DOI: 10.2478/JAS-2019-0011

sciendo

Original Article

COMPARISON OF QPCR AND MORPHOLOGICAL METHODS FOR DETECTION OF *ACARAPIS WOODI* IN HONEY BEE SAMPLES

Silvina Quintana[1,2,3]*
Nicolás Szawarski[1,2]
Gabriel Sarlo[1]
Sandra Medici[1,2,3]
Mariana Rivero[4]
Martin Eguaras[1,2]
Matias Maggi[1,2]

[1] Centro de Investigación en Abejas Sociales (CIAS). Instituto de Investigaciones en Producción Sanidad y Ambiente (IIPROSAM). Facultad de Ciencias Exactas y Naturales. Universidad Nacional de Mar del Plata
[2] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Av. Rivadavia 1917 (C1033AAJ), Buenos Aires, Argentina
[3] Instituto de Análisis Fares Taie. Mar del Plata, Buenos Aires, Argentina
[4] Centro de Investigación Veterinaria de Tandil (CIVETAN-CONICET-CIC)Facultad de Ciencias Veterinarias-UNCPBA

*corresponding authors: silquintana@hotmail.com
Received: 24 August 2018; accepted: 19 February 2019

Abstract

The honey bee tracheal mite *Acarapis woodi* is an internal obligate parasite of adult honey bees (*Apis mellifera*). The small size of the mites and location within the bee's trachea create a challenge for diagnostic identification. Detailed assessment of low-level mite infestation involves microscopic examination of the bee's tracheae, but this traditional diagnostic method takes a long time. In recent years, new molecular diagnostic techniques have been developed to identify *A. woodi* with the use of PCRs. The purpose of this study was to compare the diagnostic sensitivity of microscopic examination and qPCR method for the detection of *A. woodi* in honey bee samples. Thirty-six samples were analyzed with both methods and qPCR was more sensitive than the microscopic method to detect *A. woodi*. This work demonstrates the practical application of molecular technology as a support tool for surveys and contingency management and to provide robust surveillance data on the presence or absence of *A. woodi* in honey bee colonies.

Keywords: *Acarapis woodi, Apis mellifera*, internal control, optical microscopy diagnosis, real time PCR

INTRODUCTION

The honey bee tracheal mite *Acarapis woodi* is an internal obligate parasite of adult honey bees (*Apis mellifera*). The presence of tracheal mites in honey bee colonies can affect their survival and also lead to a reduction in pollen collection and honey production (Sammataro et al., 2013). The small size of the mites (~120–190 μm) and their location within the bee's trachea create a challenge for diagnostic identification, which has led to misdiagnoses of infestation (Evans, Pettis, & Smith, 2007). Detailed assessment of low-level mite infestations involves microscopic examination of the bee's trachea. This traditional diagnostic method is very time consuming and is based on direct visualization of *A. woodi* or of the lesions in the tracheae of bees. In recent years, new molecular diagnostic techniques have been developed to identify *A. woodi* through the use of PCRs (Evans, Pettis, & Smith, 2007; Kojima et al., 2011; Garrido-Bailón et al., 2012; Cepero et al., 2015; Delmiglio et al., 2015). Real-time PCR is a tool with high specificity and sensitivity, which allows fast 2 h detection of pathogens through the selective amplification of a specific region of

DNA to be quantified with fluorescent markers throughout the reaction (Learmount et al., 2009). The purpose of this study is to compare the diagnostic sensitivity of microscopic examination and qPCR method for the detection of *A. woodi* in honey bee samples.

MATERIAL AND METHODS

A total of forty samples of *Apis mellifera* were analyzed in this study: seventeen from Chile, two from Uruguay, three from Venezuela and eighteen from Argentina. Samples belonging to Argentina had been previously analyzed by Szawarski et al. (2017) for *A. woodi* detection. Sampling bees were collected during the month of July, because *A. woodi* populations are more abundant in bee colonies during winter (Sammataro et al., 2013). In this study, each sample consisted of sixty bees collected from each colony. The bees were scooped from inside the hive through the use of plastic cups directly from combs in the honey super or from the inner covers where older bees congregate. Samples of adult worker bees were then transferred to properly labeled wide-mouth jars containing 70% ethanol (Sammataro et al., 2013).

From each analyzed honey bee colony, thirty bees were chosen randomly for the detection of *A. woodi* through visual inspection (n= 1200 bees). This method consisted of pulling off the head and collar of the bee and examining the trachea, according to the technique developed by Milne (1948) for locating internal mites in individual bees. The bee was placed under a dissecting microscope and held prone with forceps across abdomen, and the head and the first pair of legs was scraped off with a scalpel or razor blade. The ring of the prothoracic sclerite (collar) was also removed using a fine forceps. The exposed tracheae of both sides were removed after carefully being detached from the thoracic wall. The removed tracheae were placed on a glass slide and examined for mites under a microscope.

Total genomic DNA was isolated from forty individual honey bees,one bee per colony, and then preserved in 70% ethanol with the use of the commercial kit ADN HP PCR Template Preparation (Roche Diagnostics). To verify the success of DNA extraction from samples of individual bees and lack of inhibition of the PCR reactions, amplifications of beta actin gene of *A. mellifera* were conducted (Yang & Cox-Foster, 2005). The thermal cycling conditions were one cycle of initial denaturation at 95°C for two min and forty cycles of denaturation at 94°C for 10 s, annealing at 60°C for 15 s, and extension at 72°C for 15 s. After amplification, a melting curve analysis was performed to confirm specific amplification of the PCR product. Bee samples with Ct values (threshold cycle) beta actin < 30 were considered appropriate for further analysis. Four samples, two from Uruguay and two from Chile, showed a beta actin Ct value higher than 30 and were excluded from the final analysis. PCR amplification of the mitochondrial COI gene from *A. woodi* was performed with (AcarFor) 5´-CGGGCCCGAGCTTATTTTACTGCTG-3´ and reverse primer (AcarRev) 5´-GCGCCTGTCAATC-CACCTACAGAAA-3´. The expected size of the amplicon was 162 bp (Garrido- Bailón et al. 2012). The thermal cycling conditions were one cycle of initial denaturation at 95°C for 2 min and forty cycles of denaturation at 95°C for 10 s, annealing at 57°C for 15 s, and extension at 72°C for 20 s. The specific temperature dissociation of *A. woodi* PCR product was 76°C, and its reaction efficiency was calculated at 99%. All qPCR reactions were carried out in a thermocycler Rotor Gene (Qiagen, Hilden, Germany) in a final volume of 20 µl using EvaGreen as fluorescent intercalating dye (KAPA Fast, Biosystems, Woburn, E.E.U.U.), and samples with Ct values < 30 were considered positive. To verify that the selected primers were specific to *A. woodi*, the amplified DNA fragments obtained with AcarFor/Rev primers pairs were purified and directly sequenced (ABI 3500 genetic analyzer, Applied Biosystems, Foster City, CA, USA). The sequences similarities were determined by the Basic Local Alignment Search Tool (BLAST, NCBI). To measure the agreement between the morphological technique and qPCR, the Kappa index was evaluated through the use of Epidat 3.1. A value of kappa higher than 0.75 indicated

excellent agreement while values lower than 0.4 indicated poor agreement.

RESULTS

A total of forty samples (1200 bees) were analyzed for *A. woodi* presence through morphological analysis, while thirty-six samples were analyzed through the qPCR technique. In four samples, *A. woodi* DNA was detected through qPCR (Tab. 1). Only in one sample from Chile, *A. woodi* adult females *were* detected through the morphological method (Fig. 1), subsequently confirmed through molecular detection and sequencing.

Clear DNA sequences were obtained from the sequencing of PCR products amplified by AcarFor/Rev primers in different *A. woodi* -infected honey bee samples. These sequences shared 100% identity among themselves and 87-95% identity with other such sequences of *A. woodi* deposited in the Genbankas the *A. woodi* strains found in Canada (GQ916565.1), and isolates reported in United Kingdom (FJ603296.1) and Japan (AB638409.1).

Of the thirty-six samples studied with the morphological technique and qPCR methods, one sample was positive by both (Tab. 1). The most remarkable finding was that three samples negative by morphological technique were positive by qPCR and confirmed through sequencing, which demonstrated that the molecular technique was more sensitive than the microscopic method (Tab. 1). The Kappa index

Table 1.

Concordance between diagnosis by qPCR and microscopic method of *A. woodi* in honey bee samples

	Real time PCR positive result	Real time PCR negative result	Total
Morphological technique positive result	1	0	1
Morphological technique negative result	3	32	35
Total	4	32	36

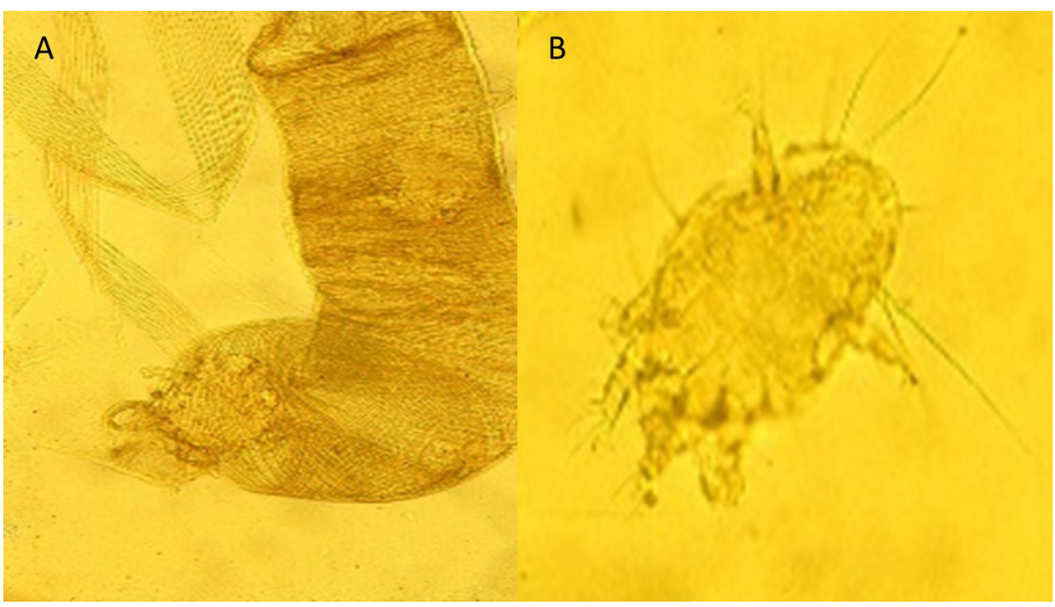

Fig. 1. Dissection of bees infected by the tracheal mite, sample from Chile. (A) Trachea which flows into the blowhole of prothorax of the body of a bee infested by *A. woodi* (400x). (B) A female individual *A. woodi* (400x).

was 0.37 (p=0.04) indicating poor agreement and that the differences between techniques were statistically significant.

Considering the microscopy technique as "gold standard", the sensitivity of the PCR was 100% (95% CI: 50-100%) and the specificity 91.67% (95% CI: 81.25-100%). The accuracy of the PCR test was 91.89% (81.75-100%). The positive predictive value of the PCR test was 25% (95% CI: 0-79.93%) and the negative predictive value was 100% (95% CI: 98.48-100%).

DISCUSSION

Detection of tracheal mites currently relies on the physical examination of bees by dissection and microscopy, but it is a very laborious procedure and requires detailed and sustained attention by the screener. This method is repetitive and requires a high level of skill and concentration from laboratory staff. Due to the repetitiveness of the method, there is high risk of worker fatigue, which may inadvertently lead to a failure to detect mites.

Until recently, there have been no PCR-based methods for the specific detection of *A. woodi.* The primers published by Evans, Pettis, & Smith (2007) have been shown to amplify other *Acarapis* species as well as other mites commonly found on honeybees. Kojima et al. (2011) published a conventional PCR method shown to amplify *A. woodi* DNA and not *A. externus*. In the present work, we used the primers described by Garrido-Bailón et al. (2012) and included a DNA extraction control. When a PCR-based method is used in routine analysis, a DNA extraction control will indicate false-negative results. DNA extraction control is critical for analyzing field-collected samples. In the particular case of honey bee field samples, such factors inhibit PCR as pollen, honey and compound eyes of bees. In this work we used the amplification of a 152 bp PCR product of honey bee beta actin gene as internal control to verify DNA quality. In fact, four samples were discarded from the final analysis because they were not suitable for *A. woodi* molecular detection.

In our study, we analyzed a total of forty samples (1200 bees) for *A. woodi* presence through morphological analysis while thirty-six samples were analyzed through qPCR. Three samples negative by morphological analysis were positive by PCR, which demonstrates that this technique shows a substantial increase in diagnostic sensitivity and the possibility to avoid false negative results since 11% of the samples had *A. woodi*, while only 2,7% were detected by the morphological technique.

This work demonstrates the practical application of molecular technology as a support tool for surveys and contingency management and also provides a robust surveillance data on the presence or absence of *A. woodi* in honey bee colonies.

REFERENCES

Cepero, A., Martín-Hernández, R., Prieto, L., Gómez-Moracho, T., Martínez-Salvador, A., Bartolomé, C., ... Higes, M. (2015). Is *Acarapis woodi* a single species? A new PCR protocol to evaluate its prevalence. *Parasitology Research*, *114*(2), 651-658. http://doi.org/10.1007/s00436-014-4229-6

Delmiglio, C., Fan, Q. C., George, S., Ward, L., Budge, G., Flynn, A., Kumarasinghe, L. (2016). Development and evaluation of a real-time PCR assay for the detection of *Acarapis woodi* (tracheal mites) in *Apis mellifera*. *Apidologie*, *47*(5), 691-702. http://doi.org/10.1007/s13592-015-0420-8

Evans, J. D., Pettis, J.S., & Smith, I.B. (2007). A diagnostic genetic test for the honey bee tracheal mite, *Acarapis woodi*. *Journal of Apicultural Research*, *46*(3), 1-5. http://doi.org/10.1080/00218839.2007.11101392

Garrido-Bailón, E., Bartolomé, C., Prieto, L., Botías, C., Martínez-Salvador, A., Meana, A., Martín-Hernández, R., Higes, M. (2012). The prevalence of *Acarapis woodi* in Spanish honey bee (*Apis mellifera*) colonies. *Experimental Parasitology, 132*(4), 530-536.

Kojima, Y., Toki, T., Morimoto, T., Yoshiyama, M., Kimura, K., Kadowaki, T. (2011). Infestation of Japanese

native honey bees by tracheal mite and virus from non-native European honey bees in Japan. Microbial Ecology, 62(4), 895-906.

Learmount, J., Conyers, C., Hird, H., Morgan, C., Craig, B., Von Samson-Himmelstjerna, G., Taylor, M. (2009). Development and validation of real-time PCR methods for diagnosis of *Teladorsagia circum-cincta* and *Haemonchus contortus* in sheep. *Veterinary Parasitoly*, *166*, 268-274.

Milne, P.S. (1948). Acarine disease of bees. *Journal of Ministry of Agriculture and Fisheries*, *54*, 473-477.

Sammataro, D., Guzman, L., George, S., Ochoa, R., Otis, G. (2013). Standard methods for tracheal mite research. *Journal of Apicultural Research*, *52*(4), 1-20. DOI: 10.3896/IBRA.1.52.4.20

Szawarski, N., Quintana, S., Levy, E., Mariano, L., Abrahamovich, A., Porrini, M., ... Maggi, M. (2017). Is *Acarapis woodi* mite currently infesting *Apis mellifera* colonies in Argentina? *Journal of Apicultural Research*, *56*(4), 387-393. https://doi.org/10.1080/00218839.2017.1339519

Yang, X., & Cox-Foster, D.L. (2005). Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. *PNAS*, *102*(21), 7470-7475. https://doi.org/10.1073/pnas.0501860102