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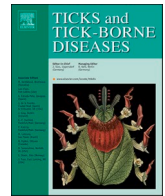
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Of fungi and ticks: Morphological and molecular characterization of fungal contaminants of a laboratory-reared *Ixodes ricinus* colony

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ABSTRACT

Establishing and maintaining tick colonies in the laboratory is essential for studying their biology and pathogen transmission, or for the development of new tick control methods. Due to their requirement for very high humidity, these laboratory-bred colonies are frequently subject to fungal contamination. In the present study, we aimed to identify the fungal species that contaminated a laboratory-reared colony of *Ixodes ricinus* through microscopic observation and molecular identification. We identified three different taxa isolated from the ticks: *Aspergillus parasiticus*, *Penicillium steckii*, and *Scopulariopsis brevicaulis*. These three species are usually regarded as environmental saprophytic molds but both direct and indirect evidence suggest that they could also be considered as entomopathogenic fungi. Although we do not have any direct evidence that the fungi isolated from *I. ricinus* in this study could cause lethal infections in ticks, we observed that once infected, heavy fungal growth coupled with very high mortality rates suggest that studying the entomopathogenic potential of these fungi could be relevant to biological tick control.

1. Introduction

Due to the altered distribution of some tick species and the emergence of several tick-borne diseases, there is an urgent need to identify new methods to combat these major vectors that impact both human and animal health (Ogden, 2017; Wikel, 2018). Current control methods, consisting largely of the application of chemical acaricides, are mostly ineffective and/or unsustainable (development of resistance, environmental impact, cost). We must broaden our understanding of tick-host interactions in order to develop more effective and sustainable control measures. It is unlikely that these will emerge unless significant progress can be made in understanding the complex tripartite relationship between ticks, pathogens, and hosts (de la Fuente et al., 2017). To this end, detailed studies on tick biology and tick-borne pathogen (TBP) transmission require the use of large numbers of live ticks raised under controlled conditions.

However, several problems routinely encountered when maintaining productive laboratory colonies doubtlessly contribute to the existing gaps in our knowledge of ticks and TBP. Establishing and maintaining

laboratory tick colonies continues to remain a challenge, and is especially difficult for hard ticks (Bonnet and Liu, 2012). Difficulties lie in the fact that ticks are obligatory hematophagous arthropods and require an animal host to survive and reproduce, that their blood meal is of long duration compared to other hematophagous arthropods and, that some species have not adapted to alternative hosts. Different techniques have been developed to feed hard ticks under laboratory conditions, either using artificial feeding systems (Kuhnert, 1996; Bonnet et al., 2007; Krober and Guerin, 2007; Gonzalez et al., 2017; Hart et al., 2018), or live animals (Ghosh and Azhahianambi, 2007; Faccini et al., 2010; Levin and Schumacher, 2016; Almazan et al., 2018; Mateos-Hernandez et al., 2020).

In addition to meeting these trophic requirements, tick enclosures must also closely mimic natural ecological conditions to ensure optimal development. Thus for each tick species, the temperature, day/night cycle variation, and the degree of humidity must be precisely controlled (Patrick and Hair, 1975; Troughton and Levin, 2007; Gray et al., 2016; Levin and Schumacher, 2016). Most tick species are highly vulnerable to desiccation and thus require very high relative humidity of above 80%.

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Such high humidity favors the development of filamentous fungi which is recognized as a major complication in tick colony maintenance. The importance of avoiding fungal contamination in laboratory tick colonies is almost systematically mentioned in reference books or papers (Ghosh and Azhahianambi, 2007; Troughton and Levin, 2007; Levin and Schumacher, 2016; Nuss et al., 2017), but, to our knowledge, none have provided a precise identification and description of the fungal species involved. Thus, following recurrent contaminations in our *Ixodes ricinus* tick colony, the purpose of the present study was, at first, to identify the causative agents responsible for these contaminations. This crucial first step is essential in order to maintain a healthy sustainable tick colony unaffected by fungi, allowing us to continue our various tick researches, including those on the potential of fungi as biocontrol agents against ticks.

2. Material and methods

2.1. Ticks

Ixodes ricinus ticks originally collected from the Sénart Forest, France (coordinates 48°40'00"N 2°29'00"E), were maintained as a "pathogen-free" colony at the tick rearing facility of ANSES (The French Agency for Food, Environmental and Occupational Health & Safety). They were reared at 22 °C with 95% relative humidity and a 12 h light/dark cycle as previously described (Bonnet et al., 2007). Ticks were engorged either on a membrane feeding system, rabbits, or sheep (Bonnet et al., 2007; Almazan et al., 2018, 2020). The ticks used in this study were those found dead in the colony, which is monitored every two days.

2.2. Microscopy

Both nymphs and female *I. ricinus* that had died one to two days prior were stored in 100% ethanol for scanning electron microscopy. None of the collected corpses displayed macroscopic evidence of filamentous fungus development. Prior to investigation, ethanol was fully evaporated overnight, in a Petri dish, and tick samples were coated with gold (Gatan Ion Beam Coater 681, USA). Samples were visualized with a Jeol JM6700 F scanning microscope (Jeol, Japan) and analyzed for the presence of fungal hyphae and spores. Image adjustment was performed in Adobe Photoshop CC 2020 (Adobe Systems Incorporated, CA, USA).

2.3. Isolation of fungal isolates and morphological identification

Fungi were isolated from additional dead *I. ricinus* females with macroscopic evidence of filamentous fungus development (moldy aspect) that appeared seven to eight days after death (Fig. 1). A total of 20 ticks were collected and further deposited on plates containing Sabouraud dextrose agar with added chloramphenicol (0.5 g/L) (Merck

KGaA, Darmstadt, Germany) to limit bacterial development. Plates were incubated at 22 °C for one week before isolates with different morphological appearances were sub-cultured under identical conditions. Macroscopic and microscopic examinations were performed for an initial identification.

2.4. Molecular identification of fungal species

To confirm initial identification, the ITS rDNA region (ITS1–5.8S–ITS2 cluster) was sequenced from isolated colonies. DNA was extracted from seven-day-old colonies using the QIAamp DNA Mini Kit (Qiagen GmbH, Germany). The ITS rDNA region, routinely used for fungal identification (Schoch et al., 2012), was amplified with the primers ITS1 (Gardes and Bruns, 1993) and ITS4 (White et al., 1990). Automated sequencing was performed at IMRB Sequencing Service (UPEC, Creteil, France) using both terminal primers. Obtained sequences were compared with known sequences listed in the GenBank nucleotide sequence database using the BLAST search option of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST), and submitted to GenBank.

3. Results

3.1. Macroscopic and microscopic examination of tick fungal contamination

Although fungal contamination was not macroscopically evident two to three days following tick death, microscopic observation revealed characteristic fungal structures with mycelial growth on tick cuticles (Fig. 2). Both regular and branched filaments were present. Chains of spores (conidia) were also detected (Fig. 2), indicating active fungal development. Fungal growth colonizing the whole tick body was visually apparent at one week after death (Fig. 1). At this stage, sporulation was intense.

3.2. Identification of fungal isolates

Moldy tick corpses systematically produced positive mycological cultures. A unique type of fungal colonies could be cultured from each tick sample, indicating that contamination of a tick was due to a single fungal type. Three colony types were detected. Macroscopic and microscopic colony examinations indicated that tick corpses were infected with *Aspergillus*, *Penicillium*, and *Scopulariopsis* genera of the subdivision Pezizomycotina (Fig. 3). Molecular analysis based on ITS sequences led to the identification of three different taxa from the ticks: *Aspergillus parasiticus* (GenBank accession number MW629712, 99.47% similarity to sequence MK165726.1), *Penicillium steckii* (GenBank accession number MW679680, 99.53% similarity to sequence

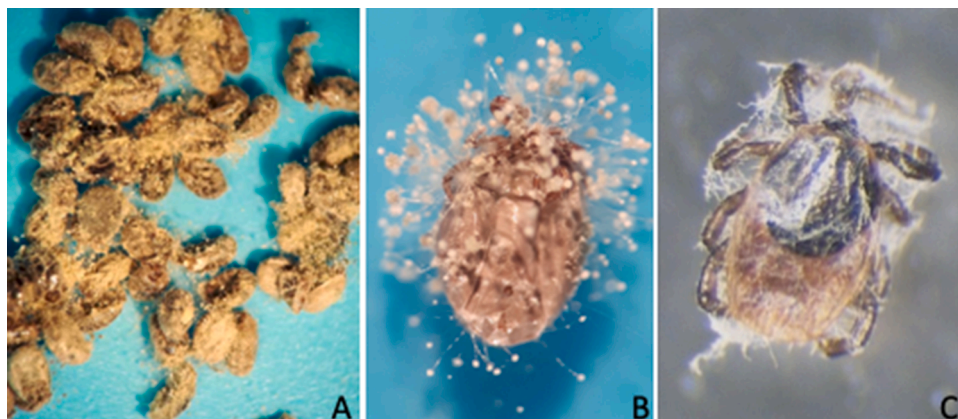


Fig. 1. Macroscopic observation of tick corpses with filamentous fungi, 7–8 days after death. A, B: engorged *I. ricinus* larvae. C: unfed *I. ricinus* female.

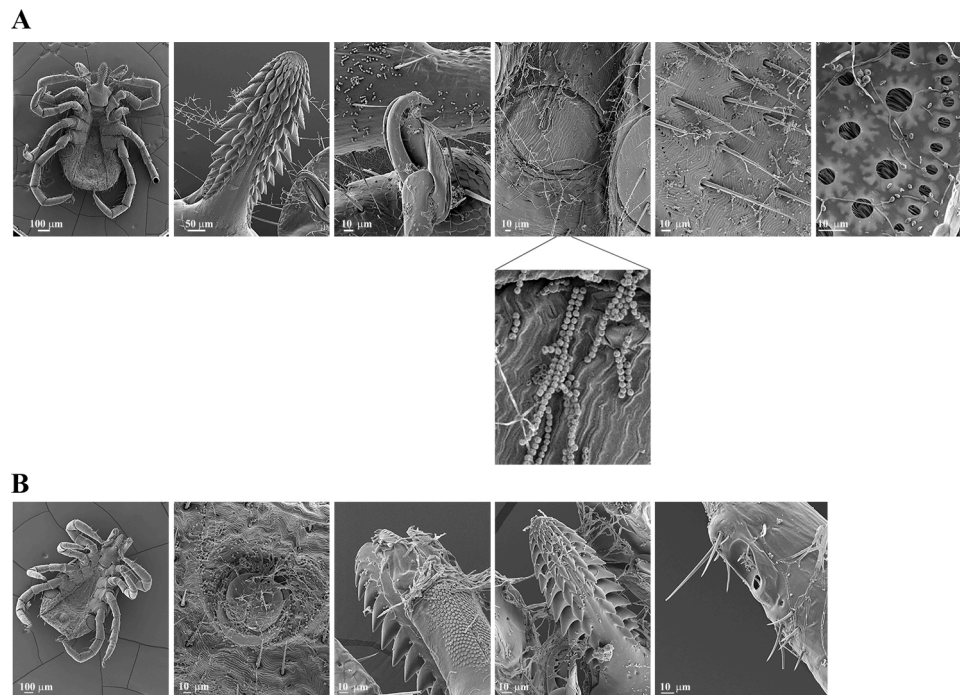


Fig. 2. Electron microscopic observation of tick corpses with filamentous fungi 1–2 days after death. Both hyphae (fungal filaments) and conidia (external asexual spores) are visible. A: ventral face (1), hypostome (2), pulvillus (3), genital aperture (4,5), cuticular sensory hairs (6), spiracle (7) of a dead *I. ricinus* female. B: ventral face (1), anal aperture (2), mouthparts (3), hypostome (4), Haller's organ (5) of a dead *I. ricinus* nymph.

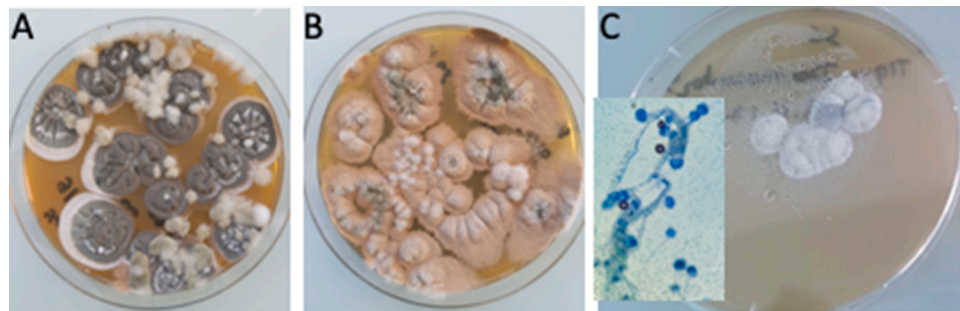


Fig. 3. Macroscopic observation of mold culture from female *I. ricinus* corpses with filamentous fungi. A: *Penicillium steckii*; B: *Scopulariopsis brevicaulis*; C: *Aspergillus parasiticus*.

MK179265.1), and *Scopulariopsis* (=Microascus) *brevicaulis* (GenBank accession number MW679678, 99.53% similarity to sequence MT609891.1).

4. Discussion

While entomopathogenic fungi, mostly focused on *Beauveria bassiana* and *Metarhizium anisopliae*, are of interest in the biological control of ticks (Kaay and Hassan, 2000; Kaaya, 2000; Lonc et al., 2014), including for *I. ricinus* (Hartelt et al., 2008; Alekseev, 2011; Wassermann et al., 2016; Szczepanska et al., 2020), they are instead the nightmare of tick breeders. Although many papers have been published on pathogenic fungi isolated from insects, there are only three studies concerning fungal infections of *I. ricinus* collected in the field (Samsinakova et al., 1974; Kalsbeek et al., 1995; Munteanu et al., 2014), and none investigating the natural contamination of laboratory-bred ticks. Following recurrent contaminations in our *I. ricinus* colony, we therefore aimed to identify the culprit fungal species. Macroscopic and microscopic observations of tick corpses followed by molecular confirmation, identified the presence of three different fungal species: *P. steckii*, *A. parasiticus*,

and *S. brevicaulis*.

Usually regarded as saprophytic fungi living in the soil, several *Penicillium* species have been isolated from insects like houseflies (Phoku et al., 2016), but also from field-collected *I. ricinus* (Samsinakova et al., 1974). *Penicillium steckii* has also been isolated from preserved *Meloe proscarabaeus* cadavers stored in a Korean museum (Lamsal et al., 2013). Although not commonly considered entomopathogenic, several species of *Penicillium* demonstrated pathogenicity to *I. scapularis* in a study aimed to identify potential entomopathogenic fungi from both soil and tick samples in the USA (Greengarten et al., 2011). In laboratory bioassays tick mortality rates ranged from 10 to 100%, depending on the fungal species, and field trials have shown that mean tick mortality in *Penicillium soppii*-treated plots was 26% higher than in control plots. These results suggest that *Penicillium* species should be given more attention as potential entomopathogenic fungi, especially in relation to ticks.

Aspergillus species are common saprophytic fungi from both outdoor and indoor environments as well as from food products, and have been isolated from both alive (Phoku et al., 2016) and dead arthropods (Kachapulula et al., 2018). Several species from this genus, including

A. parasiticus, are listed by some authors as belonging to those entomopathogenic chitinase-producing fungi that may be used in pest and vector biocontrol (Drummond and Pinnock, 1990; Lonc et al., 2014; Greeshma Rao and Narladkar, 2018), especially following the demonstration that *Aspergillus clavatus* and *Aspergillus flavus* can be as virulent as *M. anisopliae* against mosquito larvae (Bawin et al., 2016). To date and to our knowledge, only two *Aspergillus* species have been reported from natural infections of tick species. A study performed in Mexico reported that approximately 6% of engorged *Rhipicephalus microplus* females collected on cattle were infected with *A. flavus* (Miranda-Miranda et al., 2012). The authors also demonstrated that fungal spores sprayed over healthy ticks and their egg masses caused a 64% mortality rate in engorged females, as well as decreased egg mass, egg eclosion rate, and larval survival. In Spain, *Aspergillus ochraceus* infection was reported in fed *Rhipicephalus sanguineus* females collected on cottontail rabbits (Estrada-Pena et al., 1990). This natural infection completely blocks oviposition and kills the ticks, and the same effect was reproduced after experimental infection when ticks were placed under near saturated relative humidity. Finally, one *in vitro* study demonstrated that protease enzymes from soil-collected *Aspergillus sojae* and *Aspergillus oryzae* produced an efficient ovicidal effect on *Hyalomma dromedarii* eggs (Habeeb et al., 2017), where another established that chitinases extracted from *A. fumigatus*-positive soil samples caused 80% mortality in engorged *R. microplus* females (Hassan et al., 2015).

Scopulariopsis species are common saprophytic fungi found in the soil, air, plant debris, paper, dung, and moist indoor environments. Very few cases of human infection have been reported, and of those, most were caused by *S. brevicaulis* (Woudenberg et al., 2017; Perez-Cantero and Guarro, 2020). This species has also been isolated from field-collected ticks including from the cuticles of *I. ricinus* (Samsinikova et al., 1974) and *Amblyomma lepidum* (Suleiman Elham et al., 2013), and also from the cuticle and body contents of both *Dermacentor variabilis* (Yoder et al., 2003) and *R. sanguineus* (Benoit et al., 2005). In addition, it has been reported that topical exposure to *S. brevicaulis* spores has an entomopathogenic effect on all life stages of both *Hyalomma anatolicum* and *A. lepidum* (Suleiman Elham et al., 2013). *Scopulariopsis brevicaulis* pathogenicity was also demonstrated where topical application on unfed *D. variabilis* females resulted in 100% mortality after 15 days, when a marked tick mortality was also reported on *Dermacentor albipictus* eggs and larvae (Yoder et al., 2003, 2019). However, these authors also showed that the presence of *S. brevicaulis* within the integumental glands and hemocoel of *D. variabilis* protected the tick against dehydration caused by another fungus, *M. anisopliae* (Yoder et al., 2008). This latter result evokes a mutualistic symbiosis between *S. brevicaulis* and the tick, as has been suggested for other arachnids and commensal fungi (Gibbons et al., 2019). The demonstration of vertical transmission through *D. variabilis* egg contamination in the female genital tract is also in favor of such an endosymbiosis (Benoit and Yoder, 2004). Similar findings have been recently reported for the *D. albipictus* tick where vertical transmission of *S. brevicaulis* due to pre-oviposition egg exposure in the female's genital chamber was found (Yoder et al., 2019). It is also noticeable that in *in vitro* studies, *S. brevicaulis* inhibited the growth of other fungal species isolated from external tick surfaces (Benoit et al., 2009).

A key question concerning the fungi isolated from our laboratory-bred ticks is their origin. Each year, we add ticks from the field to our colony in order to maintain the genetic diversity. As these fungi are common molds in the natural environment, we cannot exclude contamination via this external source, even though attempts are made to remove superficial contamination by carefully washing each individual prior to integration into the colony. Although ticks are maintained in a Safety Level 2 laboratory with high levels of cleanliness, contaminants could potentially be introduced by staff members. Contaminants could also be acquired from the host while feeding on live mice, rabbits, or sheep, although the biting site is always disinfected before the ticks are fed. Finally, anti-fungal compounds are used to avoid

contamination in the *in vitro* feeding systems, thus this route is unlikely. (Liu et al., 2014; Oliver et al., 2016; Hart et al., 2018). Consequently, in order to maintain laboratory-reared tick colonies in good condition and without contamination it is absolutely necessary to undertake the following precautions: clean any reusable instruments with disinfectant spray before and after working with each tick cohort; clean and dry ticks derived from the field and when they have fed on animals before (re)-integrating them into the colony; limit numbers of ticks per holding container, and move freshly molted ticks into clean containers to avoid accumulation of waste that promotes fungal growth.

The other important question is whether these fungi, as opportunistic ones, invade ticks postmortem or have true entomopathogenic abilities and thus could be responsible for lethal infection in ticks. Adhesion, germination, and conidia production are recognized as the main virulence factors of entomopathogenic fungi. To colonize the host, fungi use germ tubes secreting cuticle-degrading hydrolytic extracellular enzymes to penetrate the cuticle. They then proliferate within the hemocoel until all internal tissues have been degraded, thereby killing the host. These fungi also release mycotoxins which may also be partly responsible for arthropod mortality (review in Greeshma Rao and Narladkar, 2018). In this respect, all three fungal species identified here could be considered as entomopathogenic. In fact, it is conceivable that both *P. steckii* and *S. brevicaulis*—isolated from *I. ricinus* for the first time—are capable of killing ticks when they contaminate their cuticle. Similarly, as several *Aspergillus* species can synthesize extracellular chitinase enzymes (St. Leger et al., 1993), it would be interesting to know if *A. parasiticus*—also isolated from *I. ricinus* for the first time—is able to invade live ticks as an entomopathogenic fungus. The microscopic observations made here do not indicate whether hyphae of the identified fungi are capable of penetrating the ticks. However, even if the cuticle is not penetrated, infection can occur through various tick apertures as has already been reported (Estrada-Pena et al., 1990; Samish and Rehacek, 1999). We also cannot exclude a parasitic relationship, especially because empirical observation showed that when contamination was identified on corpses, it invaded the entire tick colony. It may therefore be of interest in the future to study the entomopathogenic power of these fungi in order to assess their potential to biologically control *I. ricinus*, the most important vector affecting both human and animal health in Europe (Rizzoli et al., 2014). To our knowledge, only a few studies have investigated the efficacy of potential biological control of this tick species, reporting that biological control of *I. ricinus* may be possible. By assessing the impact of field strains of *B. bassiana*, *Paecilomyces fumosoroseus*, and *M. anisopliae*, Hartelt et al. (2008) showed that the latter fungus caused an 80% mortality rate in unfed nymphs. Wassermann et al. (2016) and Pirali-Kheirabadi et al. (2016) studied the efficacy of *M. anisopliae* blastospores against *I. ricinus* and reported a reduction of molting success by up to 90% in engorged larvae and nymphs, and a 100% mortality in fed females, respectively. Very recently, Szczepańska et al. (2020) tested several environmental and commercial strains of *Metarhizium* sp., *Beauveria* sp., and *Isaria* sp. against *I. ricinus*, and demonstrated that the most effective was an environmental strain of *M. anisopliae* with a mortality of up to 100% in unfed ticks.

In conclusion, fungal contamination is a significant hindrance in maintaining tick colonies in the laboratory, so many precautions must be taken to avoid it. However, these contaminations could also create new research avenue opportunities in tick control. In fact, it has been established that the ability of fungi to infect ticks is highly species-specific and that fungal potency and activity is largely dependent on climatic/environmental factors such as temperature and relative humidity. In that context, identification of fungal species responsible for tick mortality in laboratory-reared colonies under environmental conditions adapted to the target tick species' activity periods could represent a relevant method by which to identify promising microbial agents for biological tick control. Such a strategy could also potentially render it possible to identify more fungal species for each tick species concerned, thus avoiding potential side-effects on non-target

organisms—including insects—that are feared from fungal species with broad host ranges such as *B. bassiana* and *M. anisopliae*.

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Declaration of Competing Interests

No conflicts and competing interests exist. The authors certify that they have participated in and concur with the submission and accept responsibility for the manuscript's contents. This manuscript has not been submitted for publication in another journal.

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