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First molecular detection and genotyping of *Enterocytozoon bieneusi* (Microsporidia) in fattening pigs from Italy

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Abstract

Background *Enterocytozoon bieneusi* is the most reported microsporidian species in humans worldwide, with high prevalences also documented in animals, particularly pigs. Due to limited information on *E. bieneusi* in Italian livestock, this cross-sectional observational study aimed to investigate its prevalence, genetic diversity, and associated risk factors in pigs. For this purpose, 440 faecal samples were collected from pigs raised on 22 intensive farms in northern Italy during two sampling sessions, corresponding to the beginning and the end of the fattening cycle. A nested PCR protocol was performed to amplify the internal transcribed spacer region (ITS), followed by DNA sequencing for genotype identification. Moreover, several risk factors were evaluated as predictors of *E. bieneusi* positivity using generalised linear models.

Results In the first session, the positive samples were 197 out of 220 (89.5%); in the second, the positive samples were 82 out of 220 (37.3%). Overall, 279 out of 440 pig faecal samples (63.4%) were positive for *E. bieneusi*. In the multivariate analysis, age and faecal soiling of the animal's body were significant risk factors (p-value < 0.05), with younger animals and those with a high degree of faecal soiling showing higher prevalences. All identified ITS sequences clustered within Group 1, which comprises the main genotypes with zoonotic potential.

Conclusions This study demonstrates a high prevalence of *E. bieneusi* in Italian pigs, albeit in a single production category, supporting their role as natural reservoirs. Further research is needed to assess the parasite's presence in other pig categories, humans, environmental matrices, and food products to clarify its public health significance.

Keywords Microsporidia, *Enterocytozoon bieneusi*, Prevalence, Risk factors, Genotyping, Pigs, Zoonosis, One health

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Background

Microsporidia are a diverse group of obligate intracellular fungi infecting humans and several animal species worldwide. Among them, *Enterocytozoon bieneusi* is of particular interest, being the most prevalent species and responsible for the majority of reported human microsporidiosis cases [1, 2].

Following ingestion by an appropriate host, *E. bieneusi* spores invade host cells, mainly enterocytes, but also epithelial cells of the hepatobiliary tract, and replicate. Mature spores, which represent the infective stage, are released upon host cell rupture and subsequently excreted with faeces into the environment [3, 4]. The spores are small (about 1 μm) and have a chitinous cell wall, which confers prolonged survival and resistance under varying environmental and climatic conditions [4, 5]. Moreover, they have been detected in several matrices, including drinking water sources, wastewater treatment facilities, soil and food products [6–8]. In this regard, *E. bieneusi* and other microsporidia are included among foodborne and waterborne parasites, listed as microbial contaminants of concern by the US Environmental Protection Agency (EPA), and classified as Category B priority biological agents by the US National Institute of Allergy and Infectious Diseases (NIAID) [9–11].

In immunocompetent individuals, infection is generally asymptomatic or manifests as self-limiting diarrhoea. In contrast, immunocompromised people, such as organ transplant recipients, HIV-infected individuals, and chemotherapy patients, may develop the clinical condition known as Chronic Wasting Disease, characterised by severe symptoms including watery diarrhoea, nausea, abdominal pain, and malabsorption [12–14].

Regarding pigs, the pathogenic role of *E. bieneusi* remains unclear. Some studies report molecular detection of this microsporidian species in pigs with gastrointestinal symptoms [15–17], while others document even high prevalence rates in clinically healthy animals [18–20]. Pigs can become infected at an early age and shed spores in faeces over prolonged periods, contaminating the pens and posing an infection risk to other animals [21–24]. In humans, infection occurs via the faecal-oral route, through the ingestion of spores originating from environmental or animal sources, as well as via human-to-human transmission, particularly involving human-adapted genotypes [4, 25].

Compared to conventional microscopy, PCR-based DNA amplification provides greater sensitivity and specificity for detecting *E. bieneusi*, considering the small size of spores which may be confused with bacteria, food particles, or other microsporidia [4, 12, 25, 26]. When combined with PCR amplicon sequencing and polymorphism analysis, this technique can be used as an effective genotyping tool for tracing infection sources and transmission routes, while also assessing the zoonotic risk [12, 22].

The internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) gene is widely used for genotyping due to its variability among *E. bieneusi* isolates [14]. To date, over 685 genotypes have been identified globally and grouped into 11 genetic groups [25–27]. Of these, the first two groups (Group 1 and Group 2) comprise most of the genotypes, including the major zoonotic ones, with over 90% implicated in human infections [14]. Groups 3 to 11, on the other hand, appear more host-specific, although data on these groups remain limited [10].

Several studies have documented genotype overlap between pigs and humans, identifying genotypes such as CS-4, D, EbpA, EbpC, G, H, Henan-IV, PigEBITS5, PigEBITS7, I, and BEB4 in both hosts. This supports the hypothesis that pigs act as potential reservoirs of zoonotic genotypes, highlighting their relevance in the context of human exposure, particularly for farmers and people involved in the food production chain. Understanding the genotypic diversity of *E. bieneusi* is critical from a One Health perspective, as different genotypes can differ markedly in host specificity and zoonotic potential. In particular, those within Groups 1 and 2 account for the majority of human infections and are frequently shared with animal hosts, including pigs. Consequently, identifying the genotypes circulating in pig populations is essential for evaluating the risk of interspecies transmission and the potential public health impact [4, 10, 11, 20].

Research on *E. bieneusi* circulation in companion animals, wildlife, and livestock is increasing, highlighting variable prevalence rates [3, 4, 12]. Conversely, epidemiological studies in domestic pigs are scarce worldwide and, to the authors' knowledge, entirely absent for Italy. This gap is particularly relevant in northern Italy, where pig farming is a key livestock sector. The area is characterised by high-density intensive farming systems, with large numbers of animals, limited outdoor access, and considerable manure production, all of which can increase environmental contamination and facilitate the intra- and inter-farm transmission of environmentally resistant pathogens, such as *E. bieneusi*. In addition, age-related differences in susceptibility and management factors (e.g., hygiene, stocking density, and all-in/all-out practices) are known to influence the dynamics of enteric infections in pigs and are therefore expected to influence the risk of *E. bieneusi* circulation and transmission [20, 28].

Considering this lack of data on *E. bieneusi* circulation, the present study aimed to investigate its prevalence, genetic diversity, and associated risk factors in pigs of different age groups raised in northern Italy, with a focus

on identifying zoonotic genotypes from a One Health perspective.

Methods

Sampling and data collection

This cross-sectional observational study was carried out in northern Italy between 2023 and 2024. The minimum required sample size of 385 faecal samples was calculated using a statistical tool [29], considering a population of approximately 2 million fattening pigs in northern Italy within the selected territory, an expected prevalence of 50%, a 95% confidence level, and a desired absolute precision of 5% [30]. Faecal samples were taken from pigs raised in 22 intensive farms, located in an area particularly devoted to the pig industry, spanning Lombardy, Piedmont and Emilia-Romagna regions [31]. The farms were selected based on the willingness of the farm veterinarian and farmer to participate; all animals were commercial hybrids of Landrace and Large White breeds and appeared clinically healthy. Within farms, a random sampling strategy was applied: several pens, housing approximately 20 animals each, were chosen to ensure representativeness of the different stages of the fattening cycle. From each pen, the veterinarian randomly selected two pigs without any preferential criteria. Faeces were then collected by gloved hand from the rectal ampulla to minimise contamination (Institutional Animal Care and Use Committee of Università degli Studi di Milano, Protocol number: OPBA_90_2023). Twenty individual samples were obtained per farm: 10 from pigs at the beginning of the fattening cycle (first category; aged 3–5 months, weighing 35–50 kg), and 10 from pigs just before slaughter (second category; aged 6–9 months, weighing 150–170 kg). Overall, a total of 440 faecal samples were gathered, 220 in the first phase and 220 in the second phase of the fattening cycle. The two sampling sessions were conducted on the same farms but involved distinct batches of pigs.

Following collection, samples were placed individually in labelled plastic containers, transported to the laboratory refrigerated at +4 °C, and aliquots of each sample were stored at −20 °C for subsequent molecular analyses.

At the same time as sampling, an individual form was completed through farmer interviews, collecting information on the animals, sampling time, and farm management variables, in particular: application of the all-in/all-out system (yes/no), farm size (< 1900 animals, ≥ 1900 animals), sampling season (spring, summer, autumn, winter), age (≤ 5 months, > 5 months) and faecal soiling of the body (scores 0, 1, 2). Faecal soiling was assessed using a cleanliness score according to Classyfarm [32], a risk categorization system funded by the Italian Ministry of Health: 0, soiled body surface does not exceed 20%; 1, soiled body surface is between 20% and 50%; 2, soiled body surface exceeds 50%.

Molecular and phylogenetic analyses

DNA extraction was performed on 440 faecal samples, using approximately 200 mg of faeces per sample and the QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. The concentration and quality of the extracted DNA samples were assessed using a Nanodrop ND-1000 (Thermo Fisher Scientific, MA, USA) spectrophotometer.

DNA samples were subsequently subjected to a nested PCR protocol targeting a 389 bp fragment of the ITS region, using primers previously described [33] (Additional File 1). PCR reactions were performed in a final volume of 50 µL, containing 5 µL of 10X DreamTaq Buffer (Thermo Fisher Scientific, Life Technologies, Italy), 5 µL of 2 mM dNTP Mix (Thermo Fisher Scientific, Life Technologies, Italy), 1 µl of each primer (10 µM), 1.25 U of DreamTaq DNA Polymerase (5U/µL) (Thermo Fisher Scientific, Life Technologies, Italy), 32.75 µL of nuclease-free water (Sigma-Aldrich, Italy), and 5 µL of DNA samples (approximately 10–100 ng per reaction). For the secondary PCR, 1 µL of the primary PCR product was used as template. Each PCR run included positive controls (previously confirmed by amplicon sequencing) and non-template negative controls. The cycling conditions of the primary PCR consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of amplification at 94 °C for 45 s, annealing at 57 °C for 45 s and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The secondary PCR conditions were the same, except for a reduced number of cycles (30) and a lower annealing temperature (55 °C).

A randomly selected subset of primary PCR samples (n = 110) and all the secondary PCR amplicons were analysed by 1.5% agarose gel electrophoresis and visualised by staining with ethidium bromide. Bands of the expected size were excised from the gel and purified using a commercial kit (NucleoSpin® Gel and PCR Clean-up, Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. The purified PCR products were bidirectionally Sanger sequenced (Microsynth Seqlab, Göttingen, Germany). The resulting electropherograms were checked, and consensus sequences were manually assembled. Sequences were compared with those available in the GenBank database using the BLASTn algorithm, with accession number AF076040 as reference [34].

For the phylogenetic analysis, a total of 22 sequences, each derived from a representative positive sample per farm, were included along with a representative selection of *E. bieneusi* genotypes from the main phylogenetic groups, obtained from previous studies and downloaded

from the GenBank database (N = 34) [34]. These sequences were subsequently aligned with those obtained in the present study using MUSCLE [35] and polished with Gblocks [36]. The optimal substitution model was selected using jModelTest [37] based on the Akaike Information Criterion (AIC). A Maximum Likelihood (ML) phylogeny was then inferred with W-IQ-TREE [38] with 1000 bootstrap pseudo-replicates. The resulting tree was rooted and visually edited with the iTOL tool [39].

Statistical analysis

A sample was considered positive for *E. bieneusi* if the PCR assay yielded a positive result; at the farm level, positivity was defined as the detection of at least one positive animal. The prevalence and 95% confidence intervals (CI) of *E. bieneusi* infection in sampled pigs were calculated at the individual level, according to the categorised variables considered: application of the all-in/all-out system, farm size, sampling season, age, and faecal soiling of the body.

These variables were assessed as potential risk factors for *E. bieneusi* occurrence and included in generalised linear models (GLMs). The binary outcome (negative/positive for *E. bieneusi*) was set as the dependent variable, while the risk factors were entered as categorical independent variables in univariate models. Variables with a p-value < 0.1 were subsequently included in multivariate models developed through a backward selection procedure (significance level to remove variables from the model = 0.05), based on Akaike's information criterion (AIC) values.

Statistical analysis was performed using R Studio software (version 4.4.2, R Foundation for Statistical Computing, Vienna, Austria).

A completed STROBE-Vet checklist is provided as Additional File 2, summarising the report on study design, sampling, molecular methods and statistical analyses in accordance with STROBE-Vet guidelines [40].

Results

In the first sampling session, 197 out of 220 faecal samples tested positive (89.5%, 95% CI: 84.7–93.3), while in the second sampling session, 82 out of 220 samples were positive (37.3%, 95% CI: 30.9–44.0). Overall, E. *bieneusi* DNA was detected in 279 of the 440 analysed pig faecal samples (63.4%, 95% CI: 58.7–67.9) through PCR amplification of the ITS region. All the positive samples were detected through the secondary PCR step, while none of the samples randomly selected from the primary PCR amplification showed any visible bands.

At the farm level, all 22 farms (100%) tested positive at the beginning of the fattening cycle, whereas positivity decreased to 20 out of 22 farms (90.9%, 95% CI: 70.8–99.0) at the end of the cycle, just before slaughter.

Higher prevalence rates were observed in farms that did not apply the all-in/all-out system (73.3%, 95% CI: 64.5–81.0), in spring (84.7%, 95% CI: 78.4–89.8), and in pigs younger than five months of age (89.5%, 95% CI: 84.6–93.3). In addition, prevalence increased with the degree of faecal soiling of the pigs' bodies, with the highest rate observed in category 2, representing pigs with more than 50% of the soiled body surface (77.9%, 95% CI: 70.1–84.4) (Table 1).

Univariate analysis supported the descriptive findings: variables significantly associated with infection (p-value < 0.1) included the application of the all-in/all-out system, sampling season, age, and faecal soiling score. Specifically, a significantly higher risk of *E. bieneusi* infection was observed in pigs from farms not applying the all-in/all-out system (OR: 1.8, 95% CI: 1.2–2.9), in animals sampled in spring (OR: 4.9, 95% CI: 2.6–9.3), in younger pigs (OR: 13.05, 95% CI: 7.8–21.8), and in those with a faecal soiling score of 1 (OR: 1.8, 95% CI: 1.1–2.8) or 2 (OR: 3.4, 95% CI: 2.0–5.5) (Table 1).

In the multivariate model, only age and faecal soiling remained statistically significant predictors of *E. bieneusi* infection (p-value < 0.05). The risk of infection was significantly higher in pigs younger than five months (OR: 11.1, 95% CI: 6.0–20.5), as well as in those with a faecal soiling score of 1 (OR: 1.9, 95% CI: 1.1–3.5) or 2 (OR: 5.3, 95% CI: 2.4–11.7) (Table 2).

A total of 22 ITS sequences, each derived from one positive sample per farm, were used for phylogenetic analysis. The sequences obtained and analysed via BLAST in the GenBank database showed 97–100% identity with each other and 97.12–100% identity with the reference *E. bieneusi* genotype EbpA (accession number AF076040). Overall, the sequences were grouped into seven distinct isolates: 14 out of 22 corresponded to isolate EbITA Ss 1, 2 out of 22 to EbITA Ss 6, while the remaining sequences represented unique isolates.

Representative nucleotide sequences of the detected isolates were deposited in the GenBank database under accession numbers PV911610–PV911616.

Among the identified genotypes, EbpA, which includes strains recovered from various host species, including humans, was detected in 63.6% (14/22) of the sequences analysed. Isolate EbITA Ss 2 clustered with a currently unclassified genotype previously identified in pigs in Korea (accession numbers MN158157–MN158158). Isolate EbITA Ss 3 was assigned to genotype KWB4 (reference sequence MT984295), originally detected in wild boars in Korea [41]. Isolate EbITA Ss 6 exhibited 100% sequence identity with genotypes CC-1 and CHPR2 (reference sequences KF724905 and PP158576, respectively). No mixed infections involving multiple *E. bieneusi* genotypes were observed. For a detailed description of the identified isolates, see Table 3.

Table 1 Prevalence of *Enterocytozoon bieneusi* and univariate analysis of associated risk factors in Italian pigs

Variable	Category	Positive/Examined (*n*)	Prevalence % (95% CI[b])	OR[a] (95% CI)	*p*-value
Age	≤ 5 months	188/210	89.5 (84.6–93.3)	13.05 (7.8–21.8)	0.00*
	> 5 months (ref.)	91/230	39.6 (33.2–46.2)	1	
All-in/All-out	No	88/120	73.3 (64.5–81)	1.8 (1.2–2.9)	0.01*
	Yes (ref.)	191/320	59.7 (54.1–65.1)	1	
Faecal soiling	0 (ref.)	92/180	51.1 (43.6–58.6)	1	
	1	78/120	65 (55.8–73.5)	1.8 (1.1–2.8)	0.02*
	2	109/140	77.9 (70.1–84.4)	3.4 (2-5.5)	0.00*
Farm size	< 1900 animals	172/280	61.4 (55.4–67.2)	1.3 (0.8–1.9)	0.254
	≥ 1900 animals (ref.)	107/160	66.9 (59-74.1)	1	
Sampling season	Autumn	44/100	44 (34.1–54.3)	0.7 (0.4–1.3)	0.256
	Spring	144/170	84.7 (78.4–89.8)	4.9 (2.6–9.3)	0.00*
	Summer	54/100	54 (43.7–64)	1.05 (0.6–1.9)	0.883
	Winter (ref.)	37/70	52.9 (40.5–65)	1	
Overall positivity and prevalence		279/440	63.4 (58.7–68)	--	

[a]*OR* Odds Ratio

[b]*CI* Confidence Interval

*Values statistically significant (*p*-value < 0.1)

Table 2 Multivariate analysis of risk factors associated with *Enterocytozoon bieneusi* infection in Italian pigs

Variable	Category	OR[a] (95% CI[b])	*p*-value
Age	≤ 5 months	11.1 (6-20.5)	0.00*
	> 5 months (ref.)	1	
Faecal soiling	0 (ref.)	1	
	1	1.9 (1.1–3.5)	0.03*
	2	5.3 (2.4–11.7)	0.00*

[a]*OR* Odds Ratio

[b]*CI* Confidence Interval

*Values statistically significant (*p*-value < 0.05)

Table 3 List of *Enterocytozoon bieneusi* isolates with genotypes, phylogenetic groups, and number of samples per isolates

Isolate	Assigned genotype	Phylogenetic group	Number of samples per isolate
EbITA Ss 1	EbpA[a]	1	14
EbITA Ss 2	unassigned[b]	1	1
EbITA Ss 3	KWB4[c]	1	2
EbITA Ss 4	H[d]	1	1
EbITA Ss 5	H[d]	1	1
EbITA Ss 6	CC-1/CHPR2[e]	1	2
EbITA Ss 7	EbpA[a]	1	1

[a]Reference sequence AF076040

[b]Genotype currently not assigned, reference sequences MN158157, MN158158

[c]Reference sequence MT984295

[d]Reference sequence AF135835

[e]Reference sequences KF724905, PP158576

Sequences from the seven isolates clustered within Group 1 (Fig. 1), and all the obtained isolates belonged to subgroups 1d and 1e (data not shown). The isolates identified in this study correspond to genotypes previously reported in the literature.

Discussion

This study represents the first molecular and epidemiological survey of *E. bieneusi* in fattening pigs in northern Italy, providing valuable data on prevalence, genotypes, and associated risk factors. Some limitations include sampling restricted to intensive farms as well as the absence of other pig categories, environmental, or human data, which could influence prevalence estimates. The

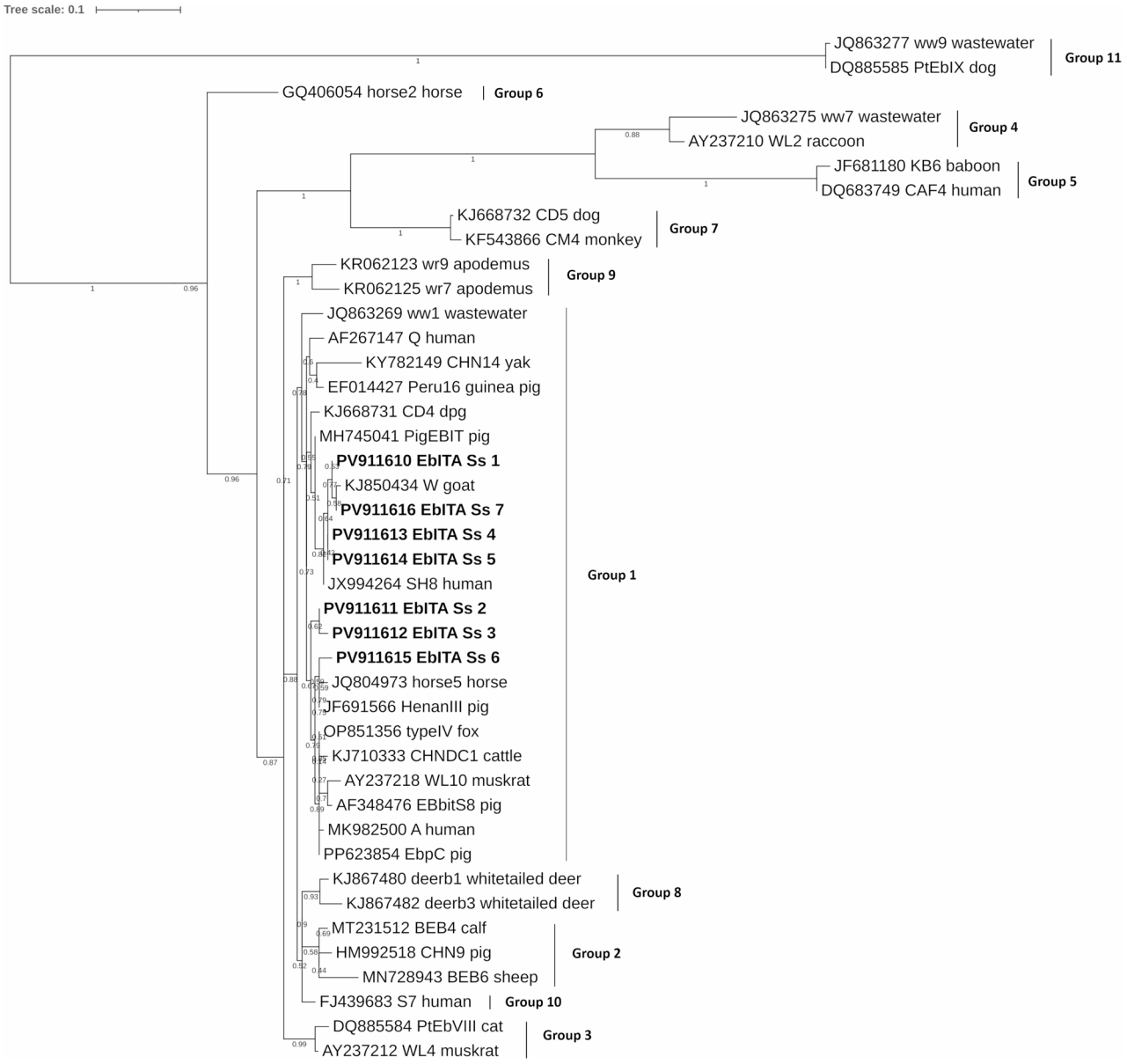

Fig. 1 Maximum likelihood phylogenetic tree of ITS sequences of *Enterocytozoon bieneusi*. Sequences obtained in this study are in bold. For each sequence, the NCBI accession numbers, genotype and host species are reported. HKY85 model was used as parameter of nucleotide substitution. Numbers on branches report bootstrap supports with 1000 pseudo-replicates. Support bootstrap values below 0.50 were omitted. The scale bar stands for proportional sequence divergence.

results can be generalised mainly to intensive Italian fattening pig farms and should be extrapolated with caution to other farming systems and pig categories.

In Italy, data on this microsporidian species remain scarce. The first report of its detection and genotyping in human patients was only recently published in 2024 [25]. Furthermore, limited information is available regarding animal hosts. To date, only one survey conducted in southwestern Europe investigated the presence of intestinal microeukaryotes in free-ranging wolves, reporting a 0% prevalence of *E. bieneusi* in Italian samples [42].

In our study, 279 out of 440 pig faecal samples tested positive, resulting in an overall prevalence of 63.4%. Molecular studies in pigs have reported prevalences ranging from 7.7% to 94%, influenced by factors such as geographic location, diagnostic protocols, production category, farm type, and the number and age of animals sampled [11, 14, 18, 21, 43–45]. Notably, most of this research was conducted in China, with fewer studies carried out in Europe, indicating a current paucity of data in this geographical area.

The type of intensive farming practiced in Italy generally provides limited outdoor access and involves high-density housing, where animals are kept in close contact. These conditions may facilitate the spread of enteropathogens such as *E. bieneusi*, which is transmitted via the faecal-oral route and produces environmentally resistant spores [4, 16, 28, 44, 46]. In this context, it is essential to implement proper hygiene and biosecurity measures at the farm level, such as the systematic application of all-in/all-out protocols, adequate deratisation plans, and strict control of water and feed sources [43, 47, 48].

According to the statistical analysis, several variables were statistically significant (p-value < 0.1) in the univariate model, including the application of the all-in/all-out system, sampling season, age, and the faecal soiling of the animal's body. Farms that did not apply the all-in/all-out system showed a higher risk of *E. bieneusi* infection, likely due to the absence of both a sanitary vacuum and systematic cleaning and disinfection of the facilities prior to the start of a new fattening cycle [31, 49]. Sampling season was also a risk significant factor, with animals showing higher infection rates in spring (OR = 4.9; P = 84.7%) compared to other seasons. Although no studies have investigated seasonality as a predictor *E. bieneusi* infection, this pattern may be linked to the biology of the spores, which favour mild climates. In this regard, several studies reported higher prevalences during temperate periods, such as spring, which are beneficial for the growth and transmission of *E. bieneusi* [50, 51]. In terms of age, pigs under five months were significantly more at risk of infection. Pigs can acquire *E. bieneusi* shortly after birth and shed spores that persist in the environment, contributing to continuous contamination of housing facilities. Moreover, animals in this age group are often newly transferred to the fattening site, a stressful event that may cause transient immunosuppression, increasing their susceptibility to infections. These factors may partly explain the marked decrease in prevalence observed between the two sampling sessions, along with the possible lower susceptibility of older pigs and the gradual reduction in environmental contamination due to routine cleaning and management practices [21, 23, 52, 53]. Faecal soiling of the body also emerged as a significant risk factor, as it may facilitate *E. bieneusi* transmission among pigs sharing the same pen, maintaining high circulation of enteropathogens and elevating the zoonotic risk for farm workers [28]. In the multivariate model, only age and faecal soiling of the body remained significant variables (p-value < 0.05). In particular, the risk of *E. bieneusi* infection was significantly higher in younger animals (under five months) and in those showing higher faecal soiling scores (scores 1 and 2).

Based on the high overall prevalence and the identified risk factors, *E. bieneusi* appears to be persistent in intensive pig farms in northern Italy. Further longitudinal studies across different Italian regions, farm types, and pig categories are needed to determine whether the observed patterns reflect stable endemicity or a recent introduction.

According to the molecular characterisation, this study revealed the presence of several known *E. bieneusi* genotypes (EbpA, H, KWB4, and CC-1/CHPR2), all belonging to the zoonotic Group 1, specifically subgroups 1d and 1e. At least two of these genotypes (EbpA and H) have been previously reported in humans. Additionally, EbpA is among the most frequently identified genotypes in domestic pigs and wild boars [10]. Although EbpA has been described as having a relatively narrow host and geographic range, its detection in humans suggests that pigs may serve as reservoirs for zoonotic transmission. Conversely, genotype H has not yet been reported in humans but does not appear to be strictly host-specific to pigs, as it has also been identified in dogs and cats [10]. For two isolates, genotype assignment could not be definitively determined. Isolate EbITA Ss 2 showed high sequence identity with two entries from pigs in Korea (MN158157–MN158158), which currently lack formal genotype classification. Isolate EbITA Ss 6 was found to be 100% identical to both genotypes CC-1 and CHPR2. These genotypes share the same sequence; however, they differ in length and have been described as distinct genotypes in the literature. For this reason, the genotype assignment of isolate EbITA Ss 6 remains unresolved. To our knowledge, this is the first report of subgroups 1d and 1e in Italy, as previous investigations have only reported subgroup 1b/c (genotype A) isolates from humans [25].

Notably, several of the genotypes identified in pigs in this study are identical or closely related to those recently reported in Italian human isolates, reinforcing the hypothesis that domestic pigs may contribute to the zoonotic transmission of E. *bieneusi*. Potential routes of transmission from pigs to humans may include direct contact with animals, handling contaminated manure, and exposure during slaughter procedures [19, 23]. In this context, workers along the food chain, particularly those who may not observe adequate hygiene practices, such as farmers, may be at greater risk of exposure, further emphasising the importance of raising awareness of occupational zoonotic risks. To date, direct evidence linking human infections to animal-derived *E. bieneusi* is still lacking; nevertheless, a survey conducted in China identified direct contact with pigs or contaminated premises as one of the most significant risk factors for microsporidian infection among HIV-positive patients [54]. Similarly, in Peru, the same genotype was detected in a

child with self-limiting diarrhoea and in guinea pigs from the same household, suggesting potential zoonotic transmission [55].

In humans, prevalences reported worldwide are around 7.9% and mainly concern patients with a compromised immune system [56–58]. In Italy, a recent survey reported a positivity rate of 0.7% in humans, but the lack of detailed information on the sampled individuals made it difficult to speculate on transmission routes and infection sources [25].

Overall, it is important to emphasise the potential for zoonotic and cross-species transmission of *E. bieneusi* from both public health and veterinary perspectives, especially considering the absence of effective vaccines or treatments for human microsporidiosis [3, 10, 19, 59]. Within this framework, veterinarians should take responsibility for developing effective biosecurity plans to prevent and limit the circulation of pathogens, as well as for educating and training personnel working in the pig industry, beginning with farmers. Moreover, given that all genotypes identified in this study belong to zoonotic Group 1, it is necessary to strengthen public health surveillance, including targeted monitoring of occupational categories. Another important aspect is the proper use of manure through adequate treatment, for example, promoting longer maturation periods, and the safe disposal of faeces to prevent environmental and water contamination, which may lead to a widespread geographical dispersion of *E. bieneusi* spores [22, 28, 44].

Conclusions

This study provides the first epidemiological evidence of *E. bieneusi* in Italian pigs, with an overall prevalence of 63.4%. The highest infection rate was observed in younger animals, suggesting that age may be an important predictor of its occurrence. Furthermore, the faecal soiling of the body was significantly associated with infection, highlighting the central role of management practices. Molecular characterisation identified five genotypes, all formerly described and classified within Group 1, including two with documented zoonotic potential and previously reported in humans.

From a practical point of view, strict hygiene and biosecurity measures are recommended to limit the circulation of *E. bieneusi* in pig farms, and awareness of zoonotic transmission should be increased among farm workers and personnel involved in the food production chain.

Future research should focus on multilocus sequence typing (MLST) to clarify the genetic diversity, host specificity, and transmission dynamics of *E. bieneusi*, while implementing a One Health surveillance approach that encompasses humans, animals, the environment, and food products to better assess public health risks.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-05234-5.

Additional file 1: Supplementary Table 1. Primers used for the molecular identification and characterisation of *Enterocytozoon bieneusi* in Italian pigs.

Additional file 2: Completed STROBE-Vet checklist in accordance with STROBE-Vet guidelines for observational research in veterinary epidemiology.

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Authors' contributions

CA: Conceptualization, Laboratory Analyses, Formal Analysis, Writing—original draft, Writing—review and editing; LV: Conceptualization, Formal Analysis, Writing—review and editing; AC: Formal Analysis, Writing—original draft, Writing—review and editing; AZ: Laboratory Analyses, Writing—review and editing; MV: Sample Collection, Writing—review and editing; MTM: Conceptualization, Formal Analysis, Writing—original draft, Writing—review and editing, Supervision. All authors have read and approved the final manuscript.

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Data availability

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All procedures for the collection of pig faecal samples were approved by the Institutional Animal Care and Use Committee of Università degli Studi di Milano ("Organismo Preposto al Benessere degli Animali," Protocol number OPBA_ 90_2023).
Informed consent was obtained from the pig farmers involved in this study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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