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Original article

Infection rate based on quantitative real-time PCR of *Melissococcus plutonius*, the causal agent of European foulbrood, in honeybee colonies before and after apiary sanitation*

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Abstract – Since 1999, the incidence of European foulbrood (EFB) in Switzerland has been rising constantly. To understand the reason for this increase, an epidemiological study of the efficacy of sanitation measures was carried out. Workers from brood nests and flight entrances at infected apiaries were collected from colonies with and without clinical symptoms in 2005–2006. In order to quantify bacterial loads, a novel real-time PCR assay was developed for *Melissococcus plutonius*. Our data show that workers from brood nests have about 20-times higher bacterial loads than those from flight entrances, suggesting that the former are more suitable for EFB-monitoring. Moreover, current sanitation measures appear to be insufficient because only three out of eight apiaries were free of *M. plutonius* one year after sanitation. While no clinical symptoms are observed below 50 000 CFU of *M. plutonius* per bee, workers can nevertheless be carriers and likely responsible for bacterial propagation.

***Melissococcus plutonius* / *Apis mellifera* / epidemiology / real-time PCR / foulbrood**

1. INTRODUCTION

Cheshire and Cheyne (1885) first described the clinical symptoms of European foulbrood (EFB) in honeybee colonies in 1885. White (1912) identified the causal agent of this disease, the Gram-positive bacterium *Bacillus pluton*. Bailey, who studied its biology and epidemiology (1956, 1957) renamed the bacterium *Melissococcus pluton* (Bailey and Collins, 1982), which was corrected to *M. plutonius* (Trüper and De'Clari, 1998). Young bee larvae (≤ 48 hours) are extremely susceptible to *M. plutonius*. EFB-infected larvae loose their healthy appearance, look undernourished and are twisted at the bottom of the cell. Their color changes from a normal pearly white to yellow, then brown and finally grayish black (Bailey, 1961). Usually, death is accelerated by secondary infection with bacteria such as *Paenibacillus alvei*, *Achromobacter eurydice* or *Enterococcus faecalis* (Bailey, 1956, 1983). *M. plutonius* is able to survive for several years when deposited on the cell wall after pupation of weakly infected larvae (Bailey and Ball, 1991). Disease outbreaks seem to be linked to colony stress conditions, such as lack of food, water or space; however, genetic factors, colony density, weather and geography may also play a role (Bailey, 1961). Interestingly, sudden death of many larvae followed by a spontaneous recovery of the colony a few weeks later has also been observed (Bailey, 1961). Diseased colonies can be treated with antibiotics (e.g. oxytetracycline (OTC)), but these only have a bacteriostatic effect on *M. plutonius* (Thompson and Brown, 2001).

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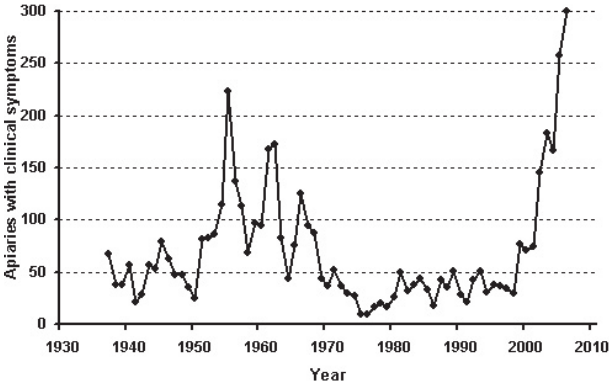

Figure 1. EFB cases in Switzerland over the last 70 years. After increased emergence of the disease in the 1950s and 1960s, the number of cases was less than or equal to 50 newly infected apiaries per year. From 1999 onwards, the number of infected apiaries has constantly risen each year, reaching 300 cases in 2006.

In Switzerland, EFB is endemic and restricted to certain areas (Forsgren et al., 2005; Belloy et al., 2007). Under Swiss law, EFB is a notifiable disease and subject to strict sanitation measures which state that infected colonies and weak colonies without clinical symptoms on an infected apiary must be destroyed. In the case of a strong colony with few clinical symptoms, the shook swarm method may be applied (Tierseuchenverordnung (TSV), RS 916.401, 27.06.1995, Art. 273/274; Zentrum für Bienenforschung, 2003). In the sixties, EFB was often treated with antibiotics, but this did not prevent an increase in the disease incidence from 0.1% to about 0.6%. Some years later, the use of antibiotics was prohibited and the incidence decreased to 0.3% or lower (≤ 50 infected apiaries per year). This situation continued until 1999; from then on, the amount of registered cases has continually risen in distinct regions of Switzerland, reaching 300 infected apiaries in 2006 (Fig. 1). Thus, the national incidence increased to 1.5%. The reasons for this increase in EFB are not well understood.

The presence of *M. plutonius* in bee colonies has been investigated and reported previously. Forsgren et al. (2005) detected *M. plutonius* in larvae and pupae without clinical symptoms. Belloy et al. (2007) demonstrated that workers act as carriers of EFB, not only among the colony, but also between colonies and apiaries. They also showed that *M. plutonius* is present in workers from colonies without clinical symptoms. Therefore, the workers seem to be better suited for epidemiological studies of EFB than brood, which is in agreement with results reported for American foulbrood (Lindström and Fries, 2005). Both EFB studies (Forsgren et al., 2005; Belloy et al., 2007) were performed with the hemi-nested PCR method developed by Djordjevic et al. (1998). The major drawback of this method is that results are strictly qualitative, not quantitative. In the present study, we developed a real-time PCR assay that enabled the quantification of *M. plutonius* in bee samples. Based on the quantitative PCR approach, an epidemiological study was conducted in order to evaluate the efficacy of the Swiss sanitation procedure for EFB.

2. MATERIALS AND METHODS

2.1. Bacterial strains

Melissococcus plutonius type strain ATCC 35311 was grown anaerobically (GENbox, bioMérieux, France) at 35 °C on EFB-agar or in EFB broth (Bailey, 1957). The media were amended prior to use with 3 μg/mL of nalidixic acid (Hornitzky and Smith, 1998). *Enterococcus faecalis* strains FAM 1793, 14032, 14034 and 15541 from the collection at our institute were grown aerobically at 37 °C in M17 Broth (Terzaghi and Sandine, 1975) amended with 5 g/L of glucose.

2.2. DNA extraction from bacterial liquid cultures

After incubation (four days for *M. plutonius* and one day for *E. faecalis*), bacterial pellets were collected by centrifugation of the liquid cultures (11 000 *g*, 5 min). The bacterial genomic DNA was extracted with the Qiagen DNeasy® Tissue Kit (Qiagen, Germany). DNA concentrations were measured with a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop, USA).

2.3. Sequencing of the *sodA* gene of *M. plutonius*

A 480-bp amplification product of the single copy *sodA* gene, which encodes the manganese-dependent superoxide dismutase in *Enterococcus* sp. strains, was obtained as described by Poyart et al. (2000). PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Germany) and both strands were sequenced. The sequencing reactions were performed with the ABI-PRISM Big Dye® Terminator v1.1 Sequencing Kit (Applied Biosystems, USA) and the sequences were read with a ABI-PRISM 310 Genetic Analyzer.

2.4. Primers and probe design for *M. plutonius*

A 79-bp fragment with the primers MelissoF (5'-CAG CTA GTC GGT TTG GTT CC-3'), MelissoR (5'-TTG GCT GTA GAT AGA ATT GAC AAT-3') and Taqman® MGB (minor groove binding) probe (6'FAM-CTT GGT TGG TCG TTG AC-MBGNFQ-3') was designed based upon the sequenced *sodA* gene (Fig. 2) by using the Primer Express®software (version 2.0, Applied Biosystems, USA). The primers were purchased from Eurogentec (Belgium) and the Taqman® MGB probe was acquired from Applied Biosystems (USA).

2.5. Real-time PCR conditions

The qPCR MasterMix No Rox (Eurogentec, Belgium) was used for all measurements. The real-time PCR was carried out in a final reaction volume of 12 μL containing 0.3 μM of each primer, 0.1 μM probe, 2 × reaction buffer and 2 μL of DNA. Amplifications were run in a Rotor-Gene™ RG3000A (Corbett Life Science, Australia) using the following program: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Real-time data were analyzed using Rotor-Gene™ software version 6.0 (Corbett Life Science, Australia). Each sample was measured in duplicate and non-template controls tested negative in all PCR runs.

2.6. Sampling and sanitation procedure

Sampling was performed at a total of 114 colonies at 11 apiaries. Worker bees were sampled from brood nests (BN, n = 251) and flight entrances (FE, n = 183) at four different time intervals. Out of these 434 samples, 127 were taken from colonies showing clinical symptoms and 307 were sampled from colonies showing no clinical symptoms. The first collection of samples was performed just before the sanitation procedures in May and June, 2005 (n = 168, BN = 83, FE = 85). The sanitation protocol requires burning of every colony showing clinical symptoms and weak colonies of the infected apiary. The remaining stocks of combs are recycled (i.e., heated at 120 °C during 30 min). Hives and beekeeping materials are disinfected (e.g., with 5% sodium carbonate or 4% sodium hydroxide). The second sampling was performed one month later (n = 104, BN = 52, FE = 52), the third collection of samples occurred in October, 2005, just before the wintering period (n = 64, only BN), and the last sampling was done in May, 2006 (n = 98, BN = 52, FE = 46). During each sampling, about 100 workers were collected in the brood nest and at the flight entrance. All samples were stored at –20 °C until further use.

2.7. Estimation of clinical symptoms of EFB

Based on a visual evaluation of the brood combs, the following evaluation scale was introduced to quantify the clinical symptoms: (0) no visible symptoms, (1) less than 10 larvae with visible symptoms, (2) 10 to 50 larvae with visible symptoms and (3) more than 50 larvae with visible symptoms. The evaluation was applied to the most infected comb side of the colony with the highest number of larvae showing visible symptoms.

Figure 2. ClustalW (1.83) alignment of the sequenced *sodA* gene of *M. plutonius* ATCC 35311^T (available under GenBank accession number EF666055) with the *sodA* gene from *E. faecalis* ATCC 19433^T (GenBank accession number AJ387912). Mismatches between *E. faecalis* and the designed *M. plutonius* primers and probe are indicated by gray shading. Primer annealing sites are indicated in bold face, the Taqman® MBG probe target site is italicised and in bold face.

2.8. DNA extraction from worker samples

Bees (12 g = ~ 100 workers) were placed in a bag with 50 mL sterile water and crushed twice for 2 min in a stomacher apparatus at high speed. The homogenate was poured into a 50 mL plastic tube and centrifuged for 15 min at 1150 *g*. The supernatant (1.5 mL) was transferred to a 2 mL tube and centrifuged at 20 000 *g* for 2 min. The supernatant was then discarded and the pellet was resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100), amended with 20 mg/mL lysozyme and incubated for one hour at 37 °C. DNA was extracted from the lysate with the EZ1 DNA Tissue kit and a BioRobot® EZ1 workstation (Qiagen, Germany). 2 µL of the extracted DNA was used as the template for real-time PCR.

2.9. Amplification control

For samples that were negative for *M. plutonius*, the β-actin gene of *Apis mellifera* was amplified to evaluate the amplification ability of the extracted DNA. For this purpose, a 121-bp fragment with the primers β-actinF (5'-CCT GGA ATC GCA GAT AGA ATG C-3'), β-actinR (5'-CAA GAA TTG ACC CAC CAA TCC ATA-3') and a Taqman® probe (5'HEX-TCA CTG CCC TAG CAC CAT CCA CCA-TAMRA-3') was designed from the β-actin gene sequence of *A. Mellifera* (GenBank accession number AB023025) using the Beacon

5 software (Premier Biosoft International, USA). The primers and probe were purchased from Microsynth (Switzerland). Real-time PCR conditions were identical to those described in section 2.5. The β-actin fragment was consistently amplified from the DNA of *M. plutonius*-negative samples with a Ct range of 24 ± 2 cycles.

2.10. Robustness of the extraction method

DNA from 10 samples (n = 100 workers) of the same colony was extracted with the BioRobot® EZ1 workstation, as described previously. The eluted DNA, either undiluted, a 10-fold dilution, or a 100-fold dilution in sterile water, was subjected to *A. mellifera* β-actin gene amplification to identify potential inhibitory components in the extraction matrix. No inhibition was observed and the Ct range in the undiluted DNA was as expected (i.e., 24 ± 2 cycles). To assess variability within the assay, 1.5 mL of the supernatant from an EFB-infected worker sample was sampled 6 times, DNA was extracted with the workstation and the DNA samples were subjected to quantitative PCR amplification of *M. plutonius* in parallel. The Ct range between the samples was 20.55 ± 0.13 cycle.

2.11. Set up of the standard curve for *M. plutonius* quantification

A four-day old liquid culture of *M. plutonius* ATCC 35311^T was 10-fold serially diluted in sterile water and dilutions were plated on EFB-agar. Colonies were counted after four days of incubation and the obtained CFU (Colony Forming Units) range was equivalent to approximately 3.5×10^7 to 3.5 CFU/mL. The same liquid culture was also 10-fold serially diluted in a *M. plutonius*-negative worker sample, which was homogenised as described in Section 2.8. DNA was extracted from the samples with the BioRobot® EZ1 workstation. The concentration, in CFU/mL obtained from the colony counts using EFB-agar were applied to the corresponding dilutions in the worker sample and a standard curve was established with 6 standards that were equivalent to 3.5×10^7 to 3.5×10^2 CFU/mL. This standard curve was included in each real-time PCR run. The slope of the linear regression curve, calculated over a 5-log range from 3.5×10^7 to 3.5×10^2 CFU/mL, was –3.353 ($R^2 = 0.99876$). This indicated an amplification efficiency of 99%. The detection limit was up to 3.5 CFU/mL, but a quantification limit was set at 100 CFU/mL since measurements were less reproducible below this threshold.

For better understanding, the quantification data originally obtained in CFU/mL were converted to CFU/bee. The results were divided by two since 100 bees are equivalent to 50 mL of homogenised bee sample.

2.12. Statistical analysis

The data (CFU/bee) were converted into logarithmic values to check for normality within design cells and homoscedasticity was tested with the Levene test. The assumptions for parametric analysis of variance were not fulfilled. Therefore, the non-parametric Kruskal-Wallis test was applied to compare the sampling points, the different levels of symptoms and the level of infection of the worker bees from brood nests and flight entrances. When comparing two sampling points or two levels of symptoms, the Mann-Whitney U-test was used with levels of significance that were adjusted according to the Bonferroni correction. The sensitivity of the threshold fixation for worker bee samples from colonies with clinical symptoms was based on a confounding matrix. The calculations were performed with Systat (version 11) and the level of significance was set at $P < 0.05$.

3. RESULTS

3.1. The *sodA* gene of *M. plutonius*

The sequence of the 442-bp amplification product of the manganese-dependant superoxide dismutase gene (*sodA*) from *M. plutonius* ATCC 35311^T is illustrated in Figure 2 and is available under GenBank accession number EF666055. Performing a BLAST search at NCBI (www.ncbi.nlm.nih.gov/BLAST) reveals 77% similarities with *Enterococcus* sp., especially *E. faecalis*, which is closely related to *M. plutonius* (Cai and Collins, 1994) and is commonly identified as one of the bacteria responsible for secondary infection in EFB (Bailey, 1963). Alignment of the sequenced *sodA* genes of *M. plutonius* ATCC 35311^T and *Enterococcus faecalis* type strain ATCC 19433^T (GenBank accession number AJ387912) indicates good discriminating primer and probe annealing sites (Fig. 2).

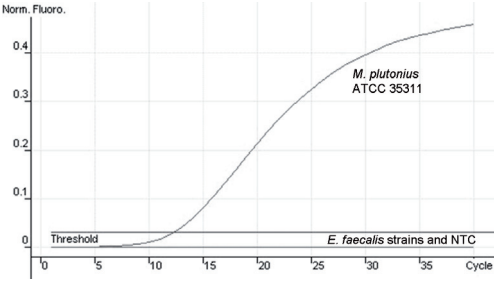

Figure 3. Real-time PCR analysis of DNA from *M. plutonius* ATCC 35311^T and four field strains of *E. faecalis* (FAM 1793, 14032, 14034 and 15541). *M. plutonius* yields a signal at a Ct value of 12.35 cycles, whereas no signals were recorded for the *E. faecalis* strains or NTC (no template control = water). 20 ng of DNA was used (for *E. faecalis* $\simeq 5.68 \times 10^6$ copies of *sodA*).

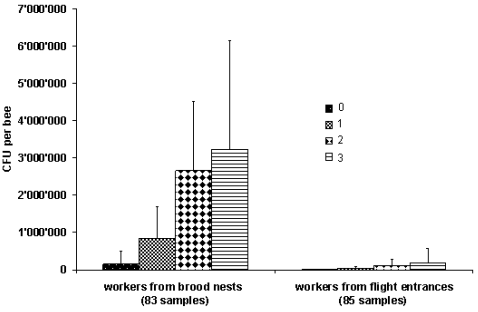

Figure 4. Quantification of *M. plutonius* in workers from brood nests and flight entrances before sanitation. Mean CFU/bee values are shown for colonies without clinical symptoms (0), with less than 10 larvae with clinical symptoms (1), with 10 to 50 larvae with clinical symptoms (2) and with more than 50 larvae with clinical symptoms (3) on the most infected comb side.

3.2. Specificity of the assay

Comparison between our novel real-time PCR system and the hemi-nested PCR system developed by Djordjedvic et al. (1998) was carried out on DNA that was previously extracted from 114 worker samples (brood nests and flight entrances) for the study reported in Belloy et al. (2007). The real-time PCR results obtained were in accordance with those obtained with the hemi-nested system (data not shown). To assure that there was not a cross-reaction with *E. faecalis*, four field strains were tested. The use of 20 ng of DNA (1 ng $\simeq 2.84 \times 10^5$ copies of *sodA* for *E. faecalis*, Paulsen et al. (2003)) in the PCR reaction did not result in a signal for the *E. faecalis* strains, whereas use of the *M. plutonius* ATCC 35311^T resulted in a signal at 12.35 cycles (Fig. 3).

3.3. Infection levels in workers from brood nests and flight entrances before sanitation

Out of 83 (brood nests) and 85 (flight entrances) sampled colonies, 29 (resp. 31 for flight entrances) colonies showed no clinical symptoms (0), 20 colonies showed less than 10 larvae with clinical symptoms (1), 18 colonies showed 10 to 50 larvae with clinical symptoms (2) and finally 16 colonies showed more than 50 larvae with clinical symptoms (3). The mean values of the CFU/bee versus the evaluation of clinical symptoms are illustrated in Figure 4. The differences between no clinical symptoms (0) and the three levels of clinical symptoms, (1), (2) and (3), were significant for workers from brood nests and flight entrances ($P < 0.05$). Workers from brood nests have, on average, 20-times higher bacterial loads than those from flight entrances. This difference in bacterial load is highly significant ($P < 0.05$). For workers from brood nests, the differences between the symptom evaluations were significant, except between symptom (2) and (3). On the other hand, the differences for workers from flight entrances were only significant when comparing no clinical symptoms (0) to the three levels of clinical symptoms, (1), (2) and (3). The differences between the clinical symptom evaluations (1), (2) and (3) were not significant.

3.4. Efficiency of the sanitation measures for workers from brood nests

At the first sampling, before sanitation, more than 50% of the colonies showed clinical symptoms in three of the 11 apiaries. Following sanitation procedure, all colonies from

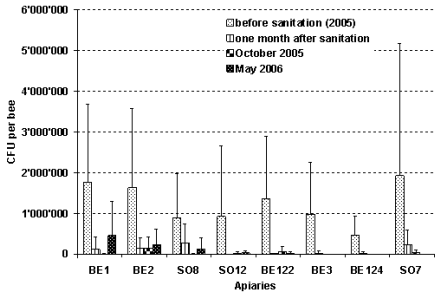

Figure 5. Mean CFU/bee values per apiary for workers of brood nests at the four sampling intervals during the 2005–2006 season. Only three apiaries (BE3, BE124 and SO7) were free of *M. plutonius* in May, 2006.

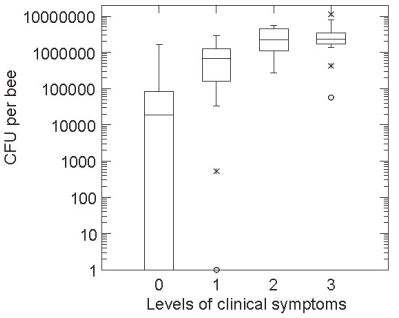

Figure 6. Median CFU/bee values from workers sampled in brood nests before sanitation versus the four levels of clinical symptoms. An artificial threshold was set at 50 000 CFU/bee; 91% of the colonies with clinical symptoms were above this value.

those apiaries were destroyed. In the remaining eight apiaries (total n = 221 samples with 91 colonies), the analysis of variance between the four sampling points was significant ($P < 0.05$). A significant decrease in the CFU/bee per colony for brood nest workers was found one month after sanitation ($P < 0.05$, Fig. 5). Likewise, a significant decrease occurred between the second and the third sampling (before wintering) ($P < 0.05$). On the other hand, the CFU/bee increased between the third and the fourth sampling, but this difference was not significant. Despite sanitation, five apiaries remained infected or were newly infected the following spring. At least one colony with new clinical symptoms was found in four apiaries (BE1, BE2, SO8 and SO12). For apiary BE 122, no clinical symptoms were observed but *M. plutonius* was detected in 2 out of 8 colonies. The BE3, BE124 and SO7 apiaries were free of *M. plutonius* one year after the sanitation.

3.5. Distribution of bacterial loads in workers from brood nests of colonies with and without clinical symptoms before sanitation

In the group of colonies without clinical symptoms, 59% of the colonies (17 out of 29) had fewer than 50 000 CFU/bee (Fig. 6). The colonies with clinical symptoms had bacterial loads above this threshold. Evaluation of the sensitivity of this artificial threshold indicated a 91% probability that colonies with clinical symptoms of the brood contained more than 50 000 CFU/bee ($P < 0.05$). Four colonies with less than 10 larvae with visible symptoms (1) were under this threshold. Indeed, one colony was negative and another had only 525 CFU/bee. Both colonies belonged to heavily infected apiaries where all of the colonies were destroyed after the first sampling. The two other colonies under this threshold had 32 850 CFU/bee and 49 200 CFU/bee, respectively.

4. DISCUSSION

In this study, we set up a novel real-time PCR system based on the manganese-dependent superoxide dismutase (*sodA*) gene. The specificity of the system was demonstrated with total concordance of results for samples analysed with the reference heminested PCR method for EFB detection and our real-time PCR system. No cross-reaction was observed with *E. faecalis* (Fig. 3), a species that is very closely related to *M. plutonius* (Cai and Collins, 1994) and which is often present as a secondary EFB infection (Bailey, 1963). Moreover, there was a good correlation between positive results for the worker bees and clinical symptoms observed in the brood.

One false negative result was observed in the colonies sampled before sanitation. This discrepancy is not due to the detection method, but to the sampled bees. These bees were obviously not infected with *M. plutonius*, despite presentation of a low level of clinical symptoms among the brood. Therefore, this real-time PCR system is a valuable molecular biological tool for monitoring EFB and enables, for the first time, the quantification of *M. plutonius* infection.

The first step of this epidemiological study was to determine which workers were optimal for the estimation of EFB infection in a colony. The sampling procedure is less laborious if a sample is taken at the flight entrance instead of opening the hive to sample workers from the brood nest. However, there are two factors that favour sampling workers from brood nests. First, the investigation showed that the defined amount of workers (i.e., 100) for analysis was not always achievable within a reasonable time window at the flight entrances, due to weather conditions and colony strength. Second, the quantification of *M. plutonius* showed that workers from brood nests contained about 20-times more bacteria than those from flight entrances (Fig. 4). This is because brood nest bees are in contact with the infected brood while flight entrance bees do not clean the cells anymore. Therefore, flight entrance bees are less suitable for monitoring EFB in apiaries than workers from brood nests. It was also clearly shown that analysis of workers from brood nests enables the detection of EFB before clinical symptoms are visible. Indeed, from 83 samples of brood nest bees taken before sanitation, 72 were positive for infection (i.e. contained more than 50 CFU/bee) but only 54 colonies showed clinical symptoms.

The above-described sanitation procedure leads to an important decrease in the average bacterial loads of colonies at the analysed apiaries (Fig. 5). By visual control of the brood, the percentage of colonies with clinical symptoms was 65% before sanitation, 10% one month later and 12% in the following spring. By comparison, when workers were analyzed with real-time PCR, the percentage of positive colonies was higher, i.e., 86.7%, 57.7% and 25.4% respectively. This clearly shows that the sanitation procedure applied in Switzerland is not sufficient to prevent new EFB outbreaks in the same apiaries the following year.

In the UK, another endemic EFB location, the sanitation procedure is quite similar to our protocol, since heavily infected colonies and small colonies with few symptoms are destroyed. The difference is that colonies likely to recover are subjected to oxytetracycline (OTC) treatment (The Bees Diseases Control Order, SI 1982 No 107, 1982). In a UK field study during the period 1994–2000, brood was visually examined and the recurrence rates for infection were about 26% for colonies in the following year (Thompson and Brown, 2001). Even though the use of OTC in Switzerland is forbidden, this recurrence is higher to that reported herein. Waite et al. (2004) showed that use of the shook swarm method for diseased colonies in combination with antibiotic treatments lowered the recurrence rate to 4.8% at the colony level. Furthermore, Thompson et al. (2006) recommended the shook swarm method without antibiotic treatment as a method for EFB control in the UK. Therefore, utilisation of the shook swarm technique in addition to our existing sanitation procedure may lower the regional recurrence rate in Switzerland. Further investigations are needed to confirm this hypothesis.

Workers of brood nests from colonies without clinical EFB symptoms may contain surprisingly high levels of *M. plutonius*. The healthy appearance of brood may be related to the good hygienic behavior of the workers (Waite et al., 2003). Nevertheless, those workers can act as carriers of *M. plutonius* (Belloy et al., 2007). Results from our study indicate that a quantification threshold of approximately 50 000 CFU/bee can be set for the emergence of clinical symptoms, since colonies showing clinical symptoms generally have bacterial loads above 50 000 CFU/bee (Fig. 6). The introduction of such a quantification threshold might constitute a rapid diagnostic tool to screen colony health status. It could be used to assess infection of colonies or apiaries near an established EFB case, which have or might develop clinical symptoms. The visual brood control which is applied today,

is very time consuming. Therefore, analysing mixed samples of workers from brood nests of several colonies in an apiary could be an interesting and efficient alternative. Further investigations are necessary to determine the quantification threshold for such mixed samples.

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Détermination du taux d'infection par PCR quantitative en temps réel de *Melissococcus plutonius*, l'agent causal de la loque européenne dans les colonies d'abeilles mellifères avant et après l'assainissement de ruchers infectés.

***Melissococcus plutonius* / *Apis mellifera* / épidémiologie / loque européenne / PCR en temps réel**

Zusammenfassung – Entwicklung der Infektion mit einer quantitativen Echt-Zeit PCR Methode von *Melissococcus plutonius*, dem Erreger der europäischen Faulbrut in Völkern von *Apis mellifera* vor und nach der Sanierung der Bienenstände. Die europäische Faulbrut (EFB) verursacht in bestimmten Regionen der Schweiz seit 1999 vermehrt Völkerverluste. Die jungen Larven werden vom Bakterium *Melissococcus plutonius*, dem Verursacher der EFB, infiziert. Die Larven sterben schnell, da sich der Erreger im Mitteldarm vermehrt und die Futteraufnahme behindert (Bailey, 1956, 1983). In der Schweiz ist EFB eine anzeigepflichtige Bienenkrankheit deren Bekämpfung von den amtlichen Veterinärbehörden überwacht wird. Die Völker mit klinischen Symptomen sowie schwache Völker werden eliminiert, die restlichen Brut- und Honigwaben eingeschmolzen und die Kasten sowie das Imkermaterial desinfiziert. In den 30 Jahren vor 1999 lag die jährliche Inzidenz der EFB unter 0,3 %. Dies waren jährlich weniger als 50 neue diagnostizierte Bienenstände. Nach einer stetigen Zunahme lag die Inzidenz 2006 bei 1,5 %. Die Gründe für diese starke Zunahme sind nicht bekannt. Zwei Untersuchungen haben gezeigt, dass Larven und Puppen, welche keine Symptome zeigen, wie auch Bienen Träger von *M. plutonius* sein können, (Forsgren et al., 2005; Belloy et al., 2007) die innerhalb des Volkes als auch zwischen den Völkern und den Bienenständen zur Ausbreitung des Erregers beitragen.
Um die Wirksamkeit der sanitären Massnahmen zur Bekämpfung von EFB zu überprüfen, wurde eine neue Echt-Zeit PCR mit dem Ziel-Gen *sodA* entwickelt, um den Befall von *M. plutonius* quantitativ erfassen zu können.
Für die epidemiologische Studie wurden 441 Bienenproben auf 11 Bienenständen im Brutnest und am Flugloch vor der Sanierung, ein Monat danach, bei der Einwinterung im Oktober 2005 sowie im Mai 2006 erhoben. Bei drei Ständen wurden wegen des starken Befalls alle Völker vernichtet und konnten nicht mehr beprobt werden. Es zeigte sich, dass die Bienen am Flugloch 20 Mal weniger mit *M. plutonius* befallen waren als denjenigen aus dem Brutnest, und diese sich daher für epidemiologische Untersuchungen weniger eignen (Fig. 4). Mit der Diagnostik von Brutnestbienen konnten vor der Sanierung 72 von 83 untersuchten Völker als befallen ermittelt werden, aber nur 54 dieser Völker zeigten klinische Symptome. Mit den eingeleiteten Sanierungsmassnahmen konnte der Befall von *M. plutonius* stark reduziert werden (Fig. 5). Dies reichte aber nicht in allen Fällen aus, um neue Ausbrüche von EFB im kommenden Frühjahr zu verhindern. Auf vier von acht sanierten Ständen konnte erneut mindestens bei einem Volk EFB mit klinischen Symptomen nachgewiesen werden. Bei den Brutnestbienen zeigte sich, dass Völker mit einem Erregerbefall von mehr als 50 000 Koloniebildenden Einheiten/Biene mit 91 % Wahrscheinlichkeit klinische Symptome aufweisen (Fig. 6). Dies könnte für die Durchführung einer Frühdiagnose oder die Umgebungskontrolle bei Fällen von EFB auf der Basis von Bienenproben sehr hilfreich sein.

***Melissococcus plutonius* / *Apis mellifera* / Epidemiologie / Europäische Faulbrut / Echt-Zeit PCR**

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