Short communication. Control of American foulbrood disease in Argentine commercial apiaries through the use of queens selected for hygienic behaviour

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Abstract

American foulbrood (AFB), caused by the bacterium *Paenibacillus larvae* is a serious disease of honey bees worldwide that inflicts considerable economic losses on beekeepers. The aim of this investigation was to determine the spread of AFB in a commercial apiary headed by queens selected for hygienic behaviour (HB), and to estimate the agreement between microbiological methods for the isolation of *P. larvae* from honey samples (HS) or bee samples (BS). All work was undertaken in a commercial apiary that showed visual signs of AFB infection. Hives positive for AFB were isolated from the apiary and the queens of the remaining hives replaced by others selected for HB. The prevalence of AFB was then recorded by monitoring visual signs of the disease, and via the isolation of *P. larvae* spores from HS and BS. The hives showed no clinical signs of AFB although HS testing showed 50% to harbour *P. larvae* spores after one year. This percentage decreased to 26% by the end of the study. The spore loads of the HS and BS were low (between 0.33 and 5 CFU/plate for HS and 0.33 and 12 CFU/plate for BS). No agreement was seen between the two microbiological isolation methods as assessed by the Kappa coefficient ($P > 0.05$). Sixty percent false negatives were recorded for the HS method. The BS method was more sensitive and is the best option for the early diagnosis of AFB; this, plus the use of HB lines may be sufficient for the control of this disease.

Additional key words: *Apis mellifera*, honey bees, microbiological methods, *Paenibacillus larvae*, prevalence.

Resumen

Comunicación corta. Prevalencia de la loque americana en un apiario comercial encabezado por reinas seleccionadas por comportamiento higiénico

La loque americana (AFB) es una enfermedad causada por *Paenibacillus larvae* que ha provocado importantes pérdidas económicas a la apicultura. El objetivo del trabajo fue evaluar la difusión de AFB en un apiario comercial encabezado por reinas seleccionadas por comportamiento higiénico (HB) y estimar la concordancia entre dos métodos microbiológicos de aislamiento de esporas de *P. larvae* a partir de muestras de miel (HS) y abejas (BS). El trabajo se realizó en un apiario comercial que presentó signos clínicos de AFB; las colmenas que tuvieron diagnóstico positivo fueron retiradas del apiario y a las restantes se les recambió las reinas utilizando líneas seleccionadas por HB. Se monitoreó la prevalencia de AFB mediante la presencia de signos clínicos y el aislamiento de esporas de *P. larvae* a partir de HS y BS. Las colmenas no presentaron signos clínicos de AFB, sin embargo, el 50% de las mismas fueron positivas al aislamiento de *P. larvae* luego de un año, este porcentaje disminuyó al 26% al finalizar el ensayo. La carga de esporos presentes en las muestras HS y BS de la colmenas fue baja, registrándose entre 0,33 y 5 CFU/placa para HS y 0,33 y 12 CFU/placa para BS. No hay concordancia entre los métodos de aislamiento ($P > 0,05$). Los resultados de HS arrojaron un 60% de falsos negativos. Para un diagnóstico temprano de AFB, el método de aislamiento a partir de abejas es más sensible que el de miel. Nuestros resultados muestran que, con la detección temprana de la enfermedad y la utilización de genética HB sería suficiente para controlar AFB.

Palabras claves adicionales: aislamiento microbiológico, *Apis mellifera, Paenibacillus larvae,* prevalencia.

American foulbrood (AFB), caused by the bacterium *Paenibacillus larvae,* is a serious disease of honey bees worldwide that inflicts considerable economic losses on beekeepers. In Argentina it was first recorded in 1989 (Alippi and Núñez, 1991); since then a prevalence of 30% has been recorded in some areas and losses for beekeepers have been important (Del Hoyo *et al*., 1993). During the 1990s, AFB control programs were developed to reduce the prevalence of the disease. These consist of shaking infected beehives (Del Hoyo *et al*., 2001), the use of colonies selected for hygienic behaviour (HB) (Palacio *et al*., 2000), and the disinfection of contaminated beehive materials (Del Hoyo *et al*., 1998a,b). Beekeepers that have used these control methods have been successful in reducing the incidence of the disease to 1-3% (Figini *et al.,* 2005). However, in some areas of the country, the use of HB lines has not been incorporated into commercial beekeeping.

Once AFB becomes established in a region its eradication is very difficult (Matheson and Reid, 1992). Early diagnosis is therefore important for preventing its spread. According to Goodwin *et al*. (1993) three possible states of infection exist: 1) contamination: *P. larvae* is present in the hive but causes no ill effects, 2) subclinical infection: *P. larvae* adversely affects at least the larvae, although no disease is apparent to observers, and 3) clinical infection: *P. larvae* adversely affects the larvae and visible signs of AFB are apparent. Methods exist for the identification of hives with subclinical infection that require the inspection of either honey samples (HS) (Hanssen, 1984; Hornitzky and Clark, 1991; Hornitzky and Nicholls, 1993; Alippi, 1995; Nordstrom and Fries, 1995) or bee samples (BS) (Hornitzky and Karlovskis, 1989), although it is unknown which is most sensitive (Hansen and Brodsgaard, 1999). Del Hoyo *et al.* (2001) report that in hives infected with AFB, BS samples cultured on Agar J plates return greater numbers of *P. larvae* colony forming units (CFU) than HS samples. However, the differences were not significant when the *P. larvae* spore load was reduced by shaking and the hives showed no clinical signs of disease.

The examination of honey for viable spores may, however, be of value in tracing disease outbreaks (Hornitzky and Clark, 1991). Some companies that market queens and package bees improved for hygienic behaviour (HB) in Argentina, periodically check for AFB and routinely analyse honey samples as part of their efforts to prevent the disease.

However, it is difficult to predict the relationship between the number of spores present in honey and the appearance of the disease (Hansen and Brodsgaard, 1999).

Several authors have reported HB lines to be tolerant to AFB (Spivak and Gilliam, 1993; Palacio *et al.*, 2000) but fail to note the *P. larvae* spore loads of the examined hives.

The aims of the present work were to: i) determine the spread of AFB in a commercial apiary using queens selected for HB, and ii) to estimate the agreement between the numbers of *P. larvae* spores determined by the HS and BS methods.

An apiary in the Buenos Aires province with 32 productive hives was selected; no antibiotic treatments had been administered in this apiary for 10 years. The colonies were established in brood chambers with 10 combs. Honey supers were added during the summer. The first health inspection was performed at the beginning of spring in 2002. On November 5th, two colonies with visual symptoms of AFB were detected. Honey samples were collected from these colonies and cultured in the laboratory to test for the presence of *P. larvae* spores; values of over 200 CFU/plate were recorded. These hives were withdrawn from the apiary and the remainder homogenized and requeened, introducing queens from the PROAPI Genetic Improvement Program – a breeding program initiated in 1992 to select colonies for HB (Palacio *et al.,* 2000).

On December 5th 2002 all hives were visually inspected for signs of AFB. Honey samples were taken from each in the spring and autumn of 2002-2004. In the spring of 2003 and autumn of 2004, further samples of 30 nurse bees were taken from the broodnest frames of each hive.

The method of Alippi *et al.* (2004), with modifications, was used to isolate viable *P larvae* spores from HS. Three plates were prepared with MYPGP agar containing nalidixic acid (6 µg mL^{-1}) and incubated at $37 \pm 1°C$ in a 10% CO_2 atmosphere.

The 30 bees from each hive were prepared according to Hornitzky and Karlovskis (1989). Three plates were prepared with MYPGP agar containing nalidixic acid (6 µg mL^{-1}) and incubated as above. These were examined

after 4, 7 and 14 days and the number of *P. larvae* CFU recorded. Colonies were identified by their distinct colony morphology, Gram stain and catalase reaction (Alippi, 1991). Results were expressed as mean CFU for the three plates.

The percentages of hives positive for *P. larvae* in the spring and autumn were compared using the McNemar's test and the agreement between the micro-biological methods examined by calculating the Kappa coefficient. All calculation were performed using SAS software (1995).

No clinical signs of AFB were observed in any of the requeened hives. In the autumn of 2003 the percentage of hives positive for *P. larvae* was significantly greater than that observed at the beginning (P = 0.03) (Table 1). No significant differences were detected between the spring and autumn of 2003 (P = 0.47). Afterwards the prevalence of the disease decreased but not significantly so (P = 0.07).

The peak of subclinical infection (40-50% of hives) occurred 4-12 months after the initial withdrawal of the hives showing clinical signs of AFB; it then decreased. Hornitzky (1998) artificially infected five of an apiary's 20 colonies and found that approximately 90% showed peak subclinical infection three months later. The prevalence of infection decreased after the removal of diseased colonies in week 19.

In the spring of 2002, *P. larvae* was isolated from HS from five hives. Two of these samples returned 30 CFU/plate; the other samples held fewer spores (X_{min} = 1 CFU; X_{max} = 7 CFU). The lowest spore conta-mination levels were observed in the last sampling period (0.33 and 5 CFU/plate). Each CFU corresponds to approximately three viable spores per gram of HS. Only between 6% (Digman and Stahly, 1983) and 7.6% (Nordstrom and Fries, 1995) of inoculated spores germinate on MPYGP. At the end of the experiment, the largest quantity of viable spores per hive was 15

Table 1. Percentage of hives positive and negative for *Paenibacillus larvae* according to honey samples collected over a period of 17 months

Period	Culture (−)	Culture (+)	P-value[1]
Spring 2002	83.3	16.6	—
Autumn 2003	60	40	0.03
Spring 2003	50	50	0.47
Autumn 2004	73.3	26.6	0.07

[1] P-value: Comparison of positive cultures (%) with the previous measurement. McNemar Test.

Table 2. Isolation of *Paenibacillus larvae* from honey and bee samples in the spring of 2003 and the autumn of 2004

	Honey	Bees	
		Negative	Positive
Spring 2003	Negative	6 (40%)	9 (60%)
	Positive	3 (20%)	12 (80%)
Autumn 2004	Negative	9 (40.9%)	13 (59.1%)
	Positive	3 (37.5%)	5 (62.5%)

per gram of honey (5 CFU/plate), representing appro-ximately 197.4-250 total spores per gram of honey. The spore load in the hives was therefore very low.

Table 2 summarises the isolation of *P. larvae* from the HS and BS. Only one hive remained negative for the bacterium in both HS and BS over the entire sampling period. In the spring of 2003, nine hives negative in terms of their HS samples were positive in terms of their BS samples. This means that a 60% (9/15) of false negatives occurred by HS method with respect to the BS method. In the autumn this percen-tage was similar (59.1%). Using the BS method, 33.3% false negatives were recorded (3/9) (with respect to the HS method) in the spring and 25% (3/12) in the autumn. No agreement between the HS and BS methods was seen in the spring (Kappa = 0.200; P > 0.05) or autumn (Kappa = 0.0244; P > 0.05) [Kappa values of 0.4 to 0.5 indicate a moderate level of agreement (Kabay, 1995)].

Given its lower number of associated false negative results, the BS method appears to be more sensitive than the HS method; it therefore provides a better tool for the early diagnosis of *P. larvae*, for the prevention of outbreaks, and for studies of AFB prevalence in apiaries. In the BS method the numbers of *P. larvae* CFU/plate obtained were X_{mi} = 0.33 and X_{max} = 12. Each CFU represents approximately 12.5 viable spores per gram of bees. The hive with the largest number of spores (X = 12 CFU/plate) therefore had 150 viable spores per gram of bees. Borracci *et al*. (2003) working *in vitro* with *P. larvae* strains from the Buenos Aires province, recorded a lethality of 93-95% after inocu-lations of 150 spores per larvae when the latter were < 48 h old. Although direct extrapolations cannot be made, it is probable that in the hives of the present experiment with approximately 150 viable spores per gram of bees, some larvae would be affected by the disease. However, since bees selected for HB were used it is likely that these larvae were removed (Rinderer

and Rothenbuhler, 1969; Spivak and Gilliam, 1993; Palacio *et al*., 2000); certainly, no visible signs of AFB were detected. According to Palacio (2005), hygienic colonies detect and remove sick young more quickly than non-hygienic ones. This may explain the low levels of contamination found: the bees eliminated the affected brood before the causal agent reached the sporulation phase. After inoculating AFB into hygienic colonies headed by queens instrumentally inseminated with the semen of drones from hygienic colonies, Spivak and Reuter (2001) found that some colonies recovered from the disease. In the present study, the queens were naturally mated and the apiary was not diseased; the spores in these colonies may not have been in sufficient numbers to initiate clinical infection. The results therefore suggest that the early detection of the disease, the withdrawal of affected hives, and the use of HB lines, might be sufficient to control AFB. It may therefore be possible to manage apiaries without antibiotics and to preserve honey quality.

Acknowledgements

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