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DIARRHOEA-CAUSING ENTERIC PROTIST SPECIES IN INTENSIVELY AND EXTENSIVELY RAISED PIGS (SUS SCROFA DOMESTICUS) IN SOUTHERN SPAIN. PART I: PREVALENCE AND GENETIC DIVERSITY

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Diarrhoea-causing enteric protist species in intensively and extensively raised pigs (*Sus scrofa domesticus*) in Southern Spain. Part I: Prevalence and genetic diversity

Running Head: Diarrhoea-causing protists in Spanish farmed pigs

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SUMMARY

Numerous protist species are shared between humans and pigs. Among those, *Giardia duodenalis*, *Cryptosporidium* spp., and *Balantioides coli* have a clear public and animal health significance. For other such as *Enterocytozoon bieneusi* and *Blastocysti*s sp., their impact in animal health has not been fully stablished. Little information is currently available on the molecular diversity of these protists in swine populations. To fill this gap, we molecularly assessed *G. duodenalis*, *Cryptosporidium* spp., *B. coli*, *Blastocystis* sp., and *E. bieneusi* in faecal samples from Iberian and Large White pigs raised under different (intensive and/or extensive) management systems in southern Spain. A total of 151 extensively raised Iberian pigs, 140 intensively raised Iberian pigs, and 184 intensively raised Large White pigs were investigated. *Blastocystis* sp. was the agent most prevalently found (47.8%), followed by *B. coli* (45.5%), *G. duodenalis* (10.7%), *E. bieneusi* (6.9%), and *Cryptosporidium* spp. (5.5%). *Blastocystis* sp. was significantly less prevalent in intensively raised Iberian pigs (22.9%) than in their extensively raised counterparts (51.0%) or in intensively raised Large White pigs (64.1%). A significant higher prevalence was found for *G. duodenalis*), *Cryptosporidium* spp. , and *E. bieneusi* in Large White pigs than Iberian pigs. *Balantioides coli* was similarly distributed (40.0–51.1%) in all three investigated swine populations. Sequence analyses revealed the presence of *G. duodenalis* assemblage E, two *Cryptosporidium* species (*Cryptosporidium scrofarum* and *Cryptosporidium suis*), *B. coli* (genotypes A and B), *Blastocystis* sp. (ST1, ST3, and ST5), *E. bieneusi* (EbpA, EbpC, EbpD, O, and a novel genotype named PigSpEb2). Novel genotype PigSpEb2 was found alone or in combination with EbpA. Data suggest a widespread exposure to protist enteroparasites in domestic pig populations irrespectively of breed and raising management system. Many of the species/genotype identified have zoonotic potential and might represent a public health concern.

KEYWORDS: *Giardia duodenalis*; *Cryptosporidium*; *Balantioides coli*; *Blastocystis*; *Enterocytozoon bieneusi*; Large White pig; Iberian pig; Spain; transmission; genotyping.

1. INTRODUCTION

Pigs are suitable reservoirs for a large number of infectious diseases by bacterial (e.g., *Brucella suis*, *Escherichia coli*, *Salmonella* spp., *Streptococcus suis*), viral (e.g., Influenza A virus, Hepatitis E virus) and parasitic (e.g., *Ascaris suum*, cysticercosis by *Taenia solium*, *Trichinella spiralis*) agents, representing an important zoonotic and food safety concern in many regions of the world (Ramirez, 2018). Among enteropathogenic protists, the protozoan *Giardia duodenalis* (syn. *G. intestinalis*, *G. lamblia*) and *Cryptosporidium* spp., the ciliate *Balantioides coli*, the stramenopile *Blastocystis* sp., and the microsporidia *Enterocytozoon bieneusi* are the main species infecting livestock including pigs (Hublin et al., 2021; Ponce-Gordo and García-Rodríguez, 2021; Ryan et al., 2014; Ryan and Zahedi, 2019; Santín and Fayer, 2011). Infections by *G. duodenalis* and *Cryptosporidium* spp. have a direct impact on growth rates in young livestock and are associated with important economic losses (Armson et al., 2009; Klein et al., 2008; Geurden et al., 2010; Santin, 2020; Schubnell et al., 2016). Transmission is predominately faeco-oral via direct contact with infected hosts or their faecal material, or indirect through the ingestion of contaminated water of food.

Giardia duodenalis has a major public and animal health significance in terms of gastrointestinal disease, being able to infect a wide host range, including mammals, birds, and amphibians (Dixon, 2021; Feng and Xiao, 2011). *Giardia duodenalis* is currently recognized as a species complex that includes eight distinct genotypic assemblages (A to H) with marked differences in host specificity and range. Assemblages A and B have a

loose host specificity and are frequently reported in humans, livestock, companion animals, and wildlife. In contrast, assemblage C and D primarily occur in canids, E in livestock, F in cats, G in rodents, and H in marine pinnipeds (Lasek-Nesselquist et al., 2010; Feng and Xiao, 2011). Assemblage E is typically the most prevalent assemblage detected in domestic pigs (Ryan and Zahedi, 2019; Sprong et al., 2009), although assemblages A, B, C, D, and F have also been sporadically documented in such production animals (Feng and Xiao, 2011; Minetti et al., 2014).

At least 46 *Cryptosporidium* species are currently recognized as taxonomically valid (Ježková et al., 2021; Zahedi et al., 2021), of which host-adapted *C. suis* and *C. scrofarum*, zoonotic *C. parvum*, and to a much lesser extent, *C. felis*, *C. muris*, *C. tyzzeri* and *C. andersoni* have been identified in pigs (De Felice et al., 2020; Petersen et al., 2015; Pettersson et al., 2020; Němejc et al., 2013; Wang et al., 2020). *Cryptosporidium* species distribution in infected pigs seems to follow an age-related pattern, although no associations between species and clinical signs or intensity of oocyst shedding have been demonstrated (Němejc et al., 2013).

Balantioides coli, formerly known as *Balantidium coli*, can parasitize a wide diversity of mammal species including pigs, cattle, sheep, goat, camels, and equids (Ponce-Gordo and García-Rodríguez, 2020). *Balantioides coli* is the only ciliate known to infect humans, with domestic pigs and wild boars (*S. scrofa ferus*) being regarded as the main reservoir hosts for human infections (Solaymani-Mohammadi and Petri, 2006). Swine infections by *B. coli* are mostly asymptomatic. Molecular genotyping studies are scarce and limited by the lack of informative genotyping tools. To date, three *B. coli* genotypes (A to C) have been proposed. Genotypes A and B have been mainly reported in swine, and genotype C in non-human primates (Ponce-Gordo et al., 2008).

The stramenopile *Blastocystis* sp. is a ubiquitous intestinal protist that infects/colonizes a broad range of mammalian and avian species (Hublin et al., 2021). It is also considered the most widespread non-fungal microeukaryote present in human stool samples (Andersen and Stensvold, 2016). Phylogenetic analysis of the small subunit of the rRNA (*ssu* rRNA) gene of *Blastocystis* sp. has allowed the identification of 27 subtypes (ST1-ST17, ST21, ST23-ST31) for which full-length (or near full-length) sequences are available and are therefore considered as legitimate STs (Stensvold and Clark, 2020; Maloney and Santín, 2021; Maloney et al., 2021). In pigs, nine subtypes have been reported (ST1-ST5, ST6, ST7, ST10, and ST15) (Hublin et al., 2021; Süli et al., 2021). Except for ST15, all these subtypes have been identified in humans and are considered zoonotic (Khaled et al., 2020; Stensvold and Clark, 2020). Among them, ST5 is by far the most prevalent identified subtype in pigs, a fact that strongly suggests that pigs are the natural host for this subtype. *Blastocystis* carriage in pigs does not seem to be associated with pathogenicity (Quilez et al., 1995).

Based on polymorphisms of the internal transcribed spacer (ITS) of the *ssu* rRNA gene, over 500 genotypes of *E. bieneusi* have been described and distributed in 11 phylogenetic groups (Li et al., 2019; Li and Xiao 2020). Group 1 and 2 include zoonotic genotypes that have been identified in humans and animals, whereas Groups 3–11 contain host-adapted genotypes (Li et al., 2019). In pigs, at least 139 genotypes have been described worldwide, of which D, EbpA, EbpC, G, H, and O are the most prevalent (reviewed by Li et al., 2019; Dashti et al., 2020). Most of genotypes found in pigs have been reported in humans, suggesting pigs are reservoir of zoonotic genotypes and may play a key role in the transmission of *E. bieneusi* to humans (Li et al., 2019).

Whereas *B. coli* and *Cryptosporidiu*m spp. are well-recognized diarrhoea-causing pathogens in pigs, the pathogenic role of *G. duodenalis*, *Blastocystis* sp., and *E. bieneusi*

137 are not fully stablished. Little information is currently available on the epidemiology of
138 the above-mentioned microorganisms in farmed pig populations in Spain. To bridge this
139 gap of knowledge, we present here novel data on the occurrence and genetic diversity of
140 common enteric protist species in extensively and intensively raised Iberian pigs and
141 Large White pigs in southern Spain.
142
143 **2. MATERIALS AND METHODS**
144 **2.1. Ethical statement**
145 This study was carried out in accordance with Spanish legislation guidelines (RD 8/2003)
146 and with the International Guiding Principles for Biomedical Research Involving Animals
147 issued by the Council for International Organization of Medical Sciences and the
148 International Council for Laboratory Animal Science (RD 53/2013).
149
150 **2.2. Study area and sampling**
151 The study was conducted in the North area of Córdoba province (Andalusia,
152 Southern Spain; 36°N–38°600 N, 1°750 W–7°250 W). Most pig farming in this area is
153 extensive, with Iberian pigs (a traditional breed of the domestic pig *Sus scrofa domesticus*
154 that is native to the Iberian Peninsula) wandering in semi-freedom from 3–18 months in
155 agroforestry systems, a characteristic habitat consisting of Mediterranean holm-oak and
156 cork-oak pastures (Garrido-Fernández and León-Camacho, 2019), where this species
157 shares natural resources with other domestic and wild species. Intensive pig farms raising
158 both Iberian pigs and Large White pigs are also present in the region.
159 Intensively and extensively managed pig farms investigated in the present survey
160 were simply randomly selected by the Environmental Department of the Regional
161 Government of Andalusia for previous studies aiming to determine the prevalence of

Hepatitis E virus (HEV) infection taking advantage of routine veterinary inspections (Lopez-Lopez et al., 2018; Risalde et al., 2020). Individual rectal faecal samples from Iberian and Large White pig were collected between October 1st and November 30th 2015. Sampled animals did not show evident clinical manifestations, including diarrhoea.

In this study we retrospectively analysed genomic DNA extracted from a total of 475 faecal samples from extensively and intensively raised Iberian and Large White pigs in 23 farms. Samples were deliberately selected to match the sex and age group of the investigated animals. Genomic DNA was purified using the QIAamp cador Pathogen Mini Kit (QIAGEN, Hilden, Germany), as described elsewhere (Risalde et al., 2020). Blinded DNA samples were submitted to the National Centre for Microbiology (Majadahonda, Spain) for downstream molecular investigation of enteric protist species.

2.3. Molecular detection and characterisation of *Giardia duodenalis*

To detect *G. duodenalis*, a real-time PCR (qPCR) protocol was used to amplify a 62-bp region of the *ssu* rRNA gene of the parasite (Verweij et al., 2003). The reaction mixture (25 µL) contained 3 µL of template DNA, 12.5 pmol of each primer Gd-80F and Gd-127R, 10 pmol of probe (Table S1), and 1X TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA). Amplification reactions were performed in a Corbett Rotor-Gene 6000 qPCR cycler (QIAGEN). Cycling conditions were: an initial hold step of 2 min at 55 ºC and 15 min at 95 ºC, followed by 45 cycles of 15 s at 95 ºC and 1 min at 60 ºC.

Giardia duodenalis isolates with qPCR cycle threshold (Ct) values ≤32 were reassessed by sequence-based multi-locus genotyping of the genes encoding for the glutamate dehydrogenase (*gdh*), beta-giardin (*bg*), and triose phosphate isomerase (*tpi*) proteins of the parasite. A semi-nested PCR was used to amplify a 432-bp fragment of

187 the *gdh* gene (Read et al., 2004). PCR reaction mixtures (25 µl) included 5 µl of template
188 DNA and 0.5 µM of the primer pairs GDHeF/GDHiR in the primary reaction and
189 GDHiF/GDHiR in the secondary reaction (Table S1). Both amplification protocols
190 consisted of an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of 95
191 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a final extension of 72 °C for 7 min.
192 A nested PCR was used to amplify a 511 bp-fragment of the *bg* gene (Lalle et al.,
193 2005). PCR reaction mixtures (25 µl) consisted of 3 µl of template DNA and 0.4 µM of
194 the primers sets G7_F/G759_R in the primary reaction and G99_F/G609_R in the
195 secondary reaction (Table S1). The primary PCR reaction was carried out with the
196 following amplification conditions: one step of 95 °C for 7 min, followed by 35 cycles of
197 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min with a final extension of 72 °C for 7
198 min. The conditions for the secondary PCR were identical to the primary PCR except that
199 the annealing temperature was 55 °C.
200 A nested PCR was used to amplify a 530 bp-fragment of the *tpi* gene (Sulaiman
201 et al., 2003). PCR reaction mixtures (50 µl) included 2–2.5 µl of template DNA and 0.2
202 µM of the primer pairs AL3543/AL3546 in the primary reaction and AL3544/AL3545 in
203 the secondary reaction (Table S1). Both amplification protocols consisted of an initial
204 denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 50 °C for
205 45 s and 72 °C for 1 min, with a final extension of 72 °C for 10 min.
206
207 **2.4. Molecular detection and characterisation of *Cryptosporidium* spp.**
208 To detect *Cryptosporidium* spp., a nested-PCR protocol was used to amplify a 587
209 bp fragment of the *ssu* rRNA gene of the parasite (Tiangtip and Jongwutiwes, 2002).
210 Amplification reactions (50 µl) included 3 µl of DNA sample and 0.3 µM of the primer
211 pairs CR-P1/CR-P2 in the primary reaction and CR-P3/CPB-DIAGR in the secondary

212 reaction (Table S1). Both PCR reactions were carried out as follows: one step of 94 °C
213 for 3 min, followed by 35 cycles of 94 °C for 40 s, 50 °C for 40 s and 72 °C for 1 min,
214 concluding with a final extension of 72 °C for 10 min.
215
216 **2.5. Molecular detection and characterisation of *Balantioides coli***
217 Detection of *B. coli* was attempted by a direct PCR assay to amplify the complete
218 ITS1–5.8s-rRNA–ITS2 region and the last 117 bp (3´ end) of the *ssu*-rRNA sequence of
219 this ciliate (Ponce-Gordo et al., 2011). The assay uses the primer set B5D/B5RC (Table
220 S1). PCR reactions (25 µl) consisted of 2 µl of template DNA and 0.4 µM of each primer.
221 PCR conditions were as follows: 94 °C for 10 min; 30 cycles of 94 °C for 1 min, 60 °C
222 for 1 min, 72 °C for 1 min, and a final extension for 5 min at 72 °C.
223
224 **2.6. Molecular detection and characterisation of *Blastocystis* sp.**
225 Molecular detection of *Blastocystis* sp. was achieved by a direct PCR protocol
226 targeting a fragment of 600 bp of the *ssu* rRNA gene of the protist (Scicluna et al., 2006).
227 The assay uses the pan-*Blastocystis*, barcode primer set RD5/BhRDr (Table S1). The
228 amplification conditions consisted of one step of 95 °C for 3 min, followed by 30 cycles
229 of 1 min each at 94, 59 and 72 °C, with an additional 2 min final extension at 72 °C.
230
231 **2.7. Molecular detection and characterisation of *Enterocytozoon bieneusi***
232 Detection of *E. bieneusi* was conducted by a nested PCR protocol to amplify the
233 internal transcribed spacer (ITS) region as well as portions of the flanking large and small
234 subunit of the ribosomal RNA gene as previously described (Buckholt et al., 2002). The
235 outer EBITS3/EBTIS4 and inner EBITS1/EBITS2.4 primer sets (Table S1) were used to
236 generate PCR products of 435 and 390 bp, respectively. Cycling conditions for the

primary PCR consisted of one step of 94 °C for 3 min, followed by 35 cycles of amplification at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 40 s, with a final extension at 72 °C for 10 min. Conditions for the secondary PCR were identical to the primary PCR except only 30 cycles were carried out with an annealing temperature of 55 °C.

The direct, semi-nested, and nested PCR protocols described above were conducted on a 2720 Thermal Cycler (Applied Biosystems). Reaction mixes always included 2.5 units of MyTAQ™ DNA polymerase (Bioline GmbH, Luckenwalde, Germany), and 5× MyTAQ™ reaction buffer containing 5 mM dNTPs and 15 mM $MgCl_2$. Laboratory-confirmed positive and negative DNA samples for each investigated parasite species were routinely used as controls and included in each round of PCR. PCR amplicons were visualized on 2% D5 agarose gels (Conda, Madrid, Spain) stained with Pronasafe nucleic acid staining solution (Conda).

2.8. Sequence and phylogenetic analyses

Positive PCR products were directly sequenced in both directions using appropriate internal primer sets (Table S1). DNA sequencing was conducted by capillary electrophoresis using BigDye® Terminator chemistry on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Raw sequencing data in both forward and reverse directions were viewed using the Chromas Lite version 2.1 sequence analysis program (https://technelysium.com.au/wp/chromas/). The BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare nucleotide sequences with sequences retrieved from the NCBI GenBank database. Generated DNA consensus sequences were aligned to appropriate reference sequences using the MEGA 6 software (Tamura et al., 2013) for species confirmation and genotype identification. *Blastocystis*

sequences were submitted at the *Blastocystis* 18S database (http://pubmlst.org/blastocystis/) for subtype confirmation and allele identification.

Enterocytozoon bieneusi nucleotide sequences obtained in this study as well as *E. bieneusi* appropriate reference retrieved from GenBank to include all *E. bieneusi* groups were aligned with the Clustal W algorithm using MEGA X (Kumar et al., 2018). 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 using MEGA X (Kumar et al., 2018).

The sequences obtained in this study have been deposited in GenBank under accession numbers MW657751–MW657752 (*G. duodenalis*, *gdh* and *tpi* loci), MW835325–MW835329 (*Cryptosporidium* spp.), MW648341–MW648347 (*Balantioides coli*), MW647154–MW647170 (*Blastocystis* sp.), and MZ506845–MZ506849 (*E. bieneusi*).

2.9. Cloning of *Enterocytozoon bieneusi* DNA

When *E. bieneusi* mixed genotype infection within a specimen was suspected from the chromatogram sequence traces, the secondary PCR products were cloned using the TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA, USA) and transformants were selected from each specimen (four clones from each specimen), PCR-amplified, and sequenced in both directions using M13 forward and reverse primers. Briefly, amplicons were purified using Exonuclease I/Shrimp Alkaline Phosphatase (ExoSAP-IT Express, Affymetrix Inc., Santa Clara, CA, USA), and sequenced in both directions using primers utilized for PCR screening in 10 µl reactions, Big Dye™ chemistries, and an ABI 3130 sequencer analyser (Applied Biosystems). Sequence chromatograms of each strand were aligned and examined with Lasergene software (DNASTAR, Inc., Madison, WI, USA).

286

287 **2.10. Statistics analysis**

288 The prevalences of *G. duodenalis*, *Cryptosporidium* spp., *B. coli*, *Blastocystis* sp.,
289 and *E. bieneusi* in the global and different pig populations investigated were calculated
290 using the proportion of positive samples with respect to the total number of samples
291 examined with 95% confidence interval (95% CI). In Iberian pigs, the prevalence was
292 also calculated by farming management system (intensive *versus* extensive). We used the
293 Pearson's χ^2 test when the expected values of at least 80% of the cells in a 2x2 contingency
294 table to be greater than 5. When these conditions were not verified, we compared the
295 qualitative variables via the Fisher's exact test. Analyses were carried out using SPSS
296 statistical software package version 18.0 (IBM Corporation, Somers, NY, USA).

297

298 **3. RESULTS**

299 **3.1. Study population**

300 A total of 475 animals were included in the study. Of these, 31.8% (151/475) and
301 29.5% (140/475) were extensively and intensively raised Iberian pigs, respectively, and
302 38.7% (184/475) were intensively raised Large White pigs. Sows and fattening pigs
303 represented 57–83% and 17–44% of the sampled Iberian and Large White pigs,
304 respectively (Table 1).

305

306 **3.2. Prevalence of enteroparasites**

307 The overall prevalence rates of the enteroparasite species investigated in the
308 present survey are shown in Table 2. *Blastocystis* sp. was the agent most prevalently
309 found (47.8%, 95% CI: 43.2–52.4), followed by *Balantioides coli* (45.5%, 95% CI: 40.9–
310 50.1), *G. duodenalis* (10.7%, 95% CI: 8.1–13.9), *E. bieneusi* (6.9%, 95% CI: 4.8–9.6),

311 and *Cryptosporidium* spp. (5.5%, 95% CI: 3.6–7.9). *Blastocystis* sp. was significantly less
312 prevalent in intensively raised Iberian pigs (22.9%, P <0.001) than in their extensively
313 raised counterparts (51.0%) or in intensively raised Large White pigs (64.1%). Large
314 White pigs were significantly more infected by *G. duodenalis* (15.2%, P = 0.004),
315 *Cryptosporidium* spp. (8.2%, P = 0.047), and *E. bieneusi* (12.5%, P <0.001) than Iberian
316 pigs irrespectively of their managing system (10.4–21.2%, 4.6–13.1%, and 8.1–18.2%,
317 respectively). Finally, *B. coli* was similarly distributed (40.0–51.1%) in all three
318 investigated swine populations (P = 0.121)

319 Comparison of protist prevalence rates according to pig breed (Iberian pigs *vs*.
320 Large White pigs) and management system (intensive *vs*. extensive farming) is shown in
321 Table 3. Large White pigs were significantly more infected by *G. duodenalis* (P = 0.012),
322 *Blastocystis* sp. (<0.001), and *E. bieneusi* (<0.001) compared to Iberian pigs.
323 Management system only affected the prevalence of *Cryptosporidium*, significantly
324 higher in intensively than in extensively raised animals (P = 0.023). No statistically
325 significant differences in protist infection rates were observed between fattening pigs and
326 sows (data not shown).

327 The prevalences of *G. duodenalis*, *Cryptosporidium* spp., *Blastocystis* sp., *B. coli*,
328 and *E. bieneusi* in each of the 23 farms sampled in the present study are shown in Table
329 S2. Overall, *G. duodenalis* was detected in 69.6% (16/23) of the sampled farms,
330 *Cryptosporidium* spp. in 39.1% (9/23), *Blastocystis* sp. in 95.7% (22/23), *B. coli* in 91.3%
331 (21/23), and *E. bieneusi* in 34.8%, (8/23). All farms were infected by at least one protist
332 species.

333 Overall, 64.6% (307/475) of the pigs investigated were infected/colonized by at
334 least one protist species. Table S3 summarizes the distribution of single and mixed protist
335 infections in pigs. The most prevalent double infection found was *Blastocystis* sp. + *B.*

coli (38.1%, 117/307). The most prevalent triple infection found was *G. duodenalis* + *Blastocystis* sp. + *B. coli* (5.9%, 18/307). The most prevalent quadruple infection found was *Blastocystis* sp. + *Cryptosporidium* spp. + *E. bieneusi* + *B. coli* (1.6%, 5/307).

3.3. Molecular characterization of *Giardia duodenalis* isolates

A total of 51 DNA isolates yielded a positive result for *G. duodenalis* by qPCR. The generated cycle threshold (Ct) values had median values of 32.8 (range: 27.4–39.9). Of them, 60.8% (31/51) has qPCR values ≥32. To maximize time and resources, only DNA isolates with qPCR Ct values <32 (n = 20) were assessed for genotyping and sub-genotyping purposes at the *gdh*, *bg*, and *tpi* loci. Two DNA isolates were successfully amplified at the *gdh* locus, and a third one at the *tpi* locus. All 20 DNA isolates investigated failed to yield a PCR product at the *bg* locus. Nucleotide sequence analysis allowed the identification of assemblage E in the three amplified DNA isolates at the *gdh* or *tpi* loci. The main features of these sequences are summarized in Table 4. Two of the identified assemblage E sequences were from fattening pigs and the remaining one from a sow. Both fattening pigs were Large White pigs belonging to the same farm (GenBank accession numbers MW657751 and MW657752), whereas the sow was an intensively raised Iberian pig (isolate of insufficient quality to be deposited in GenBank). Sequences generated at the *gdh* locus contained single nucleotide polymorphisms (SNPs) in the form of ambiguous (double peak) positions and transitional substitutions (T/C, A/G) at similar frequency rates. In contrast, transitional substitutions (T/C, A/G) prevailed over double peaks in the only sequence generated at the *tpi* locus. In the latter sequence a transversional T/G mutation associated with amino acid change at the protein level was identified (Table 4).

3.4. Molecular characterization of *Cryptosporidium* spp. isolates

A total of 26 DNA isolates yielded amplicons of the expected size by *ssu*-PCR. Sequence analysis revealed the presence of two *Cryptosporidium* species, namely *C. scrofarum* (61.5%, 16/26) and *C. suis* (38.5%, 10/26) (Table 4). Out of the 16 sequences assigned to *C. scrofarum*, 14 of them showed 100% identity with reference sequence KF597534. The remaining two sequences varied from KF597534 by two SNPs each that involved ambiguous (double peak) positions in all cases. Similarly, nine out of ten sequences identified as *C. suis* had 100% identity with reference sequence AF115377, with the remaining one differing from it by a single SNP involving a double TA nucleotide deletion at positions 687 and 688 (Table 4).

Cryptosporidium suis was predominantly found in intensively raised Large White pigs (60.0%, 9/15), whereas *C. scrofarum* accounted for most of the *Cryptosporidium* infections detected in intensively (87.5%, 7/8) and extensively (100%, 3/3) raised Iberian pigs. Half of the *Cryptosporidium* infections detected (13/26, 9 infections by *C. suis* and four infections by *C. scrofarum*) were identified in a single farm of intensively raised Large White pigs (Table S4).

3.5. Molecular characterization of *Balantioides coli* isolates

The vast majority (93.0%, 201/216) of the swine samples that tested positive for *B. coli* by *ssu*-PCR delivered complex sequence patterns involving large numbers of heterozygous positions that made difficult their unequivocal assignment to a given genotype. Using the classification system proposed by Ponce-Gordo et al. (2008), unambiguous sequencing data were available for 15 *B. coli* isolates, revealing the presence of genotypes A (13.3%, 2/15) and B (86.7%, 13/15) (Table 4). The two *B. coli* isolates identified as genotype A were identical to reference sequence AM982724. Out of

386 the 12 sequences confirmed as genotype B, four had 100% identity with reference
387 sequence AM982725, whereas the remaining eight sequences differed from it by 1–5
388 SNPs. Most SNPs detected corresponded to ambiguous (double peak) positions (Table
389 4).
390
391 **3.6. Molecular characterization of *Blastocystis* sp. isolates**
392 Nucleotide sequence analyses of the 227 *ssu*-PCR products that generated
393 readable sequence data allowed the identification of three *Blastocystis* subtypes (ST)
394 including ST1 (7.5%, 17/227), ST3 (1.3%, 3/227), and ST5 (91.2%, 207/227) (Table 5).
395 An additional 84 isolates yielded faint bands on gel electrophoresis associated with poor
396 quality sequences. Because the nature of these samples could not be confirmed by Sanger
397 sequencing, they were conservatively considered as negative for *Blastocystis* sp.
398 *Blastocystis* ST1 and ST3 were only detected in extensively and intensively Iberian pigs
399 raised in single (but independent) farms; ST5 was the only detected *Blastocystis* ST
400 circulating in Large White pigs.
401 Allele calling using the *Blastocystis* 18S database revealed the presence of alleles
402 4 and 38 within ST1 and ST3, respectively. All 17 sequences assigned to allele 4 were
403 identical to reference sequence MT898451, and all three sequences identified as allele 38
404 were identical to reference sequence JF792495 (Table 5). A much higher intra-subtype
405 genetic diversity was observed within *Blastocystis* ST5 isolates, where alleles 17 (0.5%,
406 1/207), 115 (8.7%, 18/207), 119 (4.8%, 10/207), 153 (56.5%, 117/207) and 16+17 (1.9%,
407 4/207) were identified. The remaining 57 ST5 isolates (27.5%) could not be assigned to
408 a given allele due to insufficient sequence quality or the presence of ambiguous (double
409 peak) positions during chromatogram inspection (Table 5). Sequences assigned to allele
410 115 were grouped into two distinct genetic variants that differed by 3–4 SNPs (all of them

411 associated with heterozygous positions) from reference sequence AB107964. Out of the
412 117 sequences identified as allele 153, a total of 49 were identical to reference sequence
413 MN945414. The remaining 68 sequences were grouped in nine distinct genetic variants
414 differing by 2–4 SNPs from reference sequence MN945414. All the SNPs identified
415 within allele 153 sequences corresponded to clear double peak positions (Table 5).
416 Finally, four sequences were identified as having mixed alleles infections involving
417 alleles 16+17 based on peak splitting at the ambiguous position 136 of reference sequence
418 MN945414 (Table 5).
419
420 **3.7. Molecular characterization of *Enterocytozoon bieneusi* isolates**
421 Nucleotide sequences analysis of the ITS region allowed the identification of four
422 known *E. bieneusi* genotypes including EbpA (66.6%, 22/33), EbpC (12.1%, 4/33), EbpD
423 (6.1%, 2/33), O (6.1%, 2/33) and a mixed EbpA+EbpD infection (3.0%, 1/33). In
424 addition, a novel genotype (named PigSpEb2) was also identified alone (PigSpEb2: 3.0%,
425 1/33) or as mixed EbpA+PigSpEb2 infection (3.0%, 1/33) (Table 6). Genotype PigSpEb2
426 nucleotide sequence differs by a SNP (A to G) at position 195 in the ITS region with
427 genotype EbpC (AF076042) (242/243, 99% similarity). The accuracy of the novel
428 genotype PigSpEb2 sequences was confirmed in two independent PCR and sequencing
429 reactions and using direct Sanger sequencing as well as sequencing after cloning. Mixed
430 infections involving two genotypes were identified using cloning.
431 The distribution of *E. bieneusi* genotypes according to breed and management
432 system of the pigs that tested positive to the parasite was shown in Table S4. Despite
433 being the most frequent genotype found, EbpA was only detected in intensively raised
434 pigs (Large White: 73.9%, 17/23; Iberian: 33.3%, 3/9). The very same trend was also true
435 for genotype EbpD and O. In contrast, EbpC was detected in Iberian pigs irrespectively

436 of their management system but was absent in Large White pigs. Mixed infections
437 involving known and novel *E. bieneusi* genotypes were detected in intensively raised
438 animals only (Table S4). Both mixed infections were identified in a single farm of
439 intensively raised Large White pigs presenting the highest infection rates by
440 *Cryptosporidium* spp., and *E. bieneusi* found in the present survey (Table S4).
441 Phylogenetic analysis showed that all genotypes identified in the present study
442 clustered together within the potentially zoonotic Group 1 (Figure 1).
443
444 **4. DISCUSSION**
445 Data presented here confirm that infections by *G. duodenalis*, *Cryptosporidium*
446 spp., *B. coli*, *Blastocystis* sp., and *E. bieneusi* are common in domestic pigs, regardless
447 their breed (Iberian or Large White pigs) and management system (intensive or
448 extensive). Regarding protozoa, the overall prevalence rate of *G. duodenalis* (10.7%)
449 detected was in range with those reported in previous Spanish studies in extensively raised
450 Iberian pigs (17.2%) in the same region (Rivero-Juarez et al., 2020) and in intensive pig
451 farms (7%, overall value) in north-western Spain (Reinoso and Becares, 2008). *Giardia*
452 *duodenalis* infections in pigs have been reported in the range of 0.1–17% in other
453 European countries including Denmark (Maddox-Hyttel et al., 2006), Germany (Epe et
454 al., 2004), and Norway (Hamnes et al., 2007). *Cryptosporidium* infections rates reported
455 here varied from 2% in extensively raised pigs to 6–8% in intensively-managed pigs.
456 These prevalences are in the lower range of those reported in porcine populations from
457 European countries such as Czech Republic (22%, Němejc et al., 2013), Denmark (3%–
458 72%, Petersen et al., 2015), Germany (0.1%, Epe et al., 2004), Ireland (11%, Zintl et al.,
459 2007), Norway (8%, Hamnes et al., 2007), Poland (28%, Rzeżutka et al., 2014), Spain
460 (8%, Rivero Juárez et al., 2020), and Sweden (25%, Pettersson et al., 2020). Finally, *B.*

461 *coli* is primarily regarded as an opportunistic parasite of pigs (Schuster and Ramirez-
462 Avila, 2008). The *B. coli* prevalence rate found in the surveyed swine population (45.5%)
463 was slightly lower than that (52.7%) previously reported by our research group in
464 extensively raised Iberian pigs (Rivero-Juarez et al., 2020).

465 The stramenopile *Blastocystis* sp. was the most prevalent enteroparasite detected
466 in the present study (47.8%). This rate is lower than that previously identified (73.1%) in
467 extensively raised Iberian pigs (Rivero-Juarez et al., 2020). It should be noted that the
468 figure provided here is a conservative estimation, as only sequences confirmed by Sanger
469 sequencing were considered as true positive for *Blastocystis*. Microscopy-based
470 prevalence rates ranging from 7–47% have been previously described in intensively
471 raised Large White pigs in other Spanish regions (Quilez et al., 1995; Navarro et al., 2008;
472 Köster and Carmena, 2020). Prevalence values ranging from 20–83% have also been
473 documented in other European countries including Czech Republic, Denmark, Germany,
474 and Serbia (reviewed in Hublin et al., 2021).

475 As in the case of *Cryptosporidium* infections, the microsporidia *E. bieneusi* was
476 more prevalently found in intensively (6–13%) than in extensively (<1%) raised pigs.
477 These figures are lower than those (21–23%) reported by PCR in other Spanish pig
478 populations in the autonomous regions of Andalusia (Dashti et al., 2020) and
479 Extremadura and Castile-Leon (Galván-Díaz et al., 2014). Higher *E. bieneusi* prevalence
480 rates have been consistently documented in porcine populations in the Czech Republic
481 (94%, Sak et al., 2008), Germany (10%–32%, Dengjel et al., 2001; Reetz et al., 2009),
482 Slovakia (19%, Valenčáková & Danišová, 2019), and Switzerland (35%, Breitenmoser et
483 al., 1999).

484 Of note, all *G. duodenalis*, *Cryptosporidium* spp., *B. coli*, *Blastocystis* sp. and *E.*
485 *bieneusi* infections found in the present study were identified in asymptomatic animals.

In the case of *G. duodenalis*, high qPCR Ct values were predominantly observed, what could suggest chronic infections and/or low parasite burden. This situation would also explain the low amplification success rate obtained at the *gdh*, *bg*, and *tpi* loci, all three characterised by limited sensitivity associated to their single-copy nature. It should be also noted that infected but asymptomatic animals can still actively shed infective cysts/oocysts/spores of the above mentioned protist species that could contribute to environmental contamination as well as result on occupational disease in individuals such as farmers, veterinarians, and butchers exposed to risk factors arising from work activity.

A major contribution of this study is the description of the genetic diversity of *G. duodenalis*, *Cryptosporidium* spp., *B. coli*, *Blastocystis* sp. and *E. bieneusi* isolates circulating in the surveyed swine population. Thus, assemblage E was the only *G. duodenalis* genetic variant found infecting pigs. This result agrees with previous findings describing assemblage E as more prevalent than assemblage A in Iberian pigs (Rivero-Juarez et al., 2020). The above-mentioned study is, to the best of our knowledge, the only attempt to genotype *G. duodenalis* isolates of swine origin conducted in Spain to date. Two *Cryptosporidium* species (*C. scrofarum*: 62%, *C. suis*: 38%) were identified in the surveyed swine population. Similar proportions (68% *vs*. 32%, respectively) have been found in Danish organic pig farms (Petersen et al., 2015). The opposite trend (*C. suis*: 67%, *C. scrofarum*: 33%) was documented in suckling piglets in Norway (Hamnes et al., 2007). Both *Cryptosporidium* species were reported at similar rates (48%–51% *vs*. 38%–48%, respectively) in pigs of all ages in the Czech Republic (Němejc et al., 2013) and in intensively farmed pigs in Ireland (Zintl et al., 2007). In these surveys, porcine infections by *C. muris* and *C. parvum* were sporadically detected. *Cryptosporidium parvum* was also identified at low rates in pig herds raised in Poland (Rzeżutka et al., 2014). Very little information is currently available on the genetic diversity of *B. coli*, for which three

genotypes (A to C) have been proposed to exist (Ponce-Gordo et al., 2008). Data presented here indicates that *B. coli* genotype B was six-fold more frequent than genotype A in Spanish domestic pigs. Interestingly, genotype B sequences presented a much higher degree of genetic variability (in the form of deletions, ambiguous positions, and transitional mutations) than the sequences assigned to genotype A, at least at the ITS marker. More research should be conducted to ascertain the genetic diversity of this ciliate parasite at other, more informative, loci.

Sequencing data from *Blastocystis* isolates confirmed the predominance of ST5 in pig populations previously found in Spain (Rivero-Juarez et al., 2020) and other countries (reviewed in Hublin et al., 2021). A large molecular diversity was observed within ST5 sequences, as demonstrated by the identification of alleles 17, 115, 119, 153, and mixed 16+17. This genetic variability was particularly evident within alleles 115 and 153 sequences, where 2 and 10 different genetic variants (all involving double peaks at chromatogram inspection) were identified, respectively. It is likely that some of these ambiguous positions correspond indeed to unresolved (by Sanger sequencing) *Blastocystis* mixed subtype infections (Maloney et al., 2019). Interestingly, zoonotic ST1 and ST3 were also identified in specific, independent farms. Whether this finding represents a true intra-herd transmission of the parasite or the result of a zoonotic event of anthropic origin, it remains to be elucidated.

In the present study, four known (EbpA, EbpC, EbpD, and O) and one novel (named PigSpEb2) *E. bieneusi* genotypes were found circulating in Spanish pigs. Genotypes EbpA, I, O, PigEb4, PigHN-II, and PigSpEb1 have been previously described in porcine populations in the country thus far (Dashti et al., 2020; Galván et al., 2014). Therefore, this study represents the first description of genotypes EbpC and EbpD in this host in Spain. EbpA was the *E. bieneusi* genotype most prevalently found, being present

(alone or in combination with other genotypes) in 73% of the infections. This result agrees with those obtained in other European countries including the Czech Republic (95%, Sak et al., 2008), Germany (60%, Dengjel et al., 2001), and Switzerland (43%, Breitenmoser et al., 1999). The finding of genotype O was also interesting, as this genetic variant had only been reported in pigs in two European countries to date, Germany (Dengjel et al., 2001; Reetz et al., 2009) and Spain (Dashti et al., 2020). Similarly, genotypes EbpC and EbpD had been detected previously in European porcine populations in Germany and Switzerland (Breitenmoser et al., 1999; Reetz et al., 2009). Of note, genotypes EbpA, EbpC, EbpD, and O identified in Iberian and Large White pigs in this study, have been found in humans and other animals (Li et al., 2019), indicating that Spanish pigs are hosts of potentially zoonotic genotypes.

Overall, parasitic infection rates were higher in intensively raised animals than in extensively raised ones, indicating that crowding conditions favour the transmission of pathogens at the farm level. This was particularly evident for *E. bieneusi* mixed infections, only detected in pigs raised under intensive management systems. Indeed, a single farm raising White Large pigs harboured the highest infection rates for two of the pathogens investigated here (*Cryptosporidium* spp.: 33%, *E. bieneusi*: 48%), strongly suggesting that appropriate management practices are key in minimizing the risk of infection by these pathogens. Intensive farming is associated with higher stocking density (space/animal, animals/pen, or animals/room) that is likewise associated with higher transmission rate of pathogens included those transmitted via the faecal-oral route, as the protist included in this study. Due to the lack of effective drug treatment or vaccine to treat most protist parasites surveyed here, management measurements become critical to control spread of these pathogens. Among the management practices associated with intensive production are batch production and the all in/all out system. To aid controlling

parasite transmission in intensive farming it will be key to adequate clean, disinfect and dry animal enclosures prior to the arrival of new groups (replacement or self-replacement) of pigs. Additional biosecurity measures may include the implementation of rodent and insect control programs, bird-proof nets in windows, sanitary ford, water sources, chlorination water practices, feed source and delivery systems, carcass storage, change of boots and presence of fences (Simon-Grifé et al., 2013) Veterinarians and farmers have also a responsibility to develop a holistic approach to biosecurity to prevent transmission of zoonotic diseases. That means measurements to control not only spread of pathogens within the herd but also to those working in swine facilities must comply with safe treatment and disposal of pig faeces. The advent of molecular diagnostics and epidemiology has provided a better understanding of the complex protist transmission, however, there is still a need of further molecular epidemiological surveys in pigs to better elucidate the mode and risk of transmission of protist in swine populations.

Spain has the largest pig population in Europe and is the fourth largest pork producer in the world behind China, the United States and Germany. Pig production is an important driver of the Spanish economy, accounting for around 36.4% of total animal husbandry production (MAPAMA, 2021). Data presented here, although geographically restricted and limited to a relatively low number of farms, indicate widespread distribution of the selected enteroparasites in pig farms in southern Spain. A national surveillance program should be implemented to determine the frequency and distribution of enteroparasites of public veterinary health relevance in Spain.

CONCLUSIONS

Prevalence and epidemiological data presented here suggest a widespread exposure to *G. duodenalis*, *Cryptosporidium* spp., *B. coli*, *Blastocystis* sp., and *E. bieneusi*

586 in domestic pig populations in southern Spain. The study provided also relevant
587 molecular epidemiological information. A large genetic diversity was observed within
588 *Blastocystis* ST5, a subtype for which pigs are thought to be natural hosts. Within *B. coli*,
589 genotype B was the predominant genetic variant of the parasite circulating in pigs,
590 exhibiting a high degree of intra-genetic variability. Similarly, five distinct *E. bieneusi*
591 genotypes (one of them novel) were identified in pigs, all of the belonging to the group
592 1, four reported previously in humans, suggesting that swine may play a relevant role in
593 the transmission of this microsporidia to humans. These findings may have relevant
594 public health implications, as asymptomatic pigs can act as disregarded sources of human
595 and farm animal infections by diarrhoea-causing protist species.
596

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615

616 **CONFLICT OF INTEREST**

617 The authors have no conflict of interest to declare.

618 **DATA AVAILABILITY STATEMENT**

619 The data that supports the findings of this study are available within the main body of the
620 manuscript.
621

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FIGURE CAPTIONS

Figure 1. Phylogenetic relationships among *Enterocytozoon bieneusi* genotypes identified in this study and reference *E. bieneusi* genotypes to include all current groups as inferred by a neighbour-joining analysis of the ITS rRNA gene sequence. Genetic distances were calculated using the Kimura two-parameter model. Nucleotide sequences determined in this study are identified with circles. Black circles represent novel genotype PigSpEb2 identified in this study. Bootstrap values lower than 75% are not displayed.