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Variability in germination and in temperature and storage resistance among *Paenibacillus larvae* genotypes

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Abstract

There are several methods for cultivation of *Paenibacillus larvae*, the causative agent of American foulbrood (AFB) in honey bees. Protocols for detection of sub-clinical levels of the bacterium from honey and bee samples include heat treatment of samples. The main objective of this study was to investigate if there is variability in temperature resistance among *P. larvae* genotypes, potentially leading to biased diagnose and disease monitoring. The variation in germination and proliferation ability among type collection ($N = 4$) and field isolates ($N = 4$) of *P. larvae* representing four different genotypes was investigated. Results demonstrate a significant variability between *P. larvae* genotypes in germination rate on solid media as well as in endospore resistance to heat treatment and storage. It is concluded that strains of different genotypes should be included in evaluation of standard laboratory protocols for cultivation of *P. larvae* to avoid bias in disease monitoring and quantification of the pathogen.

Keywords: American foulbrood; Endospores; Genotypes; Germination; *Paenibacillus larvae*

1. Introduction

American foulbrood (AFB) is a severe bacterial disease affecting apiculture worldwide. AFB is lethal to infected honey bee larvae and can be devastating to colonies and apiaries if proper control measures are not carried out. The etiological agent, *Paenibacillus larvae* (Genersch et al., 2006) is an endospore-forming, Gram-positive rod. Bacterial sub-typing has demonstrated considerable diversity within the species, and at least four distinct genotypes have been described (Ashiralieva and Genersch, 2006). Traditional diagnosis of AFB is based on observation of clinical symptoms and microbial cultivation of material from infected colonies. Several diagnostic protocols have been presented, all including heat treatment of samples to avoid contamination of other bacteria and stimulate germination (Table 1).

The number of bacterial cells in a solution can be measured either by microscopic count (total cell count) or by counting colonies growing on artificial

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Table 1
Temperature and duration of heat treatment in various investigations of the presence of *Paenibacillus larvae* spores in samples of honey and adult bees

Publication	Heat treatment of bee samples	Heat treatment of honey samples
Pernal and Melathopoulos (2006)	85 °C for 15 min	85 °C for 15 min
Hansen (1984)	–	88–92 °C for 5 min
Hornitzky and Karlovskis (1989)	80 °C for 15 min	–
Dingman and Stahly (1983)	–	65 °C for 15 min
Schuch et al. (2001)	–	80 °C for 15 min
Nordström et al. (2002)	85 °C for 10 min	88 °C for 10 min
Hornitzky and Clark (1991)	–	80 °C for 15 min
Alippi et al. (2004)	–	85–90 °C for 10 min
Lindström and Fries (2005)	91 °C for 10 min	–
Genersch and Otten (2003)	–	90 °C for 6 min
Steinkraus and Morse (1996)	–	80 °C for 10 min
Goodwin et al. (1996)	93 °C for 20 min	–
Nordström and Fries (1995)	–	80–82 °C for 10 min
Shimanuki and Knox (1988)	–	80 °C for 10 min

media (viable count). Plate counts can be highly unreliable, and microscopic counts typically reveal far more spores than germinate on any given culture medium. Thus, total spore number can be underestimated by several orders of magnitude (Madigan et al., 2003). Selection of media, choice of temperature treatment as well as awareness of the discrepancy between total cell count and viable cell count are important when performing studies on *P. larvae*. Moreover, the documented genetic variability between isolates raises the question if methods used will yield genotype specific results. Thus, the primary goal of the current study was to investigate if the response to various heat treatments is strain and/or genotype specific. Additionally, we investigated if the germination rate and endospore storage durability is genotype specific.

2. Materials and methods

2.1. Bacterial strains

Eight strains of *P. larvae* were used in the experiment representing the four different genotypes described using ERIC primers (Genersch et al., 2006). Four of the isolates were commercially available reference or type collection strains: ATCC 9545 (ERIC I), LMG 16252 (ERIC III), LMG 16247, LMG 14427 (ERIC IV). The remaining four strains were isolated from samples of Swedish honey bee brood with symptoms of AFB (field strains), and deposited at the Culture Collection of the University of Gothenburg: CCUG 48979, CCUG 48978 (ERIC I), CCUG 48972, CCUG 48973 (ERIC II).

Bacterial strains were cultivated on MYPGP-agar plates as described by Nordström and Fries (1995), and incubated at 35 °C and 5% CO_2 for 10–14 days to obtain sporulation. Fresh spore suspensions (stock solutions) were prepared by suspending bacterial colonies in sterile 0.9% NaCl. Suspensions were checked under microscope to confirm presence of endospores and absence of vegetative cells. The stock spore suspensions were stored at 4 °C.

2.2. Colony and microscopic count

A 10-fold dilution series from each stock suspension was made by successive dilutions to a final dilution of 10^{-6}. Ten microliters of the dilutions were spread over the surface of an agar plate using a sterile spreader. Each dilution was inoculated onto three plates to obtain a mean plating value for colony forming units (CFU). Plates representing the dilution with countable (30–100) CFU were used in the final estimation of the number of viable spores in the undiluted stock suspension.

A direct microscopic count of the spore suspensions was made in sub-samples of the stock suspensions using a Helber Bacteria Counting Chamber (Hawksley), according to the manufacturer's recommended protocol. Counting was made using a