

Characterization of *Bacillus larvae* white, the causative agent of american foulbrood of honey-bees. First record of its occurrence in Argentina

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SUMMARY

American foulbrood caused by *Bacillus larvae* white is recorded for the first time on brood combs of Argentinian hives. The identification of the causative agent was based on disease symptomatology, morphological characters, pathogenicity tests and physiological and biochemical reactions. Studies by scanning electron microscopy showed the occurrence of large flagellar bundles of *Bacillus larvae* strains growing in biphasic BL medium. An electron microscope survey of the surface configuration of bacterial spores was also made.

RESUMEN

Caracterización de *Bacillus larvae* white, agente causal de la loque americana de las abejas. Primer registro en la Argentina.

La loque americana de las larvas de las abejas (*Apis mellifera* L.) fue registrada sobre panales de cría por primera vez en la Argentina. La enfermedad es ocasionada por la bacteria *Bacillus larvae* white y su identificación se basó en la sintomatología clínica, en los resultados de las pruebas de patogenicidad y en las características morfológicas, fisiológicas y bioquímicas de las cepas bacterianas aisladas. Se efectuaron estudios de microscopía electrónica de barrido constatándose la presencia de grandes atados de flagelos bacterianos (Figura 1) en cultivos de *B. larvae* desarrollados en medio BL bifásico. También se determinó la configuración superficial de las esporas de *B. larvae* vistas al microscopio electrónico de barrido, las cuales presentaron una superficie lisa y se hallaron libres, sin restos de esporangio (Figura 2).

During the autumn of 1989 many hives with a patchy appearance of their brood combs were detected in colonies in the area of Tandil (Buenos Aires province). Sunken cappings with a greasy look and partially

open cells could also be seen. When the cappings were removed, dead larvae could be drawn out into a dark ropy material longer than 2 cm. Larval remains varied between light coffee to dark brown color with a glue like

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consistency. When the comb was held in the sunlight, dark brown scales firmly attached at the lower side of the cells were seen. Scale material showed a greenish fluorescence when exposed to longwave UV light. In some cases it was possible to see "tongues" of dead pupae (false tongue) protruding from the scales across the cells. Diseased combs had a characteristic sour odor.

The above mentioned symptomatology and preliminary microscopic observations lead us to consider this disease as American foulbrood (AFB) one of the most serious of the brood diseases. AFB is found almost everywhere with the exception of some African, Asian and Latin American countries (10). The only report for Argentina was the isolation of *Bacillus larvae* spores from two honey samples retained in Denmark (7).

The purpose of the present work was the isolation and characterization of the causative organism, *Bacillus larvae* and the examination of brood samples and bacterial smears by scanning electron microscopy (SEM).

This is the first scientific report of the disease in Argentinian hives.

MATERIALS AND METHODS

Isolation

Scales and ropy material containing *B. larvae* spores were suspended in 5 ml of sterile distilled water. After 15 min the supernatant was decanted, replaced with 5 ml of sterile distilled water and held at 80-85°C for 20 min in order to kill the non-spore-forming bacteria. Larval remains were aseptically extracted and aliquotes of each suspension were sown in Bailey and Lee's (BL) germination medium N° 5 (2) or J semi-solid medium (containing 0.1%

agar) (6). The tubes were incubated at 36-37°C for 48-72 h. The sub-surface vegetative growth were then transferred to the surface of J solid medium of brain heart infusion agar + thiamin (BHI) (12). Inoculated plates were incubated at 36-37°C for 2 days or until bacterial growth could be detected. Honey samples from diseased colonies were examined for *B. larvae* viable spores according to the procedure of Hansen & Rasmussen (8).

Morphological characters

Smears of ropy material mixed with distilled water were negatively stained with nigrosin (10% w/v) and observed under an oil immersion objective. Bacterial smears from scales of diseased larvae were fixed, stained and examined by the modified hanging drop method (11). Samples of young cultures grown on J agar (6) were air dried and stained by Hucker's modification of the Gram stain (4).

Scanning Electron Microscopy

Selected isolates were grown for 48 h on biphasic BL agar (14) and for 10 days on J-agar, suspended in sterile distilled water, transferred to 5 x 5 mm glass pieces, air dried, fixed in 5% glutaraldehyde at pH 7.4 in 0.2 M phosphate buffer for 12 h at 4°C, then washed three times (30 min) in 0.2 M phosphate buffer (pH 7.4). Then they were dehydrated in a 50, 75, 95 and 100% acetone series.

Scales and ropy material from brood combs were first washed in physiological saline and fixed and dehydrated as previously described.

All the specimens were coated with a 1 µm film of gold and examined under a Jeol JSM T100 scanning electron microscope.

Physiological and biochemical test

Giordani's modification of the Holist milk test (5) was performed on scales and diseased larvae.

The methods of Gordon et al. (6) and Sheath (13) were used to test for Voges-Proskauer reaction (VP), catalase reaction, hydrolysis of starch, liquefaction of gelatin, utilization of citrate, decomposition of caseine, ability to withstand serial transfer in nutrient broth, growth at 20°C and reduction of nitrate to nitrite.

Production of acid from carbohydrates was tested in J broth with the glucose replaced by 0.5% of the test substrate (6). The indicator used was 0.04% alcoholic bromocresol purple mixed with a drop of the culture after 14 days incubation (13). The carbohydrates assayed were: L (+) arabinose, D (+) glucose, D (-) mannitol, D (+) xylose and D (+) trehalose.

Inoculation test

Inoculation tests were performed on 0-24 h old larvae according to Bailey and Lee's method (2). Suspensions of spores of *B. larvae* (1×10^9 spores \times ml⁻¹) in a 25% v/v sterile solution of honey in water were used. One hundred cells containing larvae less than 24 h old were inoculated with 5 μ l of the spore-honey-water solution by means of a microsyringe. Each larva received a final concentration of about 10^6 spores. Fifty control larvae were inoculated with a sterile solution of honey in water (25% v/v), each larva received 5 μ l.

Larvae to be examined by SEM were removed at random before capping and their dissected guts fixed, dehydrated and coated as previously explained. Larvae not taken for SEM were left in the experimental colony until

sealed in their cells. Then, the comb was incubated at 36-37°C. After 30 days, all larvae were examined for clinical symptoms and spores of *B. larvae*.

RESULTS

Morphological characters

The bacteria isolated were Gram (+) and motile with peritrichous flagella. The cells were rod-shaped, measuring 0.5-0.6 μ m by 2.3-5.0 μ m. Spores were ellipsoidal, central to terminal, 0.6-0.7 μ m in diameter and 1.3-1.5 μ m in length, thick rimmed and refractile under phase-contrast.

Spores from scales and diseased larvae exhibited Brownian movement when tested by the hanging drop method.

Bacterial colonies growing on J-medium were whitish to grayish about 3-4 mm in diameter after 48 h, flat and with rough surfaces and irregular edges. Colonies on BHI and BL media were smaller than those on J-medium.

Bacteria isolated from honey samples showed similar cultural characteristics than those isolated from brood combs.

Scanning electron Microscopy

Vegetative cells and flagellar bundles of various sizes developed after 72 h of growth in biphasic BL agar (Figure 1).

Spores were ellipsoidal, highly refractile and appeared mainly free, with a smooth surface configuration (Figure 2).

Spores without traces of sporangia and smooth surfaces were observed on ropy material samples. No spores were found on scale preparations.

Physiological and biochemical test

Scales gave a positive reaction to Giordani's modification of the Holst milk test. In some cases, ropy material gave negative results.

All the strains were catalase and Voges-Proskauer negative and were unable to withstand serial transfer in nutrient broth or to grow at 20°C. They liquefied gelatin but did not hydrolyse starch.

Cascin was decomposed and nitrates were reduced to nitrites. Citrate was not used.

Acid was produced aerobically from glucose and trehalose. No acid was produced from arabinose and xylose and variable results were obtained from mannitol.

Inoculation tests

Bacillus larvae spores germinated in the mid-gut contents of larvae up to 2-days-old. SEM observations of larval mid-guts showed the presence of vegetative rods covering the epithelium in larvae of 5 to 10 days-old. No bacteria were observed in mid-guts of controls.

Thirty days after the inoculation, untouched cappings were removed and dead larvae giving a positive stretch-test were found inside the cells.

Forty seven days after the inoculation, scales with the same characteristics to those seen in cases of AFB were found attached at the lower side of affected cells.

Bacterial spores from ropy material and scales exhibited a Brownian movement as observed by the hanging drop technique in samples up to 30-days old.

Bacteria with the same characteristics of *B. larvae* were reisolated from artificially inoculated combs.

DISCUSSION

The symptoms of AFB observed in the field and obtained by experimental inoculations were similar to those described by others (1, 2, 5, 11, 12).

The morphological characters and physiological and biochemical properties of the pathogen were the same as those for *B. larvae* as previously reported (1, 2, 6, 7, 9, 13, 14).

SEM observations of flagellar bundles were similar to those described by Wilson & Combs (14). Spores showed smooth surface contours as seen in scanning electron photomicrographs, these surface configurations were in agreement with the description of Bulla et al. (3) and is a distinct character of *B. larvae*.

Results reported herein demonstrate that the isolated bacterium from brood combs and honey samples was *Bacillus larvae* White, the causal agent of AFB of honey bees. This is the first recorded case in Argentina, although it should be pointed out that Hansen (7) in his study of the incidence of AFB in foreign honeys retailed in Denmark, found 2 Argentinian samples (out of four samples) containing *B. larvae* spores.

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FIGURE 1: Scanning electron micrograph of vegetative cells of *Bacillus larvae* showing bundles of flagella formed in biphasic BI. agar BAR 1 μ m.

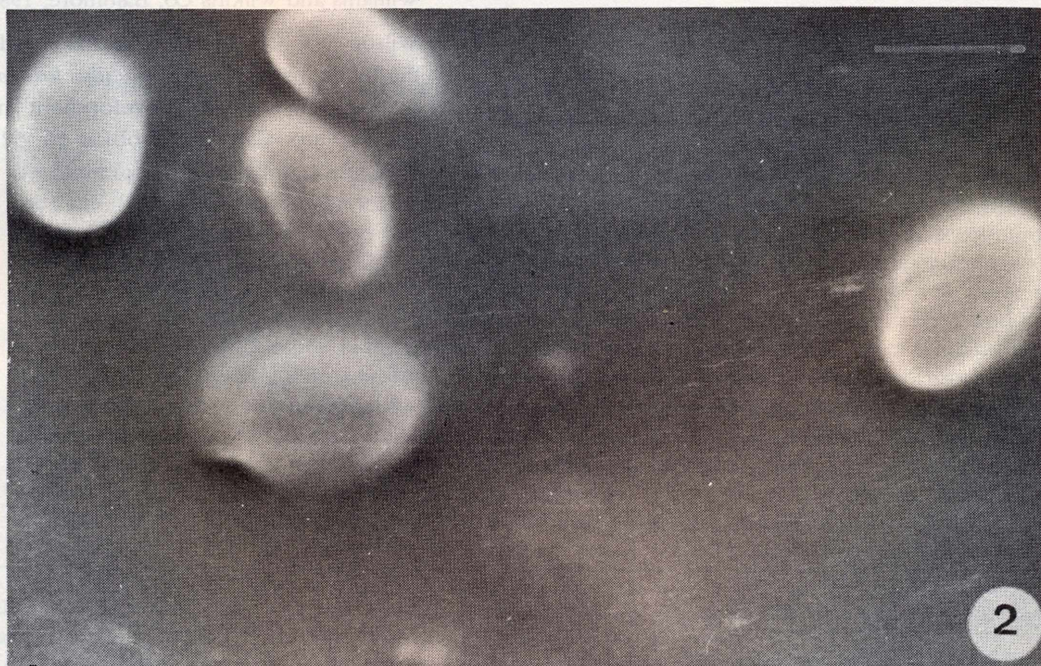


FIGURE 2: Scanning electron micrograph of free spores of *B. larvae* with smooth surfaces and without sporangia remnants. BAR: 1 μ m.

REFERENCES

1. Bailey L. Capítulo 4: Bacterias. p.: 28-43. In: Patología de las Abejas. Editorial Acribia, Zaragoza, 1984.
2. Bailey L, Lee DC. *Bacillus larvae*: its cultivation in vitro and its growth in vivo. J. Gen. Microbiol. 29: 711-717, 1962.
3. Bulla LA, St Julian G, Rhodes RA, Hesseltine CW. Scanning electron and phase-contrast microscopy of bacterial spores. Appl. Microbiol. 18: 490-495, 1969.
4. Doetsch RN. Chapter 3: Determinative methods of light microscopy pp. 21-33. In: Gerhardt P, Murray RGE, Costillow RN, Nester EW, Wood WA, Krieg NR, Phillips GB. Manual of methods for general bacteriology. American Society for Microbiology, New York, 1981.
5. Giordani G. Malattie della covata. Peste americana pp. 17-42. In: Giordani G, Vecchi MA. Nozione pratiche sulle malattie delle api. 2° ed. Edizione L'Apicoltore d'Italia, Roma, 1973.
6. Gordon RE, Hynes WC, Pang CH-N. The genus *Bacillus*. Agriculture Handbook N° 427. USDA, Washington, 1973.
7. Hansen H. The incidence of the foulbrood bacterium *Bacillus larvae* in honeys retailed in Denmark. Tidsskr. Planteavl 88: 329-336, 1984.
8. Hansen H, Rasmussen B. The investigation of honey from bee colonies for *Bacillus larvae*. Tidsskr. Planteavl 90: 81-86, 1986.
9. Lloyd JM. Simplified laboratory diagnosis of American Foulbrood disease. J Apic. Res. 25, 55-57, 1986.
10. Nixon M. Preliminary world maps of honey-bee diseases and parasites. Bee World 63, 23-42, 1982.
11. Shimanuki H. Chapter 3: Bacteria. pp. 43-61. In: Morse E (Ed.) Honey bee pests, predators and diseases. Cornell University Press, 1978.
12. Shimanuki H, Cantwell GE. Handbook for the diagnosis of Honey bee diseases, parasites and pests. Plant Protection Institute, Agricultural Research Service. Beltsville, Maryland, 1978.
13. Sneath PHA. Section 13: Endospore-forming Gram-positive rods and cocci. pp. 1104-1125. In: Krieg, NR Holt JG (Eds) Bergey's Manual of Systematic Bacteriology vol 2. The Williams and Wilkins Co, Baltimore, 1984.
14. Wilson WT, Combs LE. Flagellar bundles of the honey bee pathogen *Bacillus larvae*, their occurrence, size and development in vivo and in vitro. Can. J. Microbiol. 16: 521-526, 1970.