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An integrated management strategy to prevent outbreaks and eliminate infection pressure of American foulbrood disease in a commercial beekeeping operation

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ABSTRACT

The bacterial disease American Foulbrood (AFB), caused by the Gram-positive bacterium *Paenibacillus larvae*, is considered the most contagious and destructive infectious disease affecting honeybees world-wide. The resilient nature of *P. larvae* bacterial spores presents a difficult problem for the control of AFB. Burning clinically symptomatic colonies is widely considered the only workable strategy to prevent further spread of the disease. Antibiotic use is banned in EU countries, and although used commonly in the U.S. and Canada, it only masks symptoms and does not prevent the further spread of the disease. Not surprisingly, there is an increased demand for chemical-free strategies to prevent and control of AFB. The aim of this study was to implement a management program with a long-term perspective to reduce infection pressure and eliminate AFB outbreaks. The study was conducted within a commercial beekeeping operation in central Sweden that has previously experienced re-occurring AFB outbreaks. For 5 years, *P. larvae* were cultured from adult bee samples taken in the fall. The following spring, any identified sub-clinically infected colonies were shaken onto new material and quarantined from the rest of the beekeeping operation. After the first year clinical symptoms were not again observed, and during the 5 years of the study the proportion of apiaries harbouring *P. larvae* spores decreased from 74% to 4%. A multinomial regression analysis also clearly demonstrated that the proportion of infected colonies with the highest levels of spore counts disproportionately declined so that by the end of the study the only remaining infected apiaries were in the lowest spore count category (the three higher spore count categories having been eradicated). These results demonstrate the importance of management practices on AFB disease epidemiology. Early detection of subclinical spore prevelance and quarantine management as presented here can provide an effective sustainable chemical-free preventive solution to reduce both the incidence of AFB outbreaks and continued transmission risk at a large-scale.

1. Introduction

The bacterial disease American Foulbrood (AFB), caused by the Gram-positive bacterium *Paenibacillus larvae*, is considered the most contagious and destructive infectious disease affecting honeybees world-wide (Genersch, 2010). Young honeybee larvae become infected by ingesting as few as ten *P. larvae* spores in contaminated food (Brødsgaard et al., 1998; Hitchcock et al., 1979). The spores then germinate and proliferate in the midgut, invade the larval tissue and kill the host larva (Genersch et al., 2005; Yue et al., 2008). As the infection proceeds, the larval remains degrade to a brownish semi-fluid glue-like mass emitting a characteristic foul odour. This 'ropy' mass and foul odour are the primary clinical symptom for field diagnosis of AFB. The remains of the larva finally form a hard scale in the bottom of the cell that contains billions of extremely tenacious bacterial spores that drive the transmission of this disease within and between colonies (Lindström et al., 2008a, 2008b). These bacterial spores can be viable for decades, resist disinfection and survive adverse conditions such as high temperatures or UV exposure (Hasemann, 1961). This resilient nature of *P. larvae* bacterial spores presents a difficult problem for the prevention and control of AFB.

With its highly contagious nature and high virulence, AFB disease causes huge economic losses to apiculture (Genersch, 2010). The world organization for animal health (OIE) considers the disease to be of socioeconomic importance in the international trade of bees and bee products (Alippi, 2014). In many countries AFB is a notifiable disease and is required by law to be reported to the relevant government authorities. Burning the symptomatic diseased colonies is widely

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considered the only workable control method to prevent the further spread of disease and is the usual legal requirement, at least in most European countries (Alippi, 2014; Genersch, 2010). Antibiotics are commonly used as a prophylactic treatment in several countries including the U.S. and Canada (Evans, 2003; Genersch, 2010). However, antibiotic treatment is not a sustainable strategy since it only masks AFB symptoms and does not eliminate the bacterial spores that drive the spread of the disease (Oldroyd et al., 1989). Current legislation has banned antibiotic use in most European countries to treat AFB as they have the potential to leave residues in honey (Bargańska et al., 2011; Hammel et al., 2008; Lopez et al., 2008). Moreover, antibiotic resistant *P. larvae* isolates have been detected in the U.S., Canada, and Argentina (Alippi et al., 2007; Evans, 2003; Miyagi et al., 2000). Not surprisingly, there is an increased demand for alternative, natural strategies for the prevention and control of AFB which has produced extensive studies testing the application of essential oils, plant extracts, propolis, probiotics, to name a few (Alonso-Salces et al., 2017). Unfortunately, the resilient nature of *P. larvae* spores may limit the efficacy of these alternative approaches.

American foulbrood is transmitted horizontally between colonies at a local scale by drifting (adult bees carrying *P. larvae* spores move between colonies) and by robbing (adult bees steal honey from weaker contaminated colonies; Goodwin et al., 1994; Hornitzky, 1998). These natural transmission routes are however significantly enhanced and facilitated through standard beekeeping (Fries and Camazine, 2001; Genersch, 2010). Common practices such as exchanging brood frames and bees between colonies and keeping numerous hives in close proximity accelerates the spread of AFB disease and are much more important transmission routes than natural drifting or robbing (Fries and Camazine, 2001; Lindström et al., 2008b). Therefore, improved management strategies should be the logical solution to reducing AFB epidemics. One strategy is to isolate sick colonies to prevent the opportunity for the disease to spread further. This quarantine strategy has been effective in New Zealand for the control of AFB outbreaks in beekeeping operations (Goodwin, 2006). In apiculture, quarantine strategies can occur at 2 levels: hive quarantine where no material is swapped between hives; or apiary quarantine where each apiary is managed separately with no interchanging of equipment between apiaries (Goodwin, 2006). However, a quarantine strategy is only as good as the detection techniques used to identify the diseased colonies in the first place. Early detection is critical. Optimally, spores would be detected in colonies before clinical symptoms occur and this is best achieved through culturing the bacteria from samples of adult bees (Forsgren and Laugen, 2014; Lindström and Fries, 2005).

The aim of this study was to monitor the epidemiology of AFB disease over several years to evaluate the efficacy of a preventive management strategy. The strategy was designed to incorporate early disease detection with apiary quarantine in order to reduce both the incidence of AFB outbreaks and the potential for further spread of the disease within a large-scale commercial beekeeping operation and to present a sustainable preventive chemical-free solution to mitigate AFB disease.

2. Materials and methods

2.1. Background and first inspection for clinical symptoms of AFB

This study was conducted in collaboration with a local commercial beekeeping operation just outside Uppsala in central Sweden (covering a geographical area of approximately 30 km^2) that has experienced repeated AFB outbreaks over several years prior to this study (Lindström and Fries, 2005). In the first year of the study the beekeeping operation contained 56 apiaries with about 10–12 colonies in each apiary (Table 1). In May 2012, apiaries suspected to be infected with AFB disease were subjected to visual inspection of the brood for clinical symptoms. A total of 58 colonies in 5 apiaries (12 in 3 apiaries and 11 in the other 2 apiaries) were inspected and clinical symptoms of AFB were observed in 11 of the 58 inspected colonies while the remaining inspected colonies (n = 47) showed no visual symptoms of disease in the brood. All the symptomatic colonies (n = 11) were burned in accordance with Swedish legislation.

2.2. Annual monitoring of P. larvae spore levels

The study began with the first samples of adult bees taken during the brood-less period just before the colonies overwintered in October 2012. The samples comprised three hundred adult bees collected from each colony. Composite apiary samples of 100 bees were created by pooling 8–10 bees from each colony sample from within the same apiary (Table 1). The apiary composite sample was used for the initial screening of bacterial spores by microbial culturing, which was performed over the following winter months. Bacterial spore counts, presented as Colony Forming Units (CFU) were separated into categories based on the relation with the hypothetical potential for the colony to develop clinical symptoms (Table 2). If spore levels were high in the apiary composite sample (category 3, 4 or 5; Table 2), then 100 bees per colony (from the individual colony samples) within that apiary were investigated for bacterial spore counts determined by microbial culturing.

2.3. Quarantine management strategy

Any individual colony that was identified with high levels of bacterial spores (category 3, 4 and 5; Table 2) was placed in quarantine management the following spring (March-April) while the brood rearing was still minimal. The quarantine management strategy involved two steps: 1) shaking the adult bees onto new uncontaminated hive material while the infected hive material was destroyed (including all wax, brood and stores of honey and pollen); and 2) using completely separated equipment from the remaining part of the beekeeping operation. The adult bees shaken onto new hive material were temporarily fed sugar solution for early support until they could forage themselves. When the quarantined colonies started rearing brood they were visually inspected for clinical symptoms of AFB.

Fall sampling and spring quarantining procedures were continued as described above for a total of 5 years, 2012–2016, including all the colonies of the beekeeper's expanding operation (Table 1). New colonies were established within the beekeeper's operation from the colonies that were free of bacterial spores (Table 1; category 1, Table 2).

2.4. Cultivation of P. larvae from bee samples

Paenibacillus larvae was cultured from adult bees for early detection of AFB having been proven to be the most diagnostically accurate method in relating spore counts to clinical symptoms (Forsgren and Laugen, 2014).

The bacterium was cultivated from samples of bees on MYPGP-agar plates as previously described by Nordström and Fries (1995). Samples of 100 adult worker bees were crushed in 20 mL of sterile 0.9% NaCl in a filter grinding bag (Neoreba®). The fluid produced was centrifuged for 10 min at 27,000 g and the resulting pellet was re-suspended in 2 mL sterile NaCl, heat shocked at 85 °C for 10 min and spread out over 3 MYPGP-agar plates (10 µL each). The numbers of *P. larvae* colonies were counted on each plate after an incubation period of 7 days at 36 °C in 5% CO_2 and a mean value for the 3 plates calculated. Since 10 µL of the bee suspension was streaked on each plate, the CFU per bee was calculated by multiplying by a factor of 200 (since 100 bees was used in the 2 mL suspension). All *P. larvae* isolates from this study over the entire study period were of the same genotype (ERIC-1).

Table 1
The total numbers of apiaries and colonies that were sampled over the 5 years of this study. All colonies within each apiary were sampled.

	2012	2013	2014	2015	2016
Total number of assayed apiaries (composite samples)	56	67	76	87	90
Total number of sampled colonies	572	784	802	894	945
Total number of apiaries where spores were found and it was necessary to further assay individual colonies	13	5	3	6	0
Total number of colonies assayed individually	156	45	34	49	0

Table 2
Quarantine CFU categories based on the relation between the number of *P. larvae* bacterial spores (CFU) and the hypothetical probability of exhibiting clinical disease.
Adapted from Table 3 in: Goodwin, 2006.

CFU category	Mean number of CFU per plate	CFU per 100 bees
1	0	0
2	1-10	1-2000
3	11-50	2001-10000
4	51-200	10001-40000
5	> 200	> 40000

2.5. Statistical analyses

A multinomial logistic regression was used to analyse the probability of changes in CFU categories of apiaries (using apiary composite samples) over the 5 years of the study. A multinomial was used instead of an ordered logistic regression, because the proportional odds assumption for the ordered logistic was violated (chi-square p = 0.01). Analyses were implemented in R (R Core Team, 2017) using the 'mlogit' package (Croissant, 2013). The CFU categories were used as a 5-level category variable, with category 1 being apiaries with no detectable CFUs - this category was used as the reference level in the analysis. To avoid any bias associated with using incomplete time series from specific apiaries, only the apiaries with data from all years of the study were included (n = 45).

3. Results

The early detection and quarantine strategy applied to this commercial beekeeping operation was effective at significantly reducing the amount of detectable *P. larvae* spores, e.g. the infection pressure, throughout the entire operation (Table 3; Fig. 1). This management strategy also prevented any reoccurrence of AFB disease outbreaks. After the initial screening in 2012, where 11 colonies with AFB clinical symptoms were destroyed, no clinical symptoms were found again throughout the beekeeper's entire operation over the following 4 years of the investigations, or since.

Table 3
The estimated multinomial logistic regression coefficients for the model explaining CFU category relative to the year of the study (where CFU category 1 is the reference level) for 45 apiaries. Since parameter estimates are relative to the reference category, these can be interpreted as: for a unit change in the predictor variable (i.e. year) this is the expected logit change in the category of interest (i.e. CFU categories 2–5) relative to the reference group. Thus, for each passing year we expect the probability of an apiary being in any category other than category 1 to decline (with the parameter estimates showing the logit magnitude of this relative decline).

CFU category	Parameter	Estimate ± SE	t-statistic	P-value
2	Intercept	1.29 ± 0.31		
	Year	−0.83 ± 0.11	7.65	< 0.0001
3	Intercept	−1.85 ± 0.85		
	Year	−0.59 ± 0.29	1.98	0.046
4	Intercept	−1.83 ± 1.20		
	Year	−0.97 ± 0.52	1.85	0.063
5	Intercept	0.17 ± 0.62		
	Year	−1.26 ± 0.31	4.10	< 0.0001

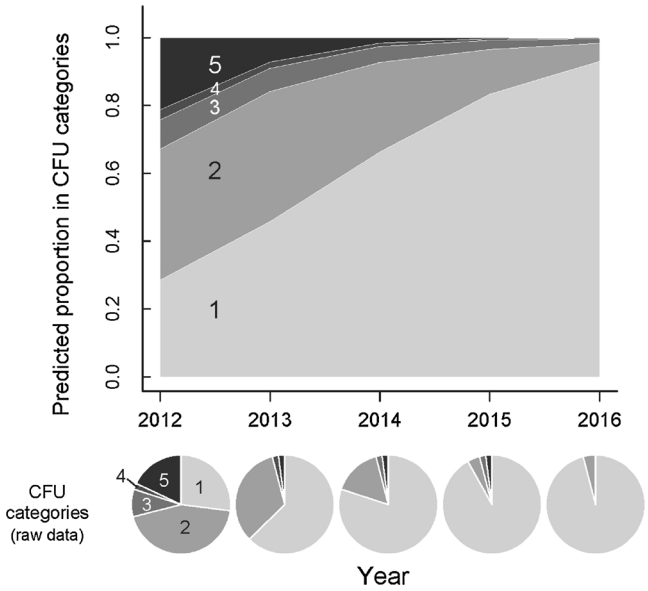

Fig. 1. Predicted proportion of apiaries in the five different CFU categories (above) compared to the raw data (below) during the study period from 2012 to 2016 (n = 45). The predictions are generated from the multinomial logistic regression analysis (for parameter estimates and their SEs see Table 3).

In the fall 2012, 73% of the apiaries harboured *P. larvae* spores with 29% of them with high level bacterial spore counts (categories 3, 4 and 5; Fig. 1). Already after the first year of applying the quarantine strategy, the percentage of apiaries harbouring *P. larvae* spores was reduced to 37% in the fall 2013 with only 4% of the apiaries having high levels of bacterial spores belonging to categories 3, 4 and 5 (Fig. 1). By the end of the study in 2016, only 4% of the apiaries harboured *P. larvae* spores and all these apiaries had low levels of spores (category 2), with no apiaries falling into categories 3, 4 or 5 (Fig. 1).

From each of the composite apiary samples only a small proportion of the colonies actually contained high levels of *P. larvae* bacterial spores (Table 1), whereas the rest of the colonies in the same apiary were free of bacterial spores or had very low levels. In the 11 clinically diseased colonies that were destroyed the first year, there was large variation in the CFU values from cultured bee samples ranging from roughly 20 400 to 25 934 000 CFU/ 100 bees.

4. Discussion

The epidemiology of AFB has been modeled (Datta et al., 2013) and studied using molecular and genetic tools to track the spread and locate the source of disease epidemics at a landscape scale (Ågren et al., 2017; Mill et al., 2014; Pentikäinen et al., 2009). This study demonstrates that epidemiological monitoring can also be used to evaluate the efficacy of a disease management program and provides practical insight into the epidemiological process of disease transmission in order to develop a preventive control strategy.

The results of this study further show that early detection and

quarantine management strategies can provide an effective sustainable non-chemical solution to reduce both the incidence of AFB outbreaks as well as the continued transmission risk. The commercial beekeeper in this study has had a history of reoccurring AFB outbreaks (Lindström and Fries, 2005), where the destruction of clinically diseased colonies was required on a regular basis. In large beekeeping operations it is common to have reoccurring outbreaks of AFB disease (Lindström and Fries, 2005; Pentikäinen et al., 2009) since *P. larvae* bacterial spores can remain infective for many years accumulating in the hive material (Hasemann, 1961). Over the course of this 5 year study, clinical symptoms were no longer observed after the first screening in 2012. More importantly, the proportion of apiaries harbouring *P. larvae* spores decreased from 74% to 4% demonstrating that early detection together with an altered management regime, with the goal to reduce the overall infection pressure, can significantly reduce the risk of disease transmission to further colonies, apiaries, or even beekeepers. From an epidemiological perspective, this study demonstrates that AFB disease dynamics respond significantly to altered management practices and that localized disease eradication is possible.

The early detection of sub-clinical AFB infections was an important component in the efficacy of the preventive control regime of this study, as previously predicted (Datta et al., 2013; Forsgren and Laugen, 2014). In an attempt to identify an early detection threshold value of *P. larvae* spore counts that would relate to clinical symptoms, Gende et al. (2011) found that all of their examined clinically symptomatic colonies had spore counts above 3000 CFU / bee. In our study, there was very large variation in *P. larvae* bacterial spore counts in the colonies identified with clinical symptoms in 2012, ranging between 204–259 340 CFU / bee. This suggests that finding a practical early detection threshold will not be a simple task. The relationship between the number of *P. larvae* bacterial spores per bee with the stage of the disease in colonies has been well studied and a variety of factors have been implicated to be involved in the disease dynamics including prior outbreaks, beekeeping practices, drifting, robbing, environment and even the presence of other pathogens (Fernández et al., 2010; Zuur et al., 2009). Additionally, different strains of *P. larvae* have different virulence (Genersch et al., 2005) and individual bees and colonies can have differing degrees of susceptibility (Spivak and Reuter, 2001), all affecting the establishment of the disease and the onset of clinical symptoms.

When high levels of *P. larvae* spores were detected in an apiary composite sample, the individual colony samples from that apiary were cultured. In all cases, *P. larvae* was detected in only 1–2 colonies while all other colonies from the same apiary (10–12 colonies) had low to undetectable levels. Natural transmission pathways such as drifting or robbing are density dependent with more frequent AFB outbreaks in high colony density (Lindström et al., 2008b), which is typical within apiculture and was the case with the beekeeping operation in this study. Therefore, it was expected to see a higher proportion of colonies within a contaminated apiary actually containing *P. larvae* bacterial spores. The fact that this was not the case suggests that the drifting is not a relevant transmission route for disease outbreaks even at an extreme local scale between hives in close proximity. This result supports the notion that beekeeping practices are likely the primary transmission route causing disease (Fries and Camazine, 2001; Lindström et al., 2008b).

While it is becoming common knowledge that beekeeping practices facilitate the spread of AFB, operational practicality is often prioritized over disease prevention management. As with any farmed species, the destruction of animals should always be considered a last resort when all other measures to prevent the further spread of the disease are ineffective (Datta et al., 2013). Burning diseased colonies is not a sustainable nor economically viable solution to mitigate AFB and preventive management strategies applied to not yet clinically disease colonies would provide a much more economically viable approach (Pernal et al., 2008). Quarantine strategies probably work best in beekeeping operations with stationary apiaries like the one in this study but can also be incorporated in migratory beekeeping if sub-clinical colonies are quarantined instead of being transported for pollination services, facilitating the spread of the disease over long distances (Ågren et al., 2017; Pentikäinen et al., 2009). A consensus among beekeepers to employ these preventive strategies could effectively reduce the incidence of AFB epidemics at a national or even international level.

A possible limitation to this management strategy, both from a preventive or elimination approach, is that it requires extra effort in organization and logistics planning from the beekeeper. However, the benefit of eliminating disease causing spores from a commercial operation, as accomplished in this study, or preventing the establishment of AFB within the operation, should outweigh this small drawback.

Most advice to beekeepers focuses on controlling the disease once it is found. Sweden has a national inspection system but it is based on identifying clinical symptoms by examining brood, which is often too late and the colony must be destroyed. The OIE manual only provides instructions for laboratory methods for detecting AFB and has no guidelines or advice for practical measures for beekeepers (Anonymous, 2016). This study combines early detection of the causative bacteria through an active surveillance of spore levels with a practical quarantine system to effectively exterminate the disease in a big beekeeping operation.

Preventive AFB disease management requires effective ways to identify sub-clinical infections and then taking appropriate measures to prevent the further spread of the disease to additional individuals. Culturing *P. larvae* bacterial spores from samples of adult bees, as done in this study, provides an effective way to assess the transmission risks between colonies (Forsgren and Laugen, 2014). Sampling and culturing the bacteria in the autumn enables preparation over the winter months to plan and implement management strategies early in the spring before the disease develops further when brood production begins in the colony. Incorporating this early detection with a low cost quarantine management strategy proved to be highly effective at reducing reoccurring AFB disease outbreaks as well as nearly eliminating the potential transmission risk between colonies, apiaries and other beekeepers, offering a sustainable chemical-free solution to combat AFB.

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