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 esporas presentes en las muestras HS y BS de las 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.

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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 requenched, 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±1°C in a 10% CO₂ 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

Abbreviations used: AFB (American foulbrood), BS (bee samples), CFU (colony forming units), HB (hygienic behaviour), HS (honey samples), MYPGP (Müller-Hinton, yeast extract, acid potassium phosphate, glucose, sodium piruvate).
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 microbiological 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 unequeened 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 contamination 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 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 inoculations of 150 spores per larva 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 2008).

### 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   | 6 (40%) | 9 (60%) |     |
|               | 3 (20%) | 12 (80%) |     |
| Autumn 2004   | 9 (40.9%) | 13 (59.1%) |     |
|               | 3 (37.5%) | 5 (62.5%) |     |
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.

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**References**

ALIPPI A.M., 1991. A comparison of laboratory techniques for the detection of significant bacteria of the honey bee, *Apis mellifera*, in Argentina. J Apic Res 30, 75-80.

ALIPPI A.M., 1995. Detection of *Bacillus larvae* spores in Argentinean honeys by using a semi-selective medium. Microbiol 11, 343-350.

ALIPPI A.M., NÚÑEZ L., 1991. La loque Americana en Argentina. Vida Apícola 49, 20-24. [In Spanish].

ALIPPI A.M., REYNALDI F.J., LÓPEZ A.C., DE GIUSTI M.R., AGUILAR O.M., 2004. Molecular epidemiology of *Paenibacillus larvae* larvae and incidence of American foulbrood in Argentinean honeys from Buenos Aires province. J Apic Res 43, 135-143.

BIRRACCI S.E., CHACANA P.A., PALACIO M.A., TERZOLO H.R., 2003. Experimental production of American Foulbrood. XXXVIII Apimondia International Apicultural Congress. Ljubljana, Slovenia. August 24-29. p. 955.

DEL HOYO M.L., BASUALDO M., FIGINI E., CARRASCO C.L., 1993. Ocuencia de *Bacillus larvae* white en colonias de *Apis mellifera* L. Proc IX Reunión y Asamblea Anual de Asociación Argentina de Veterinarios de Laboratorios de Diagnóstico. Tandil, Argentina. pp. 34-35. [In Spanish].

DEL HOYO M., BASUALDO M., TORRES J., BEDASCARRASBURE E., 1998a. Use of DHT-equipment for disinfection of AFB-contaminated beehive materials in Argentina. Am Bee J 138, 738-740.

DEL HOYO M., BASUALDO M., BEDASCARRASBURE E., 1998b. A method to test the efficiency of a disinfecting process with hot paraffin for AFB control. Am Bee J 138, 741-742.

DEL HOYO M.L., BASUALDO M., LORENZO A., PALACIO M.A., RODRÍGUEZ E.M., BEDASCARRASBURE E.L., 2001. Effect of shaking honey bee colonies affected by American foulbrood on *Paenibacillus larvae* spore loads. J Apic Res 40, 65-69.

DIGMAN D.W., STAHLHY D.P., 1983. Medium promoting sporulation of *Bacillus larvae* and metabolism of medium components. Appl Environ Microbiol 46, 860-869.

FIGINI E., LORENZO A., BEDASCARRASBURE E.L., 2005. Manejo de la colmena para control de Loque Americana sin la utilización de antibióticos. Campo y Abejas VIII, 32-33. [In Spanish].

GOODWIN R.M., PERRY J.H., HAINES H.M., 1993. American Foulbrood disease Part II: subclinical infections. New Zeal Beekeeper 218, 7-9.

HANSEN H., 1984. The incidente of the foulbrood bacterium *Bacillus larvae* in honeys retailed in Denmark. Tidsskrift Planteavl 88, 329-336.

HANSEN H., BRODSGAARD C.L., 1999. American Foulbrood: a review of its biology, diagnosis and control. Bee World 80, 5-23.

HORNTZKY M.A.Z., 1998. The spread of *Paenibacillus larvae* subsp *larvae* infections in an apiary. J Apic Res 37, 261-265.

HORNTZKY M.A.Z., KARLOVSKIS S., 1989. A culture technique for the detection of *Bacillus larvae* in honey bees. J Apic Res 28, 118-120.

HORNTZKY M.A.Z., CLARK S., 1991. Culture of *Bacillus larvae* from bulk honey samples for the detection of American Foulbrood. J Apic Res 30, 13-16.

HORNTZKY M.A.Z., NICHOLS P.J., 1993. *J medium is superior to sheep blood agar and brain heart infusion agar for the isolation of Bacillus larvae* from honey samples. J Apic Res 32, 51-52.

KABAY M.J., 1995. Evaluation of the culture of honey to detect American foulbrood. Aust Vet J 72, 33-34.

MATHESON A., REID M., 1992. Strategies for the prevention and control of American Foulbrood. Am Bee J 132, 399-475.

NORDSTROM S., FRIES I., 1995. A comparison of media and cultural conditions for identification of *Bacillus larvae* in honey. J Apic Res 34, 97-103.

PALACIO M.A., 2005. Relación *Apis mellifera-Ascaphera apis*. Estudio del comportamiento higiénico. PhD Thesis. Universidad Nacional de Mar del Plata, Argentina. 216 pp. [In Spanish].

PALACIO M.A., FIGINI E., RUFFENEGO S., RODRÍGUEZ E.M., DEL HOYO M.L., BEDASCARRASBURE E.L., 2000. Changes in a population of *Apis mellifera* L selected
for hygienic behaviour and its relation to brood disease tolerance. Apidologie 31, 471-478.
RINDERER T.E., ROTHENBUHLER W.C., 1969. Resistance to American Foulbrood in honey bees. X. Comparative mortality of queen, drone and worker larvae. J Inv Pathol 13, 81-86.
SAS, 1995. SAS/STAT User’s guide. Version 6, 4th ed, SAS Institute, Cary, NC, USA.

SPIVAK M., GILLIAM M., 1993. Hygienic behavior of honey bees and its application for control of brood diseases and varroa. Part 1: Hygienic behavior and resistance to American foulbrood. Bee World 79, 124-134.
SPIVAK M., REUTER G.S., 2001. Resistance to American foulbrood disease by honey bee colonies Apis mellifera bred for hygienic behavior. Apidologie 32, 555-565.