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Detection of *Lotmaria passim* in honeybees from Emilia Romagna (Italy) by cultural method

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Abstract:
Lotmaria passim is considered an emerging field of study in honeybee pathology, since it can threaten the health of the colony leading to a higher mortality rate. However, there is a lack of knowledge regarding the diffusion of this trypanosomatid in Italy. In this study, we highlight the presence of *L. passim* in the province of Bologna through its culture isolation from honeybee guts and microscopic observation.

Keywords: *Apis mellifera*, *Lotmaria passim*, trypanosomatid, cultural method, morphological identification, honeybee pathogens

1. Introduction

The family Trypanosomatidae (Euglenozoa, Kinetoplastea) consists of 25 genera with a wide host range, including vertebrates and insects (Maslov et al., 2013; Kostygov et al., 2021). Until a few years ago, in honeybee, the report of monoxenous trypanosomatids in the digestive system was limited to only two species: *Leptomonas apis* Lotmar 1946, no longer reported, and *Crithidia mellificae* Langridge & McGhee, 1967. The presence of trypanosomatids in *Apis mellifera* has been increasingly recognized, in particular after the description of the species *Lotmaria passim* (Schwarz et al., 2015), which nowadays is considered as the most widespread bee trypanosomatid all over the world (Arismendi et al., 2016; Stevanovic et al., 2016; Vargas et al., 2017; Williams et al., 2019 Castelli et al., 2019; Ribani et al., 2021; Michalczyk & Sokół, 2022). It has been suggested that trypanosomatids infestation could be related to an impairment of bee immune system, a nutritional deficit and an alteration of bee behavior (Buendía-Abad et al., 2022; Gómez-Moracho et al., 2020; Liu et al., 2020; Lukeš et al., 2018); however, the details of the pathogenic effects are still not fully understood.
Recently, studies have begun to deepen the interaction of *L. passim* with other well-known bee pathogen: Arismendi et al. (2020) have explored the possible synergic effect on the survival of honeybees infected at the same time by *Nosema ceranae* and *L. passim*; Quintana et al. (2021) have

reported the finding of *L. passim* in the mite *Varroa destructor*, while Nanetti et al. (2021) in *Aethina tumida*. Despite its wide range of distribution, there is a lack of knowledge about the real diffusion in the bee colonies of *L. passim*, and in particular few studies have evaluated its distribution in Italy (Bordin et al., 2022; Cilia et al., 2022; Ribani et al., 2021).

At present, methods based on DNA assay are the most used to detect the presence of *L. passim* (Arismendi et al., 2016; Castelli et al., 2019; Cilia et al., 2022; Michalczyk & Sokół, 2022; Stevanovic et al., 2016), and few publications have reported the isolation of the parasite directly from the honeybee's intestine (Schwarz et al., 2015; Buendía-Abad et al., 2021; Ribani et al., 2021). In the present preliminary study, we aimed to detect the presence of trypanosomatids in *A. mellifera* from different apiaries of Emilia-Romagna region (Italy) through its direct isolation from the intestine of single honeybees and microscopic observation.

2. Material and Methods

Five apiaries have been included in this study: four (A, B, C, D) belonging to the same beekeeper are in three municipalities of the province of Bologna, and one belonging to a different beekeeper in the province of Rimini. The apiaries A and B are in the suburban area of Bologna (54 m above sea level), the apiary C is located in Argelato (25 m a.s.l.), a town rich of cropland, while the apiary D is located in Pianoro (200 m a.s.l.), surrounded by semi-intensive vineyards. The last is an organic apiary in the municipality of Montescudo (province of Rimini, 209 m a.s.l) in an area characterized by semi-intensive cultivations. All the samples were collected in spring and summer 2022.

From each apiary of the province of Bologna, 6 colonies were selected, and 15 honeybees were sampled from each colony, while in the apiary of the province of Rimini 3 colonies were selected and 21 honeybees were sampled from each one. The specimens were collected from the external frames in all the colonies investigated. The search for intestinal flagellates was performed on single guts sampled from each honeybee using the culture method previously reported (Ribani et al., 2021). Briefly, the gut of each honeybee was dipped and grinded in 0.5 mL of supplemented DS2 medium and incubated at 26 °C. Wet mount slides with 10 µL of each culture were observed with light microscope at 3 and 7 days after the incubation, to verify the presence of free active flagellates. Some cultures were maintained by subculture steps every 4–10 days in fresh medium (ratio 1:5).

Morphological observation insights and image acquisition were performed on some positive cultures, in May-Grunwald Giemsa stained slides, at 400 × and 1000 × magnification through Leica DMLS light microscope (Leica, Wetzlar, Germany), equipped with a digital camera Nikon DS-Fi2 with imaging software NIS Elements 4.10.01 (Nikon, Tokyo, Japan). The scanning electron

microscopy (SEM) analysis was also performed as follows: pelleted cells were fixed with 3% glutaraldehyde in phosphate buffer (for 2 h at room temperature, then overnight at 4 °C), washed three times in PBS, dehydrated in a graded ethanol series and dried with hexamethyldisilazane. Subsequently, samples were mounted on aluminum stubs, sputter coated with gold-palladium using a SC7620 Mini Sputter Coater (Quorum Technologies) and observed using a Phenom XL G2 Desktop SEM operating at 10 kV. Axenic cultures of reference strains of *C. mellifica* (ATCC 30254) and *L. passim* (Ribani et al., 2021) maintained on the same medium at the same condition of incubation were used for morphological comparisons.

3. Results and Discussion

To microscopical examination, the presence of trypanosomatid flagellates was observed in several cultures of bee gut, mostly after 7 days of incubation. Overall, of the 360 bees analyzed from the Bologna province, 81 (22.5%) were positive for flagellates in culture [CI 95%; 18.19 - 26.81]. The total positivity rate was similar to the ones of each apiary (Table 1). No positivity occurred in the samples from the province of Rimini.

Apiary	n. examined colonies from each apiary	n. examined honeybees for each colony	average number of positive honeybees for each colony (min-max)	number of honeybees for each apiary	positive honeybees for each apiary (number and %)	confidence interval IC 95%
Bologna A	6	15	3.5 (2-5)	90	21 (23.3%)	14.6 – 32.0
Bologna B	6	15	3.5 (0-7)	90	21 (23.3%)	14.6 – 32.0
Bologna C	6	15	2.6 (0-10)	90	16 (17.7%)	9.8 – 25.3
Bologna D	6	15	3.8 (0-9)	90	23 (25.5%)	16.5 – 34.5
Rimini	3	21	0	63	0	0

Table 1. Maximum and minimum number of honeybees positive to flagellate morphologically identified as *Lotmaria passim* for each apiary, positivity frequencies and confidence interval in the apiaries.

Morphological observations performed either in wet slides or in slides stained with May Grunwald-Giemsa showed predominant elongated and tear-drop shaped cells typical of a promastigote morphotype. The cells narrowed posteriorly to a short caudate (tail-like) extension, often with a characteristic ‘‘nose” of the posterior end (Figure 1A, B, and C), consistent with the description of

L. passim (Schwarz et al., 2015; Ribani et al., 2021; Buendía-Abad et al., 2022). These characteristics were confirmed by SEM observation of some specimens. Moreover, in wet mounts, these trypasonomatids were actively moving. The morphology observed in the isolates, also in wet slides, clearly differed from that of a *C. mellificae* strain (ATCC 30254) grown in the same culture medium and incubation temperature (Figure 1- D, E, and F), used as a control. Moreover, *C. mellificae* showed much slower movements. Therefore, we can assume that the isolates observed in the present study can be attributable to *L. passim*.

To date, prevalence studies available in the literature have been carried out using molecular methods such as PCR (Bartolomé et al., 2018; Bordin et al., 2022; Stevanovic et al., 2016), or real time PCR (Arismendi et al., 2022; Cilia et al., 2022; Vejnovic et al., 2018; Xu et al., 2018) that quantified the parasitic load of *Lotmaria* on pools of bees. To our knowledge, no study evaluated the frequency of positive subjects within an infected colony. This information could be useful to better understand the degree of the parasite's diffusion among cohabiting bees, and to define in more detail the sample size needed to evaluate the presence/absence of the parasite. Moreover, albeit time consuming, isolation methods allow to observe the presence of living flagellates, amplifying their number (observation after 7 days gave us the highest number of positives), thus allowing to verify the actual colonization of the bee intestine and to obtain strains which could be further studied.

In the present paper, *L. passim* was absent in the bees of the organic apiary from the province of Rimini, while it was observed in all four apiaries tested in the province of Bologna. This high frequency may be due to the fact that these latter apiaries were held by the same beekeeper, who is used to exchange frames and brood between colonies. A wide variability was observed among the colonies of the same apiary, from negative ones to colonies with up to 66.6% of positive bees. This variability could explain the failure to find positive apiaries in Emilia Romagna by Cilia et al. (2022) by PCR on pools of ten bees from each colony. Differently, Ribani et al. (2021) reported in the same region a percentage of 88% of honey samples from different apiaries positive to *L. passim* DNA, suggesting a high diffusion of the parasite in the region.

Due to the limited number of colonies analyzed in the present study, it would not be appropriate to consider the results indicative of the prevalence of *L. passim* in the apiaries of Emilia-Romagna. Nevertheless, the current study provides evidence of the presence of bees actually colonized by this trypanosomatid in this region, and underlines the need for a deeper investigation regarding the epidemiology of this flagellate in Italy.

Declarations of interest: none.

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223 Figure captions:

224 Figure 1: Wet slides (A, D), May Grunwald-Giemsa-stained slides (B, E), and SEM (C, F) images

225 from cell cultures on Insectagro DS2 medium of a field isolate of *Lotmaria passim* (A, B, C) and

226 *Crithidia mellificae* ATCC 30254 (D, E, F). Bar = 10 micron (A-D and F) and 15 micron (C).

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