above 90% | | | | |
|---|---|---|---|---|---|
| | 100% | 99.7% | 99.0% | 95.0% | 90.0% |
| | | | | | ($\times 10^6$ spores/m*l*) |
| 1/1 | 58.00 | 59.33 | 76.33 | 111.67 | 128.67 |
| 1/20 | 2.90 | 2.97 | 3.81 | 5.58 | 6.43 |
| 1/30 | 1.93 | 1.98 | 2.54 | 3.72 | 4.29 |
| 1/40 | 1.45 | 1.48 | 1.91 | 2.79 | 3.22 |
| 1/60 | 0.97** | 0.99** | 1.27 | 1.86 | 2.14 |
| 1/80 | 0.73 | 0.74 | 0.95** | 1.40 | 1.61 |
| 1/100 | 0.58 | 0.59 | 0.76 | 1.12** | 1.29 |
| 1/120 | 0.48 | 0.49 | 0.64 | 0.93 | 1.07** |
| 1/140 | 0.41* | 0.42 | 0.55 | 0.80 | 0.92 |
| 1/160 | 0.36 | 0.37* | 0.48 | 0.70 | 0.80 |
| 1/180 | 0.32 | 0.33 | 0.42 | 0.62 | 0.72 |
| 1/200 | 0.29 | 0.30 | 0.38* | 0.56 | 0.64 |
| 1/220 | 0.26 | 0.27 | 0.35 | 0.51 | 0.59 |
| 1/240 | 0.24 | 0.25 | 0.32 | 0.47 | 0.54 |
| 1/260 | 0.22 | 0.23 | 0.29 | 0.43 | 0.50 |
| 1/280 | 0.21 | 0.21 | 0.27 | 0.40 | 0.46 |
| 1/300 | 0.19 | 0.20 | 0.25 | 0.37* | 0.43 |
| 1/320 | 0.18 | 0.18 | 0.23 | 0.35 | 0.40 |
| 1/340 | 0.17 | 0.17 | 0.22 | 0.33 | 0.38* |

*dillution limit suggested as a level observable at least 1 spore per microscopic field.
**the samples recognizable about 3 spores per field, and detectable within 10 second through microscopic observation by the magnification of 600 ×.

tical use as those estimated.

Herein recommended simplistic mass inspection system of silkworm moth without centrifuge by conditioning the number of a lot for about 40 moths (for 1 box of egg production). It is suggested that these methods will promote efficiency and save the facillities and labor, as well as the time needed for the practice of moth inspection

## 적    요

관행 모아검사법의 문제점을 개선하기 위하여 미립자병 감염 모아에 존재하는 병원포자 함량을 조사하고 경란전염성을 검정하는 동시에, 모아검사의 효율성을 고려한 검사시료의 희석한계를 조사하고 합리적인 모아검사단위의 설정과 검사방법의 개선을 시도하였다. 그 결과 표준형 및 야생형 *Nosema bombycis*는 장려잠품종에서 차대잠의 80% 이상의 경란 전염이 확인되었으며, 감염모아 1두당 *N. bombycis* 포자의 수는 평균 7×10⁸ 로서, 검사용 모아 100 마리 중 감염아 1마리가 혼재하여도 배율 600의 현미경 검사에서 포자가 검출되었다. 집단검사를 위한 모아검사단위의 최대한계는 감염모아 허용비율이 0%일 때 140 두, 0.5%일 때 160 두, 1%에서는 200 두였으나, 신속성과 감염아 허용비율 등의 조건을 충족할 수 있는실용적 검사단위는 60두 이내로 산정하였다. 관행의 보통잠종 제조에서 규정하는 감염아 허용비율은 0.5%로서 모아검사 단위가 35 내지 40 두이므로, 원심분리에 의한 집포자 조작이 생략되어도 시야당 병원포자의 검출빈도가 향상되어 모아의 감염 여부를 식별하기에 충분하였다. 따라서, 본 결과는 기술 및 설비 등 여건이 미흡한 지역이나 개발도상국에서 실행할 수 있는 모아검사 체계의 이론적 근거를 확립하는 동시에 잠업 선진국에서도 모아검사에 요하는 설비와 시간 및 비용을 절감할 수 있는 방안을 제시하였다.

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