Enterocytozoon bieneusi (Microsporidia): Identification of novel genotypes and evidence of transmission between sympatric wild boars (Sus scrofa ferus) and Iberian pigs (Sus scrofa domesticus) in Southern Spain

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Running Head: *Enterocytozoon bieneusi* in Spanish swine

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SUMMARY

Microsporidia is a phylum of obligate emergent intracellular protist-like fungi pathogens that infect a broad range of hosts including vertebrates and invertebrates. *Enterocytozoon bieneusi* is the most common cause of microsporidiosis in humans, affecting primarily immunosuppressed patients but also reported in immunocompetent individuals. Epidemiological information on the presence and molecular diversity of *E. bieneusi* in livestock and wildlife in Spain is limited. Therefore, the occurrence of this microsporidia was investigated in sympatric extensively reared Iberian pigs (n = 186) and free ranging wild boars (n = 142) in the province of Córdoba, Southern Spain. Forty-two Iberian pigs (22.6%) and three wild boars (2.1%) were found *E. bieneusi*-positive by PCR. In Iberian pigs, occurrence of *E. bieneusi* was significantly higher in sows than in fattening pigs (31.6% *vs.* 11.4%; p = 0.001). Five genotypes were identified in Iberian pigs, four previously reported (EbpA, PigEb4, O, Pig HN-II) and a novel genotype (named PigSpEb1), while only two genotypes were identified in wild boars, EbpA and novel genotype PigSpEb1. All five genotypes identified belong to Group 1 suggesting zoonotic potential. This study constitutes the first report on the occurrence and molecular characterization of *E. bieneusi* in Iberian pigs and wild boars. The identification of two genotypes with zoonotic potential in sympatric Iberian pigs and wild boars suggests that *E. bieneusi* can be potentially transmitted between those two hosts, but also implies that they may act as natural sources of microsporidia infection to other hosts including humans.

KEYWORDS: *Enterocytozoon bieneusi*; Iberian pig; wild boar; Spain; transmission; genotyping.

1. INTRODUCTION

Microsporidia is a diverse phylum of obligate intracellular and spore-forming pathogens that infect a wide range of invertebrate and vertebrate hosts (Stentiford et al., 2016). Currently, Microsporidia comprises 200 genera and 1,500 described species, of which 17 are infective to human beings (Weiss and Becnel, 2014). Among them, the most frequently reported species is *Enterocytozoon bieneusi* (Santín, 2015). Infections with *E. bieneusi* are mostly limited to the gastrointestinal tract and are typically associated with chronic diarrhoea and wasting syndrome in immunocompromised individuals such as cancer patients, organ transplant recipients, and the elderly (Matos et al., 2012). In immunocompetent subjects, clinical symptoms are usually self-limiting diarrhoea and intestinal malabsorption (Lobo et al., 2012). In addition to humans, *E. bieneusi* has been described in a wide variety of animals including mammals and birds that can serve as reservoirs of the pathogen (Santín and Fayer, 2011). Transmission routes of *E. bieneusi* are not completely elucidated, but infections are thought to result from faecal-oral transmission of spores from direct contact with infected humans or animals, or through ingestion of contaminated food and water (Stentiford et al., 2016).

Based on sequence analyses of the internal transcriber spacer (ITS) region of the rRNA gene, more than 400 genotypes of *E. bieneusi* have been described and allocated into 11 distinct genetic groups (Santín, 2015; Li et al., 2019b). Groups 1 and 2 contain potentially zoonotic genotypes, whereas the remaining (groups 3–11) comprise host-adapted genotypes associated with specific animal species (Li and Xiao 2019; Li et al., 2019b).

First report of *E. bieneusi* in pigs (*Sus scrofa domesticus*) was on a farm in Switzerland (Deplazes et al., 1996); subsequent epidemiological studies have documented the occurrence of the pathogen at infection rates ranging from 10–94% in

apparently healthy pigs globally (Table 1). Consequently, over 130 *E. bieneusi* genotypes have been identified in this host, including genotypes also reported in humans such as D, EbpA, EbpC, G, H, I, or O (Table 1), suggesting that pigs may act as reservoirs of human infections and can represent a public health concern (Li et al., 2019a). In contrast, information on *E. bieneusi* in wild boars (*Sus scrofa*) is far more limited. Only two studies, reporting a prevalence rate ranging from 4–41%, have been carried out in free-living animals in central Europe and in captive animals in China (Němejc et al., 2014; Li et al., 2017). To date, 26 *E. bieneusi* genotypes have been identified in wild boars, being the zoonotic EbpC and EbpA the most commonly reported (Table 2).

At least 34 swine pathogens including viral, bacterial, and parasitic agents have been demonstrated to cause clinical disease in livestock, poultry, wildlife, and humans (Miller et al., 2017). In the Iberian Peninsula, extensively reared Iberian pigs (traditional autochthonous breed of the domestic pig raised along Southern Spain and Portugal) share habitat (Mediterranean forest), water, and feeding resources (mainly acorn of *Quercus* spp. trees) with wildlife such as wild ungulates, mesocarnivores, leporids, and wild boars (Kukielka et al., 2016; Rivero-Juarez et al., 2018). Overlapping of domestic and sylvatic transmission cycles may facilitate cross-species disease transmission between free-living animal and livestock species, and vice versa (Ruiz-Fons et al., 2008). This situation represents a significant livestock industry problem associated to important economic loses, in addition to a public veterinary health concern (Gortázar et al., 2010). Although several epidemiological studies have reported the occurrence of *E. bieneusi* swine infections around the world (Table 1), only a single study has investigated the presence of this microsporidia in Spanish pigs (Galván-Diaz et al., 2014). No information is currently available in the country regarding the presence and

molecular diversity of *E. bieneusi* in wild boars. Therefore, this survey was carried out to determine the presence and genotype diversity of *E. bieneusi* in sympatric Iberian pig (raised in extensive conditions) and wild boar populations in Southern Spain, to evaluate cross-species transmission between both populations.

2. MATERIALS AND METHODS

2.1. Ethical statement

This study was carried out in accordance with Spanish legislation guidelines (RD 8/2003) and with the International Guiding Principles for Biomedical Research Involving Animals issued by the Council for International Organization of Medical Sciences and the International Council for Laboratory Animal Science (RD 53/2013).

2.2. Study area and sampling

The study was conducted in the north area of Córdoba province (Andalusia, southern Spain; 36°N–38°600 N, 1°750 W–7°250 W). Most pig farming in this area is extensive, with animals wandering in semi-liberty. The Iberian pig is an autochthonous breed of the Iberian Peninsula derived from the *Sus mediterraneus* with a characteristic habitat called "dehesa" consisting of Mediterranean holm-oak and cork-oak pastures (Garrido-Fernández and León-Camacho, 2019). Extensively farmed Iberian pigs represent around 10% of national pig farming production in Spain, and the southwestern regions contain approximately 80% of Spain's Iberian pig farms. Iberian pigs are extensively raised from 3 to 18 months under "dehesa" agroforestry systems, sharing natural resources with other domestic and wild species, mainly wild boar (Parra et al., 2003). For Iberian pigs, sow and fattening pigs share space and are managed under the same conditions.

126 Iberian pig farms included in this study were randomly selected by the
127 Environmental Department of the Regional Government of Andalusia for a previous
128 study aiming to determine the prevalence of Hepatitis E virus infection (Lopez-Lopez et
129 al., 2018). Population density of Iberian pigs in the study area in the 2015-2016 period
130 was 100–150 individual/km^2. Only extensive farms were sampled as those are the ones
131 with higher rate of contact with wild boars. The number of pigs to be randomly sampled
132 at each farm was calculated to ensure a 95% probability of detecting at least one
133 positive animal, assuming a minimum prevalence of 5%. This represented a sample size
134 of 40 animals per farm. Individual Iberian pig rectal faecal samples were collected
135 between October 1th and November 30th 2015.

136 For wild boars, individual rectal faecal samples were collected on animals
137 legally abated in the study area during the 2015/2016 hunting season (October 15th to
138 February 15th). Population density of wild boars in the study area in the 2015-2016
139 period was 3.0–3.5 individual/km^2. These wild boar samples were previously used to
140 determine the prevalence of Hepatitis E virus infection in this host (Rivero-Juarez et al.,
141 2018). The age of these animals was determined based on tooth eruption. Animals ≤24
142 months old were classified as young, and those over 2 years old as adults.

143 For the purpose of this study we retrospectively analysed DNA extracted from a
144 total of 328 faecal samples from extensively reared Iberian pigs (n = 186) and wild
145 boars (n = 142) sharing the same feeding areas (Figure 1). Genomic DNA was purified
146 using the RNeasy Plus Mini Kit (QIAgen, Hilden, Germany). Samples were deliberately
147 selected to match the sex and age group of the investigated animals.
148

149 **2.3. PCR and sequence analysis**

To identify *E. bieneusi*, a nested PCR protocol was used to amplify the ITS region as well as portions of the flanking large and small subunit of the ribosomal RNA gene as previously described (Buckholt et al., 2002). The outer (EBITS3 and EBTIS4) and inner (EBITS1 and EBITS2.4) primer sets were used to generate PCR products of 435 and 390 bp, respectively. Negative and positive controls were included in every PCR run. The amplicons of the second PCR were examined on 2% D5 agarose gels stained with Pronasafe (Conda, Madrid, Spain).

All amplicons of the expected size were directly sequenced in both directions with the internal primer pair in 10 µl reactions using Big Dye™ chemistries and an ABI 3730xl sequencer analyser (Applied Biosystems, Foster City, CA). Raw sequences were examined with Chromas Lite version 2.1 software (http://chromaslite.software.informer.com/2.1) to generate consensus sequences. These sequences were compared with reference sequences deposited at the National Center for Biotechnology Information (NCBI) using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The established nomenclature system based in ITS nucleotide sequence was used to determine *E. bieneusi* genotypes (Santín and Fayer, 2009). Sequences generated in the present study were deposited in the GenBank public repository database under accession numbers MN699287-MN699293.

2.4. Statistics analysis

The prevalence of *E. bieneusi* in Iberian pig and wild boar populations was calculated using the proportion of positive samples with respect to the total number of samples examined with 95% confidence interval (95% CI). In Iberian pigs, the prevalence was also calculated by animal type (sows versus fattening pigs), and in wild boars by gender (male versus female), and age (juvenile versus adult). These categorical

variables were expressed as numbers of cases (percentages) and the frequencies were compared using the χ^2 or Fisher's tests. The statistical significance was established at a *p*-value of less than 0.05. Analyses were carried out using SPSS statistical software package version 18.0 (IBM Corporation, Somers, NY, USA).

2.5. Phylogenetic analysis

Sequences obtained in this study as well as *E. bieneusi* sequences previously identified in livestock, wildlife, and environmental samples in Spain and appropriate reference sequences retrieved from GenBank were aligned with the Clustal W algorithm and the evolutionary distances between them were estimated using MEGA 6 (Tamura et al., 2013). Phylogenetic interference was carried out by the Neighbor-Joining (NJ) method as previously described (Saitou and Nei 1987). Genetic distance was calculated with the Kimura parameter-2 model.

3. RESULTS

Forty-two (22.6%, 95% CI: 16.8%–29.3%) of 186 Iberian pigs and three (2.1%, 95% CI: 4.0%–6.0%) of 142 wild boars examined were PCR-positive for *E. bieneusi* (Table 3). Prevalence of *E. bieneusi* was significantly higher among Iberian pigs than among wild boars (22.6% versus 2.1%; $p < 0.001$). In Iberian pigs, sows showed higher prevalence than fattening pigs (31.6% versus 11.4%; $p = 0.001$). In wild boars, no correlation was found between infection and gender and age (Table 3).

Nucleotide sequences analysis of the ITS region revealed five distinct genotypes circulating in Iberian pigs, including four previously reported (EbpA, O, PigHN-II and PigEb4) and a novel genotype (named PigSpEb1) (Table 4). Mixed infections involving genotypes EbpA+PigEb4 were detected in four Iberian pigs (Table 4). In those four

Iberian pigs, double peaks were observed at chromatogram inspection in the nucleotide sequences obtained from two independent PCR reactions for each sample. EbpA was the most prevalent genotype found in 52.4% of the *E. bieneusi* positive samples, followed by genotype O identified in 19% of the positive samples. Novel genotype PigSpEb1 was found in three animals (7.1%, Table 4). In wild boars two of the genotypes identified in Iberian pigs, EbpA and PigSpEb1, were found in two and one samples, respectively (Figure 2 and Table 4). Genotype PigSpEb1 nucleotide sequence differs only in one nucleotide in the ITS region with genotype CAM5 (99.6% similarity) reported previously in Bactrian camels (*Camelus bactrianus*) in China (MG602795; Qi et al., 2018). The accuracy of the novel genotype PigSpEb1 sequence was confirmed in two independent PCR and sequencing reactions.

Phylogenetic analysis showed that all genotypes identified in the present study clustered together within the potentially zoonotic Group 1 (Figure 3).

4. DISCUSSION

In Spain, *E. bieneusi* has been described in humans in immunocompromised patients (del Aguila et al., 1997a,b; López-Vélez et al., 1999; Lores et al., 2002b) and immunocompetent individuals (Abreu-Acosta et al., 2005; Galván et al., 2011), as well as in a wide range of animals host that includes companion, production, and free-living animal species (del Aguila et al., 1999; Lores et al., 2002a; Haro et al., 2005,2006; Galván-Diaz et al., 2014; Santín et al., 2018). In addition, spores of *E. bieneusi* have been identified in environmental (water) samples (Galván et al., 2013). In the present survey, *E. bieneusi* was identified in 23% and 2% of the investigated Iberian pigs and wild boars, respectively. Higher prevalence in pigs than wild boars is likely due to differences in animal population densities, susceptibility or risk of exposure to the

parasite. This constitutes the first report of *E. bieneusi* in wild boars in Spain. So far, *E. bieneusi* has only been reported in wild boars in Poland, Austria, Czech Republic, Slovakia (Němejc et al., 2014) and China (Li et al., 2017; Feng et al., 2020). In pigs, *E. bieneusi* has been frequently reported worldwide (Table 1), and there is only one study in Spain that has reported, in apparently healthy farmed pigs, the presence of *E. bieneusi* (Galván-Diaz et al., 2014). The *E. bieneusi* infection rate (23%) found in Iberian pigs was similar to the prevalence reported in pigs in Spain, but lower than prevalence reported in other countries including Brazil (59%), China (31–90%), Czech Republic (94%), Germany (33-67%) Japan (33%), Switzerland (35%), Thailand (28%) and the United States (32%) (Table 1). Lower prevalences of *E. bieneusi* were identified in other studies in China (16–18%), Egypt (13%), Germany (10%), Slovakia (19%), South Korea (14%), and Thailand (16-21%) (Table 1).

The prevalence found in wild boars (2%) in the present study was lower than those (4–42%) reported in previous studies conducted in Central Europe and China (Table 2). In Spain, the presence of *E. bieneusi* in wildlife has previously been documented in wild carnivores including European badgers (*Meles meles*, 23%), beech martens (*Martes foina*, 11%) and red foxes (*Vulpes vulpes*, 9–14%) (Galván-Diaz et al., 2014; Santín et al., 2018).

Five distinct genotypes were identified in Iberian pigs, four genotypes previously reported, EbpA (also identified as F), O, PigEb4, PigHN-II, and a novel genotype named as PigSpEb1. In domestic pigs in Spain, only genotype I (not found in this study) had been reported so far (Galván-Diaz et al., 2014), therefore this represents the first identification of genotypes EbpA, O, PigEb4, and Pig HN-II in extensively reared Iberian pigs in the Spain. Two genotypes, EbpA and novel PigSpEb1, were identified for the first time in Spanish wild boars.

EbpA, the most prevalent genotype in this study in Iberian pigs and wild boars, is commonly reported genotypes in pigs worldwide (Zhou et al., 2020). It was also the most abundant genotype in a survey in wild boar in Central Europe (Němejc et al., 2014) and the second genotype most frequently identified in captive wild boars in China (Li et al., 2017). EbpA has also been found in humans, in HIV-infected patients in Nigeria (Akinbo et al., 2012), in two hospitalized children in China (Wang et al., 2013), and in immunocompetent individuals in Czech Republic (Sak et al., 2008). In addition, EbpA has also been reported in non-human primates, pigs, cattle, dogs, goats, horses, mice, birds, and wild boars (Li et al., 2019b) (Table 2). Genotype O, the second most prevalent genotype identified in Iberian pigs in this study, was first reported in a pig in Germany (Dengjel et al., 2001), and later identified in domestic pigs in Brazil, Germany, Thailand, and China (Dengjel et al., 2001; Leelayoova et al., 2009; Reetz et al., 2009; Li et al., 2014a; Zhao et al., 2014; Fiuza et al., 2015; Wan et al., 2016). It has also been identified in other hosts including non-human primates, sheep, cattle, horses, and dogs in China (Karim et al., 2014,2015; Zhao et al., 2015; Li et al., 2016; Qi et al., 2016). In addition, O genotype has been identified in one HIV-infected adult patient in Thailand (Leelayoova et al., 2006), suggesting that this genotype may have zoonotic potential at least in immunosuppressed patients. PigEb4 was initially described in domestic pigs in Brazil (Fiuza et al., 2015), but years later detected also in pigs and sheep in China (Chen et al., 2018; Wang et al., 2018b; Li et al., 2019c). The only information currently available for genotype PigHN-II, identified in this study in 2 Iberian pigs, is that an identical ITS nucleotide sequence obtained from a pig isolate was deposited in GenBank under accession number MF406105.

EbpA and novel PigSpEb1 genotypes were simultaneously detected in Iberian pigs and wild boars. This is, to the best of our knowledge, the first molecular

epidemiological evidence of the occurrence of cross-species transmission of *E. bieneusi* between both sympatric host populations that share the same habitat, including food and water resources. At present, both the frequency and directionality of these events remain unknown and should be further investigated in future studies. It should be emphasized that interspecies interactions between livestock and wildlife have been proved frequent in several Spanish regions, particularly at water points (Kukielka et al., 2013; Triguero Ocaña et al., 2019). In addition, wild boar home range is variable depending among other factors of food availability and reproductive status, and this movement allows their interaction with not only Iberian pigs but also with other domestic animals (cattle or sheep) and wild ungulates (red deer) that also live in the dehesa. The identification of *E. bieneusi* in sympatric Iberian pigs and wild boars support the spill over potential of *E. bieneusi* (and other Microsporidia) when domestic and sylvatic transmission cycles of the parasite overlap, representing a public veterinary health threat that should be taken into consideration. Indeed, farm workers in contact with swine faecal material (manure) or hunters that handle carcasses may be exposed to this parasite. In addition, both pork/Iberian pig and wild boar meat are very valuable in Southern Spain, and during the evisceration process at slaughterhouses or hunting activities cross contamination can occur. Dynamic exchange of other swine pathogens including porcine parvovirus, hepatitis E virus, *Brucella* spp. or *Toxoplasma gondii*, among others, have been previously reported and some may result in transmission from those reservoirs to humans through ingestion of infected food products (Ruiz-Fons et al., 2008; Pavio et al., 2016). Monitoring the presence of *E. bieneusi* in stool samples from humans in close contact with domestic and feral swine and/or their faeces should be carried out to assess the true zoonotic potential of this pathogen.

CONCLUSIONS

This study constitutes the first report of the presence and molecular characterization of *E. bieneusi* in extensively reared Iberian pigs and free-living wild boars sharing the same habitat in Spain. Our results contribute to our understanding of the epidemiology of *E. bieneusi* in livestock and wildlife in Spain and suggest, for the first time, an *E. bieneusi* transmission between domestic (Iberian pigs) and sylvatic (wild boars) hosts. The presence of novel PigSpEb1 and potentially zoonotic genotype EbpA in both hosts represent the first report of these genetic variants in Spain, indicating that Iberian pigs and wild boars may act as suitable hosts of the parasite and may play a role in the transmission of *E. bieneusi* to other animals, including humans.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available within the main body of the manuscript.

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636

637 **FIGURE CAPTIONS**

638 **Figure 1.** Geographic location of the province of Córdoba in Southern Spain (upper
639 map) and the sampling sites where faecal specimen from Iberian pigs and wild boars
640 were collected (lower map). Red and black filled figures represent PCR-positive and
641 PCR-negative results to *Enterocytozoon bieneusi*, respectively.
642

643 **Figure 2.** Venn diagram depicting the distribution of *Enterocytozoon bieneusi*
644 genotypes identified in the present survey among Iberian pigs and wild boars.
645 Overlapping circles indicate shared genotyped between both host species.
646
647 **Figure 3.** Phylogenetic relationships among *Enterocytozoon bieneusi* genotypes
648 identified in this study and known *E. bieneusi* genotypes previously identified in
649 Spanish human, animal, and environmental samples, as inferred by a neighbor-joining
650 analysis of the ITS rRNA gene sequence. Genetic distances were calculated using the
651 Kimura two-parameter model. Nucleotide sequences determined in this study are
652 identified with red filled circles. Red open circles represent sequences from human,
653 animal or environmental origin previously reported in Spain. Asterisks indicate
654 sequences identical to the provided GenBank accession number. Blue filled circles
655 represent sequences identified in wild boars from countries other than Spain. Bootstrap
656 values lower than 75% are not displayed.
657