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Original article

Spatial distribution of *Melissococcus plutonius* in adult honey bees collected from apiaries and colonies with and without symptoms of European foulbrood*

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Abstract – In Switzerland, the incidence of European foulbrood (EFB), caused by the Gram-positive bacterium *Melissococcus plutonius*, has increased dramatically between 1997 and 2005 but the epidemiology, including the transmission of *M. plutonius*, remains poorly understood. In this study, the distribution of *M. plutonius* among bees originating from apiaries and colonies with and without symptoms of EFB was evaluated using a specific and sensitive hemi-nested PCR. In more than 90% of colonies without EFB symptoms located in apiaries with EFB symptoms, the bees were carriers of *M. plutonius*. In apiaries without EFB symptoms, but near apiaries with EFB symptoms, bees carrying *M. plutonius* were detected in about 30% of the colonies. In regions without European foulbrood history, all bee samples were negative. The proportion of adult bees carrying *M. plutonius* in colonies without symptoms appeared to increase when the distance to apiaries with clinical EFB symptoms decreased.

European foulbrood / *Melissococcus plutonius* / epidemiology / adult honey bee / symptom

1. INTRODUCTION

European foulbrood (EFB) is a disease of honey bee larvae (*Apis mellifera*) caused by the Gram-positive bacterium *Melissococcus plutonius* (Bailey, 1956, 1982). It is widespread in most honey producing countries (Matheson, 1993) including Switzerland where the incidence has increased dramatically between 1997 and 2005 without apparent reason (Forsgren et al., 2005) but the epidemiology of the disease remains poorly understood.

Typical symptoms of EFB are diagnosed in the field by visual inspection of brood combs. The symptoms consist of 4–5 day old brown dead larvae, displaced in the cells under various degrees of decomposition (Bailey and Ball, 1991). Several alternative methods have been described for detection of *M. plutonius* including microscopic examination after bacterial staining (Hornitzky and Wilson, 1989), ELISA (Pinnock and Featherstone, 1984), PCR (Djordjevic et al., 1998) and cultivation of the bacterium from diseased larvae (Bailey and Ball, 1991).

Recently, Forsgren et al. (2005) demonstrated a non-ubiquitous presence of *M. plutonius* in larvae and honey in the brood nest originating from colonies with and without symptoms of EFB. In this study, we evaluated the distribution of *M. plutonius* among colonies with and without symptoms using samples of adult bees to understand the spatial

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distribution of the pathogen in the host population. We used a specific and highly sensitive hemi-nested PCR established by Djordjevic (1998) and recently further developed for detection of *M. plutonius* in honey bees and their products (McKee et al., 2002) to analyse adult bees for the presence of the bacterium.

2. MATERIALS AND METHODS

2.1. Bee Samples

Bees were collected from 16 colonies in two apiaries without EFB symptoms in an EFB-free area (without EFB history), and from 64 colonies in 11 different apiaries in an area of Switzerland where EFB is present. Of the 11 apiaries in areas with EFB, six were diagnosed with clinical signs of EFB. These six apiaries contained a total of 32 colonies, 12 without clinical symptoms and 20 with clinical symptoms of EFB. The remaining 5 apiaries, also containing a total of 32 colonies, were all free from clinical symptoms of EFB.

From each colony (n = 80) four matchboxes full of bees (approximately 80 to 100) were sampled from the brood nest as well as at the hive entrance and frozen at –20 °C. From these samples, a subsample of 25 bees was used for the PCR analyses.

2.2. Extraction of bacterial DNA

Bacterial DNA was extracted from a pool of 25 bees. Bees placed in 20 mL of sterile water were crushed in a stomacher for 3 min at high speed. The resulting liquid was centrifuged at 1150 *g* for 15 min and 3 mL of the supernatant was centrifuged at 12000 *g* for 2 min. Target DNA was subsequently extracted from the bacterial pellet following the protocol for isolation of Gram-positive bacteria of the Qiagen DNeasy Tissues Kit (Qiagen, Basel, Switzerland). DNA was eluted in 120 μL of elution buffer (Qiagen).

2.3. Hemi-nested PCR

Two μL of DNA extraction was used as a template for the *M. plutonius* hemi-nested PCR developed by Djordjevic et al. (1998) and slightly modified by McKee et al. (2002). As a positive control, DNA from the *M. plutonius* type strain (ATCC 35311) was used. DNA free reactions were also included as negative control.

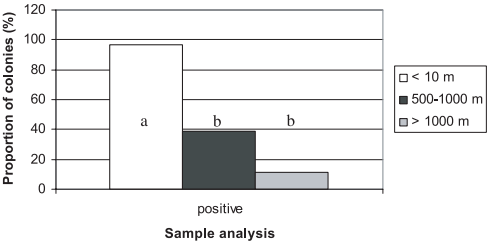

Figure 1. Proportion of samples positive for *Melissococcus plutonius* in colonies within apiaries with clinical EFB present (< 10 m, 6 apiaries, 32 colonies) and in non-symptomatic apiaries at two different distance categories (500–1000 m, 3 apiaries, 23 colonies; >1000 m, 2 apiaries, 9 colonies). Bars with different lettering are significantly different (significant differences were found between bee samples harvested at <10 m and bee samples harvested between 500 and 1000 m (Chi-square value = 22.5, df = 1, $P < 0.01$) and between bee samples harvested at <10 m and bee sample harvested at a distance largest than 1000 m (Chi-square value = 30.16, df = 1, $P < 0.01$) but not when samples were harvested between 500 and 1000 or further than 1000 m (Chi-square value for colonies between 500 and 1000 m and colonies > 1000 m = 2.36, df = 1, $P > 0.05$)).

3. RESULTS

Distribution of *M. plutonius*

There was a clear influence of the spatial distribution of infected hosts (colonies with clinical EFB symptoms) on the incidence of adult bee samples positive for *M. plutonius*. The proportion of samples that tested positive for *M. plutonius* in non-symptomatic colonies was significantly higher in apiaries with cases of clinical disease (11 among 12, Tab. I) compared to colonies in apiaries where no clinical disease symptoms were recorded (10 among 32, Chi-square value = 12.77, df = 1, $P < 0.001$, Tab. I). There was a tendency that as the distance to colonies with clinical disease symptoms increased the proportion of samples testing positive for *M. plutonius* decreased (Fig. 1), although the effect of distance was not significant (Chi-square value for colonies between 500 and 1000 m and colonies > 1000 m = 2.36, df = 1, $P > 0.05$) (Fig. 1).

Table I. Number of adult bee samples positive for *M. plutonius* in brood nest samples and hive entrance samples in apiaries and regions with and without European foulbrood (EFB).

region	apiaries	number of apiaries	number of colonies					
			without visual EFB symptoms			with visual EFB symptoms		
			N	brood nest bees positive	hive entrance bees positive	N	brood nest bees positive	hive entrance bees positive
without EFB	without EFB	2	16	0	0			
with EFB	with EFB	6	12	11	9	20	20	20
	without EFB	5	32	10	7			

N = total number of colonies samples of each sample type.

None of the 16 colonies from the two apiaries located in an EFB free area showed positive results in the specific PCR, neither in brood nest samples nor in bees collected at the hive entrance (Tab. I). In the six apiaries with clinical symptoms of EFB, represented by 32 colonies, all bees collected from the brood nest and from the hive entrance in colonies with symptoms (n = 20) did contain the bacterium (Tab. I). Among the colonies without EFB symptoms (n = 12), but located in apiaries with EFB symptoms, only one sample from the brood nest (11 positives among 12 samples) and three samples from the hive entrance (9 positives among 12 samples) gave no PCR amplification (Tab. I). Only one of the 12 symptom-free colonies in apiaries containing EFB symptoms tested negative both from the brood nest and hive entrance bee samples. There was no significant difference in the efficacy of detecting *M. plutonius* in samples of bees collected from the brood nest compared to bees collected at to the hive entrance in apiaries with EFB and without EFB (Chi-squared values = 1.2 for brood nest samples from apiaries with EFB compared to samples from apiaries without EFB and 0.72 for hive entrance samples from apiaries with EFB compared to samples from apiaries without EFB, df = 1, $P > 0.05$, Tab. I), although we observed more positive results for brood nest samples (Tab. I).

In the five apiaries without clinical symptoms of EFB, but located in an EFB area, represented by 32 colonies, seven and ten samples from the hive entrance and the brood nest respectively, were positive in the PCR analysis (Tab. I). In only one apiary in this area, all bee samples were negative and gave no PCR amplification.

4. DISCUSSION

We failed to detect *M. plutonius* using a specific and sensitive PCR method to detect *M. plutonius* in adult bees from colonies located in an area free of EFB. This result confirms that the bacterium is not ubiquitously distributed (Forsgren et al., 2005) and suggests that a large geographical area without EFB symptoms potentially may be considered to be free from *M. plutonius*.

Analysis of bees from colonies with EFB symptoms showed a strong correlation between the presence of diseased larvae and *M. plutonius* in adults; all brood nest and hive entrance bees tested positive. Compared to results obtained by Forsgren et al. (2005), our results indicate that in colonies with clinical symptoms, the bacterium is more distributed among the bees compared to among the larvae. Furthermore, from apiaries with EFB symptoms, only one out of 12 bee samples collected from colonies without symptoms failed to produce positive PCR (8%). In spite of the limited number of apiaries tested (6, Tab. I), this demonstrates the possibility that almost all colonies in apiaries with EFB symptoms contain bees that are carriers of the bacterium.

The bacterium was detected among colonies from apiaries without EFB symptoms. This finding demonstrates clearly that the bacterium can be present in bees from colonies without symptoms, which is congruent with earlier reports (Pinnock and Featherstone, 1984). There was a tendency that the proportion of adult bees carrying *M. plutonius* in colonies without symptoms increased with decreasing distance to apiaries with clinical EFB symptoms. Indeed, within an apiary containing some colonies with EFB symptoms, where colonies are located relatively close together, more than 90% of the colonies were carriers of *M. plutonius.* In comparison, 39% of colonies from apiaries without EFB symptoms that neighboured an apiary with clinical cases of EFB (distance 0.5 to 1 km), were carriers of the bacterium in adult bee samples.

The density of a susceptible host population will largely determine if infectious disease develop into an epidemic (Watanabe, 1987). However, a density threshold is not only dependent on the host density, but also on the spatial distribution of hosts (Brown and Bolker, 2004). In domesticated honey bees, host density can be measured on two different levels: within apiary density of colonies, and the density of apiaries. For pathogens whose dispersal scale is comparable to the spacing of individual hosts (here colonies within apiaries), the smaller individual scale must be considered (Brown and Bolker, 2004). However, the distribution of apiaries must also be considered to understand disease transmission in honey bees. In the present investigation apiaries were randomly sampled from the population of apiaries in the respective regions. This means that there may have been clinically diseased colonies within flight distance, and even closer to colonies with EFB symptoms than recorded here. Nevertheless, we demonstrated the importance of the spatial structure for disease transmission within apiaries. There were also indications (Fig. 1) that the distance from clinically diseased apiaries may influence the probability of detecting *M. plutonius* in bee samples. However, this aspect needs to be confirmed with further studies, where all colonies within a certain area are analysed for the bacterium, and inspected for EFB clinical symptoms. Potentially, the high density of honey bee colonies promotes transmission of pathogens, such as *M. plutonius*, and may partly explain why EFB at the moment is a major problem for bee health in Switzerland.

For a better understanding of the intra- and inter-colony transmission of *M. plutonius* as well as inter-apiary transmission, further investigations using molecular techniques such as real-time PCR would be useful. Modelling the system using quantitative transmission data within and between apiaries should be pursued to be able to predict, and possibly prevent, disease outbreaks.

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Répartition spatiale de *Melissococcus plutonius* chez des abeilles (*Apis mellifera*) adultes prélevées dans des ruchers et des colonies avec ou sans symptômes de loque européenne.

loque européenne / *Melissococcus plutonius* / épidémiologie / symptôme clinique / ADN bactérien

Zusammenfassung – Regionale Verbreitung von *Melissococcus plutonius* bei adulten Honigbienen aus Bienenständen und Völkern mit und ohne Symptome der Europäischen Faulbrut. Die Anzahl Fälle europäischer Faulbrut (EFB), ausgelöst durch das Bakterium *Melissococcus plutonius*, haben in der Schweiz seit 1997 stark zugenommen. Die bis dahin publizierten wissenschaftlichen Beiträge beschreiben vor allem die Symptome der EFB und/oder die Analyseverfahren zur Bestimmung des Erregers *M. plutonius*. Über die Epidemiologie der Krankheit hingegen ist wenig bekannt. Die selektive Infektion von Larven und die sporadische Kontamination von Honig durch *M. plutonius* innerhalb eines Volkes sind bekannt. In dieser Untersuchung wurde die Verteilung des Erregers auf Bienen aus Völkern ohne klinische Symptome und solchen mit eindeutigem Krankheitsbild mit Hilfe einer sensitiven "heminested" PCR Methode (Djordjevic, 1998; McKee, 2002) untersucht.

In einem Gebiet mit starkem EFB Befall wurden auf 11 Bienenständen aus 64 Völkern Brutnest- und Flugbienenproben gesammelt. In analoger Weise wurden Proben von 16 Völkern auf zwei Ständen einer sauerbrutfreien Region erhoben. Die DNA des Bakteriums wurde aus einer Probe von 25 Bienen extrahiert und durch eine "heminested" PCR-Methode amplifiziert.

In allen Bienenproben aus Völkern mit Symptomen konnte der Erreger mittels PCR nachgewiesen werden. 90 % der Bienen gesunder Völker auf Ständen, die auch Völker mit klinischen EFB Symptomen beherbergten, waren Träger von *M. plutonius*. Auch in unauffälligen Nachbarständen enthielten 30 % der Bienenproben EFB Erreger. Der Befall der Bienen scheint mit zunehmender Distanz zum Infektionsherd abzunehmen. Auf den Bienenständen aus der Region ohne EFB-Geschichte konnte das Bakterium in keiner Bienenprobe nachgewiesen werden. Dies zeigt, dass *M. plutonius* nicht ubiquitär vorhanden ist. Zum gleichen Resultat führte eine Untersuchung von Forsgren et al. (2005) bei Brut- und Futterkranzproben.

Zwei Faktoren, welche die Verbreitung einer Krankheit innerhalb einer Population beeinflussen, sind einerseits die Wirtesdichte und anderseits die räumliche Verteilung. Das grösste Befallsrisiko tragen deshalb die Völker des gleichen Standes, gefolgt von denjenigen der Nachbarstände je nach Distanz (Abb.). Die relativ hohe Bienendichte in gewissen Regionen der Schweiz könnte die Verbreitung des Erregers unterstützen und teilweise auch die aktuelle, epidemische Ausbreitung der EFB in der Schweiz erklären. Die Bienen sind Träger und Überträger von *M. plutonius*. Deshalb trägt mit Sicherheit Räuberei zur Verbreitung der Krankheit bei. Um die epidemiologischen Kenntnisse über EFB zu verbessern und im speziellen das Übertragen innerhalb eines Volkes und zwischen den Völkern zu studieren, ist ein quantitatives Erfassen des Erregers mit "realtime" PCR notwendig.

***Melissococcus plutonius* / europäische Faulbrut / Epidemiologie / Symptom / adulte Honigbiene**

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