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Proceeding Paper

Presence and Distribution of Human-Related Microsporidian Spores across Different Topsoil Areas across Alcalá de Henares (Spain) †

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Abstract: Humans can potentially be exposed to *Enterocytozoon bieneusi* and *Encephalitozoon* spp. from topsoils when playing or spending time in recreational areas. Two hundred and seventy-seven topsoil samples were collected across Alcalá de Henares (Spain) in July 2017: one hundred and fifty-five urban, sixty industrial and twelve from a public garden. Simultaneous detection was performed using a SYBR Green real-time PCR following the appropriate extraction of DNA with Fast-Prep for Soil®. The organic matter content (OM), pH, electrical conductivity (EC) and soil texture (percentages of sand, clay and silt) were also determined. *E. bieneusi* was detected in nine (five urban, four industrial); meanwhile, *Encephalitozoon* spp. was detected in 22 topsoil samples (sixteen urban, six industrial; ten *E. intestinalis*, nine *E. intestinalis*/*E. hellem* and three *E. cuniculi*). The presence of *E. bieneusi* was associated with urban soils that presented lower EC (0.50 vs. 0.71 dS/m; p-value = 0.0110), as this factor may provide a richer environment for the survival of spores. The presence of microsporidian spores was higher in those topsoils with a higher OM content for *E. bieneusi* (6.96% vs. 4.98%; p-value = 0.0342) and *E. intestinalis*/*E. hellem* in one of the four quadrants into which the urban area was divided (5.54% vs. 3.12%; p-value = 0.0007). *E. intestinalis* is present in industrial topsoils with significantly lower contents of sand (14.5% vs. 21.74; p-value = 0.00003) but higher contents of silt (78.5% vs. 64.9%; p-value = 0.0229), which might be attributed to the differences in the capacity of topsoils to retain moisture, depending on their texture. Moreover, the provision of enough oxygen might play a role in the higher presence of *E. intestinalis*/*E. hellem* in urban topsoils with lower contents of clay (11% vs. 19%; p-value = 0.0200). A better understanding of these potential associations is critical in selecting appropriate decontamination techniques and strategies to prevent and minimise human exposure.

Keywords: microsporidia; urban topsoils; physicochemical properties; soil texture; risks; Alcalá de Henares

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1. Introduction

Microsporidia are a diverse group of unicellular obligate intracellular parasites that have been recently classified as fungi, either as a basal branch or as a sister group [1].

Phylogenetic studies suggest that microsporidia are closely related to a group of obligate intracellular parasites called the Cryptomycota or Rozellomycota [2]. About 1400 microsporidia species distributed within 200 genera that appear to infect all animals have been described [3]; however, to date, only nine genera have been shown to be capable of producing infections in humans [4]: *Vittaforma*, *Anncaliia*, *Pleistophora*, *Encephalitozoon*, *Enterocytozoon*, *Trachipleistophora*, *Tubulinosema*, *Endoreticulatus* and *Microsporidium*. Within these genera, seventeen species are related to human infection [5], the most relevant being *Enterocytozoon bieneusi* and *Encephalitozoon* species, which include *E. intestinalis*, *E. hellem* and *E. cuniculi*.

Human-related microsporidia species can affect both immunocompromised and immunocompetent individuals and produce oval-shaped microscopic spores that are highly resistant to harsh environmental conditions [1,4]. Although it is considered that microsporidia are ubiquitous in the environment, their diversity in soils and sand has been little explored, despite the literature suggesting that there could be a wide diversity of microsporidian species in soils and composts [3]. Thus, Ardila-Garcia et al. [3] identified 22 novel microsporidian sequences in soils, sands and composts collected from the Vancouver metropolitan area (BC, Canada) and Union Bay Natural Area in Seattle, USA.

Despite the fact that humans, especially children and immunodeficient individuals, could be potentially exposed to *Enterocytozoon bieneusi* and *Encephalitozoon* spp. from topsoils when playing or spending time in green and recreational areas in urban environments, previous studies have not detected these species in sands collected from playgrounds in parks in southern Madrid (Spain) [6]. However, these authors explored the presence of microsporidian spores using microscopic methods. As a result, we carried out a preliminary study using molecular methods and described, for the first time, spores of *E. bieneusi* and *Encephalitozoon* spp. in topsoils collected from urban parks across Alcalá de Henares (central Spain, northeast of Madrid) [7].

The main aim of this study was to study whether the physicochemical properties and/or the texture of the Alcalá's topsoils monitored across the different urban parks in the urban city and green areas and parks from an adjacent industrial site in Alcalá de Henares would impact the presence and distribution of *Enterocytozoon bieneusi* and *Encephalitozoon* spp.

2. Material and Methods

A total of 227 topsoil samples (0–3 cm depth) were collected in July 2017 across different urban (155) and industrial (60) areas in Alcalá de Henares (Spain) and in a public garden in the city centre (12), following previous methods described by our team, Peña-Fernández et al. [8]. Briefly, topsoil samples were dried at room temperature for 2 weeks, ground and sieved with a 2 mm sieve to remove stones, coarse materials and other debris. The collected topsoil samples were further prepared and concentrated as follows. Samples were kept in 15–20 mL of PBS (1% sterile) for 24 h in agitation to obtain a homogenised supernatant. After 24 h, supernatants were collected in sterile 50 mL Falcon tubes and centrifuged at 3000 rpm for 30 min. Pellets were collected and suspended in 5 mL of sterile PBS (1%); aliquots were kept at −80 C for the molecular analysis.

DNA was extracted by disrupting the spores using Fast-Prep for Soil® following the manufacturer's methodologies (MP Biomedicals, Solon, OH, USA), as described by Da Silva et al. [9]. PCR inhibitors were removed using a QIAquick PCR kit (QIAGEN, Chastsworth, CA, USA), followed by a SYBR Green real-time PCR according to the methods described by Polley et al. [10]. Species identification was based on the melting temperature (Tm) of the amplicons, as described by Andreu-Ballester et al. [11]. Spores of *Encephalitozoon intestinalis* (Cali et al.) from Hartskeerl et al. (nasal isolate from an HIV-seropositive individual; ATCC® 50507™; https://www.atcc.org/products/50507, accessed on 29 November 2023) and *Encephalitozoon cuniculi* from Levaditi et al. (urine from an AIDS patient; ATCC® 50602™; https://www.atcc.org/products/50602, accessed on 29 November 2023) were used as positive controls. A positive control was also used for *E. bieneusi*, which was kindly provided by the parasitology group at the University of San Pablo CEU (Spain).

Topsoils were also further screened for organic matter content (OM), pH, electric conductivity (EC) and texture (percentages of sand, clay and silt), according to the Spanish official methodology for soil analysis [12]. Briefly, the pH and EC were measured in suspension using a 1:2.5 (w/v) ratio of soil and deionised water; the OM content was analysed using the Walkley–Black method; and the soil texture was determined using a Bouyoucos densitometer [13].

Statistical analyses were performed using the free software R-project, version 4.1.0 [14]. Significance scores were based on Student's *t*-test. Differences were considered statistically significant at *p*-values lower than 0.05.

3. Results and Discussion

E. bieneusi was detected in nine topsoil samples (five urban, four industrial); meanwhile, *Encephalitozoon* spp. was detected in twenty-two topsoil samples (sixteen urban, six industrial; ten *E. intestinalis,* nine *E. intestinalis/E. hellem* and three *E. cuniculi*). Our results are in line with a pilot study performed by our group in 2016, in which these species were detected in twenty-eight samples (topsoil and sand from a kid's playground) collected from five parks across Alcalá city [15]; with a preliminary test performed in which only sixty of all these samples were processed [16]; and with our previous presentation [7]. To our knowledge, this is the first report reporting the presence of human-related microsporidian spores in topsoils from Alcalá de Henares in urban and industrial areas and the first time that these emerging human parasites have been detected and further explored in an urban area in the Madrid region. Thus, Dado et al. [6] did not observe spores of microsporidia after processing six hundred and twenty-five sand samples collected from 67 parks in southern Madrid. However, this could be attributed to the fact that these samples were only processed using microscopical methods. The type of sample, i.e., sand, might also affect the presence of these emerging species. Thus, similar reports exploring the presence of helminth eggs have reported higher frequencies in samples collected across sports fields and park playgrounds than from fenced sandpits [17].

The lower presence of *E. bieneusi* in Alcalá topsoils *versus* the presence of *Encephalitozoon* spp. might be explained by unknown behavioural and/or characteristics of their spores in topsoils, as *E. bieneusi* is known to affect a large variety of vertebrate hosts, so a higher frequency was expected, as urban animals, including mammals and birds, could act as potential reservoirs, which would facilitate the environmental contamination of cities and its continuous transmission. Thus, Galván-Díaz et al. [18] observed a higher frequency of *E. bieneusi* than *E. intestinalis* in faeces from different animal species collected in Spain, including Madrid.

The presence of *E. bieneusi* was associated with urban soils that presented lower EC (0.50 vs. 0.71 dS/m; *p*-value = 0.0110), as this factor may provide a richer environment for the survival of spores. The presence of microsporidian spores was mostly more intense in those topsoils with higher OM contents, irrespective of the area. Thus, significant differences were observed for *E. bieneusi* spores when considering all the topsoils monitored together (6.96% vs. 4.98%; *p*-value = 0.0342) and for *E. intestinalis/E. hellem* in one of the four quadrants into which the urban area was divided (5.54% vs. 3.12%; *p*-value = 0.0007). The OM content can modify the physical and chemical properties of the soil by changing its texture and preserving the soil moisture [19], which might contribute to the presence of these spores.

The presence of any of these parasitic species did not exhibit a statistical relation with the pH of the topsoils (range = 6.25–7.95) for any of the areas monitored. This could be attributed to the fact that the optimum pH range for most soil microorganisms has been described to be from 5 to 8 [20].

In relation to the topsoil textures, *E. intestinalis* was present in industrial topsoils with a significantly lower content of sand (14.5% vs. 21.74%; *p*-value = 0.00003) but a higher content of silt (78.5% vs. 64.9%; p-value = 0.0229), which might be attributed to the differences in the capacity of topsoils to retain moisture depending on their texture [19]. These authors have found lower frequencies of protozoan and helminth eggs in soils with higher percentages of sand than in those with a high presence of silt and clay, which they explain as being due to the poor retention of water in sandy soils. However, spores of *E. intestinalis*/*E. hellem* were more present in those topsoils with lower contents of clay in the main urban area (11% vs. 19%; p-value = 0.0200). Paller and De Chavez [21] have observed more eggs from soil-transmitted helminths in sandy soils than in clayey soils, which they explain as being due to an insufficient provision of oxygen in clayey soils for egg development. Thus, the provision of enough oxygen might impact the persistence of microsporidian spores in topsoils, which should be further explored. Clear information on the impact of soil physicochemical factors and texture on the presence and distribution of emerging human-related microsporidian spores is necessary to identify the appropriate public health interventions and decontamination options/techniques to protect human health and minimise potential exposures [22].

4. Conclusions

To our knowledge, this is the first report discussing the presence and distribution of spores of *E. bieneusi* and *Encephalitozoon* species (*E. intestinalis*, *E. hellem*, *E. cuniculi*) in topsoils across urban and industrial locations in Alcalá de Henares and the first report reporting the presence of these emerging zoonotic parasites in soils from the Madrid region. A better understanding of these potential associations is critical to selecting appropriate decontamination techniques and strategies to prevent and minimise human exposure.

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