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Infectivity of Two Isolates of Microsporidia on Silkworm (*Bombyx mori* L.) in South Sulawesi

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Abstract

Pebrine disease caused by *Nosema bombycis* is a detrimental disease of *Bombyx mori* and a serious limiting factor in sericulture industry that can even thwart the cocoon harvest. This study aims to determine the infectivity of two isolates of *N. bombycis* from two different seed sources (locally produced, PebLok and imported seeds, PebCin) in South Sulawesi. Spore suspension of *N. bombycis* (1×10^8 spores/ml aquadest) was evenly smeared on mulberry leaf surfaces. Early third and fourth larval instars of *B. mori* were fed with the smeared leaves. Insect mortality and symptom development were observed every 24 hr. Research results show that PebCin and PebLok were more infective at instar 3 and instar 4, respectively, although virulence of both isolates were relatively equal with cumulative mortality of more than 60%. Infection of both isolates caused a longer period of larval development for more than 10 days compared to uninfected larvae which was related to increased duration for each instar.

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Keywords

Bombyx mori
Infectivity
Nosema bombycis
PebCin
PebLok

Introduction

South Sulawesi is the center of silk production in Indonesia, but for the last five years, production was declining steadily. One of the main problems is the presence of pebrine disease. The disease caused by *Nosema bombycis* Naegeli (Microsporidia: Nosematidae). Pebrine disease had also destroyed the natural silk industry in Europe, particularly France and Italy in the mid-19th century (Franzen, 2008; Yup-lian, 1991; Rahmathulla et al., 2012). In 1845, France cocoon production was 26,000 tons and fell to 4,000 tonnes in 1853, later in 1865 only then the cause of the France and Italy silk industry collapse was revealed which related to the pebrine disease (Bhat et al., 2009). More than 40% of infection on female parents of silkworm were reported in Southeast Asia including Indonesia and Vietnam (Japan Overseas Cooperation Volunteers, 1975). Furthermore, the level of attacks in the last five years has reached more than 60% and even it frequently causes harvest failures in Indonesia (BPA, 2015).

Pathogenicity is absolute whether pathogenic organisms causing disease in its host or not, while the virulence is a quantitative measure of the ability to cause disease. Infectivity is the ability of an organism to cause infection (Shapiro-Ilan et al., 2012). Silkworm farmers in South Sulawesi use locally produced and imported eggs. In both of seed sources, infection of the microsporidia is unavoidable. Therefore, a study was conducted to determine the infectivity of microsporidianobtained from the two sources of silkworm seeds.

Materials and methods

Isolation and purification of microsporidian spores

Two isolates of microsporidia were isolated from silkworm larvae (*N. bombycis*) grown from seeds obtained from two different sources. Microsporidian PebLok isolate was from local seed source and PebCin isolate was from import seed source. Heavily infected larvae were crushed in distilled water and large silkworm and mulberry debris were removed by filtration on 2 cheesecloth layers. The supernatant was discarded and centrifuged 3000 rpm for 15 min, and the spore pellet was re-suspended to 10 ml using sterile distilled water, and then the suspension was re-centrifuged. This procedure was repeated 3 times, spores were washed in distilled water until the pellet was white. Finally, 10 ml spore solution was pipetted and poured into a hemocytometer for spore counting. The spores were counted under a light microscope at x40 magnifications (Kermani et al., 2013). Spore suspensions of 1×10^{8} ml^{-1} were prepared by diluting with distilled water, and then stored at 4°C until further use.

Preparation of silkworms infected with Microsporidia

The silkworm larvae of BS09 were reared under room conditions with an average temperature of 25.9°C and humidity of 87.4%, 12 hrs of light and 12 hrs of dark. The silkworm larvae of the 2nd molt (third instar) and 3rd molt (fourth instar) were orally inoculated by feeding with mulberry leaves that were previously smeared with *N. Bombycis* spores.

Results and discussion

Disease occurrence

A day after fed with *N. bombycis* spore, no obvious symptoms were shown by the larvae. On the third day after infection then the larvae showed symptoms, i.e., slower larval movements, sluggish, reduced appetite and stunted development. These symptoms continued until the next moulting period. In the third instar inoculation, mortality occurred at the third day after infection (DAI) and was earlier in PebCin compared to PebLok (Fig. 1).

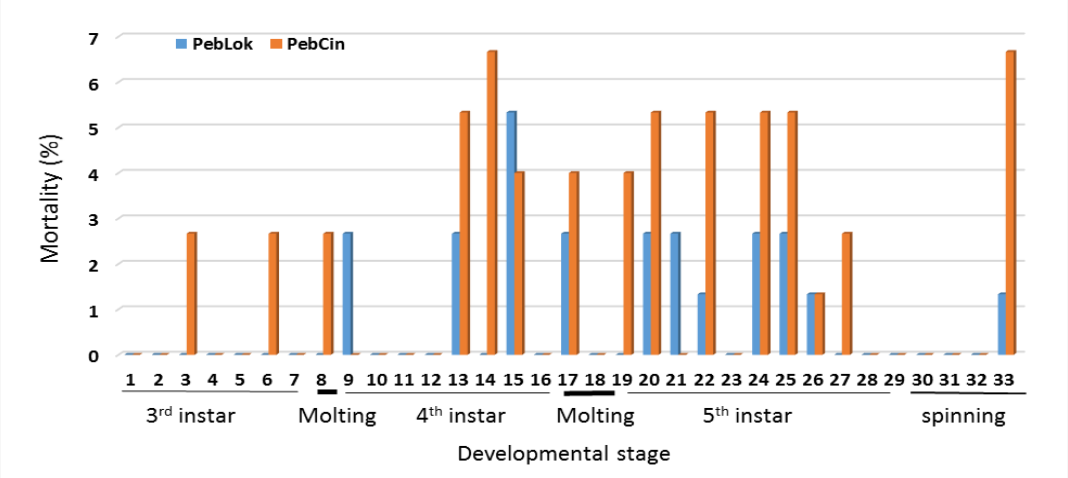

Fig. 1: Silkworm mortality after infection of two isolates of *N. bombycis* with different strains at third instar inoculation.

Death of larvae were observed almost every day, but the highest mortality occurred in PebCin at 14thDAI or in the late instar 4 period and at 33rd or in the late spinning, while the highest mortality in PebLok was at 15thDAI. In the fourth instar inoculation (Fig. 2), the opposite happened on mortality where highest mortality in PebLok occurred at third DAI which was earlier than in PebCin. Although the occurrence of larvae death was observed every day, the highest mortality occurred at the time of the last molt. This continued and the highest mortality in PebLok occurred on day 11, 12 and 13 DAI or when entering fifth instar while the highest mortality in PebCin was at the 14th DAI (5thinstar). The new external symptoms were observed after day 10 DAI and mortality of larvae increased in both inoculation instar 3 or 4. Histopathological study by Jyothi and Patil (2011) shows the midgut has formed sporoblast phase on the third day after infection. Hossain et al. (2012), sporo plasma gradually increased in size and developed into meronts 24-48 hrs after inoculation. Infection by the most pathogenic strain of *N. bombycis* in India showed mortality occurred on day 10 after infection and can occur at all stages of larval development which is described by Rao et al. (2004).

The incubation period is the period starting from the entry of the pathogen into the host's body until the occurrence of symptoms or signs of the disease (Nataraju et al., 2005). The incubation period, or the latent period is a period from an infection or pathogen inoculation until the commencement of larval mortality (Begum et al., 2004). Bhat et al. (2009), reported that the larvae become infected during early instar stages 1 and 2 will be dead in instar 5, but if the infection occurs during the 4th or 5th instar, larvae can still survive and form cocoons. Further observation by Bhat and Nataraju (2005) showed that inoculated larvae at 4th instar with a concentration 1×10^5 spores ml^{-1} can survive until pupa formation phase.

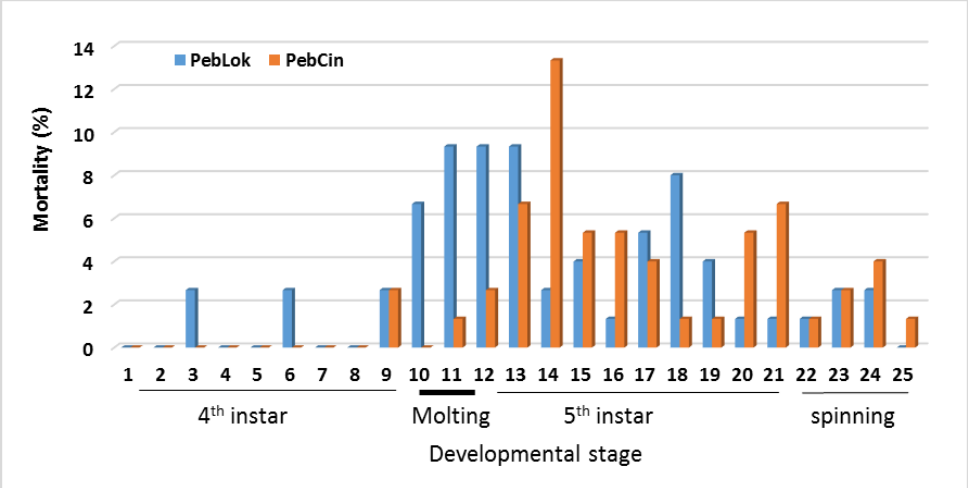

Fig. 2: Silkworm mortality after infection of two isolates of *N. bombycis* with different strains at fourth instar inoculation.

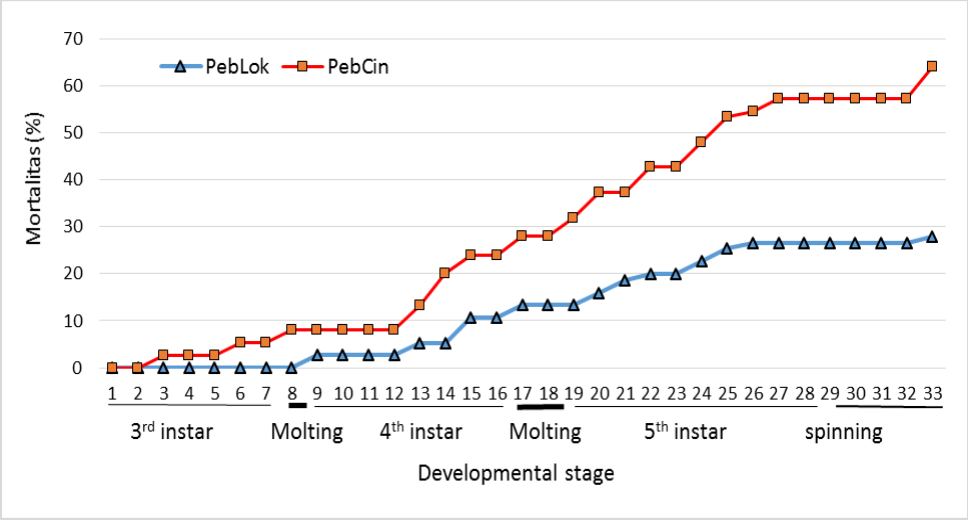

Fig. 3: Cumulative mortality of silkworms after infection of two isolates of *N. bombycis* with different strains at third instar inoculation.

Cumulative mortality of silkworms after inoculation of two isolates at instar 3 shows higher figure in PebCin (64%) than in PebLok which only showed cumulative mortality of 28% (Fig. 3). Contrary, in the fourth instar inoculation, PebCin cumulative mortality was 65% while PebLok reached 77% (Fig. 4). Infectivity is a manifestation of the severity of the disease that can be measured on the infected host. Estimates of mortality or the mortality rate of the host is one measure of the infectivity of the pathogen (Thomas and Elkinton, 2004). Sharma et al. (2014) suggests that infection of 1×10^6 spores ml^{-1} in early instar 3 showed cumulative mortality of larvae to become pupa of 50.66%. Infection of *N. bombycis* on the first day of instar 2 resulted in cumulative mortality of larvae entering the cocoon forming phase of 41.0% (Bhat

et al., 2009). In addition, study of Tu et al. (2011) shows mortality up to 61.11% at infectivity of *N. bombycis* was at concentration of 10^7 ml^{-1}. This result similar to Rao et al. (2004) that suggests that at a dose of 10^7 spores ml^{-1} mortality can reach more than 50% after infection. While studies of Jyothi and Patil (2011) showed that fourth instar mortality was 86% at a concentration of 1×10^6 spores $larva^{-1}$, infectivity can reach 90% at a concentration 1.52×10^6 spores ml^{-1} (Chakrabarty et al., 2012).

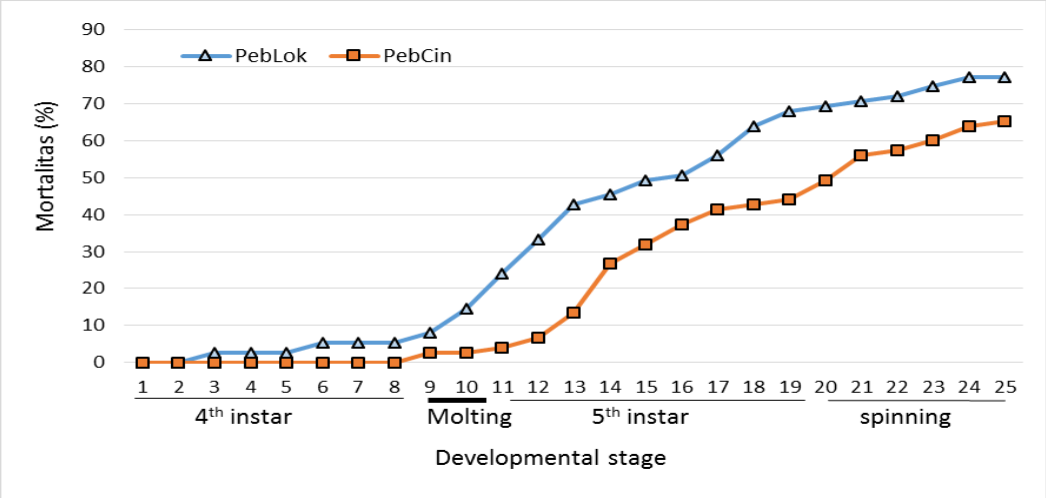

Fig. 4: Cumulative mortality of silkworm after infection of two isolates of *N. bombycis* with different strains on fourth instar inoculation.

Disease progression

As response to the infection of microspores, a change occurred on the duration of each instar development stage. Table 1 shows that the duration of larval development of infected larvae were longer than in the not infected larvae, i.e., longer 2.5 days in the third instar; 3 days in the fourth instar; 2.5 days in the fifth instar and 2 days in the cocoon formation phase (spinning) resulted in overall increase of 10.5 days compared to the uninfected larvae. According to Solter et al. (2012), effects that are typical on silkworm larvae that sublethal for infection of *N. bombycis* is extended period for larval development due to suspended ecdysis process.

Table 1. Larval and spinning stages of silkworms infected with *N. bombycis* strain PebLok and PebCin on 3rd or 4th instar.

Instar	Treatment			Control (no infection)		
	Eating stage (day)	Moulting (day)	Larval stage (day)	Eating stage (day)	Moulting (day)	Larval stage (day)
3	6.0	1.5	7.5	4.0	1.0	5.0
4	7.0	2.5	9.5	4.5	1.5	6.0
5	9.5	-	9.5	7.0	-	7.0
Spinning	5.0	-	5.0	3.0	-	3.0
Total			31.5	Total		21.0

Conclusion

Strain PebCin is more ineffective at the third instar, with cumulative mortality of more than 60%. Strain PebLok infective at the fourth instar but the virulence of two isolates of *N. bombycis* were relatively equal in the cumulative larval mortality of more than 60%. Both isolates resulted in a longer duration of larval development of about 10 days longer compared to uninfected due to increased duration for each instar stage.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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