

Prognostic value of using bee and hive debris samples for the detection of American foulbrood disease in honey bee colonies

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Abstract – The objective of this study was to compare how well the detection of *Paenibacillus larvae* in samples of live bees or in accumulated winter debris collected from the bottom of beehives relates to symptoms of American foulbrood in honey bee colonies. Fifty-eight colonies in one commercial beekeeping operation were inspected for disease symptoms and assayed for *P. larvae* using culture-based techniques and PCR. The results show that culture-based techniques are more accurate than recently published PCR methods for detecting the bacterium in clinically diseased colonies, and that the prognostic value of bacterial colony counts from bee samples is superior to colony counts from debris. However, if the objective is to monitor the prevalence of the bacterium irrespective of disease symptoms, the preferable method is PCR analysis of accumulated winter hive debris.

American foulbrood / *Paenibacillus larvae* / culture / PCR / logistic regression

1. INTRODUCTION

The honey bee larval disease American foulbrood (AFB) is one of the major pathogenic threats to beekeeping. The disease is highly contagious, capable of killing infected colonies and causing large economic losses worldwide. AFB is caused by the Gram-positive, spore-forming bacterium *Paenibacillus larvae* (Genersch et al. 2006). Spores are only infectious to young larvae that become infected by consuming spore-contaminated food (Hitchcock et al. 1979). As few as 10 spores are enough to infect and kill a larva (Brødsgaard et al. 1998; Woodrow 1942). The most conspicuous

symptom in colonies suffering from clinical AFB disease is irregular brood capping, with capped and uncapped cells scattered irregularly across the brood frames. The caps are dark, sunken and often punctured, emitting a ‘foul’ odor. The brown, glue-like remains of the dead larvae in these cells form a highly characteristic ropey thread when pulled out with a wooden stick or a match, which is a reliable field diagnosis for AFB. The remains of the larvae finally form a hard scale in the bottom of the cell. Such scales contain millions of bacterial spores that remain viable for decades (Genersch 2010; Hasemann 1961).

Traditional diagnosis of AFB is based on the observation of clinical symptoms in the hive as described above (De Graaf et al. 2006) and microbial cultivation of material from infected colonies. Several diagnostic protocols have been

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published for the cultivation of *P. larvae* from extracted honey (De Graaf et al. 2006; Nordström and Fries 1995; Pernal and Melathopoulos 2006), beehive debris (Bzdil 2007; Titera and Haklova 2003) and adult bees (Gillard et al. 2008; Lindström and Fries 2005). A direct comparison between these showed that cultivating the bacteria from live bees relates better to colony-level disease symptoms than cultivating from honey samples (Lindström and Fries 2005; Nordström et al. 2002). Culture-based laboratory *P. larvae* assays are definitive for confirming the diagnosis of AFB suspected colonies, but may be less suitable for surveillance and epidemiological studies. Early detection of sub-clinical levels of *P. larvae* in an apiary or beekeeping practice is imperative for effective preventative disease control. One common objection to culture-based AFB diagnosis methods has been that many spores do not germinate in vitro. For instance, Dingman and Stahly (1983) concluded that only 6 % of *P. larvae* spores germinate under culture conditions. A more recent study (Forsgren et al. 2008) also shows that there is a significant variability in the germination rate on solid media between different *P. larvae* genotypes.

In recent years, a number of PCR-based protocols have been published as an alternative to traditional cultivation-based techniques for the diagnosis, characterization and quantification of *P. larvae*. Conventional PCR has been used to detect *P. larvae* in brood, foulbrood scales and in honey (Alippi et al. 2004; Bakonyi et al. 2003; Dobbelaere et al. 2001; Lauro et al. 2003) as well as in beehive debris (Ryba et al. 2009). More recently quantitative, real-time PCR-based methods have been developed for the quantification of *P. larvae* in honey and larvae (Han et al. 2008; Martinez et al. 2010).

Several of these molecular protocols were developed for the detection of *P. larvae* DNA in vegetative bacterial cells cultivated on agar medium or in total DNA isolated from larvae with an active infection (Dobbelaere et al. 2001; Lauro et al. 2003). However, for epidemiological and quantitative studies it is imperative to be able to detect not only the vegetative cells but also the bacterial spores from different materials such as honey, debris, adult bees and wax. There are specific protocols available for

the extraction of genetic material from the endospores using commercially available extraction kits (Ryba et al. 2009).

Analysis of the debris collected from the bottom of the beehive is a simple and non-invasive method for assessing the health status of a bee colony. However, it is not known how well the published *P. larvae* hive debris assays, either PCR-based (Ryba et al. 2009) or culture-based (Titera and Haklova 2003), relate to colony-level disease symptoms, compared to similar assays of adult bees. Moreover, we still know relatively little about the consistency of culture- or PCR-based assays for the detection and quantification of *P. larvae* from other types of honey bee colony samples.

The main objectives of this study were therefore to: (1) evaluate how well two types of beehive samples (adult bees and hive debris) relate to actual disease symptoms in the colony (2) to compare two methods for assaying bee and debris samples, by real-time PCR and by microbial cultivation on semi-selective media. By sampling adult bees and debris from the same hives, assessing the samples with the two different methods and applying separate logistic regressions to the data, this study provides the first statistical evaluation of some of the current diagnostic methods of AFB.

2. MATERIALS AND METHODS

2.1. Sampling

A total of 58 colonies in five honey bee (*Apis mellifera* L) apiaries in a commercial beekeeping operation in Uppsala County, Sweden were subjected to visual brood inspection for AFB symptoms in May 2012. Two types of samples were collected from these colonies; adult bees from the brood nest and hive debris. Two to three hundred bees were shaken into a plastic bag, killed by freezing, and kept frozen until processed. The debris was collected from the bottom board representing the accumulated hive debris from the winter period. Disease symptoms were observed in 11 of the inspected colonies, but in several cases only a few diseased larvae were found.

In the rest of the colonies ($n=47$) there were no visual symptoms of disease in the brood.

2.2. Cultivation of *P. larvae* from bees

The bee samples were cultivated on MYPGP-agar plates as described by Nordström and Fries (1995). In brief, 100 worker bees were placed into a filter grinding bag (Neoreba®), and 20 mL of sterile 0.9 % NaCl was added. The bees were crushed and the fluid produced centrifuged for 10 min at 27,000×g. The resulting pellet was resuspended in 2 mL sterile NaCl, heat shocked at 85 °C for 10 min and spread onto 3 MYPGP-agar plates (10 µL each). The plates were incubated at 36 °C in 5 % CO₂ for 7 days and the numbers of *P. larvae* colonies were counted. The data was normalized and presented as colony forming units (CFU) per bee.

2.3. Cultivation of *P. larvae* from hive debris

The hive debris was processed using the Tween 80 method (Bzdil 2007). Briefly, 1 g of debris was mixed with 8.5 mL sterile water and 0.5 % Tween 80 (pre-warmed to 70 °C), carefully mixed and immersed for 30–60 min in a 70 °C water bath. The tubes were removed from the water bath and the samples settled for 2 h (room temperature) before 2 mL of the liquid was transferred to a new tube and mixed with an additional 2 mL of sterile water. The samples were mixed thoroughly, heated at 85 °C for 10 min and spread onto 3 MYPGP-agar plates (10 µL each). The plates were incubated at 36 °C in 5 % CO₂ for 7 days and the numbers of *P. larvae* colonies were counted. The data is presented as CFU per gram hive debris.

2.4. Comparison of DNA extraction methods

A pilot experiment was performed to evaluate two previously published extraction protocols for isolating genomic bacterial DNA from *P. larvae* spores (Martinez et al. 2010; Ryba et al. 2009). A stock spore suspension of *P. larvae* (CCUG 48979 Forsgren et al. 2008) in sterile ddH₂O was prepared and checked under a phase-contrast light microscope (×1,000) to confirm presence of endospores and absence of vegetative cells. The spores were counted

in a Helber Bacteria Counting Chamber (Hawksley, UK) and tenfold dilutions (10⁷, 10⁶, 10⁵ and 10⁴ spores/mL) of the suspension were prepared. Bees and hive debris from healthy colonies were spiked with different concentrations of *P. larvae* spores (Table 1). Briefly, one whole bee or 1 g debris was crushed in 1 mL of spore suspension, before being subjected to DNA extraction using two commercial extraction kits, QIAamp® DNA Mini Kit (Qiagen)(Ryba et al. 2009) and UltraClean Soil DNA isolation kit (Mo Bio Laboratories Inc., Martinez et al. 2010), following the manufacturer's instructions.

2.5. Bacterial DNA extraction from bee samples

One hundred worker bees were placed into a filter grinding bag (Neoreba®), and 20 mL of sterile ddH₂O was added. The bees were crushed and 1 mL of the fluid produced was immediately subjected to DNA extraction using the QIAamp® genomic DNA isolation mini kit for Gram-positive bacteria (Qiagen). The DNA was eluted with 100 µL elution buffer and stored at -20 °C until further use.

2.6. Bacterial DNA extraction from hive debris

One gram of debris was subjected to DNA extraction following the protocol of Ryba and co-workers (Ryba et al. 2009). Briefly, 1 g of debris was mixed with 5 mL of sterile, distilled water and shaken at room temperature for 1 h. One hundred microliters of the suspension was immediately subjected to DNA extraction using the QIAamp® genomic DNA isolation mini kit for Gram-positive bacteria (Qiagen). The DNA was eluted with 100 µL elution buffer and stored at -20 °C until further use.

2.7. Real-time PCR for *P. larvae* 16s RNA sequence

The quantitative real-time PCR assay used here was modified from Martinez et al. (2010). Briefly, the reaction contained 10 µL of SsoFast™ EvaGreen® Supermix (Bio-Rad), 0.4 µM each of primers PL2-Fw and PL2-Rev, 2 µL of template, and the final

Table I. Results from PCR performed on bee and debris spiked with *P. larvae* spore suspensions (10^3 , 10^4 , 10^5 , and 10^6 spores). Bacterial DNA was isolated using two commercial kits (A=UltraClean Soil DNA isolation kit, B=QIAamp® DNA Mini Kit). Six 100 μ L aliquots of each spore solution was extracted and subjected to PCR. Numbers indicate how many aliquots out of the six aliquots assayed produced PCR product.

Extraction method	A	B	A	B	A	B	A	B
Spore conc.	10^6	10^6	10^5	0^5	10^4	10^4	10^3	10^3
Bee+spores	6/6	6/6	6/6	4/6	nd	nd	nd	nd
Debris+spores	6/6	6/6	6/6	6/6	6/6	6/6	5/6	5/6
<i>P. larvae</i> spores only	6/6	6/6	6/6	6/6	5/6	6/6	1/6	6/6

reaction volume was adjusted to 20 μ L with nuclease-free water. A negative control containing water instead of DNA template was included in each run. All real-time qPCR reactions (standards, unknown samples and controls) were performed in duplicate in neighboring wells on the sample plate. The results reported are an average of the duplicates. The amplification and data acquisition was carried out using a CFX Connect® (Bio-Rad) real-time PCR machine under the following cycling condition: enzyme activation step, 98 °C for 2 min, PCR cycling (40 cycles of 98 °C for 5 s (denaturation), 58 °C for 5 s (annealing/extension) and data collection. The identity of the amplified product was confirmed using a melting curve analysis, by raising the temperature from 55 to 95 °C in 0.5 °C increments with a hold of 1 s at each increment. The assay specificity and the presence/absence of non-specific amplification products were determined through the melting temperature(s) (T_m) of the amplified product(s). Each set of PCR assays included serial dilutions of DNA extracted from known concentrations of *P. larvae* spores (total microscopic count) as external standards, for relating the qPCR data to spore counts (Forsgren et al. 2008). Standard curves were prepared by using serial dilutions of target DNA ranging from 10^2 to 10^8 as quantification standards in every run. The quantification data was normalized to tissue or debris weight and converted to bacterial cells per bee or bacterial cells per gram debris.

2.8. Statistical analyses

To determine if the sample type (adult bees vs. debris) influenced the amount of bacteria detected with the two assessment methods (culture vs. PCR), we used two-sample *t* tests to test for difference in means, and *F* tests to test for differences in variances.

To evaluate the effectiveness of the four combinations of sample types and detection methods in detecting AFB-symptomatic colonies, we fitted separate logistic regressions to each of the four combinations, using the statistical package R, version 3.0.0 (R Core Team 2013). We fitted the health status of the hive, healthy or diseased, as a binary response variable, and the \log_{10} -transformed amount of bacteria detected by each of the four combinations as the predictor. We evaluated the predictive power of the four combinations from the model output, through the Akaike's Information Criterion (AIC) statistic. A lower AIC value for a particular combination of detection method and sample type indicates a better fit of the model to the data, and therefore a superior ability to predict clinical symptoms. However, the AIC score is only a relative measure of model performance and does not give the absolute goodness-of-fit of a model. Therefore, we present two additional criteria for evaluating the method-sample type combinations. First, the amount of variation explained by each model is presented by a pseudo- R^2 (Nakagawa and Schielzeth 2012), given by formula 1—(residual deviance/null deviance). R^2 takes values between 0 and 1, and the higher the values; the more variation in the data is explained by the model. Second, to get an estimate of the detection sensitivity for each combination of sample type and detection method, we calculated the predictor value at which 95 % (95-quantile; q_{95}) of the colonies were diagnosed with clinical symptoms, using the function

$$q_{95} = \frac{-\ln\left(\frac{1}{0.95-1}\right) + b}{a}$$

where *a* is the slope and *b* is the intercept of each logistic regression. A low q_{95} -value predicted from a specific model means that a low amount of

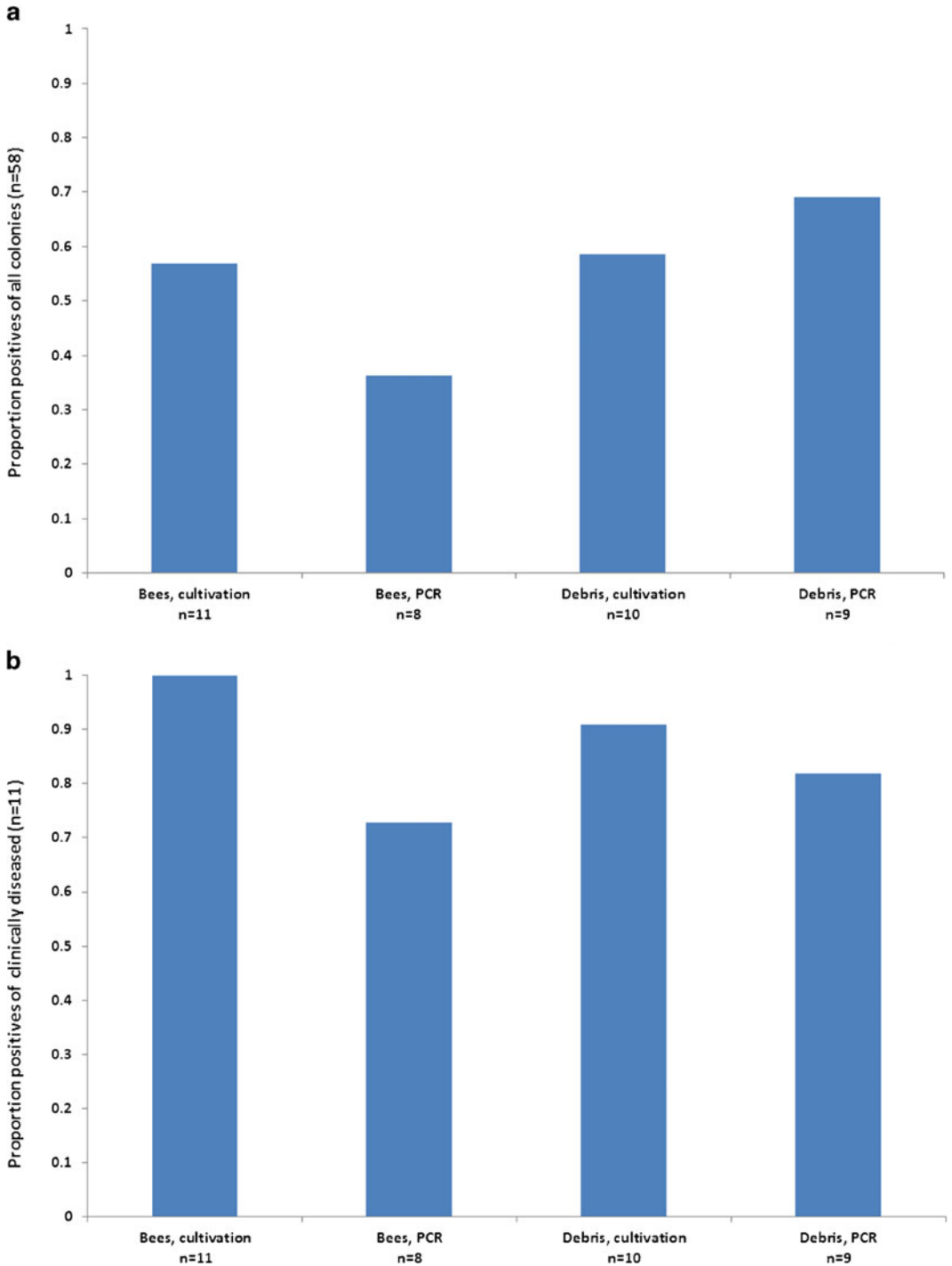


Figure 1. Number of colonies where *P. larvae* was detected using (1) bee samples and culture, (2) bee samples and PCR, (3) hive debris and culture and (4) hive debris and PCR, calculated as a proportion of all assayed colonies (a) and as a proportion of colonies with clinical samples (b).

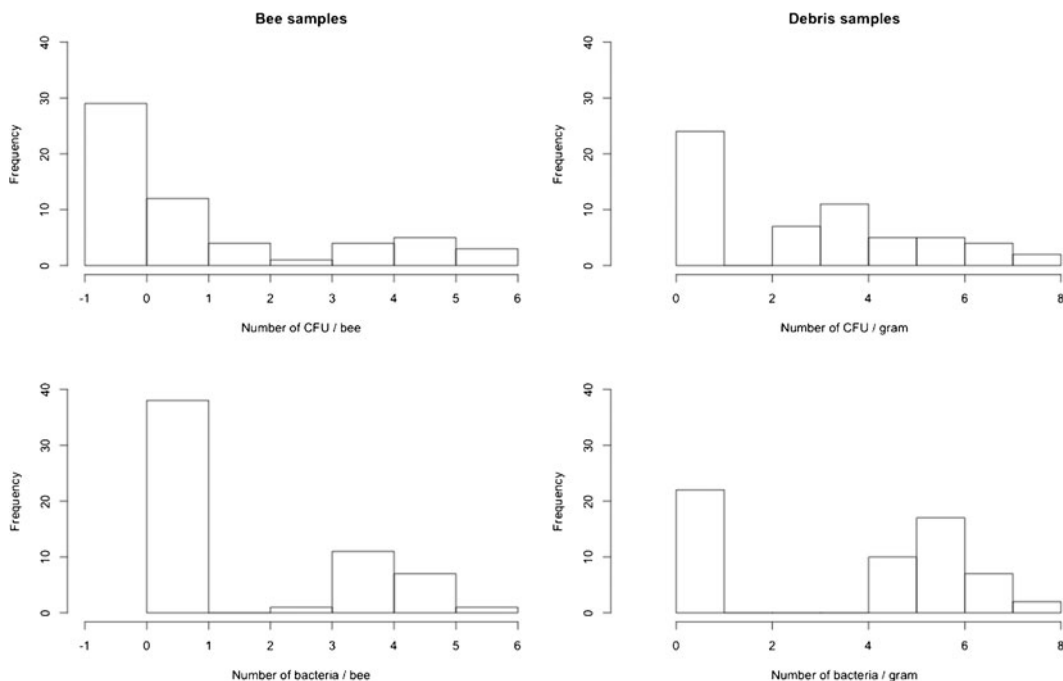


Figure 2. Frequency plot showing the occurrence of \log_{10} -transformed number of bacteria from two different sample types; adult bees (*left panels*) and hive debris (*right panels*), extracted with two different analysis methods; culture (*upper panels*) and PCR (*lower panels*).

bacteria is necessary for detecting clinically diseased colonies. Consequently, an assessment method with a lower q_{95} -value is more sensitive than a method with a high q_{95} -value.

3. RESULTS

3.1. Cultivation of *P. larvae* from bees versus debris

Eleven of the inspected colonies showed clinical signs of AFB. All the adult bee samples (11/11) and all but one debris sample (10/11) from the diseased colonies were positive for *P. larvae* when cultured as described above (Figure 1b). Moreover, in all the sampled colonies, irrespective of disease symptoms, 34 out of 58 debris samples and 33 out of 58 bee samples were found positive for *P. larvae* when cultured (Figure 1a). A Welch two-sample t test showed that significantly more

bacteria could be detected in the debris samples than in bee samples, using the culture method (mean 2.56 and 1.09, respectively, $t=-3.74$, $DF=103.69$, $P=0.0003$; Figure 2). The two sample types also differed in their variance, with the debris samples generally showing more variance than the bee samples (F test; $F=0.52$, $DF=57$, $P=0.015$).

3.2. Real-time PCR detection of *P. larvae* from bees versus debris

A Welch two-sample t test showed that on average, there were significantly more bacteria detected in the debris samples than in bee samples, using qPCR (mean 3.46 and 1.35, respectively, $t=-4.72$, $DF=101.50$, $P=7.4 \times 10^{-6}$; Figure 2). The sample types also differed in their variance, with the debris samples generally showing more variance than the bee samples (F test; $F=0.48$, $DF=57$, $P=0.006$).

3.3. Comparison of DNA extraction methods

There were clear differences between the two extraction methods regarding the efficiency in extracting DNA from *P. larvae* spores. The QIAamp® DNA Mini Kit proved to be more efficient for DNA isolation from pure *P. larvae* spore suspensions, with six out of six extracted aliquots of the 10³ suspension producing a PCR product, whereas product was only detected from 1 out of 6 bacterial DNA templates of the same concentration extracted using the UltraClean Soil DNA isolation kit (Table I). Hence, the bee and debris samples in this study were extracted using QIAamp® DNA Mini Kit, according to the manufacturer’s instructions. However, when a healthy bee spiked with bacterial spore suspension was extracted, the UltraClean Soil DNA isolation kit was superior to the QIAamp® DNA Mini Kit (see Table I), indicating a better efficiency in removing inhibitory compounds from the bee extract.

3.4. Predicting health status of colonies from amount of bacteria

The accuracy of predicting colony health status using bacterial amounts was higher for bee samples than for debris samples, as indicated by the better model fit (lower AIC value and higher R² value), irrespective of the detection method used (Table II). Moreover, for each sample type, culturing bacteria is always superior to PCR. Plotting the fitted models (Figure 3) shows that the combination of bee samples and culture-based assays (steepest slope) is far better at predicting colony health status than the three other combinations (shallower slopes). The combination also had the lowest level of bacteria needed to predict 95 % of diseased colonies (Table II).

4. DISCUSSION

Our results clearly demonstrate that if the objective is to find clinically diseased colonies, the prognostic value of bacterial colony counts from bee samples is superior to bacterial colony

Table II. Model output from logistic regressions of the probability of detecting American foulbrood disease with four different combinations of sample types (adult bees vs. debris), and assessment methods (culture vs. PCR). All models have 56 degrees of freedom.

Method	Sample type	Intercept		Slope		ResDev	q ₉₅	AIC	R ²				
		Estimate	Std	Estimate	Std								
		z	p	z	p								
(a) Culture	Bees	-4.9529	1.4409	-3.437	0.000587	1.6434	0.4569	3.597	0.00032	16.09	4.81	20.09	0.71
	Debris	-5.2218	1.4651	-3.564	0.000365	0.9710	0.3028	3.207	0.00134	33.57	8.41	37.57	0.22
(b) PCR	Bees	-2.7025	0.6459	-4.184	2.86e-05	0.6162	0.1962	3.141	0.00168	44.15	9.16	48.15	0.40
	Debris	-3.0917	0.9774	-3.163	0.00156	0.3818	0.1821	2.097	0.03599	49.87	15.81	53.87	0.11

ResDev residual deviation, q₉₅ the value of the predictor (amount of bacteria) for which 95 % of the infected hives have been detected by each combination of assessment method and sample type, R² is a pseudo-R² calculated from formula 1—(ResDev/Null Deviance)

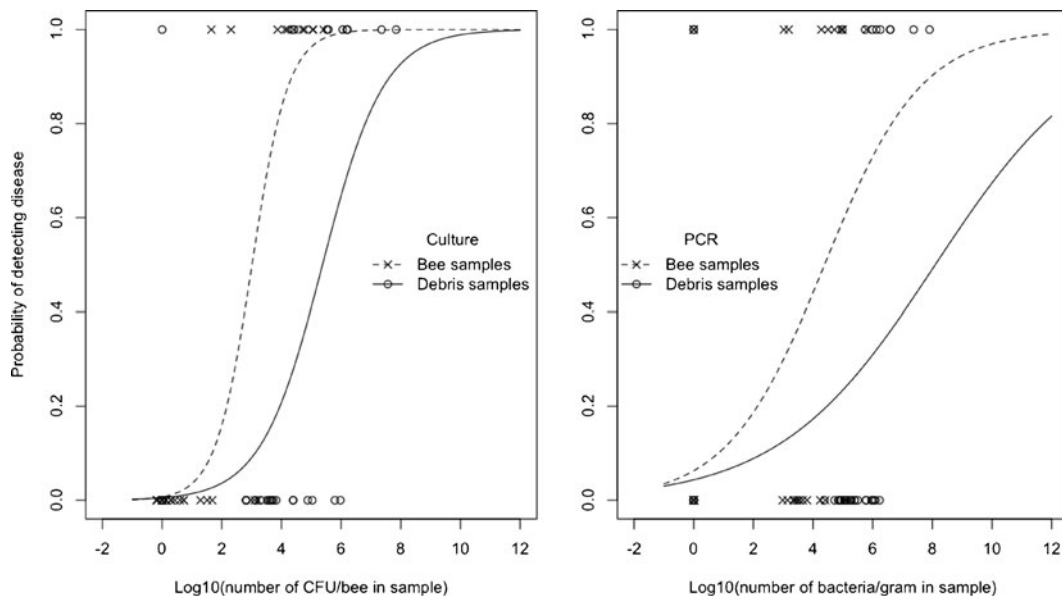


Figure 3. Raw data and fitted logistic regressions showing the probability of detecting American foulbrood disease) as a function of \log_{10} -transformed number of bacteria in sample, using two different assessment methods; culture (*left panel*) and PCR (*right panel*) and two different sample types; adult bees (*crosses and dotted lines*) and PCR (*open circles and solid lines*).

counts from debris. All the bee samples from visibly diseased colonies contained detectable *P. larvae* spores using cultivation, whereas one sample of debris gave a false negative result (i.e., disease symptom in the colony, but no colonies produced using microbial cultivation). However, using bacterial colony counts for the quantification of *P. larvae* in hive debris and adult bee samples revealed significantly higher spore loads in the debris (Figure 2). This is not surprising since bees collected from the brood chamber represent a “snapshot” of the actual disease status of the brood, whereas hive debris collected during the wintering period reflects an historical accumulation of bacterial spores from the colony during that time. However, in all the sampled colonies combined, irrespective of disease symptoms, the results for the two materials are comparable, with 34 out of 58 debris samples and 33 out of 58 bee samples found positive for *P. larvae* when cultured (Figure 1a).

Significantly more spores were detected in the hive debris than in the bee samples not only when

colony counts were used, but also using the PCR assay. It is likely that the bacterial loads are higher in hive material accumulated for months than in live bees whose life-span is measured in weeks. Moreover, the probable presence of PCR-inhibitors in adult bee samples and the difficulty to extract bacterial DNA from the endospores may lead to a general underestimation of bacterial loads in all sample materials, using PCR-based methods.

Culture-based detection methods allow the quantification of microorganisms with considerable precision, but PCR-based assays are often said to be superior for many reasons. However, quantification of PCR products should not necessarily be equated with the number of organisms present in the original sample (Vanechoutte and Van Eldere 1997). Amplification of targets from clinical samples may have rather limited sensitivity, in contrast to what is often reported from research studies (Vanechoutte and Van Eldere 1997). This can clearly be seen by our data where we can detect *P. larvae* in some of the sampled bee colonies using

bacterial colony counts but not with the PCR method. This may depend on a variety of factors such as inhibitors of the PCR reaction and/or failures in the extraction protocols (Wilson 1997). Rusenova et al. (2012) reported low sensitivity in their PCR assay when using standard DNA extraction protocols. Incubation of the samples in a rich nutrient medium for 1 h at 37 °C considerably improved the protocol, presumably because the spore envelope is altered and the bacterial DNA has become more accessible for extraction.

Martinez et al. (2010) reported an assay detection limit of 2 *P. larvae* spores g⁻¹ honey contrasting to earlier reports of a detection limit of 10⁵ spores g⁻¹ of honey (Ryba et al. 2009) and 283 spores g⁻¹ of honey (Alippi et al. 2004). These differences may very well be due to differences in extraction methods, highlighting the possibility that comparative evaluation of DNA extraction and purification may be more important than comparisons of different amplification methods. Our comparison of two commercial extraction kits revealed large differences in their efficiencies for isolating bacterial DNA from pure *P. larvae* spore suspensions. Much to our surprise, DNA isolated with the UltraClean Soil DNA isolation kit failed to consistently produce a PCR product when used on suspensions with less than 10³ spores mL⁻¹, whereas QIAamp® DNA Mini Kit showed to be much more effective for the isolation of pure bacterial DNA (Table 1), but was less effective when bee or debris was added. Possibly, the UltraClean Soil DNA isolation kit is more effective for the removal of polymerase inhibitors, but more work needs to be done to further evaluate the DNA extraction and purification of *P. larvae* spores.

Bee samples were more accurate than debris samples for predicting colony health status using *P. larvae* quantification, irrespective of detection method used. The combination of using pooled samples of adult bees and bacterial colony counts on MYPGP-agar to assess the bacterial load is superior to any of the other combinations (Figure 2). This is probably due to: (1) samples of adult bees better reflects the actual health status of the bee colony, and (2) the superiority of culture-based assessment of the bacterial loads to the PCR-based protocols used here, due to the detection

limitations in the nucleic acid amplification (Vanechoutte and Van Eldere 1997). However, it should be noted that the predictions from the logistic regressions were based on data from only 11 colonies for which clinical symptoms were observed. These results should therefore be validated through follow-up evaluations of molecular vs. cultivation detection techniques involving a larger sample size of symptomatic colonies.

To conclude, analyzing adult bee samples comparing PCR assay and culture, CFU counts proved to be superior. *P. larvae* was detected in 100 % of the bee samples from diseased colonies using cultivation whereas the bacteria was only detected in 8 out of 11 colonies using PCR assay, presumably depending on polymerase inhibitors in the bees (Boncristiani et al. 2011) and/or failures the DNA extraction protocols (Wilson 1997). Assaying bee samples using PCR was the least sensitive method for detecting symptoms of AFB when looking at the total number of bee colonies assayed (Figure 1a). However, using the PCR method on debris samples revealed not only the highest bacterial levels but also the highest proportion of infected bee colonies (40 out of 58 investigated bee colonies). If the objective is to detect sub-clinical levels of *P. larvae* irrespective of disease symptoms, hive debris and PCR is clearly the material and method preferred. However, in order to apply and improve any PCR assay for the detection and quantification of *P. larvae* spores in various bee related materials, such as adult bees, wax, debris, and pollen, DNA extraction and purification techniques need to be compared and validated.

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Valeur du pronostic établi à partir d'abeilles et de débris de la ruche dans la détection de la loque américaine dans les colonies d'abeilles

***Paenibacillus larvae* / AFB / culture / PCR / surveillance sanitaire / régression logistique**

Die prognostische Aussagekraft bei der Verwendung von Bienen und Gemüll für den Nachweis der Amerikanischen Faulbrut in Bienenvölkern

Amerikanische Faulbrut / *Paenibacillus larvae* / Kultur / PCR / logistische Regression

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