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Detection and quantification of *Paenibacillus larvae* spores in samples of bees, honey and hive debris as a tool for American foulbrood risk assessment

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Abstract

American foulbrood (AFB) is a severe bacterial brood disease of honey bees (*Apis mellifera* L.) caused by the spore-forming bacterium *Paenibacillus larvae*. Low-levels of *P. larvae* in honey bee colonies are very widespread and can be considered endemic, whereas AFB occurs in clinical form only when a certain level of spores is reached in the colony. We studied for the first time the relationship between the level of the wintry contamination by *P. larvae* in materials taken from the hive and the onset of the disease in the following spring. In ten apiaries, from 125 colonies without signs of AFB, samples of adult bees, honey from the brood chamber and hive debris were collected in winter and cultured to detect *P. larvae* spore levels. The colonies were then checked in spring, until the end of May, for AFB symptoms. The performance of tests based on bees, honey and hive debris was evaluated, with respect to the results of clinical examination, by determination and comparison of sensitivity (Se), specificity (Sp) and positive predictive value (PPV) at different cut-off thresholds. Using the presence or absence of spores as a cut-off threshold, the examination of debris and bees showed a good Se (100%), while the Se of honey was limited (81.25%). At the same cut-off threshold, debris showed a greater Sp than that of bees (57.80% vs 7.34%). At increased cut-off thresholds, the Se was always higher for bee examination than for debris examination, whereas Sp and PPV were always higher for debris than for bees. The results showed that the levels of *P. larvae* spores in adult bees and, above all, in hive debris can be a useful tool for AFB risk assessment. The identification and proper management of the colonies with an increased risk of developing the disease is crucial for reducing the impact of AFB.

Key words: *Paenibacillus larvae*, American foulbrood, honey bees, honey, hive debris, ROC curve analysis.

Introduction

American foulbrood (AFB), a disease affecting honey bees and caused by the spore-forming bacterium *Paenibacillus larvae* (Genersch *et al*., 2006), is one of the most serious concerns in honey bee pathology and causes considerable economic losses to beekeepers worldwide (Ellis and Munn, 2005).

Molecular characterisation of *P. larvae* based on rep-PCR with enterobacterial repetitive intergenic consensus (ERIC) primers allows the identification of four different genotypes designated ERIC I-IV. These four genotypes have different phenotypic characteristics and differ in virulence (Genersch *et al*., 2006). Moreover, spores of strains belonging to these four genotypes show different germination abilities on culture media and different responses to heat treatment (Forsgren *et al.*, 2008).

American foulbrood affects only the larval stages of honey bees and, if left untreated once clinical symptoms have emerged, often causes colony death (Hansen and Brødsgaard, 1999).

Clinically, the disease is characterized by darkened brood combs with a mottled appearance, a sour smell, and greasy, sunken and perforated cell caps, but the most typical symptom is the transformation of the dead larvae into a ropy mass that forms a characteristic viscous thread if a matchstick is inserted into the cell and then pulled out.

If not removed, the dead larvae dries into a blackish scale tightly adhering to the walls of the cells (Hansen and Brødsgaard, 1999).

Some colonies can contain relatively large numbers of spores over several seasons without showing clinical symptoms of AFB (Hansen and Rasmussen, 1986; Hornitzky and Clark, 1991; Steinkraus and Morse, 1992; Fries *et al*., 2006).

The diagnosis of AFB is based on the recognition of clinical signs and on the identification of the pathogenic agent by means of laboratory techniques (Anonymous, 2016). However, the clinical examination has some clear limitations. First of all it can be laborious and time consuming, particularly for large beekeeping operations or for territorial monitoring, moreover, in the initial stages, by visual inspection, the disease may remain undetected (Lindström and Fries, 2005).

In any case clinical examination, as also occurs for other brood diseases (Gaggìa *et al*., 2015), does not enable the detection of colonies with asymptomatic infections.

Subclinical infections may lead to recurrences of the clinical forms in apiaries and contribute to the horizontal transmission of the infection from one hive to another (Lindström and Fries, 2005). Hence, identifying colonies with high spore loads allows the implementation of preventive measures to control the onset and spread of the disease (von der Ohe, 1997).

Today, standard methods based on microbiological and biomolecular techniques are available for the detection and quantification of the spore load in adult bees and hive materials such as honey, wax and wax debris (de Graaf *et al*., 2013; Anonymous, 2016).

The examination of honey is the most frequently used method (Hansen, 1984; Hansen and Rasmussen, 1986; Hornitzky and Clark, 1991; Steinkraus and Morse, 1992; Alippi, 1995; von der Ohe, 1997; de Graaf *et al.*, 2001), although the honey does not always reflect the infection status of sampled colonies but rather the presence or absence of the infection during the period in which the nectar was collected (Nordström *et al.*, 2002). Hence, by culturing samples of honey, it is possible to obtain false negative results, with no growth of *P. larvae*, from clinically diseased colonies (Nordström *et al.*, 2002; Gillard *et al.*, 2008).

Samples of adult bees better reflect the infection status of the colony at the time of sampling, and false negative results from colonies with clinical symptoms of AFB are highly improbable (Lindström and Fries, 2005; Lindström, 2008).

The level of *P. larvae* spores in adult bees and honey stored near sealed brood can provide information about the presence of disease symptoms in honey bee colonies. This relationship has been investigated by Goodwin *et al.* (1996), Fernandez *et al.* (2010) and Gende *et al.* (2011) for the bees and by Ritter (2003) for the honey. These authors found correlations between certain spore values in adult bees or in honey stored in the brood chamber and the presence of AFB, and they related levels of *P. larvae* spores to observations of disease symptoms.

Methods for the detection of *P. larvae* spores in wax debris collected at the bottom of the hive have been developed in the Czech Republic (Titera and Haklova, 2003; Bzdil, 2007; Ryba *et al.*, 2009). These methods have shown a good ability to identify colonies infected by *P. larvae*.

Forsgren and Laugen (2014) showed that the culture method from bee samples is superior to the culture method from debris in identifying clinically diseased colonies. All bee samples collected from diseased colonies contained detectable *P. larvae* spores, whereas in one colony with disease symptoms, the debris culture gave a negative result. However, if the objective is to detect colonies infected with *P. larvae*, irrespective of disease symptoms, the bee and debris culture-based techniques yielded comparable results.

The aim of this study was to compare the abilities of culture-based techniques using adult bees, honey and hive debris to predict the onset of AFB.

We assessed the performance of culture-based techniques using adult bees, honey and hive debris sampled in winter from individual hives with respect to the results of clinical examinations carried out in the spring of the same colonies.

Materials and methods

Apiaries and sampling procedures

The study was carried out in ten apiaries located in the Emilia Romagna Region, in Northern Italy, with a total of 165 hives (table 1).

In the foraging season before the experiment, the apiaries were characterized by different situations regarding the presence of AFB. In seven out of ten apiaries, the disease was found via clinical examination, and the prevalence of diseased colonies ranged from 10% to 43%. The clinical diagnosis was based on the presence of ropy larval remains or scales in cells with concave, dark and, sometimes, perforated capping. Larval remains or scales in uncapped cells were also checked.

The diagnosis was confirmed after the laboratory isolation of *P. larvae* from larval remains or scales.

After the diagnosis, the diseased colonies were killed and burned together with the infected materials.

In the remaining three apiaries, clinical symptoms of AFB were not observed.

At the beginning of the trial, no diseased colonies were present in all apiaries, and during the experiment, preventive measures were taken to minimise the transmission of *P. larvae* spores from one hive to another via beekeeping activities. The inspection of the hives was performed with disposable gloves, and the hive tool was scorched with a gas burner before visiting a new hive. Exchanges of combs and bees between hives were avoided and honey for feeding bees was not employed.

For the trial, 125 out of 165 hives were used (table 1). Samples of debris, bees and honey were collected from each hive according to the procedures and times described below.

Table 1. Geographic coordinates, number of total, sampled and diseased colonies for each apiary.

Apiaries	Geographic coordinates	Number of colonies	Number of examined colonies	Number of diseased colonies	Detection of clinical symptoms		
					March	April	May
1	44°16'42"N 11°16'14"E	10	7	1	-	1	-
2	44°31'26"N 11°20'59"E	15	12	2	-	2	-
3	44°35'37"N 10°51'24"E	14	12	2	-	1	1
4	44°40'24"N 10°35'10"E	10	9	0	-	-	-
5	44°30'41"N 10’43'55"E	16	14	3	-	2	1
6	44°47'32"N 11°14'00"E	13	11	0	-	-	-
7	44°38'08"N 10°05'43"E	23	20	8	-	7	1
8	44°37'43"N 11°12'17"E	20	10	0	-	-	-
9	44°37'08"N 11°15'28"E	24	19	0	-	-	-
10	44°23'41"N 11°15'15"E	20	11	0	-	-	-
	Total	165	125	16	-	13	3

To collect debris, plastic sheets were placed at the bottom of the hive at the beginning of December, and after approximately 40 days, the sheets were picked up, folded and individually packed in closed bags to be delivered to the laboratory, where they were frozen at –21 °C until analysis.

Bees and honey were collected at the half of January.

Each bee sample consisted of >50 living bees taken from the central combs of the brood chamber, placed in a plastic jar and frozen at –21 °C as soon as possible.

Together with the bee samples, approximately 50 g of honey per hive was collected.

To reduce the effect of eventual uneven spore distribution, sealed honey was taken by scooping with the edge of a plastic jar along the surface of three combs for each colony.

The honey was stored at room temperature until analysis.

Analytical procedures

B e e s

The assays were performed as reported by Goodwin *et al.* (1996), with some modifications.

Fifty adult bees were crushed in a stomacher bag with 100 mL sterile water at the maximum speed for 6 min. Then, the homogenate was filtered through sterile gauze and transferred using a glass funnel to a 50-mL test tube. After shaking with a vortexer, the suspension was heated in a water bath at 85 °C for 15 min to inactivate the thermosensitive contaminants. The sample was plated onto two plates (100 µL/plate) of MYPGP (Mueller-Hinton broth, yeast extract, potassium phosphate, glucose and pyruvate) agar (Dingmann and Stahly, 1983) supplemented with nalidixic acid (3 µg/mL) (Hornitzky and Clark, 1991) and pipemidic acid (10 µg/mL) (Alippi, 1995). These two antibiotics allow the growth of *P. larvae* but inhibit the growth of other spore-forming *Paenibacillus* and *Bacillus* species.

H o n e y

The honey samples were preheated to 50 °C to facilitate the separation of the wax and a more homogeneous dispersion of spores after shaking. Five grams of honey was diluted with 5 mL of sterile distilled water. After shaking with a vortexer, the water suspension was heated in a water bath at 85 °C for 15 min. The sample was plated onto five plates (100 µL/plate) of MYPGP agar.

D e b r i s

The assays were performed as described by Bassi and Galletti (2016).

Briefly, 1 g of debris was placed in a 15-mL test tube with a sealing cap containing 9 mL of sterile distilled water. After vigorous shaking by hand for 30 s, the suspension was heated in a water bath at 85 °C for 15 min. Immediately after the heat treatment, the suspension was poured into a stomacher bag with a lateral filter, and the filtered liquid was transferred with a disposable pipette into another test tube. The sample was plated onto five plates (100 µL/plate) of MYPGP agar.

The culture plates were always incubated at 37 °C in an atmosphere with 10% CO_2 and examined after three and eight days.

For each sample from two to five colonies with a *P. larvae*-like morphology were tested for catalase reaction and the catalase-negative colonies were subjected to Gram staining for confirmation. Rarely, and only in doubtful cases, the isolates were identified as *P. larvae* by PCR performed as described by Bassi *et al*. (2015).

When counting the colonies was not possible due to rapid growth of spore-forming bacteria or due to the presence of very large numbers of *P. larvae* colonies, ten-fold dilutions from the initial homogenate were prepared and then cultured with the procedures previously described.

After the enumeration of *P. larvae* colonies, the number of viable spores was calculated and expressed as colony forming units (CFU) per bee or per gram (honey and hive debris).

Clinical inspections

From the beginning of March to the end of May, the colonies were checked at three-week intervals by visual inspection for the presence of clinical symptoms of AFB.

The clinical examination was carried out with a great care on all brood combs. The suspected cells were inspected in order to detect larval remains at ropy stage or scales. For the confirmation of the visual AFB diagnosis, larval remains with glue-like consistency and scales were cultured for the isolation of *P. larvae*. Also dead larvae, in capped or uncapped cells, with atypical lesions (e.g. watery consistence) were subjected to laboratory investigations.

The results of the clinical examination were recorded simply as the presence or absence of disease symptoms. The diseased colonies were destroyed after the diagnosis of the disease.

Data analysis

We calculated sensitivity (Se), understood as the ability to detect diseased colonies, and specificity (Sp), understood as the ability to detect not diseased colonies, for each material at different contamination thresholds by using the results of clinical inspections as references. To compare the performance of the three diagnostic tests, we used receiver operating characteristic (ROC) curves and positive predictive value (PPV) calculated at specific cut-off thresholds.

The ROC curves provide a synthetic description of the ability of the test to distinguish between diseased and non-diseased colonies (accuracy). The extension of the area under the ROC curve (AUC) is an indicator of the accuracy of the diagnostic test: the greater the AUC, the greater is the discriminant power of the test. The differences between AUCs can be used to compare the accuracy of different diagnostic tests. We used DeLong's test (DeLong *et al.*, 1988) to compare the AUCs.

The PPV of a diagnostic test indicates the probability that an individual with a positive test result is really infected. In particular, in our case, it indicates the probability that a colony with a positive result in the culture test carried out in winter will be diseased in the spring and is expressed as the ratio between the number of true

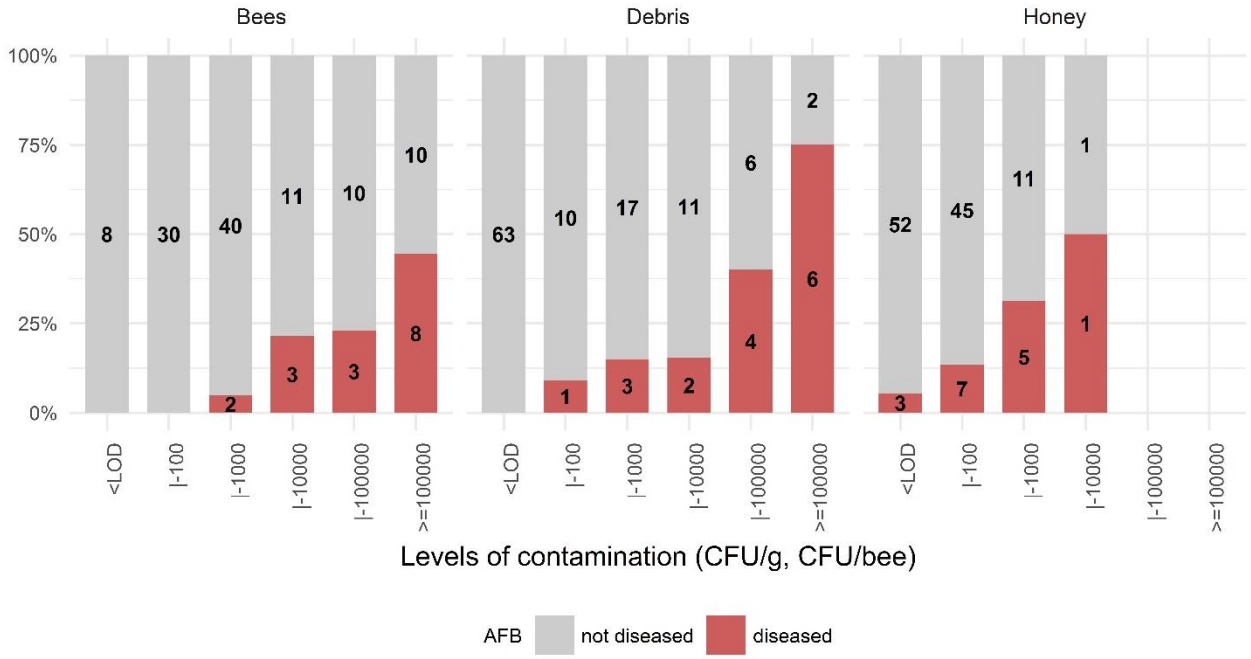

Figure 1. Percentage distribution of samples categorised by clinical outcome, grouped by different levels of contamination (CFU/g, CFU/bee). Number of samples is shown inside the bar.

positives and the total number of positives, considering as true positives the results matching the development of the disease in the spring.

All the analyses were performed with R 3.2.2 (Robin *et al.*, 2011; R Core Team, 2015).

Results

The limit of detection (LOD) of the employed culture-based methods was 20 CFU/g for debris samples, 10 CFU/bee for bee samples and 4 CFU/g for honey samples.

P. larvae spores were present in 117 samples of bees, 70 samples of honey and 62 samples of debris, out of 125 samples of each type.

In the spring, disease symptoms were observed in 16 out of 125 colonies (12.8%) belonging to five apiaries. The diseased colonies were detected in April (13) and May (3) (table 1).

In all diseased colonies by the dead larvae only strains of *P. larvae* genotype ERIC I were isolated.

Also in infected but asymptomatic colonies only strains of *P. larvae* genotype ERIC I by samples of bees, honey and debris were isolated.

The analytical results of bees, honey and hive debris, grouped in six classes of contamination (one negative class [< LOD] and five positive classes) and categorised by clinical outcome, are shown in figure 1. The proportion of diseased colonies varies depending on the level of contamination. In particular, all the colonies with debris contamination <LOD were negative for AFB, while some positive colonies were found at each level of contamination; the colonies with bee contamination <LOD or with low contamination (<100 CFU/bee) showed no symptoms of disease; finally, at each level of honey contamination, some AFB-positive colonies were found, even in cases with negative analytical results.

In figure 2, the ROC curves show the Se and Sp values of each material at various cut-off thresholds. The AUCs were calculated with 95% confidence intervals; AUC can be seen as the probability that a diseased colony has a greater test value than a non-diseased colony.

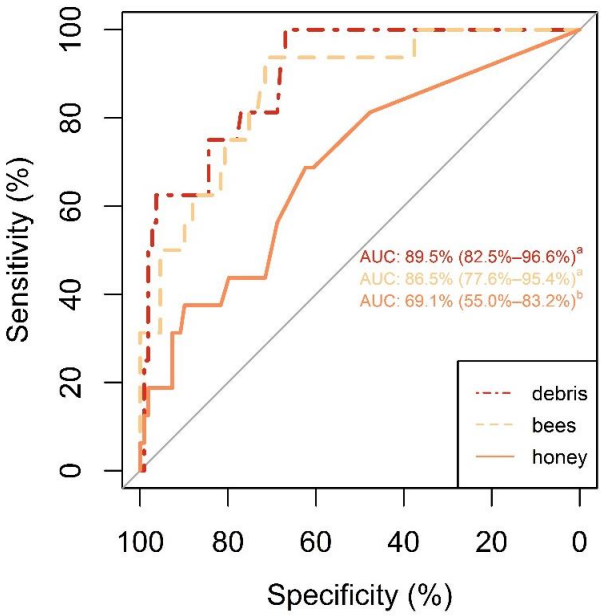

Figure 2. ROC curves with AUC values (95% confidence intervals are shown in parentheses). The different superscript letters indicate statistically significant differences ($P < 0.05$).

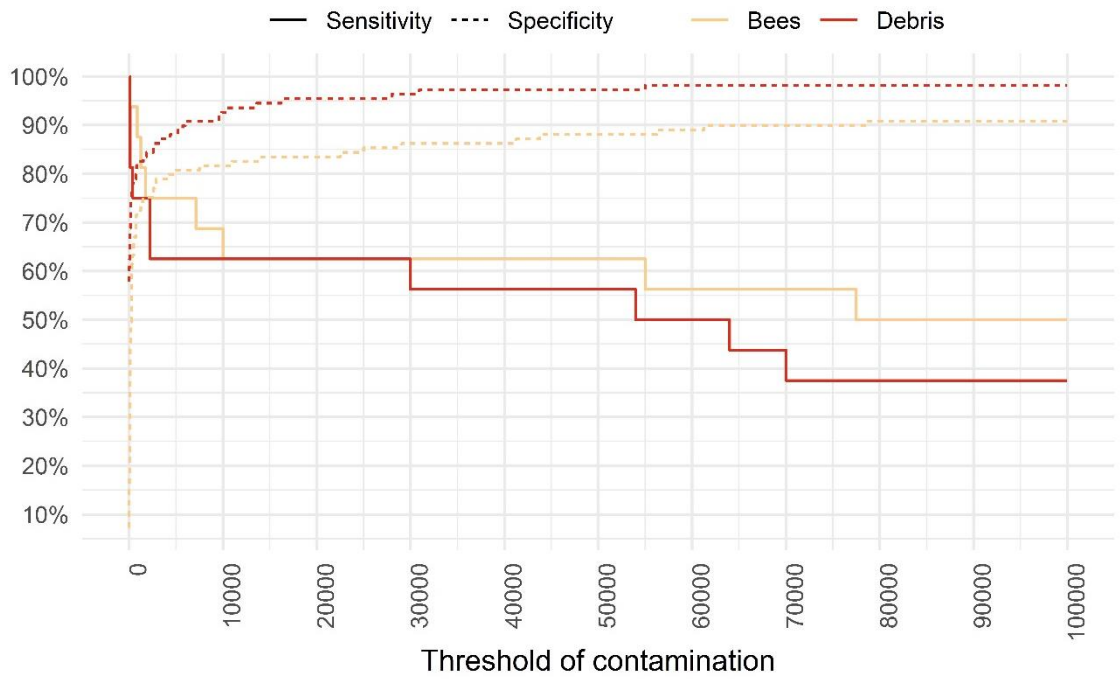

Figure 3. Sensitivity-specificity plot for debris and bees at different cut-off thresholds.

The AUCs of debris and bees had similar values (89.5% and 86.5%, respectively) and did not show significant differences (DeLong's test, $p = 0.45$). Conversely, the AUC value of honey (69.1%) was significantly smaller than those of the other two tested materials (DeLong's test, $p < 0.01$).

Because a diagnostic test is considered appropriate if the AUC is ≥ 70% (Swets, 1988), we decided to exclude honey from the subsequent data analysis.

In figure 3 Se and Sp percentages of bees and debris against the possible cut-off thresholds are shown.

As a consequence of the high proportion of positive samples (117/125) the examination of adult bees is a more sensitive method, but the Sp is low because it produces a higher proportion of false-positive results. Compared with debris, bees had a higher Se and a lower Sp at all cut-off thresholds, conversely the debris examination is less sensitive but more specific than the bees examination because it produces a lower proportion of false-positive results.

In table 2, values of Se and Sp obtained at determined cut-off thresholds are shown. With the LOD as the cut-off threshold, the Se is 100% for both debris and bees, while the Sp is 58% for debris and 7% for bees.

The PPV is another parameter considered to evaluate and compare the performance of bees and debris examination.

The PPVs related to the disease prevalence detected in our study (12.8%) are shown in table 2. Debris showed better performance than bees in terms of PPV, due to the higher Sp of this material. When the cut-off threshold increases, the PPV also increases. In particular, this effect is evident in the debris, for which the PPV is 75% at a cut-off threshold of 50,000 CFU /g (table 2).

Discussion and conclusion

Some important factors are involved in determining the outcome of *P. larvae* infection in honey bee colonies.

The strength of the colony, proportion of adult bees to brood, differences in nectar flow and pollen collection among the colonies in the apiary are factors than can affect the development of AFB (Hansen and Brødsgaard, 1999). However the colony resistance, mainly due to the level of hygienic behaviour (Spivak and Reuter, 2001), and the different virulence of *P. larvae* strains or genotypes (Genersch *et al.*, 2005) are the

Table 2. Sensitivity (Se), specificity (Sp) and positive predictive value (PPV) corresponding to the selected cut-off thresholds for debris and bees.

Cut-off threshold	Debris			Bees		
CFU/g – CFU/bee	Se (%)	Sp (%)	PPV (%)	Se (%)	Sp (%)	PPV (%)
LOD	100	58	26	100	7	14
100	94	67	29	100	35	18
1000	75	83	39	87	72	31
10000	62	93	56	69	82	35
50000	56	97	75	62	88	43

factors with the greater impact on the outcome of *P. larvae* infection.

So the onset of overt AFB not only depends on the *P. larvae* spore levels, but the presence of a high number of spores is a necessary condition (even if sometimes not sufficient) and a clinical syndrome occurs only when a certain level of spores is reached in honey bee colonies (Anonymous, 2012).

The present work aimed to study the relationship between the number of *P. larvae* spores detected in bees, honey and debris collected in winter and the onset of AFB in the same colonies during the following season.

We considered only the cases of AFB that occurred from the beginning of the season to the end of May, as these cases were more likely related to contamination by *P. larvae* in bee colonies during winter.

The results obtained show that disease symptoms are related to *P. larvae* spore levels in the hive; the higher the number of spores detected in winter, the greater is the probability that the colony develops the disease in spring (figure 1).

It is known that the microbiological examination of honey samples is of limited diagnostic value as samples from symptomatic colonies can give false negative results with no growth of *P. larvae* (Hornitzky and Clark, 1991, Nordström *et al.*, 2002, Gillard *et al.*, 2008).

In our case three colonies with the honey negative for *P. larvae* in winter developed AFB in the spring whereas no colony with bees and debris negative for *P. larvae* developed the disease in the same period. Therefore the honey showed a limited Se, and this limits its use also for predictive purposes.

According to the classification proposed by Sweets (1988), the AUC values demonstrate a good test accuracy for both bees and debris, in contrast, the honey examination shows a moderate accuracy (figure 2).

The comparison between debris and bees shows some different features with regard to the accuracy of diagnostic methods. These differences are expressed by different values of Se and Sp.

At any cut-off threshold of contamination the bee examination produces a lower proportion of false-negative results and is therefore more sensitive while the debris examination produces a lower proportion of false-positive results and is more specific.

In theory, the optimal cut-off can be considered the one that maximises the sum Se + Sp. In our case, this value corresponds to 76-80 CFU/g for debris (Se 100%, Sp 66%) and 764-875 CFU/bee for bees (Se 94%, Sp 71%).

In practice, depending on the purpose or the epidemiological situation present in the apiaries or in the territory in which the test will be used, appropriate cut-off thresholds can be chosen to favour the Se, thus minimising the number of false negatives, or to favour the Sp, thus reducing the number of false positives.

Regarding the accuracy of the methods the comparison of AUCs values shows no statistically significant differences between debris and bees, but the debris examination seems to be more useful for predicting the development of AFB because of its higher Sp and, consequently, higher PPV.

The probability that to a positive test result corresponds the disease onset is greater for debris examination than for bees examination.

Moreover the debris sampling is not destructive, is easy to perform, requires little time, is a non-invasive sampling and can be performed without opening the hives even in the winter.

These preliminary results are promising and should be confirmed on a larger sample size of colonies, including also colonies with *P. larvae* genotype ERIC II infections and colonies with mixed infections (ERIC I + ERIC II) because in the colonies used in our work only infections by strains of *P. larvae* genotype ERIC I were demonstrated.

Based on these results, knowledge of *P. larvae* spore load in materials taken from the hives, particularly the debris, can be a useful tool for AFB risk assessment.

The identification of the colonies with an increased risk of developing the AFB is essential because it allows beekeepers to take appropriate measures to prevent the onset of the disease and the spread of the infection.

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