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Searching for an American foulbrood early detection threshold by the determination of *Paenibacillus larvae* spore load in worker honey bees

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Abstract

In the present study, the relationship between *Paenibacillus larvae* (White) spore load per adult bee and the appearance of American Foulbrood (AFB) symptoms in the colony was investigated. A total of 44 beehives from 8 apiaries located in Sardinia island (Italy) were involved in this research. Adult bee samples were collected from colonies either showing or lacking the disease symptoms, and the average number of *P. larvae* spores per adult bee was determined through microbiological culture, followed by bacterial identification through a combination of morphological, biochemical and genetic assays.

Correspondence between the detection of disease symptoms in the hives and the number of spores per adult bee samples was obtained. In line with previous studies, the outcome of the present investigation supports the employment of adult bee spore load determination as a tool to get information on the actual health status of the colony. This allows an early detection of AFB disease, especially when clinical symptoms in colonies are still not observable. In addition, the results suggest that a level around 3,000 *P. larvae* spores per adult bee, obtained with our experimental conditions, might represent a threshold for the appearance of clinical symptoms in the colony.

Key words: American foulbrood, diagnosis, spore load, adult honey bees, *Apis mellifera ligustica*.

Introduction

American foulbrood (AFB) represents a fatal brood infection for the honey bees, with special regard to the European honey bee, *Apis mellifera* L. (Genersch, 2010), while the Asian honey bee, *Apis cerana* F., owing to its high degree of hygienic behaviour, appears resistant (Chen *et al*., 2000). Due to its high pathogenic potential and difficult eradication, once it is established in the apiary, this disease may cause entire colony death (Hansen and Brodsgaard, 1999). The affected hives undergo a progressive depopulation and as a result of a general weakening effect, colonies become more susceptible to robbery by stronger and uninfected colonies from the same apiary or from neighbouring ones. In this way, disease spread is favoured.

The etiological disease agent is the spore-forming bacterium *Paenibacillus larvae* (White) (Genersch *et al*., 2005), characterized by the production of highly resistant spores (Shimanuki, 1997). The infection process normally encompasses the germination of spores after ingestion by larvae fed on contaminated food (Bamrick and Rothenbuhler, 1961). On the other side, spores are unable to act significantly inside adult bee midgut, so that they are apparently harmless to this insect stage. Therefore, worker bees performing the nest tasks may operate as asymptomatic spore carriers in the apiary, as a consequence of their spore ingestion or body surface contamination. In addition, these adults may represent spore-vectors among different colonies or apiaries, as a result of their drifting or robbing behaviour (Lindström *et al*., 2008b). On the other hand, from a diagnostic point of view, the detection of spores in adult bees may represent an important tool to prevent the development and the spread of this very dangerous infectious disease (Lindström, 2006).

According to several studies, *P. larvae* spore detection in adult bees is regarded as a more sensitive diagnostic method in comparison to honey or immature stage analyses in the colony (Nordström *et al*., 2002; Gillard *et al*., 2008; Lindström *et al*., 2008a). Detecting and monitoring the number of spores per adult bee might represent the first step toward the prevention of American foulbrood development in the colony. In addition, adult spore loads data would allow gathering information on the hygienic behaviour of the colony (Fernandez *et al*., 2010).

The purpose of this work was to gain deeper understanding of the relationship between the number of spores per adult bee and the presence/absence of clinical disease symptoms in the honey bee colonies. In addition, an attempt to identify an AFB early detection threshold by the determination of *P. larvae* spore load in adult bees was made.

Materials and methods

Surveys and sampling of bees

During 2009 and 2010, adult bee (*Apis mellifera ligustica* Spinola) samples were collected from Sardinian (Italy) bee-hives in order to detect the presence and to

quantify the abundance of *P. larvae* spores, combining standard bacteriological and molecular approaches. Honey bee samples were collected from a total of 44 colonies kept in Dadant hives belonging to 8 apiaries, 3 of which located in the North (Berchidda 1, Berchidda 2, Capocaccia) and 5 in the South (Carbonia, Ingurtosu, Montevecchio, Musei, San Giovanni) of the island, during September-November 2009 and October-November 2010. Either colonies with AFB clinical symptoms or apparently healthy, were sampled.

Each colony was thoroughly inspected and all brood combs of the nest were visually screened for the absence or the presence of cells showing typical clinical symptoms, such as larval remains and dried-in scales. After the visual exam, samples of approximately 100 adult bees from each beehive were collected from combs with unsealed brood frames, thereby including worker bees performing tasks in the nest as cell cleaning or tending brood (Nordström, *et al*., 2002). Samples were stored at −20 °C before being subjected to microbiological analysis.

Isolation of spores from bees

Spores were isolated from bees, following a method proposed by Hornitzky and Karlovskis (1989) with several modifications. Thirty bees from each sample were randomly taken for the laboratory analyses and homogenized in 10 ml phosphate buffer (PBS: Na_2HPO_4, KH_2PO_4, NaCl) pH 7.2, using a stomacher for two minutes at 230 rpm. The resulting homogenate was then placed in a 90 ºC water bath for 10-15 minutes, and shaken by vortex for 30 seconds.

In order to quantify viable spore abundance, serial dilutions (1:2 and 1:10) of the bee homogenates were prepared and their aliquots (100 µl) were plated on MYPGP agar Petri dishes (Dingman and Stahly, 1983), supplemented with 9 g/l nalidixic acid to inhibit *Paenibacillus alvei* (Cheshire et Cheyne) growth, and incubated at 37 ºC in microaerophilia (5-10% CO_2). The plates were checked after 2 and 4 days to count the number of *P. larvae*-like colony forming units (CFU) for each dilution, which permitted to calculate the number of viable spores per adult bee. These analyses were replicated three times.

After counting, *P. larvae*-like colonies were purified twice on MYPGP agar supplemented with nalidixic acid before being stored at 4 °C on Nutrient Broth (Difco) containing 20% glycerol. These colonies were subjected to the following specific identification analyses.

Identification of *Paenibacillus larvae*

Morphological observations of bacterial colonies and single cells under a light microscope were routinely conducted in order to screen for *P. larvae* isolates (Schaad, 2001). On the basis of their morphological characteristics, *P. larvae*-like isolates (3 per bee sample) were selected and submitted to biochemical and genetic analyses.

After preliminary catalase test (Heyndrickx *et al*., 1996) and Gram staining (Beveridge, 2001), bacterial isolates grown on MYPGP agar, were characterised by the API 20E kit (bioMérieux, France), specifically designed for Enterobacteriaceae identification, following manufacturer's instructions. For this purpose, a plastic strip holding twenty mini-test tubes was inoculated with a suspension of the bacterial culture that, as checked with a densitometer, had a turbidity corresponding to McFarland 2. After incubation for 24 and 48 h at 37 °C, colour reactions were read and converted to the seven-digit code, also known as Analytical Profile Index (API), employed for the bacterial identification (Kilwinski *et al*., 2004).

P. larvae specific identification was confirmed also by genetic analyses. The isolates were routinely grown on MYPGP agar supplemented with nalidixic acid. After 48 h, total DNA was extracted with Dneasy Blood & Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's instructions, and used as a template for Polymerase Chain Reactions (PCRs) with specific primers PL 4 (5'-TCAGTTATAGGCCAGAAAGC-3') and PL 5 (5'-CGAGCGGACCTTGTGTTTCC-3') (Piccini *et al*., 2002) which amplifies a 700 bp fragment of the *P. larvae* 16S rRNA gene. PCR was performed in a final volume of 25 µl containing 0.75 µl of 1.5 mM $MgCl_2$, 2.5 µl of 10 x PCR buffer, 0.5 µl of 100 µM deoxynucleotide triphosphate mix, 1 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA) and 0.4 µM of each primer. All PCRs were conducted with an initial denaturation phase (1 min) at 95 °C followed by 30 amplification cycles involving 93 °C (1 min), 60 °C (30 s), 72 °C (1 min) and a final extention step (5 min) at 72 °C, using a MJ MINI BIO-RAD Thermocycler. PCR products were visualized by electrophoresis in 0.8% (w/v) agarose gels stained with SYBR Safe (Invitrogen, Eugene, Oregon, USA) DNA gel staining and visualized using a UV-Transiluminator (GEL DOC 2000 BIO-RAD).

A *P. larvae* strain isolated from a bee hive in Calabria (Italy) and previously characterized was used as a control.

Statistical analysis

All statistical analyses were performed with Statgraphics Plus software (2001) with significance level set at $\alpha = 0.05$.

Data on spore number per adult bee were analysed by general linear model (GLM) of ANOVA, and means were separated by least squares means comparison (LSD test).

For a comparison between colonies with and without symptoms the means of spore number per bee were submitted to t-test.

In all cases data met the assumption of variance homogeneity and non-transformed values were used in the analyses.

Results and discussion

Almost all the samples (95%) collected from Sardinian bee hives cultured on MYPGP agar supplemented with nalidixic acid, showed typical *P. larvae* colonies. The colonies, after 4 days, were small, regular with a rough surface, flat or with a raised profile, and colour pale beige. All isolates selected for biochemical and genetic

analyses were Gram positive and catalase negative. As a result of Analytical Profile Index (API) test, they showed a typical carbohydrate acidification profile with acid from glucose and trehalose, but not from arabinose and xylose. Moreover some isolates showed variability on Ortho-Nitro-Phenil-β-D-GalactoPyranoside, on gelatin hydrolysis and utilization of rhamnose (Kilwinski *et al*., 2004).

Identification by PCR confirmed the identification results of the biochemical and physiological tests, giving the expected amplification product of 700 bp, as shown on figure 1 where PCR products from 5 different *P. larvae* isolates are compared to a control strain from Calabria. In addition to their usefulness for bacteria identification, these data represents also a further support to the reliability of PCR with specific *P. larvae* primers, as a quick and sensitive method for the AFB laboratory diagnosis, even when there are not visible disease symptoms. In line with various researches it allows to detect spores of the AFB agent from samples of either adult bees (Hornitzky, 1998; Lindström and Fries, 2005; Piccini and Zunino, 2001) or honey (Hansen, 1984; Hansen and Brødsgaard, 1999; Ritter, 2003).

In the apiary where all sampled colonies showed AFB clinical symptoms (San Giovanni and Carbonia), the average number of spores per bee was significantly higher (5,265 ± 395 and 4,511 ± 309, respectively) than that observed in the apiaries where all colonies showed no symptoms of the disease (Berchidda 1, Berchidda 2, Ingurtosu, Montevecchio, and Musei) and in the apiary of Capo Caccia where among 19 colonies screened only one showed AFB clinical symptoms (figure 2) ($df = 7$; $F = 31.35$; $P < 0.0001$). In the apiaries with no symptoms, the mean levels of spores per bee ranged between 14 ± 7 (Berchidda 1) and 1,842 ± 1,589 (Berchidda 2) while in the apiary of Capo Caccia this value corresponded to 438 ± 188.

Considering all the colonies with clinical symptoms from different apiaries as one group (17.5% of the total colonies), the average number of spores per bee was significantly higher than that observed in the group of colonies with no symptoms (82.5% of the total colonies) (4,623 ± 942 and 274 ± 665, respectively) ($t = -14.8345$ $P < 0.01$).

In addition, the number of spores per bee in colonies with clinical symptoms was always higher than 3,031 ± 1,180, while in samples collected from colonies with no symptoms it was always lower than 1,609 ± 566, with the sole exception of a sample with 3,431 ± 1,875 (figure 3). These results suggest that a level around 3,000 *P. larvae* spores per adult bee, determined with the methodologies employed in this work, might represent a threshold for the appearance of clinical symptoms in the colony.

More in general, the present investigation is in line with conclusions raised by Lindström and Fries (2005) that suggested the use of adult bee spore load as a parameter to get information on the actual health status of the colony. Other recent studies support the use of this method as an efficient tool to detect clinically diseased colonies (Nordström *et al*., 2002; Gillard *et al*., 2008; Lindström *et al*., 2008a; 2008b), but it permits also to identify colonies at sub-clinical disease stage (Lindström, 2008).

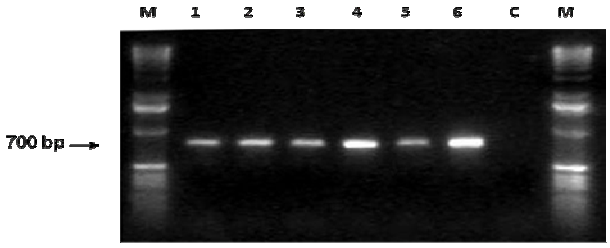

Figure 1. Agarose electrophoresis gel showing PCR products for a 16S rRNA gene fragment amplification of *P. larvae* isolates from different localities. Line 1, Musei; line 2, Calabria (positive control); line 3, Berchidda 1; line 4, Capo Caccia; line 5, Montevecchio; line 6, Ingurtosu; line C, negative control SDW; Lanes M, 100 bp ladder (Invitrogen).

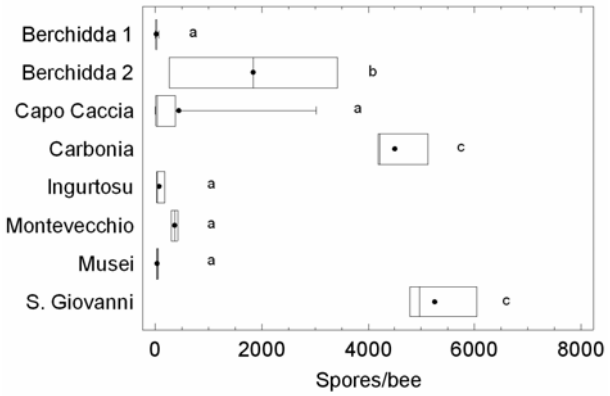

Figure 2. Number of spores per adult bee in different apiaries. The Whisker represents the maximum and the minimum of the values recorded; the points indicates the average; the left, right and middle lines of the box indicate the lower and upper quartile and the median, respectively. Bars marked with different letters indicate significant differences (GLM ANOVA, $P < 0.01$ followed by LSD test, $\alpha = 0.05$).

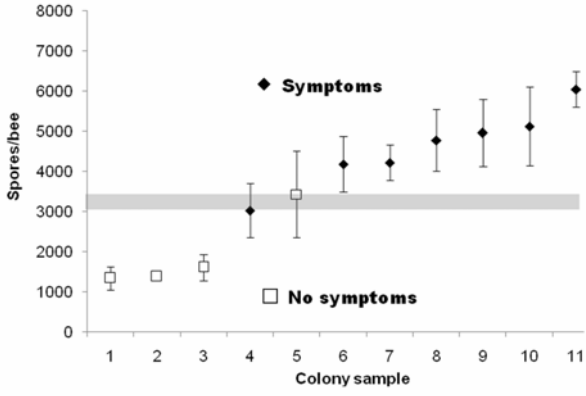

Figure 3. Mean number (± SE) of spores per adult bee in colony samples with or without American Foulbrood symptoms. Figure shows only the samples (11) with adult spore loads higher than 1,000. The other samples collected from non symptomatic colonies (33) are not represented as the relative spore loads ranged between 0 and 419. The shaded area included in the horizontal threshold line defines a range of average spore load in which both symptomatic and asymptomatic colonies can be found.

Further improvements of this detection method might involve new strategies, such as the direct DNA extraction from sampled adult bee spores, followed by quantitative 16S rRNA gene amplification (D'Alessandro *et al.*, 2007). This would allow skipping the time-consuming microbiological cultures and colony forming unit counting.

The high percentage (82.5%) of colonies at the subclinical stage of the disease we observed is representative of how early spore detection in adult bees can be helpful in determining the risk of clinical symptoms development (Nordström *et al.*, 2002; Lindström *et al.*, 2008a; 2008b). In a perspective of disease prevention, the determination of adult bee spore loads would be particularly important for yet asymptomatic colonies since symptomatic colonies are already lost for the beekeeper.

Referring to the reported relationship between spore load per adult bee and the disease stage, the determination of a threshold associated to the spread of clinical symptoms in the colony would be really valuable. In our experimental conditions this level was around 3,000 spores per adult bee. However, this value referred to the apiaries involved in this research, has to be carefully considered as more information will come from future studies conducted on colonies from different areas. In fact, there are numerous factors affecting the number of spores necessary to produce clinical symptoms, which make hard the determination of such a threshold. For instance, it is known that different *P. larvae* strains may show variable virulence levels (Genersch *et al.*, 2005) and that individual larval physiological traits may lead to different susceptibility degrees (Rothenbuhler and Thompson, 1956). At the colony level, the dissimilar hygienic behaviour of adult bees may affect the development of the disease (Spivak and Reuter, 2001). In addition, environmental factors such as pollen availability and nectar flow intensity can also play an important role (Momot and Rothenbuhler, 1971).

Previous reports (Gillard *et al.*, 2008; Lindström, 2008; Lindström *et al.*, 2008a; 2008b) focused on the distribution of *P. larvae* spores in adult bees within the hive (brood chambers, supers), or at the apiary level. More recently (Zuur *et al.*, 2009; Fernandez *et al.*, 2010), the relationship between the number of spores per bee and the disease stage in colonies from apiaries with different AFB history was studied and many factors were supposed to be implicated (i.e. outbreaks prior to the study, beekeeper management, bee drifting or robbing behaviour, the presence of other pathologies, environmental conditions). In particular, the possible influence of the Varroa management should be taken into account. In fact, the mite may cause variable degrees of colony weakening, in relation to the infestation level, making it more susceptible to the AFB development. On the other side, it may also cause a brood weight loss, which would influence directly *P. larvae* spore titre in the colony. Moreover, we cannot exclude that colonies with high *P. larvae* spore loads may not show AFB clinical symptoms as a result of the preventive use of antibiotics or of a higher intrinsic colony resistance to the disease. These factors may retard or hide the onset of the disease.

For all these reasons, more research is needed to come to a broadly applicable model of AFB development risk assessment based on a *P. larvae* spore load threshold. Considering the high variability of factors affecting symptomatology appearance in the colony, at the present state of the art, instead of a specific threshold, referring to a spore load range associated with symptoms appearance would be more realistic. Given that adult spore load monitoring in the bee colonies would be very helpful for disease prevention purposes in the apiary, a detection threshold would need to be defined case by case.

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