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Isolation of Enterobacteria and Spiroplasmas from *Apis mellifera*

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Abstract
The natural biota Enterobactericeae and Spiroplasmas was isolated from *Apis mellifera*, achieving the identification and elucidation of the genus Spiroplasma, known pathogen of *Apis mellifera* and short studied in Mexico, by using microbiologist methods, molecular techniques and atomic force microscopy. The objective of the present work was to isolate and identify Enterobacteria and Spiroplasmas from *Apis mellifera*. The isolated bacteria were *Klebsiella*, *Pseudomonas*, *Yersinia*, *Proteus* and Spiroplasmas, results suggest that *Apis mellifera* can be considered as a promissory source of secondary metabolites produced by bacteria with varying that could be used as alternative biotechnological protection crops. The identification and elucidation of the genus Spiroplasma allow deepen the still limited knowledge of this pathogen that affects one of the most important pollinators in the well-functioning of agroecosystem.

Keywords: Enterobacteraceae, Spiroplasma, *Apis mellifera*, pathogens, agroecosystems

1. Introduction
Apis mellifera is a natural pollinator considered directly or indirectly essential in food production, since approximately 75% of crops worldwide depend on pollination to produce seeds, fruits and vegetables, promoting either the yield amount or the quality of the crops [1, 2]. So the development of beekeeping offers benefit in terms of pollination, since it favors the maintenance of ecosystems, the conservation of species and generates considerable increases in the yields of agricultural crops, which are benefited by the visits of bees. However, this service is at risk due to the decrease of beekeeping due to the loss of subsidies, the poisoning by insecticides, the introduction of pests and the presence of pathogenic microorganisms for the bee, and using it as a vector disperser of diseases as caused by *Spiroplasma melliferum* [3, 4].
The studies referring to microorganisms associated with *Apis mellifera* have focused on the content of the gastrointestinal tract, basically identifying bacteria of the family *Enterobactericeae* and of the genus *Bacillus*, *Lactobacillus*, *Pseudomonas*, *Bifidobacterium*, *Corynebacterium*, *Streptococcus* and *Clostridium*, as well as fungi and yeasts [5, 6]. Around fifty species of Spiroplasmas have been reported in a wide range of hosts and as disease-causing agents in insects and plants, with insects being an important source of these bacteria, where some are entomopathogens such as *Spiroplasma melliferum* and *Spiroplasma apis* [7].
In addition to the diseases caused by the different pests that attack bees, causing losses in production, the biggest problem lies in the control methods used by the producers, which consist of the application of synthetic chemicals that leave residues and reduce the honey quality and viability of bees, for this reason the objective of this work was to isolate and identify Enterobacteria and Spiroplasmas from *Apis mellifera*.

2. Materials and Methods
2.1 Biological material sampling
The collection of *Apis mellifera* was in apiaries of the town of Santa María Coronango, Puebla-Mexico (19º 06' north latitude - 98º 15' west longitude, altitude 2200 meters, subhumid temperate climate with summer rains, temperature that oscillates between 14 ºC and 18 ºC and precipitation between 800 and 1000 mm) [8]. Sampling was randomized in the apiary consisting of 30 hives, taking 10% of the total boxes weekly for six months.

2.2. Isolation and bacterial identification from *Apis mellifera*

The specimens of *Apis mellifera* were taken to 4 ºC and then they were placed in 100 ml of nutritious broth, macerating with the help of a mortar, making independent aliquots and incubating at 30 ºC for 24 hours, followed by decimal dilutions, replanting 20 µl on nutritive agar, incubated at 30 ºC for 24 hours.

The identification was made with the API 20E ™ system (100/20 160 BIOMERIUX), a system that identifies bacteria of the family *Enterobactericeae* and Gram-negative bacilli. In addition, the oxidase and lactose tests were performed independently. From the reading of the results, a seven-figure numerical profile is obtained, and each numerical profile was introduced in the API® Identification Software, APIWEB ™.

Spiroplasmas isolation was carried out under the same scheme, changing the culture medium for the SP-4 broth, replacing 5 µl by gravity in SP-4 agar and allowing to incubate at 30 ºC for 7 days. Spiroplasma identification was performed with the PCR technique, performing DNA extraction with the ZR Bacterial DNA MiniPrep D6005 kit.

For the amplification of the 16S rRNA gene specific to the Spiroplasma genus, the primers F28 (5'GC AGA CGG TTT AGC AGG TTT GGG 3') and R5 (5'AGC ACC GAA CTT AGT CCG ACA C 3') were used to amplify a product of 271 base pairs (bp).

The reaction mixture to perform the PCR contains a final volume of 50 µl (10 µl Pyro Start ™ Fast PCR Master Mix 2X, 32 µl of water, 1.5 µl of the primer F28, 1.5 µl of the primer R5 and 5 µl of DNA to be analyzed). The amplification was performed in a Techne TC-412 thermal cycler under the following conditions: 96 ºC / 2 minutes, 30 cycles of 94 ºC / 60 seconds, 65 ºC / 50 seconds, 72 ºC / 90 seconds and a final extension at 72 ºC / 10 minutes. The PCR products were analyzed on 2% agarose gels, stained with ethidium bromide, including *Spiroplasma melliferum* control strain (ATCC 33219). The sequencing of the amplified product (271 bp) was performed in the Centro de Detección Biomolecular de la Benemérita Universidad Autónoma de Puebla.

The concentrate of the Spiroplasma samples were centrifuged at 1,200 rpm / 3 minutes and washed with distilled water three times, filtering with Millipore® EMD membrane with 0.8, 0.45 and 0.22 µm, using 4 µl of the latter filtrate, fixing it in silicon plates and proceed to observe the atomic force microscope, in order to elucidate the microstructure of Spiroplasmas.

3. Results and Discussion

The analyzed bees showed a microbiota conformed by *Klebsiella* 38%, *Pseudomonas* 32%, *Yersinia* 13% and *Proteus* 7%, these results agree with that reported by Gilliam and Taber (1991) where they report that the Enterobacteria are the most numerous microorganisms, present in the bees [9].

The microbiota reported in *Apis mellifera* is mainly housed in its digestive tract and is transmitted by the mechanisms of sociability of the species, and this microflora can play a role in the health and vitality of these organisms [10, 11].

The data obtained will allow the analysis of the traits of each of the bacterial isolates, for the benefit of the cultivation of bees, this being due to the fact that during the last ten years bee mortality cases have been reported by 30%, these reports suggest that the reasons for this disorder vary by region or country and can not be attributed to a single factor, so efforts to minimize losses must be diverse [12].

Before mentioned problem has been established that the greatest losses may be attributable to the introduction of pests and pathogens, so it is suggested the need to study and characterize the bacterial biota present in bees, as a strategy of good management and conservation of the species [13].

In the present work the genus Spiroplasma was also detected in 38% of the analyzed specimens of *Apis mellifera*, considering that these microorganisms are not part of the natural biota of the bee, but come from the surface of flowers and plants that they visit, suggesting that the prevalence of Spiroplasmas in bees is due to the transmission between hosts. It should be considered that these pathogens invade the digestive tract of bees, and have also expanded their habitat range including hemolymph, ovaries, fatty body and salivary glands [14, 15]. *Spiroplasma apis* and *Spiroplasma melliferum* are known as the causative agents of neurological diseases in bees, which is why the recent challenges related to the conservation and management of bees, as well as the problem due to the dramatic losses of colonies due to different causes such as the introduction of pests and pathogens [16, 17], being what motivates the search and study of possible pathogens, as is the case of Spiroplasmas [18, 19].

By sequencing the product amplified by PCR, a similarity of 95% was shown with the gene 16S rRNA of *Spiroplasma melliferum* (Gene bank number: KF706372.1), which is compared with Schwarz *et al*., (2014) who they report that 58% of the samples analyzed were carriers of *Spiroplasma melliferum* [15].

The present work allowed to elucidate the presence of Spiroplasmas using atomic force microscopy from the *Apis mellifera* isolates, with a structure of 12 µm in length and 0.75 µm in diameter (Figure 1), these data agree in terms of length and morphology that has been reported for Spiroplasmas, by means of transmission microscopy [20, 21]. In relation to the recent decreases in bees and the gap between the knowledge of these pathogens, due to the difficulty to be studied by conventional methods, is that the results presented here reveal important information in relation to these bacteria of interest in beekeeping due to its association with diseases in bees.

4. Conclusion

The isolation of the genus *Klebsiella*, *Pseudomonas*, *Yersinia* and *Proteus* is reported, as well as the detection of the genus *Spiroplasma*, the latter known as the pathogen of *Apis mellifera*, a report that had not been made in the Puebla-Mexico locality.

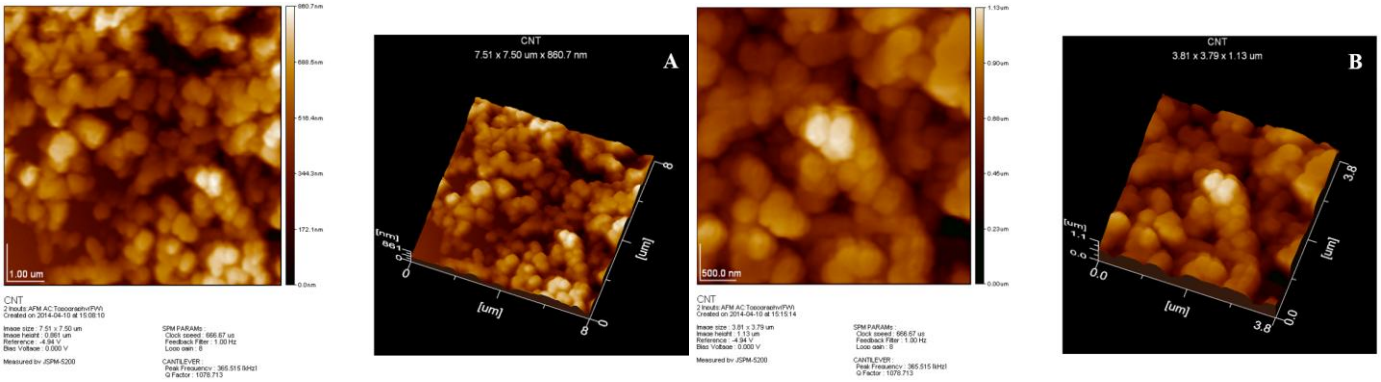

Fig 1: The individual structures with linear morphology and spherical aggregates that are phenotypic characteristics of the Spiroplasmas are shown by atomic force microscopy, in dimensions smaller than 12 µm in length and 0.75 µm in diameter, panels A and B, respectively.

5. References

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