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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 be potentially exposed to *Enterocytozoon bieneusi* and *Encephalitozoon* spp. from topsoils when playing or spending time in recreational areas. 227 topsoil samples were collected across Alcalá de Henares (Spain) in July 2017: 155 urban, 60 industrial and 12 from a public garden. Simultaneous detection was performed using a SYBR Green real-time PCR following appropriate extraction of DNA with Fast-Prep for Soil®. 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 9 (5 urban, 4 industrial) meanwhile *Encephalitozoon* spp. in 22 topsoil samples (16 urban, 6 industrial; ten *E. intestinalis*, nine *E. intestinalis*/*E. hellem* and three *E. cuniculi*). The presence *E. bieneusi* was associated within urban soils that presented lower EC for (0.50 vs. 0.71 dS/m; *p*-value = 0.0110), as this factor may provide a richer environment for the survival of the spores. The presence of microsporidian spores where higher in those topsoils with higher OM, for *E. bieneusi* (6.96% vs. 4.98%; *p*-value = 0.0342) and *E. intestinalis*/*E. hellem* in one of the four quadrants in which the urban area was divided (5.54% vs. 3.12%; *p*-value = 0.0007). *E. intestinalis* is present in industrial topsoils with significant lower content of sand (14.5% vs. 21.74; *p*-value = 0.00003) but 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 in retain moisture in function of 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 content of clay (11% vs. 19%; *p*-value = 0.0200). A better understanding of these potential associations is critical to select 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]. There has been described about 1400 microsporidia species distributed within 200 genera that appear to infect all animals [3]; however, to date, only nine genera have 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], been the most relevant *Enterocytozoon bieneusi* and *Encephalitozoon* species, which include *E. intestinalis*, *E. hellem*, *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 suggests that there would be a wide diversity of microsporidian species in soils and composts [3]. Thus, Ardila-Garcia et al. [3] has 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 humans, and specially 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 from parks from 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 on the presence and distribution of *Enterocytozoon bieneusi* and *Encephalitozoon* spp.

2. Material and Methods

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 in 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. Collected topsoil samples were further prepared and concentrated as follows. Samples were kept with 15–20 mL 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 molecular analysis.

DNA was extracted by disrupting the spores using Fast-Prep for Soil® following manufacturer methodologies (MP Biomedicals, Solon, OH, USA) as described by Da Silva et al. [9]. PCR inhibitors were removed using the QIAquick PCR kit (QIAGEN, Chastsworth, CA, USA), followed by 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.) Hartskeerl et al. (nasal isolate from an HIV-seropositive individual; ATCC® 50507™; https://www.atcc.org/products/50507) and *Encephalitozoon cuniculi* Levaditi et al. (urine from AIDS patient; ATCC® 50602™; https://www.atcc.org/products/50602) 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 the 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; OM content was analysed using the Walkley-Black method; 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 9 topsoil samples (5 urban, 4 industrial) meanwhile *Encephalitozoon* spp. in 22 topsoil samples (16 urban, 6 industrial; ten *E. intestinalis,* nine *E. intestinalis*/*E. hellem* and three *E. cuniculi*). Our results would be in line with a pilot study performed by our group in 2016, in which these species were detected in 28 samples (topsoil and sand from a kid playground) collected from five parks across Alcalá city [15], and 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 are detected and further explored in an urban area in the Madrid Region. Thus, Dado et al. [6] did not observed spores of microsporidia after processing six hundred and twenty-five sand samples collected from 67 parks from 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 sport 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 to 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] has 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 within urban soils that presented lower EC for (0.50 vs. 0.71 dS/m; *p*-value = 0.0110), as this factor may provide a richer environment for the survival of the spores. The presence of microsporidian spores was mostly more intense in those topsoils with higher OM, irrespective of the area. Thus, significant differences were observed for *E. bieneusi* spores when considered 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 in which the urban area was divided (5.54% vs. 3.12%; *p*-value = 0.0007). OM content can modify the physical and chemical properties of the soil by promoting the 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 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 significant lower content of sand (14.5% vs. 21.74%; *p*-value = 0.00003) but 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 in retain moisture in function of 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 high presence of silt and clay, which they explain 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 content of clay in the urban main 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 due to insufficient provision of oxygen that 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 from 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 select appropriate decontamination techniques and strategies to prevent and minimise human exposure.

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