Spiroplasmas Detection in Isolates of *Apis mellifera* in Puebla-Mexico

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Mollicutes are the smallest organisms capable of autonomous self-replication, and their phenotype differs from that of other bacteria in their lack of a cell wall. Mollicutes are wide spread in nature and pathogenically infect humans, other mammals, reptiles, arthropods and plants. Normally demonstrate a strict specificity to a host, as well as tissue specificity, which result from an obligate parasitic lifestyle. However, plant pathogens, including phytoplasmas, spiroplasmas and acholeplasmas, have the capability to infect a variety of plants and use insect-host vectors for transmission. The objective was detection and characterization of spiroplasmas by PCR, sequencing 16S rRNA, and atomic force microscopy, in isolates of *Apis mellifera* in Puebla-Mexico. Samples from 100 honey bee were collected and examined by PCR, sequencing 16S rRNA and atomic force microscopy. A 271 bp product was produced from the F28/R5 primer set in 38%. Alignment of the sequences showed a 95% similarity with *Spiroplasma melliferum*. Atomic force microscopy study shows the presence of spherical structures formed chains with sizes within the ranges reported for spiroplasmas.

Key words: *Apis mellifera*, spiroplasma, vector, PCR, AFM.

Spiroplasmas are prokaryotes belonging to the class Mollicutes and their traditional host are plants and insects. Due to their small cell size spiroplasmas can pass through 0.22 µm filters used in filter sterilization. Spiroplasmas are difficult to detected with light microscopy and cultivation. They are also impractical to use for detection of pathogens at low densities from fields samples[1,2].

The spiroplasmas organism showed stable vertical transmission through successives generations of the host, and exhibited negative effects on growth, reproduction, and longevity of the host, particularly in older adults[3].

The ecology of the spiroplasmas must be studied, and principal host or alternate host or both must be identified, should be of importance to agriculture or public health or as a basic microbiological model. Some spiroplasmas cause female-biased sex ratios of their host insects as a result of selective death of the male off spring during embryogenesis[3,4].

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Two species of spiroplasmas were isolated from and described as pathogens of the European honey bee, *Apis mellifera*, ~30 years ago but recent information on them is lacking despite global concern to understand bee population declines. A comprehensive survey for the prevalence of these two spiroplasmas species in current populations of honey bees using improved molecular diagnosis techniques to assay multiyear colony samples from North America (U.S.A.) and South America (Brazil). Significant annual and seasonal fluctuations of *Spiroplasma apis* and *Spiroplasma melliferum* prevalence in colonies from the U.S.A. (n=616) and Brazil (n=139) occurred during surveys from 2011 through 2013 (Schwarz *et al.*, 2014). Spiroplasmas infections in honey bees have been in Europe and Asia quite recently, due to intensive studies on the epidemiology of honey bee diseases. Despite that pathogenicity of spiroplasmas in honey bee colonies remains to be determined, others results indicated that spiroplasmas infections of the impacts on honey bee health, these bacteria tended to flourish in adult honey bees specifically during spring then vanish by summer from available serologic and microscopic detection, suggesting they opportunistically infected honey bee colonies between spring and summer via transmission from other hosts. Spiroplasmas were tentatively identified as the causative agents of neurological disease in bees specifically during the spring using the terms "Spiroplasmosis" and "May disease"[5,6].The presence of spiroplasmas in agroecosystems can influence so determinate, because the spiroplasmas may live both intracellularly and systemically via the hemolymph, with corresponding differences in pathology and mortality. Arthropod disease attributed to spiroplasmas included tremor disease in crabs, lethargy disease in beetles, and sex-ratio disorders due to significantly increased mortality of developing males in populations of fruit flies, butterflies, and beetles. Although a number of spiroplasma symbionts studied in detail are detrimental to their host, others appear to act mutualists[7,8].The aim of this study was detection and characterization of spiroplasmas by PCR, sequencing 16S rRNA, and atomic force microscopy, isolates of *Apis mellifera* in Puebla-Mexico.

MATERIALS AND METHODS

Spiroplasmas isolation

The specimens of *Apis mellifera* were macerated in medium DSM4 (pH 7.2) containing 1.5% (wt/vol) PPLO broth (Difco Laboratories), 6.5% (wt/vol) sucrose, 1.5% (wt/vol) HEPES, 0.5% (vol/vol) phenol red solution, and 10% (vol/vol) horse serum. The agar medium contained 1.5% (wt/vol) PPLO broth, 8.0% (wt/vol) sucrose, 10% (vol/vol) horse serum, and 1.0% (wt/vol) agar. The cultures were monitored daily for a change in color due to the respiratory acidification of the cultures and the titer was expressed in color-changing units (CCU/mL).

Extraction of spiroplasma DNA

Cells were cultures in medium DSM4 (30 mL) harvested by centrifugation at 10000 x g for 5 min, washed twice in SET buffer (NaCl 150 mM, EDTA 1 mM, Tris-HCl 10 mM, pH 7.5) and resuspended in SET buffer DNA extraction[9].

PCR amplification

Pair of primers produced a 271 bp fragment, forward oligo F28 (5´ CGC AGA CGG TTT AGC AAG TTT GGG 3´) and reverse oligo R5 (5´ AGC ACC GAA CTT AGT CCG ACA C 3´), which are specific for spiroplasma 16S rRNA gene[10]. The DNA extracts were used to amply fragment of the 16S rRNA gene by PCR. PCR was carried out in a final volume of 30 µL containing 5 µL template DNA, 3µL 10X STR buffer (including dNTP, Mg2+, Promega Company), 0.2 µL Taq DNA polymerase, 2µL primer mix (2 µM) and 19.8 µL sterile water. Amplification were performed starting with a 2 min template denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 50 s, extension at 72°C for 1.5 min, and final extension at 72°C for 10 min. The PCR products were assessed by electrophoresis on 2% agarose gel. *Spiroplasma melliferum* from (ATCC AS576, BSL: 1) as used as a positive control.

Sequencing 16S rRNA

Strains isolated from *Apis mellifera* were confirmed as spiroplasma by sequencing of the 16S rRNA using primers previously described. Sequence reactions were carried out using a Dye Terminator Cycle Sequencing quick start kit (Beckman Coutler) and the products were analyzed on a CEQ 8000 genetic analysis system (Beckman Coutler).

Atomic force microscopy (AFM)

Spiroplasma isolate was grown aerobically in medium DSM4 at 30ºC. The bacteria was harvested by centrifugation (10 min at 12000 x g), washed twice with desmineralized water, and resuspended in water or in 0.25 mM potassium phosphate buffer at pH 7.0.

The immobilization of spiroplasmas were suspended in water to a concentration of 10^5 per mL, after which 10 mL of this suspension was filtered through annitrocellulose membrane (Millipore) with a pore size of 0.22 µm, to immobilize the bacteria through mechanical trapping. After filtration, the filter was carefully fixed with double-stick tape onto a silicon slide and transferred to the AFM. Measurements were made at room temperature in a 0.25 mM potassium phosphate solution at pH 7.0 under an optical microscope (Nanoscope III digital instrument). V-shaped silicon nitride cantilevers from Park Scientific Instruments, with a spring constant of 0.06 N/m and a probe curvature of ~ 50 nm, were used[11].

RESULTS AND DISCUSSION

A 271 bpproduct was amplified in all spiroplasmas strains isolates (Figure 1), this PCR has shown to be a specific amplification method, which can potentially be used for the detection of spiroplasma strain from honey bee specimens. We detected 38% positive samples to spiroplasmas, our findings support that spiroplasmas are facultative symbionts and not part of the typical honey bee microbiota, but may be temporally and regionally common and thus influential to honey bee health and disease cycles[6].

In a BLAST search of the Gen Bank database, the 271 bp sequence showed greatest sequence similarity (95%) to the 16S rRNA gene of *Spiroplasma melliferum* (Gen Bank number: KF706372.1). The 16S gene sequence analyses (Figure 2) demonstrated the phylogenetic tree of the family Spiroplasma, showing the close
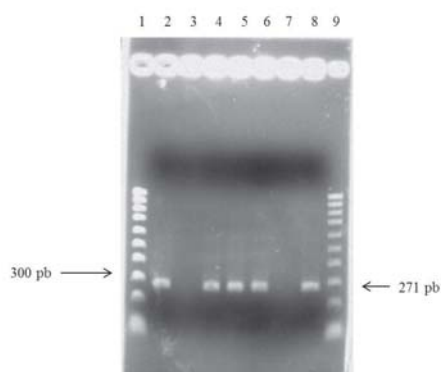

Fig 1. Electrophoretic patterns in a 2% agarose gel. (Lane 1 and 9) are markers with ladders of 100-1000 bp. (2) positive control, (3) negative control, (4, 5, 6 and 8) a 271 bp PCR product from the spiroplasmas isolates of *Apis mellifera*.

Fig 2. Phylogenetic tree, based on 16S rRNA gene sequence comparison, showing phylogenetic relationships between members of spiroplasma. Accession numbers are shown in parentheses,values 95% are shown.

relationship between the species reported and isolated in *Apis mellifera* in the present work. This result is successful because *Spiroplasma* *melliferum* is found in the feces of infected honey bees deposited on the surface of flowering plants, which serve as center of infection for healthy bees.
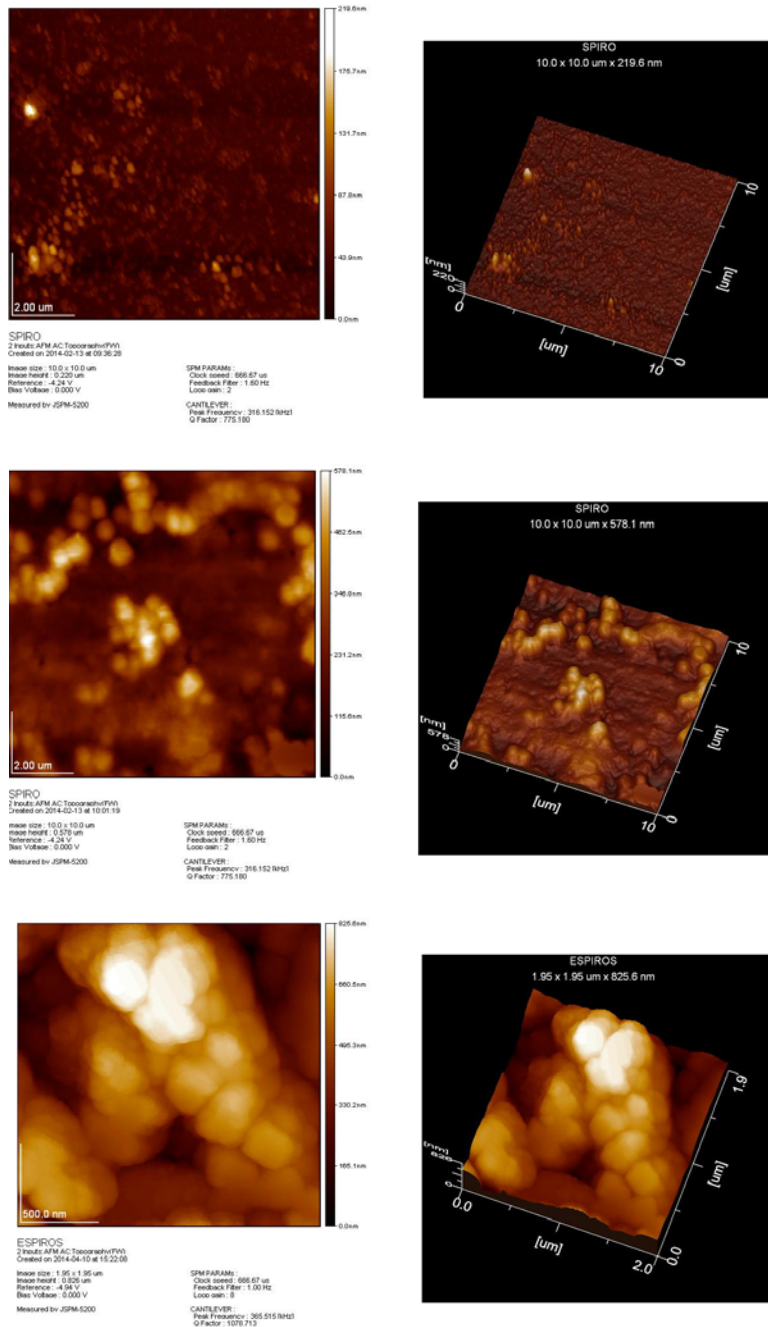

Fig 3. AFM deflection images of spiroplasmas trapped in a nitrocellulose membrane (Millipore) in pore size of 0.22 μm, (A) membrane control with an absence of structures, (B and C) sample with the culture filtrate spiroplasmas, forms of beads are observedbeing characteristic of other mollicutes, and displaying the size range reported for spiroplasmas.

Its capability to replicate in the tissues of plants or on their surfaces is not shown. *Spiroplasma melliferum* has also been found in the hemolymph of bumble bees, leafcutter bees and robber flies and in the intestinal tract of sweet bees, digger bees, and butterflies. Although the amount of *Spiroplasma melliferum* in the hemolymph of the host spectra is comparable to that in honeybees that died of spiroplasma infection, the degree of pathogenicity for its host spectra is unknown[12,13].

Because it is difficult to isolate and culture spiroplasmas, in this paper detection was implemented by AFM, a technique that has not been reported for the presence of spiroplasmas. When looking at different preparations diversity of morphologies were found and this may be related to morphological and size changes experienced by spiroplasmas during growth in liquid medium. Spiroplasma cell removed after 25, 46, 50 and 56 h of incubation at 32°C (log phase) in the two media showed no obvious differences in the frequence distribution of the various morphological forms observed earlier in *Spiroplasma citri*[14].

During that period, the only noticeable change occurred in the BSRH medium, where there appeared to be less aggregation of long helices into microcolonies and less variability in number of long helices. After long-phase growth, at time when the pH of the unbuffered BSR medium had dropped to 6.5, organisms present in this medium appeared highly aggregated and displayed altered morphology[15], and in stationary growth, cultures kept at 30°C for more than 5 days, the organisms became deformed, but some evidence of helicity persisted for many days[4]. Again, these alterations appear to be similar to morphological changes occurring in the normal aging of spiroplasma cells, and they may be associated with exhaustion of nutrients in the broth medium (Figure 3). Generally, the artificial media presents piro plasma shelically shaped, this remains an important component in the displacement of these organisms. But it has been observed in insects that may have shapeoval, sphericalor helical[16], as we present in the AFM images, being determined by the mre Bgenein some species of spiroplasmas. Spiroplasmas have even more MreB homologs, as many of these species have five MreB proteins with more divergent

sequences. Discussion of MreB is inherently complicated because of the vast diversity of MreB homologs in the bacterial and archael kingdoms. MreB homologs have been implicated in nearly every spatially organized cellular process, including cell growth, morphogenesis, polarity, protein localization, organelle positioning, division, and differentiation, as well as chromosome segregation, replication, and decatenation[17,18].

Spiroplasmas may affectits biological potential vector of positive, negative, or simply not exertany effect, which depends on environmental conditions, host plant, isolated the pathogen and vector insect species. Spiroplasmas may adversely affect the biological potential of their vectori nsects, but also other insects are favored by the infection of these pathogens, since the vectors can survive longer and produce more off spring individuals compared to uninfected insects. Spiroplasmas infection on insect vectors has implications in the incidence and spread of disease[7,19].

In conclusion, PCR technique facilitated the detection of spiroplasmas in *Apis mellifera* were collected in Puebla-Mexico. Confirmed by sequencing with 95% similarity to *Spiroplasma melliferum*. AFM allowed observing spherical structures with similarity to other mollicutes, structures having dimensions similar to those reported by scanning electron microscopy and transmission electron microcopy. Spiroplasmas detection was performed by microbiological culture, but due to the difficult to isolate, PCR, sequencing and AFM were used, remain efficient and rapid for detection of spiroplasmas.

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