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Short Communication

Detection of *Spiroplasma melliferum* in honey bee colonies in the US

Huo-Qing Zheng [a,b], Yan Ping Chen [b,*]

[a] College of Animal Sciences, Zhejiang University, Hangzhou, Zhejiang 310058, PR China
[b] USDA-ARS Bee Research Laboratory, Beltsville, MD 20705, USA

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ABSTRACT

Spiroplasma infections in honey bees have been reported in Europe and Asia quite recently, due to intensive studies on the epidemiology of honey bee diseases. The situation in the US is less well analyzed. Here, we examined the honey bee colonies in Beltsville, MD, where *Spiroplasma melliferum* was originally reported and found *S. melliferum* infection in honey bees. Our data showed high variation of *S. melliferum* infection in honey bees with a peak prevalence in May during the course of one-year study period. The colony prevalence increased from 5% in February to 68% in May and then decreased to 25% in June and 22% in July. Despite that pathogenicity of spiroplasmas in honey bee colonies remains to be determined, our results indicated that spiroplasma infections need to be included for the consideration of the impacts on honey bee health.

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1. Introduction

Spiroplasmas are small, helical, motile Eubacteria and are descendants of gram positive bacteria that lack a cell wall (Regassa and Gasparich, 2006). *Spiroplasma melliferum* and *Spiroplasma apis* are two pathogens that have been identified in Western honey bees, *Apis mellifera* (Clark, 1977; Mouches et al., 1982).

S. melliferum was first reported in honey bees during the course of an examination of honey bees for pathogenic microorganisms in Beltsville, MD, USA in 1976 (Clark, 1977, 1978; Clark et al., 1985). *S. apis* was abundantly detected in honey bees from colonies showing symptoms of "May disease" in France in early 1980s (Mouches et al., 1982, 1984, 1983). *S. apis* strain B31 was found to cause death in honey bees when injected and strain B39 when fed (Mouches et al., 1982). *S. melliferum* caused similar disease symptoms in bees when fed, but was less pathogenic at the colony level (Clark, 1978). However, current knowledge concerning the pathogenesis of spiroplasmas in infected honeybee colonies is limited and no significant bee colony losses were observed to be linked to the spiroplasma infection (Clark, 1978; Neumann and Carreck, 2010).

The recent large-scale losses of honey bee colonies in America and Europe have attracted extensive research on the epidemiology and pathogenesis of pathogenic microorganisms that cause serious diseases in honey bees (Cox-Foster et al., 2007; Neumann and Carreck, 2010). In recent years, spiroplasma infection in honey bees has been documented in China (Hui et al., 2010; Li et al., 2012), Korea (Ahn et al., 2012) and Belgium (Ravoet et al., 2013). However, the spiroplasma infection in honey bees was not reported in the past three decades in North America, with the exception of a recent work presented in a meeting (Schwarz and Evans, 2012). Here, by taking advantage of a recently published PCR detection method (Meeus et al., 2012), we examined the honey bee colonies in Beltsville, MD for spiroplasma infection.

2. Methods and materials

For colony level prevalence, ten colonies were randomly selected from an apiary and sampled in September, October, November of 2012 and January, February, May, June and July of 2013 from the experimental apiary in the Bee Research Lab of USDA-ARS, Beltsville, MD. Three additional colonies were included to replace colonies died during the study. Additional bee samples were collected from 30 colonies in February and 40 colonies in May and July of 2013 from another three experimental apiaries. The four sampling apiaries were 1–2 km away from each other and underwent the same routine beekeeping management by the same beekeepers. Thirty worker bees were randomly collected from the inner lids of hives for each colony. Only half of the colonies sampled in February were sampled in May and July due

* Corresponding author. Address: Bee Research Laboratory, USDA-ARS, Bldg. 306, BARC-East, Beltsville, MD 20705, USA. Fax: +1 301 504 8736.
E-mail address: judy.chen@ars.usda.gov (Y.P. Chen).

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to the high winter losses. Sample sizes of 10, 30, 40 and 50 gave confidences of 95% for the detection of an infection with a prevalence of 25.9%, 9.5%, 7.2% and 5.8%, respectively (Pirk et al., 2013).

The abdomen of individual bees was ground in 1 ml of sterilized water. 100 µl of homogenized solution was lysed in 1 ml of DNAzol® Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions and the genomic DNA was precipitated from the lysate with ethanol and resuspended in nuclease-free water.

From a colony showing disease symptoms with bees crawling near the entrance, twenty crawling bees were collected near the ground and another twenty bees were randomly collected from the inner lid. For individual-level prevalence, twenty bees were sampled from each of another 3 colonies that were identified with spiroplasma infection in May. These bees were individually ground and homogenized with 1 ml of DNAzol reagent.

Universal primers (BS1-976 targeting the ribosomal RNA gene for *S. melliferum* and *S. apis*) and species specific primers (As-636 for *S. apis* and Ms-160 for *S. melliferum*) were used as Meeus et al. (2012). For both the colony and individual level prevalence study, PCR was conducted in a 25 µl volume with 2 µl DNA extract, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l dNTP, 0.4 µmol/l primer and 1.25 U Recombinant Tag DNA Polymerase (Invitrogen). PCR reactions were performed at 94 °C for 2 min followed by 40 amplification cycles (30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C) and then by 10 min at 72 °C. For each sample primers targeting the honey bee β-actin gene were used to confirm DNA quality. The β-actin primer sequences were previously reported (Prisco et al., 2011). A negative control without template DNA and a positive control with *S. melliferum* genome DNA were included in each run. PCR products were analyzed on a 1.5% agarose gel and visualized by ethidium bromide staining. For sequencing purpose, the target fragment amplified by BS1-976 were purified using the Wizard PCR Prep DNA Purification System (Promega, Madison, WI) after electrophoresis and the nucleotide sequence identity of the PCR fragments was determined using a Basic Local Alignment Sequence Tool (BLAST) homology search.

3. Results

S. melliferum infection was detected in our samples with both primers BS1-976 and Ms-160 (Fig. 1). Multiple sequences were obtained from the sequencing of PCR products of BS1-976 amplified from different samples and submitted into GenBank with accession numbers (KF667482, KF706369-72). The sequence shared 100% identity with partial sequence of *S. melliferum* strain BC-3 16S ribosomal RNA gene (NCBI accession No.: NR_025756). The primers As-636 targeting the *S. apis RpoB* gene did not yield any product from any of the samples.

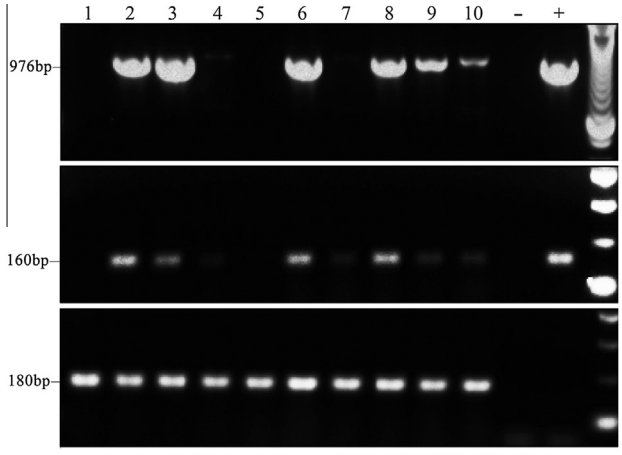

Fig. 1. Agarose gel electrophoresis of PCR products amplified by BS1-976 (top), Ms160 (middle) and honey bee actin primers (bottom). Results of 10 colonies sampled in May were shown from the first to tenth wells followed by a negative (no template) and positive (with *S. melliferum* genome DNA) control. 100 bp ladder was shown on the right.

A one-year survey showed that the prevalence of *S. melliferum* infection differed significantly between months of the year. The infection was identified in bee samples collected in February, May, June, and July (Fig. 2). Samples collected in May displayed the highest infection rate (68%) of the year and the infection rate declined to 25% and 22% in June and July, respectively. Only two out of the 40 colonies examined in February were positive. No spiroplasma infection was detected in the samples collected in September, October, November and January.

Of the 34 positive colonies in May, one showed symptoms with bees crawling on the ground in front of the hive without loss of hair, which is a typical symptom of spiroplasma infection (Mouches et al., 1984). 57.9% of the diseased crawling bees were infected with spiroplasma, which was significantly higher than the positive proportion of randomly sampled bees (35%, Chi-square test, $X^2 = 4.378$, $p = 0.036$, df = 1). This colony died in one month after the sampling.

The individual prevalence in the three selected colonies was 20%, 55%, 65%, respectively and all the three colonies were alive at the end of the study.

4. Discussion

Since the first case of spiraplasma infection in honey bees back to the 1970s, spiroplasma infection in honey bee colonies in the US has not been reported for over three decades, even with the extensive research efforts investigating the roles of pathogens/parasites on honey bee colony losses worldwide including Colony Collapse Disorder (CCD) (Cox-Foster et al., 2007; vanEngelsdorp et al., 2009). Traditional techniques to detect spiroplasmas in insects depend on culturing of hemolymph or macerated tissues, which is labor intensive and requires general precaution against fungal and bacterial contamination. In 2012, Meeus et al. reported the development of a multiplex PCR method for detection and differentiation of the mixed infection of *S. melliferum* and *S. apis* in bumble bees (Meeus et al., 2012). Recently, a comprehensive bee pathogen screening in Belgium revealed an infection with *S. apis* in 0.3% and *S. melliferum* in 4.4% of colonies sampled in July ($N = 363$) (Ravoet et al., 2013). Here, we provided the first evidence of spiroplasma infection in honey bee colonies in America since 1985.

The positive amplifications of primers BS1-976 and Ms-160 indicated *S. melliferum* infections in our samples, which was further confirmed by the partial 16S ribosomal RNA gene sequence, while *S. apis* was not detected in any of our samples by primers

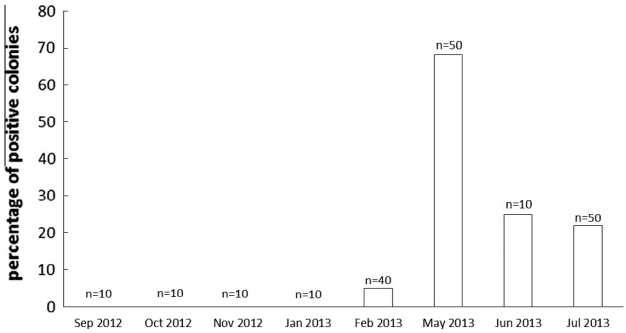

Fig. 2. Colony prevalence of *S. melliferum* infection.

As-636. This was not surprising since *S. melliferum*, but not *S. apis* was found in the same area by Clark and colleagues (Clark, 1977; Clark et al., 1985).

Although the negative detection in samples collected in September, October, November, and January could be due to the lower sample size in these months (see M & M for confidence level of sample size), our results, with much higher sample size in February, May, and July, showed a variation in the occurrence of *S. melliferum* infection in honey bee colonies in the sampling area. While only 5% of colonies were found to be positive for spiroplasma in February, 68% of colonies were positive in May. This result was in accordance with previous finding that spiroplasma disease occurred mainly from late May to early July and was commonly called "May disease" (Mouches et al., 1982). A previous study (Raju et al., 1981) showed that *S. melliferum* was found in the feces deposited on the surface of flowering plants by infected bees. It is presumably that bees would get infected while they were foraging on the contaminated nectar and pollen and then bring the pathogen to their hives. Further studies are warranted to investigate the correlation between seasonal variation in spiroplasma prevalence and honey bee foraging activities.

The higher prevalence of *S. melliferum* in diseased bees than in randomly collected bees in a diseased colony, suggested the correlation of *S. melliferum* infection to the death of the colony in one month. However, despite that typical symptom of spiroplasma disease could be observed, it was unclear that whether *S. melliferum* or other disease agents caused the death. The fact that most of the infected colonies recovered after May suggested low or short-term impact of *S. melliferum*, which was also suggested by Clark (1977) showing no productivity losses at colony level, or high resistance of honey bees to this infection.

The high variation with low or no occurrence at most time around the year and the low impact at colony level of *S. melliferum* infection, provided explanations to why it was not reported in previous studies in the past decades, regardless of the technique obstacles. However, in an era when honey bees are facing numerous threats (De la Rúa et al., 2009; Genersch, 2010; Mullin et al., 2010), the effects of spiroplasmas infections need to be considered. *Spiroplasmas* infections may only singly add to the pathology burden of honey bees or increase the vulnerability of honey bees by interaction or combination with other factors like parasites, viruses, poor nutrition and chemical residues.

Besides in honey bees, *S. melliferum* has also been found in the hemolymph of bumble bees, leafcutter bees, and robber flies as well as in the intestinal tract of sweet bees, digger bees, bumble bees, and butterflies (Alexeev et al., 2012). However, the degree of its pathogenicity has not yet been clear. The physiology of *S. melliferum* and its mechanisms of interaction with hosts remain poorly studied except for studies of its motility. The recent assembly of the *S. melliferum* KC3 genome and its proteogenomic annotation provided new molecular tools on these aspects (Alexeev et al., 2012).

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