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Brief Report

Molecular Detection of *Acarapis woodi* Using Hive Debris as Innovative and Non-Invasive Matrix

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Abstract: *Acarapis woodi* is a pathogen affecting honey bees health worldwide. Its prevalence may be underestimated due to the time-consuming traditional method for its diagnosis and the attitude in focusing the attention only onto *Varroa destructor*. New PCR techniques have allowed for the verification of the presence of *A. woodi* in 44 samples of honey bees and 11 samples of hive debris collected from 17 apiaries by the veterinary services of the Latium region (Central Italy). Overall, 9.1% of adult honey bee samples (all belonging to one apiary) and 6.3% of hive debris samples (belonging to 6 apiaries) were positive in an end point PCR and presence of the pathogen was confirmed through Sanger sequencing. Results demonstrated the potential underestimation of *A. woodi* occurrence in Italian apiaries and reported the first detection of *A. woodi* in hive debris samples.

Keywords: *Acarapis woodi*; tracheal mite; *Apis mellifera*; hive debris; diagnostic methods; Italy

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1. Introduction

Acarapis woodi is an internal parasite of the respiratory system of adult honey bees [1]. This mite belongs to the family Tarsonemidae and mainly infests the large prothoracic trachea of the worker honey bees but sometimes these parasites also infect the head, thoracic, and abdominal air sacs [2]. *Acarapis woodi* completes its life cycle within the prothoracic tracheae of newly hatched bees under 10 days of age. Reproduction occurs within the tracheae of adult bees, where female mites may lay 8 to 20 eggs. Development takes 11 to 12 days for males and 14 to 15 days for females, and a new generation of mites can emerge in two weeks [3]. The infection spreads from one bee to another by direct contact [2]. Clinical signs are not specific and include the inability to fly (K-wing), crawling around the front of the hive, and the presence of dysentery in honey bees. Moreover, at the colony level, diminished brood area, smaller bee populations, looser winter clusters, increased honey consumption, lower honey yields, and colony demise can be observed [3]. The pathogenic effects on individual bees depend on the number of parasites within the tracheae and can be attributed to mechanical injuries, obstruction of air ducts, and depletion of hemolymph [4]. The native range of *A. woodi* is uncertain. The mites were first reported from dying bee colonies in the Isle of Wight (UK) in 1921 by Rennie. Several risk factors are associated with the spread of this parasitosis worldwide, including the movement of bees between hives, swarming, robbing, migratory beekeepers, and sales of queens [5]. To date, the distribution of *A. woodi* is often described as "global," except for Sweden, Norway, Denmark, New Zealand, Australia, and Hawaii [6]. However, information on the distribution of acarapisosis is scarce and incomplete, and little is known regarding its effective prevalence in honey bee colonies in Mediterranean countries. In Italy, the

last report of *A. woodi* dates back to 1999 in honey bee colonies of the Sicilian region (unpublished data). Since then, there are no published studies aimed at evaluating the presence of *A. woodi* in Italian honey bee colonies. To fill this knowledge gap, the present study aimed to determine the occurrence of *A. woodi* in honey bee colonies of central Italy, using molecular diagnostic techniques. Moreover, debris samples were introduced as new matrices to evaluate the use of a non-invasive method for the detection of the mite.

2. Materials and Methods

2.1. Sampling Strategy

The study was carried out in *Latium* region (Central Italy) from May to June, for two consecutive years, i.e., 2018 and 2019. Overall, 44 adult honey bees and 111 hive debris were collected from 17 apiaries by the veterinary services in the context of a bee health monitoring study on voluntary basis. Honey bee samples consisted in 60 adults collected from the top of the frames. Debris samples were collected by placing a bottom board under the hive nets and collecting all the debris after 10 days. Samples were stored at −20 °C until analysis for DNA extraction.

2.2. Molecular Analyses

Adult honey bees were first homogenized in 20 mL of Phosphate Buffer Solution (PBS) 1X, and then 2 mL were centrifuged at 16,000× *g* for 5 min and the supernatant was discarded. The DNA extraction was performed with NucleoSpin® Tissue Kit following the manufacturer instructions (Macherey-Nagel; Düren, Germany). One gram per each debris was incubated for two hours at 37 °C in 10 mL PBS 1X in agitation and two mL were later subject to DNA extraction with the NucleoSpin® Tissue Kit following the manufacturer instructions (Macherey-Nagel; Düren, Germany). The quality and the integrity of the extracted DNA was determined by amplifying a fragment of 181 bp from the housekeeping gene β-actin with a real time PCR, as described by Chen et al. [7] Two specific molecular techniques for the identification of *A. woodi* were executed. The first PCR protocol amplifies a region of 180 bp of the subunit I of cytochrome oxidase (COI) and was performed as described by Cepero et al. (2015) using the following prime pairs: AWFor GGAATATGATCTGGTTTAGTTGGTC and AWRev GAATCAATTTCCAAACCCACCAATC. The cycling conditions were slightly modified as follows: 95 °C for 10 min, 50 cycles at 95 °C for 10 s, 59 °C for 45 s, 72 °C for 45 s, and a final elongation at 72 °C for 7 min. The successful outcome of the PCR was determined by using an in silico positive control, based on GenBank accession number AB634837. The detection of *A. woodi* from honeybees and debris matrices was also tested using real time PCR amplifying a sequence of 113 bp of the subunit I of COI. Reaction conditions were performed following the protocol described by Sammataro et al. (2013). Positive samples in the end point PCR were confirmed through Sanger sequencing of the 118 bp region coding for the COI gene.

3. Results

Overall, 6.3% of hive debris samples (belonging to 6 apiaries) and 9.1% of adult honey bee samples (all belonging to one apiary) were positive for the molecular detection of *A. woodi* within each PCR method. In Figure 1 is reported the agarose gel electrophoresis of some of the positive samples to the COI subunit I PCR that were than submitted to Sanger sequencing. All adult honey bee samples had a sequence identity of 95% and a query cover of 100% with the accession number HF945444.1. One debris sample had a sequence identity of 100% with accession number KX790788.1. Sequences are shown in Figure S1, marked from ITA-1 to ITA-5, and they are aligned against AB634837 (considered as the reference sequence; from Kojima et al., 2011).

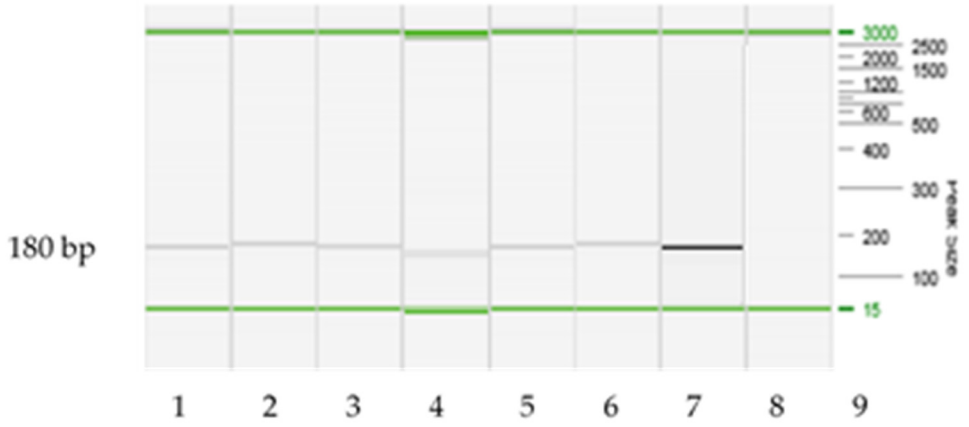

Figure 1. Gel electrophoresis of RT-PCR-amplified RNA analyzed in QIAxcel System, Qiagen. Lane 1–6, positive samples yielding amplicons of the expected size (180 bp); lane 7, positive control; lane 8, negative control; lane 9 DNA ladder.

4. Discussion

The results of the present study confirm the presence of *A. woodi* in honey bee colonies in Italy and point out the potential underestimation of its presence in Italian apiaries. In fact, in this country, acarapisosis is now overshadowed by the exponential spread of the parasitic mite *Varroa destructor*. As a result, the presence of *A. woodi* in several Italian regions is not regularly investigated. The prevalence of this parasite in Europe is relatively low: in Finland in 1991, it was slightly over 9%, rising to around 20% in the mid-1990s and then declining [8]. Similar observations were made in Greece where, over 10 years (1986 to 1995), infestation with *A. woodi* showed a decreasing trend [9]. However, the data related to the prevalence of *A. woodi* in these regions are outdated and often patchy. The prevalence of this mite in Spain in 2010–2011 (13–15.5%) increased compared to that in 2006–2007 (8.3–4%) [4,10]. This change in prevalence was not related to the different techniques employed and variation in sampling area, and varroosis treatment could be responsible for the declined trend [10]. Recent reports show the disease has spread to new regions, mainly in Asia and in the eastern regions of Russia [11].

Another factor discouraging the diagnosis of *A. woodi* in Italian apiaries is the time-consuming official method applied for its diagnosis. In fact, the conventional diagnostic method used in the surveillance program for detection of tracheal mites is the thoracic disk methods (TDM), which involves cutting a thoracic disc containing the prothoracic trachea for slide mounting and microscopic examination of the bee's tracheae [12,13]. Although TMD has a good sensitivity, allowing for the detection of even low levels of infestation (below 5%), this method is very laborious and requires a high level of skill and concentration of the laboratory staff [13]. Guanine visualization and direct enzyme-linked immunosorbent assay (ELISA) are other methods available for the detection of tracheal mites [12]. The ELISA method is sensitive enough to detect tracheal mite infestation at very low levels but lacks specificity, as cross reactivity with other proteins present in the hemolymph and in the thoracic muscles can occur [12]. Indeed, guanine visualization is an indirect method based on detecting guanine, which is the main end product of nitrogen metabolism in the mite, but its presence is only in a negligible amount in bee excretions, which could lead to potential false-negative results [12,14]. In this context, molecular techniques are more sensitive, as well as specific, especially in the case of low parasite loads. Sequencing of the amplified products of positive honey bee samples confirmed the infection by *A. woodi* since these products had an identity of 95% with the COI sequence deposited in GenBank by Cepero et al. [10] from Spanish apiaries. By contrast, only one positive hive debris sample showed an identical COI sequence with those deposited in GenBank by Ugajin. To the best of our knowledge, this is the first detection of *A. woodi* in hive debris sample. The importance of this matrix is due to its non-invasiveness because

it does not imply killing honey bees; moreover, the use of molecular techniques is faster than the official methods previously described. This new sampling method may act as a pre-clinic indicator to detect the presence of the mite in the apiaries.

The potential importance of *A. woodi* infestation should not be underestimated. In fact, it is considered that in the UK, if 30% of bees in a colony are infested, the colony will die in the following spring. However, generally in Europe, a realistic threshold could be set at 10% [12]. The mite's population size and prevalence increase during the winter, probably because of the increased longevity of bees during this season and increased clustering with cooler temperatures [15]. Heavy mite infestation affects bee metabolism leading to thermoregulatory disorders and colony death due to hypothermia [12,15]. Moreover, the pathogenic synergism between *A. woodi* and *Varroa destructor*, as well as *Nosema ceranae*, is described by several authors and it is associated with greater colony bee mortality during winter months [4,16]. Compared with the well-known bee pathogens such as *V. destructor* or *Nosema* spp., *A. woodi* has been put aside and its pathogenic role has been potentially underestimated.

5. Conclusions

The results of the present study demonstrate the presence of *A. woodi* in Italian apiaries after its apparent disappearance for 20 years. Sequencing of positive honey bee samples confirms the occurrence of *A. woodi* in honey bee colonies of Central Italy. The parasite was also detected from hive debris samples, highlighting the potential role of this matrix as a non-invasive tool useful for the diagnosis of acarapisosis. Therefore, the sensitivity and the specificity of extraction and molecular techniques must be improved on for this new matrix to allow, where appropriate, the sequencing from all of the amplified samples for further confirmation or the use of real-time PCR that avoids sequencing.

The education of beekeepers on this parasitosis as well as a control strategy against *A. woodi* should be implemented. Even if some therapeutic solutions used against varroosis are also effective against *A. woodi*, the data on the efficacy of this treatment on the tracheal mite are still limited. This preliminary study points out the potential underestimation of this pathogen in Italy; however, more exhaustive studies are needed to clarify the actual prevalence of *A. woodi* in this country and the potential relationship between its presence and colony loss during the winter months.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12062837/s1, Figure S1: Sequences obtained in our study (marked as ITA-1 to ITA-5) were aligned against the reference sequence AB634837 (line 1) and those presents in NCBI database (from line 7) using Bioedt free software.

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