

Histopathology as diagnostic tool for *Ascosphaera apis* infection in apparently healthy honey bees (*Apis mellifera ligustica*)

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Abstract

Objective: Chalkbrood is a fungal disease of honey bee brood caused by *Ascosphaera apis*. This disease is now found around the world and there are some indications that the incidence has increased in recent years. The diagnosis of Chalkbrood is based on the recognition of typical clinical symptoms and on the identification of the causative agent. Despite the numerous methods available for the detection of *A. apis*, the diagnosis of this pathology is not always easy.

Methods: Between June and July 2011 an increase in mortality of honey bees was observed to some apiaries of the Caserta province (Campania region, southern Italy). Larvae from brood samples without signs of disease and adult honey bees from the same colonies were collected and submitted to our laboratory for routine histologic evaluation.

Results and Conclusions: We demonstrate that histopathology is a sensitive and specific method for the detection and identification of *Ascosphaera apis* infection and has the additional advantage of being able to provide a diagnosis in larvae without signs of disease.

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INTRODUCTION

Chalkbrood is an invasive disease of honey bee larvae (*Apis mellifera ligustica*), caused by the fungus *Ascosphaera apis* Olive and Spiltoir [1, 2]. *A. apis* is able to infect brood of any cast (workers, drones or queens) but it does prefer drones. *A. apis* spores are ingested by young bee larvae (3-4 days of age) with their food and germinate in the gut. The mycelium then penetrates the gut wall, breaking out of the hind end of the larva's body when larvae are sealed in their cells prior to pupation. When mycelia of opposite sex are grown in close proximity, sexual reproduction occurs, with the formation of spore cysts on the outside of the dead larvae. Most of these spore cysts are ejected from the colony by the house-cleaning bees that remove dead

larvae from their cells, but many will inevitably find their way to healthy larvae via mechanical contamination on nurse bees or become lodged in food stores and especially in brood comb [3] where they remain infective for many years.

Although fatal to the larvae, *A. apis* rarely destroys a whole colony but it can cause substantial production losses. Until 1968, it was considered to be a European disease and it was only in 1971 that it became recognized as of economic importance also in the USA [4]. Chalkbrood is now found in honey bee colonies around the world and there are some indications that the incidence has increased in recent years [5]. The reasons for this increase are still unknown.

Fungal culture, microscopic examination and molecular identification (PCR) are currently the most widely used methods for the detection of *A. apis*. However, all these methods require bees with signs of disease or pathogens to be isolated and growth in culture. To the best of our knowledge, histopathological method has never been used so far for the diagnosis of *A. apis* in honey bee colonies. In this study, we describe the histological findings of chalkbrood from apparently healthy *Apis mellifera ligustica* colonies in southern Italy.

MATERIALS AND METHODS

Larvae (n=50) from brood samples without signs of disease and adult honey bees (n = 50) from the same colonies were collected between June and July 2011 in the Caserta province (Campania region, southern Italy) and fixed in 10% formalin, for histological processing. Before processing they were observed under the stereomicroscope for identification of anatomical changes and then were necropsied. Fixed samples were divided in two halves and embedded in paraffin wax. 5- μ m-thick sections were stained with haematoxylin and eosin (H&E) and observed at light microscopy. These sections were then stained with histochemical methods (Periodic Acid Schiff and Grocott) for the identification of hyphae.

RESULTS AND DISCUSSION

The stereomicroscopic and microscopic observations of the sampled honey bee (adults and larvae of any casts), did not reveal any pathologic changes in the examined organs. Only 5 young drone larvae presented numerous hyphal filaments that invaded completely the hemocoel. Their growing into fat body structures caused a slow degeneration of trophocytes and enocytes, blurring of their shape and decomposition. As reported by Carrera and co-workers (1987) [6], fungal growth was devastating: the fungus invaded, destroyed and replaced all the larval tissues except for tracheae and tracheoles (Fig. 1). The hyphae extended from visceral cavity to cuticle. A slight and local associated inflammatory reaction, composed by haemocytes and fibrinous-like material, was observed (Fig. 2). The fungal hyphae were PAS-positive and easily demonstrated by silver impregnation methods, such as Grocott's technique (Fig. 3). They were septated, 2.5-8 μ m in diameter, and showed pronounced dichotomous branching and released numerous conidia [1, 7].

Histopathological tools have been widely employed for the diagnosis of numerous infectious and parasitic diseases of animals and have proved to be sensitive and specific method for the detection and the identification of numerous pathogens, including fungi. Considering

the increasing incidences of chalkbrood in many parts of the world, including Italy, it would be important to have a sensitive and rapid technique for the detection of the causative agent. Our results demonstrate that the *A. apis* can be present in larvae of honey bees without symptoms of disease and also suggest that there is a spread of the causative agent between colonies, because positive samples are found in colonies where no symptoms of the disease can be seen. This indicates that the lack of clinical signs of *A. apis* in colonies not always reflects the actual disease status of the colony and that specimens of larvae are useful to monitor chalkbrood.

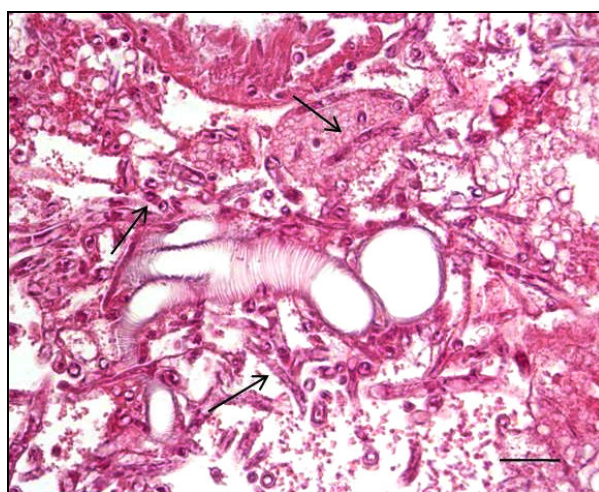


Figure 1 – Sections of honey bee larvae naturally infected by *A. apis*. Hyphae invading, destroying and replacing all larval tissues except for trachea (H&E; Scale bar: 50 μ m)

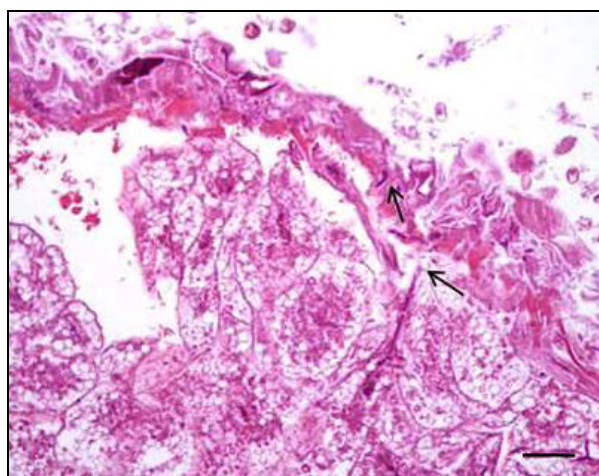


Figure 2 - Hyphae extending from visceral cavity to cuticle (arrows) (H&E, Scale bar: 50 μ m).

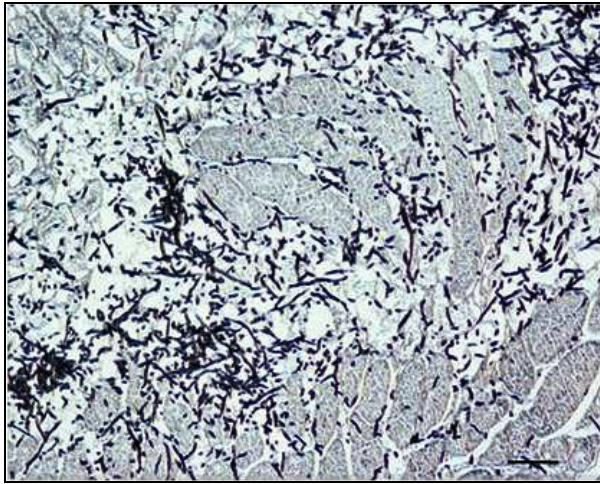


Figure 3- Fungal hyphae positive to Grocott (Scale bar: 50 µm)

It is well known that the identification of individual *Ascospaera* species is difficult and most identification are based on the size and shape of the ascomata, spore balls and conidia but unfortunately, much overlap occurs in the size of these structures, and some *Ascospaera* species will not produce sexual structures in vitro.

Our study demonstrates that histopathological method overcomes these limitations allow to differentiate *A. apis* species on morphological features and on pathologic changes. The microscopic examination of hyphal growth revealed the characteristic features of mycelium of *A. apis* as well as the ability of this fungus, in contrast to *A. aggregata*, to break down or digest chitin and actively grows through the cuticle.

In conclusion, our results also show that histopathological tool is very sensitive and efficient to

identify *A. apis* infection in asymptomatic, young larvae.

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