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Distribution of *Paenibacillus larvae* spores inside honey bee colonies and its relevance for diagnosis

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ABSTRACT

One of the most important factors affecting the development of honey bee colonies is infectious diseases such as American foulbrood (AFB) caused by the spore forming Gram-positive bacterium *Paenibacillus larvae*. Colony inspections for AFB clinical symptoms are time consuming. Moreover, diseased cells in the early stages of the infection may easily be overlooked. In this study, we investigated whether it is possible to determine the sanitary status of a colony based on analyses of different materials collected from the hive. We analysed 237 bee samples and 67 honey samples originating from 71 colonies situated in 13 apiaries with clinical AFB occurrences. We tested whether a difference in spore load among bees inside the whole hive exists and which sample material related to its location inside the hive was the most appropriate for an early AFB diagnosis based on the culture method. Results indicated that diagnostics based on analysis of honey samples and bees collected at the hive entrance are of limited value as only 86% and 83%, respectively, of samples from AFB-symptomatic colonies were positive. Analysis of bee samples collected from the brood nest, honey chamber, and edge frame allowed the detection of all colonies showing AFB clinical symptoms. Microbiological analysis showed that more than one quarter of samples collected from colonies without AFB clinical symptoms were positive for *P. larvae*. Based on these results, we recommend investigating colonies by testing bee samples from the brood nest, edge frame or honey chamber for *P. larvae* spores.

1. Introduction

Healthy honey bee colonies are essential for good honey production and effective pollination. The occurrence of infectious diseases is an important factor affecting the development of the colonies and among these diseases, the highly contagious American foulbrood (AFB) which is caused by the spore forming Gram-positive bacterium *Paenibacillus larvae* (Genersch et al., 2006). It affects the larval and pupal stage of honey bees (*Apis mellifera* L.) (Bamrick and Rothenbühler, 1961), and is a serious disease in countries worldwide (Alippi and Aguilar, 1998; Ellis and Munn, 2005), including Switzerland. All regions of this country are affected with about 100 outbreaks recorded per year over the last decade (BVET, 2008).

Because of the severity of the disease, Swiss law requires control of the infection of American foulbrood by the destruction of clinically diseased colonies. In addition, bee inspectors have to perform a visual frame-by-frame inspection of all beehives within 2 km of the infected colony. Use of antibiotics for treatment or prevention is not allowed.

With a colony density of about 4.7 colonies per km^2 in Switzerland, these surrounding inspections are labour-intensive. Moreover, diseased cells in the early stages of the infection are easily overlooked and larvae infected with some genotypes of *P. larvae* can be removed before capping of the cell, reducing the presence of clinical symptoms (Ashiralieva and Genersch, 2006).

Identification of American foulbrood pathogen can be done by several methods (Anonymous, 2004; de Graaf et al., 2006). With culture techniques, *P. larvae* spores may be detected even when no AFB symptoms were visually observed. Spores can be detected from extracted honey (Hansen, 1984; Hansen and Brodsgaard, 1999), honey taken from the brood nest (Ritter, 2003) or adult bees (Hornitzky, 1998; Lindström and Fries, 2005; Piccini and Zunino, 2001). Culturing *P. larvae* spores from honey samples is widely practised, but does not always reflect the current disease status in the colony (Kabay, 1995; Nordström et al., 2002). Sampling adult bees could yield reliable information about the actual health status oh the colony (Lindström and Fries, 2005). Depending on the season and on the type of hives, the brood chamber can be difficult to access. Lindström and Fries (2005) showed that there is no practical difference in spore load between adult honey bees caught from supers and brood

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chambers. Goodwin et al. (1996) showed that the spore load is the highest by bees taken from the brood comb, followed by bees caught from the honey frames. Bees taken from the hive entrance carried the least number of spores. Our study was performed to determine by culture method the distribution of *P. larvae* spores inside bee colonies and which material from which location inside the hive is the most appropriate for diagnosing AFB infection.

2. Materials and methods

2.1. Sampling procedure

During the years 2004 and 2005, 237 bee samples and 67 honey samples were collected during the active bee season in Switzerland from 13 hobby apiaries with clinical AFB occurrence. The apiaries contained Swiss hives, a hive system with a single brood box occupied with 13 frames. Most of the colonies had a honey chamber when we made the sampling. From each apiary, we collected samples with a maximum of four colonies with and four colonies without AFB clinical symptoms. AFB clinical symptoms, respectively, absence of clinical symptoms, were determined by visual inspection of all frames in the field according the OIE description (Anonymous, 2004). Based on this inspection, AFB positive samples were sent to the Swiss reference laboratory for bee diseases for confirmation.

Each colony sampled was visually checked for presence of AFB clinical symptoms. A total of 71 colonies (19 colonies with symptoms, and 52 without symptom) were sampled. Each bee sample consisted of 50 living bees that were immediately frozen. Samples were collected in four different locations in the hive; the hive entrance (ingoing bees), edge frame, in the middle of the brood nest and honey chamber when present. A honey sample (ca. 30 g) per colony was collected from the brood nest.

2.2. Bee samples

Fifty adult bees were crushed in a stomacher bag with 25 ml sterile water at maximum speed for 6 min and then centrifuged at 1150G for 15 min to sediment bee fragments and debris. Vegetative forms of bacteria, yeast and fungus in the supernatant were inactivated at 90 °C for 10 min. Ninety microliters of this solution was used to inoculate MYPGP-agar with nalidixic acid (3 μg/l) plates (Alippi, 1995; Dingman and Stahly, 1983; Ritter, 1996). Four plates for each bee sample were incubated at 37 °C in an atmosphere of 10% CO_2. Bacteria colonies were identified by their morphology analysis of gram-stained smears and a negative catalase test. *P. larvae* colonies were counted after an incubation of seven days. When the number of colony forming units (cfu) was higher than 500 per plate, serial dilutions were made. Dilutions providing a dense, but discrete population (100–500 cfu/plate) were counted. Ten positive plates were randomly chosen and tested by PCR (Alippi et al., 2004) to confirm their identification. Frequency and intensity of contaminations with bacteria other than *P. larvae* were recorded for each sample.

2.3. Honey samples

One honey sample was collected for each colony. It consisted of honey or sucrose stores collected on a frame with brood. Samples were stored at 4 °C until analysis. Five grams of honey were homogenized in 5 ml of sterile water at 40 °C. Centrifugation, inactivation, inoculation, incubation, identification and counting procedures were identical to those described for adult bee samples (see Section 2.2).

2.4. Data analysis

The number of colony forming units (cfu) obtained was calculated as the average of the four replicates for each bee and honey sample. Statistical analyses were performed on log transformed spore loads.

Bee colonies for which all samples showed no growth of *P. larvae* were not included in the statistical analyses to determine whether the number of spores detected from bee samples, honey samples or among the bee samples were significantly different.

One-way ANOVA ($p < 0.05$) was used to compare all samples. The Wilcoxon rank-sum test was used to determine significance of cfu difference ($p < 0.05$) between colonies with or without clinical symptoms. Pearson's χ^2 test ($p < 0.05$) was used to check for significant differences in wild contamination by other bacteria, yeast or fungus, between honey and bee samples. Sensitivity, specificity, and positive and negative predictive values were calculated according to Fletcher and Fletcher (2005). Sensitivity refers to the proportion of colonies with clinical symptoms that have a positive analysis result and the specificity refers to the proportion of colonies without symptom that have a negative analysis result. The positive predictive value is the proportion of colonies with positive test results that are correctly diagnosed.

3. Results

The PCR diagnosis confirmed for all plates selected the microscopical and biological identification of *P. larvae*. Samples collected from AFB-symptomatic bee colonies contained a significant higher number of cfu than samples collected from colonies without clinical symptoms (Wilcoxon rank-sum test. $Z = 11.65$, p-value = 0.001) (Fig. 1).

Twenty-eight bee colonies out of 71 colonies tested were free of spores (0 cfu). A total of 187 samples came from 43 beehives, where at least one sample with *P. larvae* spores was found. Analysis of these 187 samples revealed that if *P. larvae* was detected by culture method, significant differences between all types of samples were found (one-way ANOVA, $R^2 = 0.061$, df = 4, p-value = 0.02). The number of cfu was always higher in bee samples collected in the brood nest, at the edge frame, and in the honey chamber than in honey samples and bee samples from the hive entrance (Fig. 1). No differences were found between bee samples from different sites within the hive (brood nest, edge frame, honey chamber) (one-way ANOVA, $R^2 = 0.05$, df = 2, p-value = 0.75). When symptoms were visually observed, 100% of bee samples from the brood nest, edge frame and from honey chamber showed positive culture

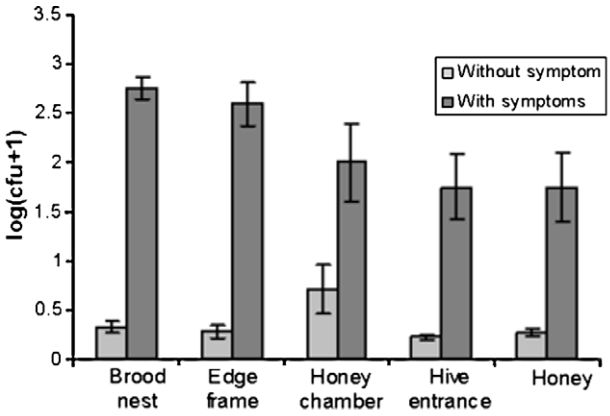

Fig. 1. Comparison of the *P. larvae* infection (cfu) between samples collecting from bee colonies with and without clinical symptoms, $n = 187$.

Table 1
The sensitivity, specificity, positive and negative predictive value for the different sample origins, $n = 304$

	Sensitivity	Specificity	Predictive value negative	Predictive value positive
Brood nest	100	79	100	64
Edge frame	100	79	100	64
Hive entrance	83	77	92	54
Honey chamber	100	63	100	50
Honey	86	78	95	54

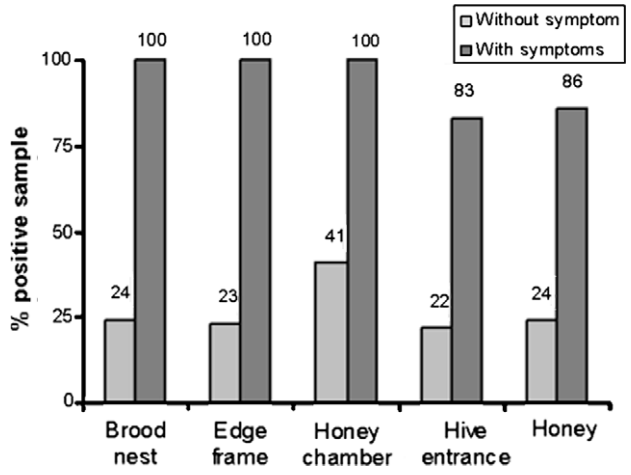

Fig. 2. Percentage of positive samples according to the origin of the samples and the presence or not of AFB clinical symptoms, $n = 304$.

results (i.e. $\geqslant 1$ cfu). In such cases, samples from the honey and hive entrance gave 86% and 83% positive results, respectively (Table 1).

Twenty-six percent of the samples collected from colonies without AFB clinical symptoms were positive for *P. larvae*. Yet, no significant difference between the five different samples collected per hive was found (Pearson's χ^2-test. $\chi^2 = 3.18$, df = 4, p-value = 0.67) (Fig. 2). Bees collected from the honey chamber were the most likely to be infected by *P. larvae* spores (41% of positive samples) but there is no significant difference between the four kinds of bee samples (Pearson's χ^2-test. $\chi^2 = 3.11$, df = 3, p-value = 0.37).

Honey samples were significantly more frequently contaminated (39%) with bacteria other than *P. larvae* or fungus than bee samples (19%) (Pearson's χ^2-test. $\chi^2 = 11.9$, df = 4, p-value = 0.018), but the number of cfu of wild contamination was not significantly different between honey and bee samples (Pearson's χ^2-test. $\chi^2 = 11.2$, df = 3, p-value = 0.189), and among the different bee samples (Pearson's χ^2-test. $\chi^2 = 0.3$, df = 3, p-value = 0.98).

4. Discussion

Our study confirms results obtained by Goodwin et al. (1993a) evidencing that colonies with clinical symptoms of AFB have a significantly higher spore load of *P. larvae* bacteria than apparently healthy colonies. This suggests that clinically diseased colonies are more likely to play an important role in the spread of AFB and that their elimination may help to contain the disease outbreak. Diagnosis based on analysis of honey samples is of limited value as only 86% of samples from symptomatic colonies were positive. This is in agreement with earlier studies (Hornitzky and Clark, 1991; Nordström et al., 2002). An explanation could be that honey may remain in beehives for several months. Therefore, the honey collected for the analysis was stored before the AFB infection had occurred.

Analysis of adult bee samples collected from the brood nest, honey chamber and edge frame allowed detection of all colonies with AFB clinical symptoms. Lindström made the same observation with bee samples collected in brood nest and honey supers (Lindström and Fries, 2005). Yet, when other parts of the hive are easier to access, because of hive construction, seasonal or apicultural aspects (e.g. presence of heavy honey chambers), bees from the edge frame or from the honey chamber may also be collected for an early diagnosis.

Bees from the hive entrance may easily be collected, but the sensitivity of *P. larvae* spore cultures from this site is reduced. This is unacceptable in a diagnostic where all clinically diseased colonies have to be detected (i.e. false negative).

Colonies may harbour *P. larvae* spores without developing AFB symptoms (Dingman and Stahly, 1983; Goodwin et al., 1993b; Hansen and Rasmussen, 1986; Lindström and Fries, 2005). As colonies with subclinical infections may play a role in the spread of AFB, decreasing the sensitivity in order to improve the specificity is not an option for obtaining a reliable primary diagnosis method.

Based on the distribution of *P. larvae*, we recommend investigating colonies by testing bee samples collected in the brood nest, edge frame, or honey supers for *P. larvae* spores. To be totally efficient, this screening must be followed by a visual inspection, frame-by-frame, of positive bee colonies in culture so as to diagnose AFB symptoms.

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