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Investigation of
Cryptosporidium spp. and *Enterocytozoon bienersi*
in wild boars from a highly anthropised area:
molecular characterisation and potential for
zoonotic transmission to domestic suids

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0. ABSTRACT

Questo lavoro di tesi verte sull'identificazione e sulla caratterizzazione molecolare di *Cryptosporidium* spp. ed *Enterocytozoon bieneusi*, parassiti a trasmissione oro-fecale appartenenti rispettivamente al phylum Apicomplexa e al phylum Microsporidia, in cinghiali selvatici cacciati in un'area antropizzata della provincia di Cremona (Lombardia). L'area di studio è di particolare interesse ecologico ed economico per l'alto tasso di urbanizzazione e di allevamenti suinicoli intensivi e per la recente crescita della popolazione di cinghiali, rappresentando, dunque, un contesto ideale per indagare le interazioni tra animali domestici e selvatici e studiare la potenziale trasmissione di agenti patogeni.

I 191 cinghiali esaminati sono stati abbattuti tra il 2024 e il 2025 nell'ambito del programma regionale di sorveglianza della fauna selvatica. I campioni fecali sono stati prelevati durante l'ispezione delle carcasse in collaborazione con l'Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna "Bruno Ubertini", sezione di Cremona. Per ogni campione sono stati raccolti dati epidemiologici riguardanti la stagione di abbattimento, il sesso e l'età dell'animale. La ricerca di *Cryptosporidium* spp. e di *E. bieneusi* è avvenuta mediante tecniche molecolari basate sull'estrazione e sulla quantificazione del DNA presente nei campioni fecali e la successiva applicazione di protocolli di nested PCR. I prodotti di amplificazione sono stati visualizzati mediante elettroforesi su gel di agarosio e le bande corrispondenti ai campioni positivi sono state escisse dal gel, purificate e sottoposte a sequenziamento. Le sequenze così ottenute sono state controllate manualmente e confrontate con sequenze di riferimento depositate in GenBank per l'identificazione della specie. Per la successiva analisi filogenetica, le sequenze di *Cryptosporidium* sono state allineate con una selezione rappresentativa di sequenze del gene SSU rRNA appartenenti a diverse specie dello stesso genere; analogamente, le sequenze di *E. bieneusi* sono state allineate con sequenze rappresentative di genotipi appartenenti ai principali gruppi filogenetici. La ricostruzione filogenetica è stata effettuata utilizzando il metodo della massima verosimiglianza (*Maximum Likelihood*) e l'albero filogenetico risultante è stato visualizzato mediante iTOL.

Per fornire una valutazione preliminare delle dinamiche di circolazione tra animali domestici e selvatici, in particolare tra suini domestici e cinghiali selvatici, i dati molecolari ottenuti nei cinghiali sono stati integrati con i dati di prevalenza di *Cryptosporidium* spp. in 22 allevamenti di suini da ingrasso del Nord Italia campionati tra il 2023 e il 2024. Per ciascuno dei 440 campioni esaminati sono stati raccolti dati epidemiologici su stagione di campionamento, sesso ed età, oltre a dati relativi alle pratiche gestionali seguite nell'allevamento. La prevalenza e la caratterizzazione

molecolare di *E. bieneusi* sono state, invece, ricavate dallo studio di Allievi *et al.* (2026), condotto sui medesimi allevamenti suinicoli inclusi nella presente tesi.

Per ciascun parassita è stata calcolata la prevalenza all'interno delle due popolazioni campionarie. La presenza di associazioni significative tra la positività all'infezione e le variabili considerate (stagione di abbattimento o di campionamento, età, sesso, dimensioni dell'allevamento, adozione del sistema tutto-pieno/tutto-vuoto, grado di imbrattamento fecale) è stata valutata mediante il test del Chi-quadro di Pearson, mentre la forza delle associazioni è stata quantificata mediante il calcolo degli odds ratio. Le variabili risultate significative nell'analisi univariata sono poi state incluse in un modello di regressione logistica multivariata. Sono stati considerati statisticamente significativi valori di $p\text{-value} < 0,05$.

Sia nei cinghiali che nei suini esaminati è stata identificata la stessa specie di *Cryptosporidium*, *C. scrofarum*, mentre i sei genotipi di *E. bieneusi* riscontrati nei cinghiali sono stati confrontati con quelli identificati nei suini nello studio di Allievi *et al.* (2026). Il confronto dei dati ottenuti ha permesso di evidenziare una netta differenza nella prevalenza dei due parassiti tra i cinghiali e i suini da ingrasso, che potrebbe essere associata alla maggiore densità e allo stretto contatto tra gli animali, condizioni tipiche degli allevamenti intensivi che possono favorire la trasmissione oro-fecale dei parassiti. L'identificazione della stessa specie di *Cryptosporidium* e di tre genotipi di *E. bieneusi* condivisi tra i cinghiali e i suini da ingrasso, inoltre, suggerisce una possibile connessione epidemiologica tra la popolazione selvatica e quella domestica. L'effettiva circolazione dei parassiti tra le due popolazioni e la sua eventuale direzionalità, tuttavia, potranno essere chiarite soltanto attraverso ulteriori approfondimenti. Sebbene il potenziale zoonotico di *C. scrofarum* appaia limitato, esso deve essere ancora pienamente stabilito; al contrario, tra i genotipi di *E. bieneusi* identificati nelle popolazioni esaminate, H, D ed EbpA sono riconosciuti come zoonotici. Il rilevamento dei due enteropatogeni nei cinghiali e la loro ampia diffusione nei suini d'allevamento (24,8% per *C. scrofarum* e 63,4% per *E. bieneusi*) sottolineano l'importanza dello sviluppo di solidi programmi di sorveglianza epidemiologica che includano la loro ricerca. L'applicazione di adeguate misure di biosicurezza negli allevamenti suinicoli intensivi, come l'adozione del sistema tutto-pieno/tutto-vuoto, rappresenta un importante strumento per limitare la diffusione dei due parassiti all'interno dell'allevamento e la loro dispersione nell'ambiente, riducendo così le potenziali occasioni di esposizione per gli animali selvatici e, nel caso dei genotipi zoonotici, per l'uomo.

I risultati di questa tesi, infine, suggeriscono un possibile ruolo epidemiologico del cinghiale nel mantenimento e nella circolazione dei parassiti esaminati nell'ambiente selvatico. Per definire in maniera esaustiva il ruolo del cinghiale nell'epidemiologia di *C. scrofarum* ed *E. bieneusi* e le sue possibili interazioni con la popolazione suina domestica saranno necessarie ulteriori indagini.

1. INTRODUCTION

The current challenges posed by globalization, urbanization and climate change have deep and complex effects on human and environmental health: on the one hand, anthropogenic activities are severely threatening biodiversity and ecosystem balance, causing eutrophication, pollution and ecological degradation; on the other hand, they are creating new, often forced, forms of interaction between humans and natural environment. This forced coexistence leads to the spread of invasive species, the extinction of less adaptable ones, and the emergence of new or previously eradicated pathogens.

In this context, wildlife is increasingly menaced by habitat loss and interference from human activities. As a result, interactions and conflicts between wildlife, humans, and livestock have become more frequent, creating a high-risk interface for the transmission of emerging, re-emerging and neglected bacterial, viral and parasitic pathogens.

The wild boar (*Sus scrofa*) is a representative example of a highly adaptable species with a considerable ecological impact. Its ability to thrive in a wide range of environments has enabled it to expand into both rural and urbanised areas, resulting in increasingly frequent contact with humans and livestock.

In order to assess the potential role of wild boars in the dissemination and maintenance of pathogens, the apicomplexan *Cryptosporidium* spp. and the microsporidian *Enterocytozoon bieneusi* – two parasites of zoonotic importance – were investigated in a population of wild boars sampled in the province of Cremona (Lombardy, Italy).

1.1. The wild boar (*Sus scrofa*)

1.1.1. Wild boar distribution and expansion

The wild boar (*Sus scrofa* Linnaeus, 1758) is an ungulate mammal belonging to the family Suidae (suborder Suiformes, order Artiodactyla) and is the wild ancestor which originated almost all current domestic pig breeds and feral pig populations through domestication processes (Larson *et al.*, 2005).

The native range of wild boars covered most of Eurasia, including regions of East Asia and North Africa, making wild boars one of the most widely distributed ungulate species. Historically and more recently, wild boars have been deliberately introduced into non-native regions, primarily for hunting purposes, contributing to the species' massive global expansion (Courchamp *et al.*, 2002;

Barrios-Garcia and Ballari, 2012). As a result, wild boars are now found in Africa, North and South America, Australia, and several Pacific Ocean islands (**Figure 1**) and are among the mammals with the widest geographical distribution (Barrios-Garcia and Ballari, 2012).



Figure 1. Worldwide distribution of *Sus Scrofa*. Gray circles indicate islands where wild boars have been introduced, while question marks indicate areas with unknown distribution (Barrios-Garcia and Ballari, 2012).

In recent years, various parts of the world, including Italy, have witnessed a rapid expansion of wild boar populations, with increasing numbers and a progressive spread into both natural and urbanised areas (Smith *et al.*, 2026). The expansion of this species has been so significant that the International Union for Conservation of Nature (IUCN) has included the wild boar on its global list of the “100 Worst Invasive Alien Species” (<https://www.iucn.org>).

The reasons behind this recent increase are linked to the species' biological characteristics. Wild boars typically have a higher reproduction rate than other ungulates – with females capable of producing up to ten piglets per litter. Moreover, as omnivore, they have a flexible and opportunistic diet. When adverse conditions occur, they are capable of migrating over long distances, up to 100-150 km, thereby spontaneously expanding their home range (Massei and Genov, 2004; Singer *et al.*, 1981). Together, these factors make this species highly adaptable to a wide variety of habitats.

In addition to their biological features, several factors contribute to the spread of these animals: abandonment of rural areas, lack of natural predators, reforestation, changes in agricultural practices, intentional reintroduction and reduced hunting pressure. Furthermore, as their geographical distribution appears to be limited mainly by harsh winters, climate change, which is leading to increasingly warmer winters, is also playing an important role (Markov *et al.*, 2022).

Urban and suburban areas have been particularly affected by this trend. Human behaviours, such as direct feeding and improper waste management, increase the tolerance of wild boars, and wildlife in general, towards human presence (Carnevali *et al.*, 2009; Pilarczyk *et al.*, 2024). Furthermore, cities and surrounding areas offer attractive food sources, places to rest, such as parks and squares, and favourable conditions for surviving the winter (Khwarahm *et al.*, 2022; Pilarczyk *et al.*, 2024). According to Castillo-Contreras *et al.* (2021), wild boars are rapidly adapting to the selective pressure of urbanisation and human presence, showing significant morphological, physiological, behavioural and metabolic adaptations. As a result of their increasing proximity to urban and rural environments, conflicts with human activities have become more frequent.

1.1.2. Ecology and environmental impact of wild boars

Wild boars are mainly vegetarian, with a diet consisting of 80-90% by plant matters: acorns, beechnuts, seeds, roots, tubers, and bulbs, which are high-energy sources of food, as well as fruits, mushrooms, and crops (Massei and Genov, 2004). To uproot the underground parts of plant, wild boars use their muzzle to overturn large areas of soil vegetation, an activity called *rooting*, which takes up most of their time. Rooting has a remarkable impact not only on belowground and surface communities, but also on other organisms, due to the physical alterations it induces in soil structure and resources availability (Barrios-Garcia and Ballari, 2012). Because of these profound effects on ecosystem processes, wild boars are often regarded as “ecosystem engineers” (Sandom *et al.*, 2012). In general, rooting has a negative impact on the abundance and diversity of plant species, reducing biodiversity in wild habitats and potentially leading to the local extinction of species (Barrios-Garcia and Ballari, 2012). It has been estimated that rooting, in areas with high wild boar densities, can reduce the herbaceous cover by as much as 80-95% (Howe *et al.*, 1981).

The damage caused by wild boars, especially in anthropic contexts, is attributable to direct foraging and related behaviours, such as intensive digging, trampling, scratching and the removal of obstacle; given the considerable size of the animal, these actions have a significant impact (Riga *et al.*, 2011). Consequently, wild boars can affect significantly agricultural activities, as crops represent an extremely rich source of food that requires minimal foraging effort. They can spoil almost every type of cultivated plant; among these, the most affected is maize, followed by rice, grapes, olives, barley, oats, chestnuts, and potatoes (Massei and Genov, 2004; Barrios-Garcia and Ballari, 2012). It is worth noting that even the stubble left after harvest can be exploited by wild boars (Barrios-Garcia and Ballari, 2012).

A minor but significant proportion of the wild boar’s diet consists of opportunistic feeding on a

wide variety of small animals and carcasses. Although the actual intake vary and is closely linked to seasonality (Wilcox and Van Vuren, 2009), it is estimated that food sources of animal origin represent on average 2-11% of the wild boar's diet, with peaks of 30% (Massei and Genov, 2004; Barrios-Garcia and Ballari, 2012). Wild boars actively predate both soil fauna (larvae, beetles, snails, earthworms, insects) and vertebrates: amphibians, reptiles, mammals, birds, indeed, are commonly found in their stomach contents (Wilcox and Van Vuren, 2009).

The impact of wild boar on animal communities is not negligible. The predation of eggs and unfledged birds has a negative effect on populations of reptiles and ground-nesting birds. Pavlov *et al.* (1981) documented severe wild boar predation on newborn lambs on a farm. In addition to direct predation, this species has an indirect negative effect on the abundance of burrowing and ground-dwelling vertebrates and invertebrates, by altering soil surface and competing for available food (Massei and Genov, 2004; Blasi *et al.*, 2014).

Beyond the ecological implications, this occasional source of food is significant because the life cycles of several parasites involve animals such as insects, earthworms, and various vertebrates as intermediate or paratenic hosts. Wild boars are therefore constantly exposed to a wide range of parasitic species capable of completing their life cycle within them (Massei and Genov, 2004; Poglayen *et al.*, 2016). Accordingly, wild boars harbour numerous parasitic, viral and bacterial pathogens, some of which with zoonotic potential, thereby acting as important reservoirs or contributing to pathogen persistence in the environment (Ruiz-Fons, 2015).

Among the main zoonotic agents associated to wild boars, those of greatest relevance to public health include the parasites *Trichinella* spp. and *Toxoplasma gondii*; the bacteria *Salmonella* spp., Shiga toxin-producing *Escherichia coli*, *Brucella suis*, *Leptospira* spp., and *Mycobacterium tuberculosis* complex; and the viruses of hepatitis E (HEV), Japanese encephalitis (JEV), Nipah virus, influenza A, and African swine fever (Ruiz-Fons, 2015; Jota Baptista *et al.*, 2023).

Exposure to pathogens associated with wild boars can occur either directly or indirectly. Direct transmission results primarily from contact with infected animals or carcasses, or from the consumption of insufficiently cooked or otherwise improperly processed wild boar meat. Consequently, hunters, butchers, and veterinarians are among the occupational groups at greatest risk of direct exposure, whereas foodborne transmission of most pathogens remains uncommon in developed countries (Ruiz-Fons, 2015). Indirect transmission, in contrast, may expose a much larger proportion of the population through contaminated food and water, arthropod vectors, or livestock and companion animals acting as intermediate hosts or sources of infection (Ruiz-Fons, 2015).

According to Jones *et al.* (2008), approximately 60% of emerging infectious diseases are zoonoses

and, within this percentage, 71.8% originate in wildlife, a number increasing over time. Given the current global distribution and demographic growth of wild boar, as well as the ongoing expansion of human settlements into rural and semi-natural environments, contacts between humans, domestic animals and wild boars are expected to increase, thereby enhancing the likelihood of both direct and indirect interspecies transmission of zoonotic pathogens (Ruiz-Fons, 2015; Figueiredo *et al.*, 2020). These considerations highlight the potential role of wild boars as a relevant source of emerging infectious diseases in humans and underscore the importance of including this species in infectious disease surveillance programmes.

1.1.3. The wild boar in Italy

Nowadays the wild boar is the most common and widespread ungulate in Italy, able to occupy a wide variety of habitats, from anthropised areas to mountainous zones (Carnevali *et al.*, 2009).

The Italian territory has always been part of this species' original areal, but direct persecution by humans, beginning in the 16th century, led to the gradual disappearance of wild boars from most regions, with consistent local extinctions (Carnevali *et al.*, 2009).

The wild boar population began to increase significantly, both in Italy and across Europe, only after the end of World War II, due to the combination of factors treated above: the reforestation of abandoned fields and pastures, the decline in number of hunters, and the depopulation of rural areas (Carnevali *et al.*, 2009; Riga *et al.*, 2011). This spontaneous expansion was facilitated by the introduction of highly prolific hybrids from Central Europe and of animals bred in specialised Italian breeding farms (Riga *et al.*, 2011; Lombardini *et al.*, 2017).

Assessing the current distribution and population size of wild boar in Italy is difficult due to the serious lack of standardised data. The most comprehensive report available, published by the Italian Institute for Environmental Protection and Research (ISPRA, <https://www.isprambiente.gov.it>), refers to the years 2001-2005. The report estimated the Italian wild boar population at approximately 600,000 individuals (Carnevali *et al.*, 2009). Moreover, it indicated that wild boar range covered 64% of Italian territory, with a continuous distribution from Liguria, through the Apennines, to Calabria, including Sardinia (**Figure 2**). In many Alpine areas and in Sicily, its presence was sporadic, likely due to recent illegal releases, which are still frequent (Monaco *et al.*, 2007).

A more recent report by ISPRA, previewed in 2023 at a conference organised by Confagricoltura, estimated that between 2015 and 2021 there were at least 1.5 million wild boars throughout the Country, a number three times higher than that documented in the previous report. These data were

later confirmed by Smith *et al.* (2026), who estimated a population of 955,936 to 1,749,584 wild boars for 2022, which corresponds to an average density of 3.18–5.85 individuals per km², one of the highest in Europe.

According to Carnevali *et al.* (2009), between 2001 and 2005 wild boars were reported in 95 of Italy's 107 provinces. Since then, their continued expansion strongly suggests that their distribution has widened further and now encompasses nearly all Italian provinces. For example, wild boars are now established in central Po Valley provinces such as Lodi, Cremona and Mantua, where they were completely absent during the 2001–2005 period (Carnevali *et al.*, 2009).

Finally, wild boars are considered responsible for approximately 80% of the total agricultural damage caused by wildlife in Italy. Between 2015 and 2021, damage to agriculture attributable to wild boars was estimated at around €120 million (ISPRA, <https://www.isprambiente.gov.it>).

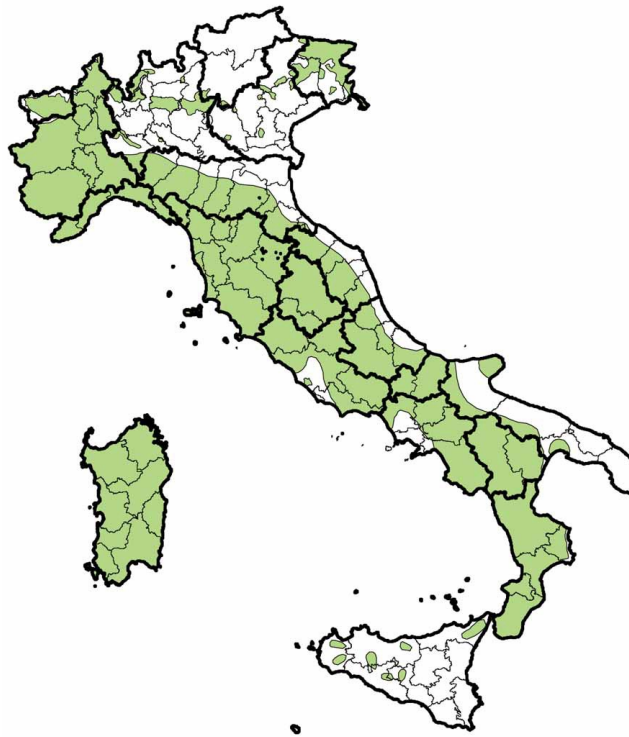


Figure 2. Geographic distribution of wild boars in Italy up to 2006
(Carnevali *et al.*, 2009)

1.1.4. Legislative framework

The main legislative framework governing wild boar management in Italy is National Law no. 157/1992, *Norme per la protezione della fauna selvatica omeoterma e per il prelievo venatorio*, (Framework Law on the Protection of Homeothermic Wildlife and Hunting). This legislation recognises the wild boar both as component of the national wildlife heritage, therefore subject to protection and conservation, and as a huntable species. According to the law, hunting is permitted

either from 1st October to 31st December or from 1st November to January 31st, depending on regional regulations. The law also provides for population control programmes, authorised by the Regions and Autonomous Provinces, when wild boars are responsible for damage to agricultural crops or pose risks to public health at local level (Law no. 157/92; Toso and Pedrotti, 2001).

Specific regional regulations further govern the local hunting calendar of wild boar and other ungulates. In Lombardy, the hunting calendar is regulated by Regional Law No. 17/2004 (as amended), which establishes that the hunting season runs from 1st October to 31st December, while selective hunting is permitted from 1st June to 31st January.

Each province is required to draft a five-year hunting management plan, which establishes the guidelines for the management of wild boars and the criteria for compensation for damage caused by the species (Law No. 157/92).

As regards measures to control wild boar expansion, there is still no coordinated and effective control plan, even though in 2003 ISPRA had already underlined the need of a harmonised, national management strategy for wild boars, based on long-term interventions and culling actions (Monaco *et al.*, 2003). By contrast, several wildlife monitoring and health control plans are in place to identify potential pathogens, with active and passive surveillance systems targeting *Brucella* spp., *Trichinella* spp., *Mycobacterium bovis* and the etiological agents of Aujeszky's disease and classic and African swine fever (IZSLER, 2026).

Although game meat consumption in Italy is generally limited and accounts for a reduced fraction of total annual meat consumption, in recent decades it has seen an overall increase, due to the growth in wild boar populations and management strategies (Ramanzin *et al.*, 2010). The health and hygiene requirements for game meat are set out in Regulation (EC) No. 853/2004, which lays down specific hygiene rules for food of animal origin.

According to the regulation, farmed wild boars may be slaughtered in the same slaughterhouse of domestic animals, ensuring however a spatial and temporal separation, and the carcasses must be inspected directly by a veterinarian. As regards wild boar harvested during hunting activities, hunters and the competent authorities are responsible for the safety and traceability of the meat: the carcasses must be examined by a qualified hunter and accompanied by appropriate documentation. In both cases, testing for *Trichinella* spp. is mandatory as part of the official inspection procedure (Regulation (EC) No. 853/2004).

1.2. *Cryptosporidium* spp.

1.2.1. Introduction

Cryptosporidium species are protozoan parasites of the gastrointestinal tract that infect a wide range of vertebrates, including livestock animals and humans (Tomazic *et al.*, 2018). The genus was first described in 1907 by the American parasitologist Ernest E. Tyzzer, who provided an accurate description of its life cycle, morphology and localisation within the intestinal tract (Tzipori and Widmer, 2008). Initially, *Cryptosporidium* spp. were considered occasional, opportunistic pathogens (Tzipori and Widmer, 2008). The clinical significance of this genus became clear only in the 1980s, when cryptosporidiosis was recognised as a cause of chronic diarrhoea and even death in immunocompromised individuals, such as HIV⁺/AIDS patients, as well as an important cause of acute diarrhoea in livestock and immunocompetent humans (Tzipori and Widmer, 2008; Ryan *et al.*, 2021b; Ryan *et al.*, 2021a).

Nowadays, *Cryptosporidium* is a well-recognised human pathogen that primarily affects children and immunocompromised adults (Ryan *et al.*, 2021; Pinto *et al.*, 2022), with an estimated prevalence of 4.3% in developed countries and 10.4% in developing countries. In developing countries cryptosporidiosis is the second leading cause of diarrhoea-associated mortality in young children after rotavirus (Pinto *et al.*, 2022). In addition, it is a major cause of enteric disease in neonatal livestock, resulting in substantial economic losses, and several *Cryptosporidium* species exhibit low host specificity and have zoonotic potential (Ryan *et al.*, 2021b; Tomazic *et al.*, 2018). Owing to its worldwide distribution and the absence of vaccines and specific treatments, cryptosporidiosis remains an important emerging cause of morbidity and mortality worldwide (Pinto *et al.*, 2022).

1.2.2. Taxonomy and life cycle

Cryptosporidium is a genus belonging to the phylum Apicomplexa, class Conoidasida, subclass Coccidia, order Eucoccidiida, suborder Eimeriorina, and family Cryptosporidiidae (Integrated Taxonomic Information System, 2026). *Cryptosporidium* species have traditionally been classified as coccidian parasites because of several similarities in their life cycle. However, molecular and biological characteristics, such as the presence of a *feeder* organelle, suggest a closer affinity with members of the subclass Gregarinia, a group of primitive parasites that infect invertebrates (Ryan *et al.*, 2021a; Pinto *et al.*, 2022). *Cryptosporidium* species have also been proposed to represent one of the early diverging lineages within the Apicomplexa (Ryan *et al.*, 2021a). Nevertheless, further

taxonomic and phylogenetic studies are needed to clarify their evolutionary position.

Cryptosporidium exhibits a complex monoxenous life cycle comprising both asexual and sexual phases (Guérin and Striepen, 2020). The parasite is transmitted via the faecal-oral route, typically through the ingestion of water or food contaminated with thick-walled oocysts, which represent the environmentally resistant and infectious stage of the parasite (Tomazic *et al.*, 2018).

The oocysts of most *Cryptosporidium* species are similar in shape and largely overlap in size, making species determination difficult or even impossible (Pinto *et al.*, 2022). They are typically spherical or ovoid, with size between 3.2 and 8.3 μm (Tomazic *et al.*, 2018; Pinto *et al.*, 2022; **Figure 3**). Two types of oocysts are produced during the life cycle: thick-walled oocysts, which are excreted in the faeces and released into the environment, and thin-walled ones, which tend to rupture within the host, thereby promoting autoinfection (Tomazic *et al.*, 2018).

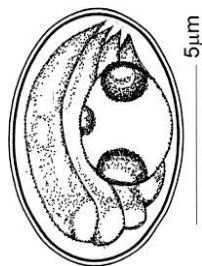


Figure 3. Schematic representation of *Cryptosporidium* oocyst (Ryan *et al.*, 2003).

The wall of thick-walled oocysts is composed of multiple layers that confer the characteristic acid-fast staining properties to *Cryptosporidium*, as well as resistance to desiccation, freezing, chlorination, and many chemical disinfectants (Tomazic *et al.*, 2018; Ryan *et al.*, 2021b). From the inside, there is a structural polysaccharide layer, a layer of cysteine-rich proteins and lipid hydrocarbon, and a glycocalyx composed of glycoproteins and acidic polysaccharides (Tomazic *et al.*, 2018). The wall is continuous except for a suture at one pole, from which, during excystation, the sporozoites exit the oocyst (Fayer *et al.*, 2008; **Figure 4**).

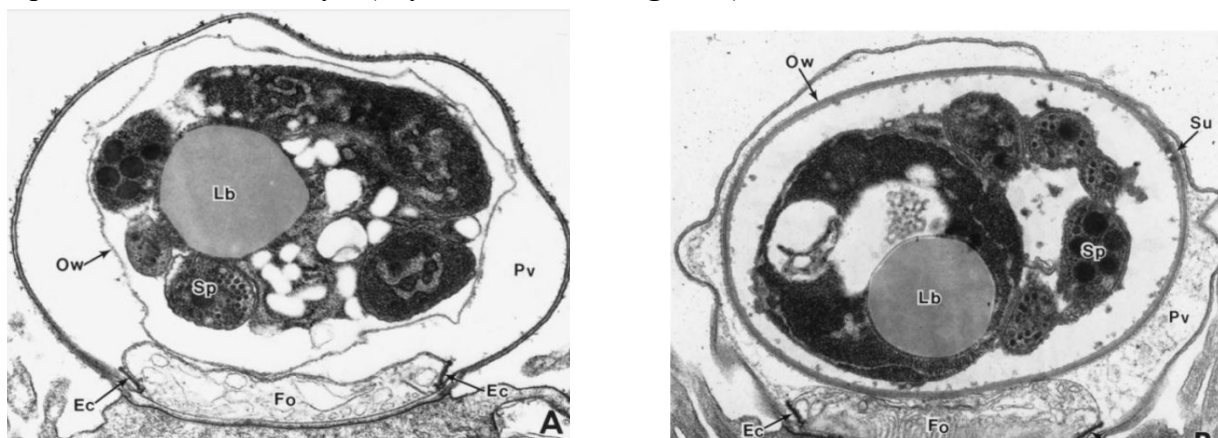


Figure 4. Transmission electron micrograph (TEM) of an intracellular section of *Cryptosporidium* oocyst. **Fo**, feeder organelle; **Lb**, lipid body; **Ow**, thick oocyst wall; **Pv**, parasitophorous vacuole; **Sp**, sporozoite; **Su**, suture at one pole (Fayer *et al.*, 2008).

Each oocyst contains four infective sporozoites, which are released during excystation, along with a residual lipid body containing amylopectin granules, a crystalline protein inclusion, ribosomes, and cytomembranes (Tomazic *et al.*, 2018; Fayer *et al.*, 2008; **Figure 4**).

The passage through the host's digestive system exposes the oocysts to various stimuli, such as changes in temperature, pH, and the presence of bile salts and proteases, which together trigger excystation. During this process, the oocyst suture opens, allowing the sporozoites to exit and invade the epithelial cells of the host's small intestine (Pinto and Vinayak, 2021; Tomazic *et al.*, 2018).

As shown in **Figure 5**, sporozoites have an elongated shape supported by a system of subpellicular microtubules and are characterised by the presence of an apical complex, the typical invasive structure of apicomplexan parasites. This complex is composed of apical rings, a conoid, and various secretory organelles, including one rhoptry, multiple micronemes, and dense granules (Tomazic *et al.*, 2018).

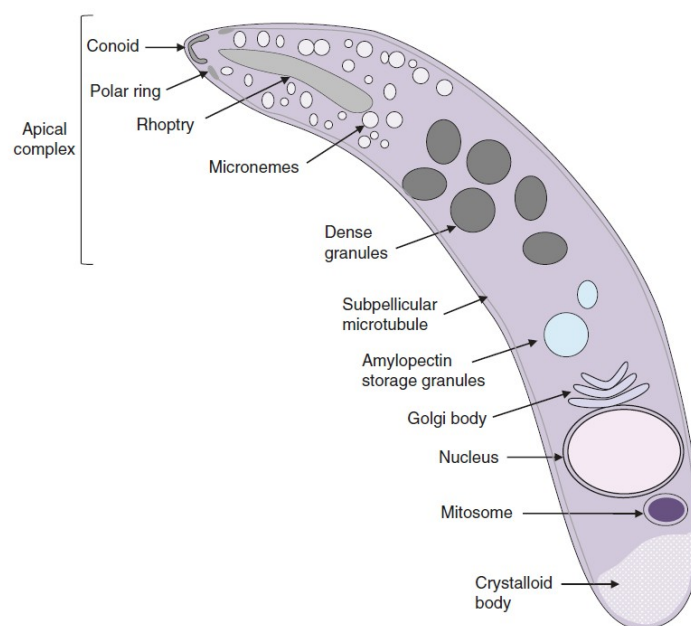


Figure 5. Schematic representation of a *Cryptosporidium* sporozoite (Tomazic *et al.*, 2018).

All infectious stages of *Cryptosporidium* express specific surface molecules and receptors involved in binding and invading the host cell, typically an enterocyte (Pinto and Vinayak, 2021). Among these, one of the most relevant is the glycoproteic complex *gp40/15*, produced through proteolytic cleavage of the precursor *gp60* (Tomazic *et al.*, 2018).

After recognition and attachment, the sporozoites bind to the apical membrane of enterocytes, between microvilli, and initiate invasion. The exact mechanism by which the sporozoite invades the epithelial cell remains unclear (Guérin and Striepen, 2020). Nevertheless, the invasion process is

known to be epicellular, as the parasite remains attached to the host cell surface in an extracytoplasmic location, enclosed within a specialised membrane-bound compartment known as the parasitophorous vacuole (Tomazic *et al.*, 2018). The compartment originates after parasite attachment, as the host cell membrane forms protrusions that surround the sporozoite. The folding of this membrane creates the feeder organelle at the host-parasite interface, a complex system of membrane invaginations that increases the surface area available for nutrient uptake (Guérin and Striepen, 2020). The rhoptries play an essential role in the formation of the parasitophorous vacuole, whereas micronemal proteins are involved in parasite attachment, host cell invasion, and gliding motility. Dense granules, in turn, are responsible for modifying the host cell following invasion (Tomazic *et al.*, 2018).

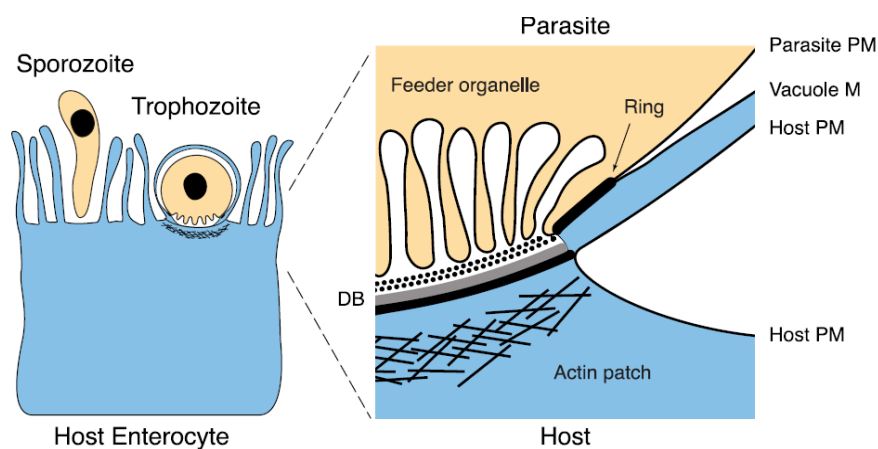


Figure 6. Complex host-parasite interface between *Cryptosporidium* and the host cell. The interface is composed of a ring-shape tight junction, a dense band (**DB**) and an actin-rich basal patch (Guérin and Striepen, 2020).

Following host cell invasion, the sporozoite differentiates into a uninucleate trophozoite. Through merogony – a form of asexual replication – each trophozoite produces a mature type I meront containing eight merozoites, which are then released into the intestinal lumen to invade adjacent epithelial cells (Pinto and Vinayak, 2021; Tomazic *et al.*, 2018). Type I merozoites can either replicate to form additional type I meront and merozoites or initiate the sexual reproduction known as gametogony, differentiating into type II meronts. Each of them contains four merozoites which then differentiate into macrogamonts, the female gamonts, and microgamont, the male gamonts (Tomazic *et al.*, 2018). While the macrogamont has a single, large nucleus and stores nutrients and wall-forming granules, the microgamont becomes multinucleated, and each nucleus is incorporated into a bullet-shaped, non-flagellated microgamete (Pinto and Vinayak, 2021; Tomazic *et al.*, 2018). The mechanism by which the male gamete recognises the female one remains unknown (Tomazic *et al.*, 2018). Nevertheless, fusion of the male and female nuclei results in the formation of a diploid zygote, which subsequently undergoes meiosis and sporogony to produce four haploid sporozoites,

each containing a single nucleus, enclosed within either a thin- or a thick-walled oocyst (Pinto and Vinayak, 2021; Tomazic *et al.*, 2018). The sporulated oocysts are then excreted in the faeces and released into the environment.

The life cycle of *Cryptosporidium* is completed within a single host and involves only one host cell type, primarily intestinal epithelial cells or, in some species, gastric or even respiratory epithelial cells (Pinto *et al.*, 2022). This characteristic distinguishes the genus *Cryptosporidium* from other apicomplexan parasites, such as *Toxoplasma gondii* and *Plasmodium falciparum*, which require multiple hosts to complete their asexual and sexual development and are capable of infecting a wide range of host cell types (Pinto and Vinayak, 2021).

The *Cryptosporidium* spp. life cycle is schematically summarised in **Figure 7**.

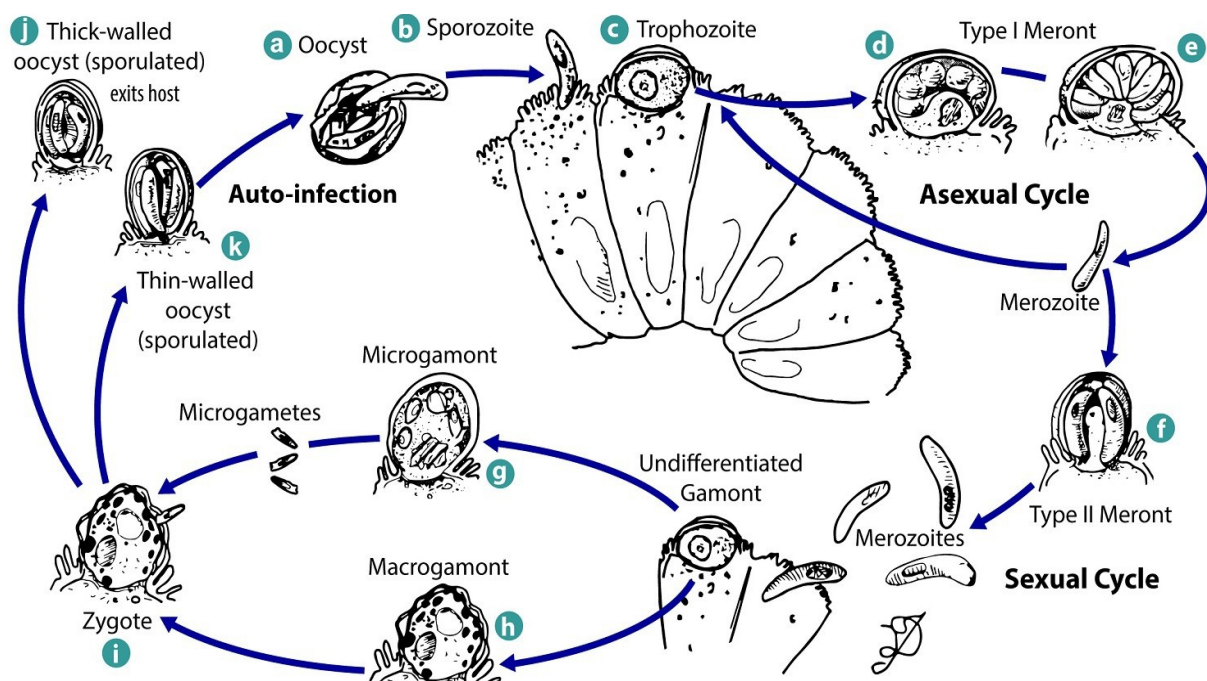


Figure 7. Schematic life cycle of *Cryptosporidium* spp. (<https://www.cdc.gov/cryptosporidium>)

1.2.3. Genome

The first *Cryptosporidium* genomes were sequenced using Sanger sequencing in 2004 and belonged to the species *C. parvum* and *C. hominis*. These genomes consisted of eight chromosomes encoding a total of 3,952-3,994 genes, with a size of 9.1-9.2 Mb (Ryan *et al.*, 2021a). Since then, 74 genome sequence assemblies for the genus *Cryptosporidium* have been published and are currently available in NCBI GenBank (Agyabeng-Dadzie *et al.*, 2024).

Genomic analysis has highlighted that *Cryptosporidium* energy metabolism is largely based on glycolysis, as the tricarboxylic acid (TCA) cycle and oxidative phosphorylation are absent (Xu *et*

al., 2004). Both aerobic and anaerobic metabolic pathways occur, whereas biosynthetic capabilities are limited due to the lack of enzymes required for the synthesis of simple sugars, amino acids, and nucleotides. Consequently, *Cryptosporidium* depends heavily on host-derived nutrients, a feature that is reflected in the large repertoire of transporter-encoding genes identified in its genome, which facilitate the uptake of essential metabolites from the host (Xu *et al.*, 2004).

1.2.4. Clinical significance

Cryptosporidiosis in immunocompetent individuals may be asymptomatic, although it is generally associated with watery diarrhoea, nausea, vomiting, fever, malnutrition, and abdominal discomfort. These clinical signs are typically self-limiting and resolve within two to three weeks (Leitch and He, 2011; Tomazic *et al.*, 2018; Ryan *et al.*, 2021b).

Three mechanisms have been proposed to explain the diarrhoea associated to cryptosporidiosis (Leitch and He, 2011): 1) *Cryptosporidium* causes malabsorption, which results in osmotic diarrhoea; 2) *Cryptosporidium* induces the generation of inflammatory products and host secretagogues; 3) *Cryptosporidium* produces an enterotoxin, which causes secretory diarrhoea.

In young children and older adults, cryptosporidiosis may cause severe diarrhoea that can persist for up to two years or longer. Persistent infection may become life-threatening and has also been associated with an increased risk of colorectal cancer (Ryan *et al.*, 2021a; Leitch and He, 2011). Furthermore, infections acquired during early childhood, particularly in malnourished children living in developing countries, may result in growth retardation and long-term cognitive impairment (Ryan *et al.*, 2021a; Leitch and He, 2011).

Immunocompromised individuals, including people living with HIV/AIDS and transplant recipients receiving immunosuppressive therapy, represent another population at particularly high risk. In these patients, cryptosporidiosis may cause profuse, persistent diarrhoea, leading to cholera-like stool volumes and severe electrolyte disturbances (Leitch and He, 2011). Moreover, extraintestinal involvement of the stomach, hepatobiliary tract, pancreas, and lungs is more common in immunocompromised patients and may occur in addition to intestinal infection (Leitch and He, 2011).

Young animals seem to be more susceptible to infection and disease, whereas adult animals are often asymptomatic (Ramirez *et al.*, 2004). The most common symptom of animal cryptosporidiosis is yellowish, watery diarrhoea, followed by dehydration, weight loss, fever, and inappetence. Most immunocompetent animals recover within 1–2 weeks (Ramirez *et al.*, 2004).

Young farm ruminants like calves, goat kids and lambs are highly susceptible to symptomatic

cryptosporidiosis, which can cause a diarrhoeic syndrome characterised by dehydration, impaired growth, weight loss, and increased mortality, resulting in significant production losses and additional economic expenses for the extra care of ill animals (Ramirez *et al.*, 2004; Ryan *et al.*, 2021b). Avian cryptosporidiosis likewise leads to the decline in productivity and is responsible for significant economic losses in the poultry industry (Ramirez *et al.*, 2004).

In pigs and horses, infections are typically asymptomatic, even in young animals, although severe *Cryptosporidium* infections in nursing piglets have been reported in Europe (Chen *et al.*, 2023; Ramirez *et al.*, 2004; Enemark *et al.*, 2003). Symptomatic cases in pigs are characterised by diarrhoea, vomiting, dehydration, reduced average daily weight gain, and a lower feed conversion ratio (Chen *et al.*, 2023).

Although mainly asymptomatic, cryptosporidiosis has also been documented in a wide range of wild animals, including wild mice, deer, feral pigs, wild rabbits, and foxes. Wildlife, therefore, provides a source of contamination for environment and surface waters, posing a risk of infection to livestock and humans (Ramirez *et al.*, 2004). Specifically, studies evaluating *Cryptosporidium* spp. prevalence in wild boar populations in Central Europe and Spain found no association between diarrhoea and infection with *Cryptosporidium* (Němejc *et al.*, 2013; García-Presedo *et al.*, 2013). This supports the hypothesis that wild boars do not exhibit clinical signs and may function as healthy carriers, contributing to the contamination of surface water, and environment (García-Presedo *et al.*, 2013).

To date, nitazoxanide is the only drug approved by the U.S. Food and Drug Administration (FDA) for the treatment of human cryptosporidiosis. This broad-spectrum antiparasitic and antiviral agent has demonstrated only limited efficacy in immunocompetent children and adults and is largely ineffective in the populations most severely affected by the disease, namely malnourished children and immunocompromised individuals (Guérin and Striepen, 2020; Ryan *et al.*, 2021b). Regarding animal cryptosporidiosis, Halocur® (halofuginone lactate) has been licensed in some countries, including the European Union, as a prophylactic agent. Its effectiveness, however, is variable, and it exhibits toxicity at high doses (Tomazic *et al.*, 2018; Ryan *et al.*, 2021b).

Up to now, there are no available vaccines against human cryptosporidiosis (Tomazic *et al.*, 2018; Ryan *et al.*, 2021b). Conversely, the vaccine Bovilis Cryptium® received marketing authorization in the European Union in 2023 for the prevention of bovine cryptosporidiosis (Timmermans *et al.*, 2023). Its active substance is the recombinant *C. parvum* glycoprotein 40 (*gp40*), a key protein involved in host cell invasion. The vaccine is administered to pregnant heifers and cows to passively transfer protective immunity to calves through colostrum (Veterinary Medicines Information website, <https://medicines.health.europa.eu/veterinary/en>).

1.2.5. Epidemiology

Cryptosporidium is transmitted via the faecal-oral route, either directly or indirectly. Direct transmission occurs through contact with an infected host (person-to-person, animal-to-person, or person-to-animal), whereas indirect transmission results from the accidental ingestion of contaminated material, including water (particularly recreational water), soil, fomites, or food (Pinto *et al.*, 2022; Tomazic *et al.*, 2018).

The remarkable resistance of *Cryptosporidium* oocysts to chlorine-based disinfectants and 70% ethanol, together with their ability to survive in the environment for up to six months, makes them particularly difficult to eradicate. Interestingly, Koh *et al.* (2013) reported that *Pseudomonas aeruginosa* biofilms not only serve as environmental reservoirs for *Cryptosporidium* oocysts but may also support their extracellular multiplication in aquatic environments.

Cryptosporidium has the potential to cause waterborne epidemics and large-scale outbreaks in both developing and developed nations (O’Leary *et al.*, 2021). Most major human outbreaks of cryptosporidiosis have been attributed to contaminated drinking water, such as the 1993 Milwaukee waterborne outbreak that affected 400,000 people (Leitch and He, 2011). Considering its widespread diffusion and its public health impact – which are often closely linked to poverty and poor hygienic conditions – in 2004 the World Health Organization included the parasite in the Neglected Disease Initiative (Certad *et al.*, 2017).

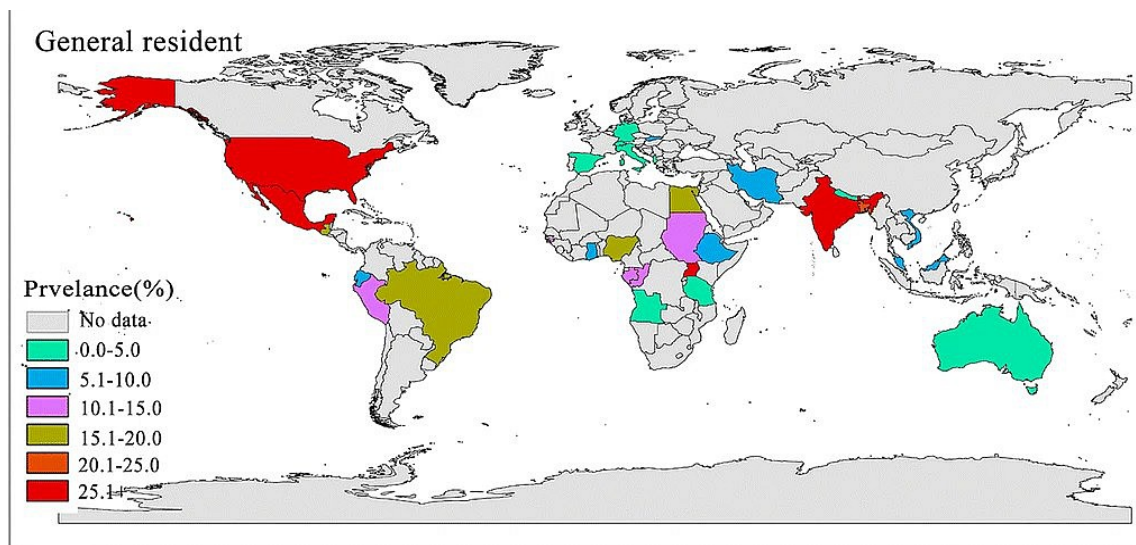


Figure 8. Prevalence of *Cryptosporidium* infection worldwide (Dong *et al.*, 2020).

According to a recent meta-analysis (**Figure 8**), the estimated overall prevalence of *Cryptosporidium* infection in humans worldwide is 7.6% (Dong *et al.*, 2020). However, prevalence rates vary significantly across countries, ranging from 0.0% to 86.0%. Low-income countries

exhibit the highest average prevalence (10.4%), compared to 4.3% in high-income countries (Dong *et al.*, 2020). Children in low-income countries are the most susceptible to cryptosporidiosis, with infection directly associated with malnutrition, stunted growth, and cognitive impairment (Guérin and Striepen, 2020; Dong *et al.*, 2020). In Bangladesh, for instance, the prevalence of *Cryptosporidium* spp. among schoolchildren is 42.5% (Dong *et al.*, 2020).

The two most predominant *Cryptosporidium* species in humans are *C. parvum* and *C. hominis*, which account for more than 95% of human cryptosporidiosis cases (Pinto *et al.*, 2022; Ryan *et al.*, 2021b).

Due to the wide host range of the genus *Cryptosporidium*, cryptosporidiosis affects a wide variety of vertebrates, from rodents to ruminants and birds (Ryan *et al.*, 2021b). According to Buchanan *et al.* (2025), the global overall prevalence of *Cryptosporidium* in cattle is 26.5%, whereas the prevalence rates in equine, buffaloes, sheep, and goats are 4.7%, 26.0%, 24.4%, and 8.2%, respectively (Hatam-Nahavandi *et al.*, 2019). Furthermore, the overall prevalence of *Cryptosporidium* spp. in wild birds has been estimated at approximately 3.96% (Wang *et al.*, 2021). Among the species susceptible to *Cryptosporidium* infection, pigs are considered one of the main reservoirs, as prevalence rates commonly reach peaks of 24-60% (Chen *et al.*, 2023; Nĕmejč *et al.*, 2013; García-Presedo *et al.*, 2013). The predominant species in pigs are *C. suis* and *C. scrofarum*, accounting for more than 90% of infections (Chen *et al.*, 2023; Ryan *et al.*, 2021b; Nĕmejč *et al.*, 2013). Both species appear to be host-specific to pigs, although sporadic human cases involving *C. suis* and *C. scrofarum* have been reported (García-Presedo *et al.*, 2013; Chen *et al.*, 2023; Ryan *et al.*, 2021b). Other species and genotypes, however, have been occasionally identified in pigs, including *C. muris*, *C. parvum*, and *C. meleagridis*, (Chen *et al.*, 2023; Ryan *et al.*, 2021b).

A recent global estimate (Chen *et al.*, 2023) indicated an overall prevalence of *Cryptosporidium* in pigs of approximately 16.3%, with considerable variability: 14.8% in Asia, 18.3% in Europe, 40.8% in Africa, 13.6% in North America, 7.1% in South America and 9.3% in Oceania. More specifically, the prevalence rates were 7.9% for *C. scrofarum* and 4.7% for *C. suis*.

Wild boars are likewise susceptible to *Cryptosporidium* infections. Several studies have confirmed the presence of *C. scrofarum* and *C. suis* in wild boars, while reporting a relatively lower prevalence compared to domestic pigs (Nĕmejč *et al.*, 2013; García-Presedo *et al.*, 2013).

1.2.6. Genetic variability and zoonotic potential

To date, 44 species of *Cryptosporidium* have been validated (Ryan *et al.*, 2021a). The definition of species is based on oocyst morphology, natural host specificity, and genetic characterization (Ryan

et al., 2021a).

As many species are morphologically indistinguishable, *Cryptosporidium* genetic diversity has been explored primarily through molecular genotyping methods (Xiao, 2010; Pinto *et al.*, 2022), which allowed the identification of more than 120 genotypes (Ryan *et al.*, 2021a).

Genotyping is typically carried out through analysis of the 18S ribosomal RNA locus (Small Subunit rRNA, SSU rRNA), which is a multi-copy gene composed of both conserved and hypervariable regions and represents the most sensitive locus for the detection of all species and genotypes (Xiao, 2010; Ryan *et al.*, 2021a). Other loci, such as the *hsp70* and *COWP*, are less used (Xiao, 2010). As these regions show high interspecific but low intraspecific variability, they are ideal for species identification and genotypic differentiation (O’Leary *et al.*, 2021).

Subtyping tools have been developed to track the transmission dynamics of *Cryptosporidium* species and genotypes between humans and animals and during outbreaks, thereby highlighting spillover and spillback phenomena (Ryan *et al.*, 2021a). The most used locus for this purpose is the *gp60* gene, which encodes a 60 kDa glycoprotein that undergoes a proteolytic cleavage to generate the *gp40/15* complex (Xiao, 2010; **Figure 9**). At the 5'-end of the *gp60* gene, there is a microsatellite-like sequence containing tandem repeats of the serine-coding trinucleotides TCA, TCG or TCT (Pinto *et al.*, 2022). Variations in the number of these repeats enable the characterization of subtype families within a species (Xiao, 2010; Pinto *et al.*, 2022). Beyond its important epidemiological relevance, *gp60* gene is crucial to parasite biology: the *gp40/15* complex is located on the surface of the parasite's apical region and is directly involved in adhesion and invasion (Xiao, 2010). As a result, in some cases, subtyping analysis can correlate the biological characteristics of the parasite with a specific subtype family (Xiao, 2010).

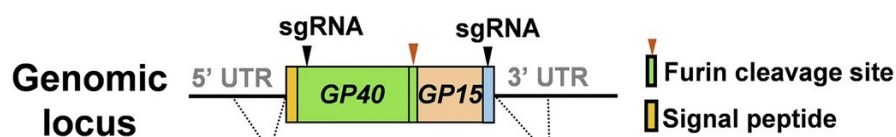


Figure 9. Schematic structure of the *gp60* locus (Li *et al.*, 2024).

The nomenclature for *Cryptosporidium* subtypes based on *gp60* sequence analysis starts with a Roman numeral indicating the species (for example, I for *C. hominis*, II for *C. parvum*), followed by a lowercase letter indicating the genotype (a, b, c...). Finally, the number of TCA (represented by the letter A), TCG (represented by the letter G), and TCT (represented by the letter T) repeats is reported. The presence of additional sequences immediately following the trinucleotide repeats is indicated by “R” (Ryan *et al.*, 2021b; Xiao, 2010).

Extensive panels including SSU rRNA, *COWP*, *hsp70*, *gp60*, and many other markers are employed in multilocus sequence typing (MLST) for epidemiological studies aimed at identifying population structure and inter- and intraspecific genetic diversity (O’Leary *et al.*, 2021). MLST is the systematic sequencing of five to seven conserved or housekeeping loci with genetic polymorphisms within an organism's genome: the sequence type, or allelic profile, is obtained when all the alleles from all loci – namely the nucleotide differences at each locus – are considered together (Li *et al.*, 2019).

Advances in genome sequencing technologies have greatly expanded genomic research on *Cryptosporidium*, paving the way for epidemiological investigations, functional genomics, protein and metabolic pathway predictions, and improved genome annotation (O’Leary *et al.*, 2021).

Among the species reported in humans, *Cryptosporidium hominis* and *C. parvum* are the most commonly identified (Ryan *et al.*, 2021b). Of the two, *C. hominis* has the highest prevalence rates in both industrialised and developing countries and is transmitted primarily through anthroponotic transmission, although it has occasionally been detected in livestock and wildlife (Ryan *et al.*, 2021b). The subtype IbA10G2 is one of the major causes of waterborne outbreaks (Ryan *et al.*, 2021b).

In contrast, the host range of *C. parvum* is wide: it is commonly reported in ungulates, wild carnivores, rodents, non-human primates, and marine mammals. Calves, sheep, goats, and other livestock represent the principal reservoirs for zoonotic transmission, making *C. parvum* the most important zoonotic *Cryptosporidium* species infecting humans, particularly in rural areas where contact with livestock is frequent (Ryan *et al.*, 2021b). *C. parvum* comprises more than 20 *gp60* subtype families, each characterised by a distinct geographical distribution and a host range of varying breadth (Ryan *et al.*, 2021b).

As regards pig species, the zoonotic potential of *C. scrofarum* and *C. suis* has not yet been fully established (Ryan *et al.*, 2021b; Chen *et al.*, 2023). A few human cases of *C. suis* have been reported in HIV-positive patients in China, Peru, and Thailand, as well as in children from Cambodia, whereas *C. scrofarum* has been documented only once in a symptomatic immunocompetent male co-infected with *Giardia duodenalis* in the Czech Republic (Ryan *et al.*, 2021b). These sporadic cases suggest a limited, although not negligible, zoonotic potential and highlight the need for further molecular investigations. Due to the current lack of a *gp60* subtyping tool for *C. scrofarum* and *C. suis*, the zoonotic potential and waterborne transmission pathways of these species remain largely unknown (Ryan *et al.*, 2021b).

1.2.7. Diagnosis

Brightfield microscopy is widely used in clinical diagnostics and involves the preparation of a stained faecal smear. The most common staining method is the modified Ziehl-Neelsen (mZN) technique, which exploits the acid-fast properties of *Cryptosporidium* to produce a marked contrast between the red-stained oocyst and the green background counterstain (Taylor *et al.*, 2016). mZN and other staining techniques developed for *Cryptosporidium* detection (such as acid-fast Kinyoun's stain and hot safrinin-methylene blue stain) are time-consuming and exhibit low sensitivity (O'Leary *et al.*, 2021). The main limitations of brightfield microscopy are the poor sensitivity, further worsened by the intermittent oocyst shedding typical of *Cryptosporidium* life cycle, and the high risk of false positives resulting from the misidentification of yeasts, fungi and bacterial spores (O'Leary *et al.*, 2021). To maximize the oocysts recovery, several concentration methods have been developed, including sedimentation and filtration (Tomazic *et al.*, 2018).

An improvement in the microscopic detection of oocysts was achieved through the introduction of immunofluorescence techniques. These methods involve incubating faecal samples with monoclonal anti-oocyst wall antibodies, conjugated with fluorescein isothiocyanate (FITC) and biotin hydrazide. The antibodies bind to parasite wall antigens and impart a distinct fluorescence to the oocysts against a dark background (O'Leary *et al.*, 2021). Despite offering higher sensitivity and specificity, these techniques are more expensive than conventional brightfield staining microscopy.

Enzyme-immunoassays (EIAs), such as ELISA, and immunochromatographic methods target parasitic antigens in faecal samples. These assays have been developed as practical, rapid, and cost-effective alternatives to microscopy, as they require no specialized instrumentation or advanced microscopy skills (O'Leary *et al.*, 2021). Compared to conventional acid-fast staining methods, EIA and ELISA kits offer significantly higher sensitivity (94-100%) and specificity (93-100%) (O'Leary *et al.*, 2021). Immunochromatographic rapid tests, which rely on the capillary migration of antigens, are even more rapid, as they eliminate reagent addition, washings, and incubation steps. Their sensitivity and specificity, however, vary considerably depending on the commercial kit used (O'Leary *et al.*, 2021). Nevertheless, both enzyme immunoassay and immuno-chromatographic tests are susceptible to generating false positive results (O'Leary *et al.*, 2021).

Molecular diagnosis relies primarily on PCR techniques, which provide a higher sensitivity than conventional microscopic and immunological methods. Molecular techniques, in addition, allow species and genotypes identification, as well as population genetic analysis (Tomazic *et al.*, 2018; O'Leary *et al.*, 2021). The amplification targets specific genomic regions, such as the small subunit

rRNA (SSU rRNA) gene and the *gp60* locus. The amplicons can be analysed via enzymatic cleavage or direct sequencing; *Restriction Fragment Length Polymorphism* (RFLP), in particular, was the first approach used for genotyping *Cryptosporidium* species, though it offered limited resolution (O’Leary *et al.*, 2021).

To date, the most employed molecular assays are conventional PCR, nested PCR, real-time PCR, and multiplex PCR, each offering distinct analytic advantages (Tomazic *et al.*, 2018; O’Leary *et al.*, 2021). Despite their superior specificity and sensibility, PCR techniques are not optimal for routine clinical diagnostics due to high costs and the high risk of contamination (O’Leary *et al.*, 2021).

1.2.8. *In vivo* animal models and *in vitro* culture systems

Most of the current knowledge on the life cycle and biology of *Cryptosporidium* spp. is based on experimentally infected animal models and *in vitro* culture systems (Pinto and Vinayak, 2021; Pinto *et al.*, 2022).

Among animal models, neonatal calves are frequently used to investigate the clinical manifestations of cryptosporidiosis and to test the therapeutic efficacy of anti-cryptosporidial drugs. Meanwhile, piglet models represent the ideal system for the analysis of human cryptosporidiosis, especially *C. hominis* infection, owing to the marked anatomical, physiological and immunological similarities between humans and pigs (Pinto and Vinayak, 2021). Given the challenges associated with large animal models, such as the stabling and the need for specific handling facilities, several mouse models, easier to manage, have been developed over time: a successful example is the mouse model developed by Sateriale *et al.* (2019), which uses a *C. tyzzeri* strain to reproduce the progression of human cryptosporidiosis in laboratory mice (Pinto and Vinayak, 2021). This species naturally infects mice and genetically resembles the species responsible for human cryptosporidiosis; to enable tracking of the infection, *C. tyzzeri* isolates were genetically engineered to create a stable transgenic strain expressing luminescent and fluorescent reporters (Sateriale *et al.*, 2019).

To reduce the use of live experimental animals, *Cryptosporidium* research has largely focused on the development of *in vitro* culture systems. Generally, the cell lines proposed over the last few decades support the complete development of the parasite, including oocyst production, but most of them are unable to sustain long-term *Cryptosporidium* infection. This limitation likely derive from the inability of these models to support the production of thin-walled oocysts and, consequently, parasite autoinfection (Pinto *et al.*, 2022). Currently, the gold standard for studying *Cryptosporidium* biology is represented by the human ileocecal adenocarcinoma cell line (HCT-8) and the human oesophageal squamous-cell carcinoma cell line (COLO-680N). COLO-680N, in

particular, provides an effective long-term culture system (Miller *et al.*, 2018). Both the cell lines, however, are carcinoma-derived or transformed; this means that their proteomic profiles and morphologies are unstable and differ from the non-transformed cells found *in vivo* (Pinto *et al.*, 2022).

Primary cell lines would offer a more accurate *in vitro* model for studying *Cryptosporidium* infection, although they present several disadvantages: they are isolated directly from tissues, thus requiring a constant supply of animals or human donors, and, unlike immortalised cell lines, they have a limited lifespan (Pinto *et al.*, 2022).

1.3. *Enterocytozoon bieneusi*

1.3.1. Introduction

Among Microsporidia, a ubiquitous group of obligate intracellular fungal parasites, *Enterocytozoon bieneusi* is by far the most frequently identified species in humans (Koehler *et al.*, 2022; Santin and Fayer, 2010). This parasite is an opportunistic pathogen reported worldwide in both humans and a wide range of animals, such as cats, dogs, horses, cattle, birds, and pigs (Li *et al.*, 2022).

Enterocytozoon bieneusi infection usually affects the enterocytes of the host with variable clinical presentation: in immunocompetent humans, it is generally asymptomatic, although it may cause acute watery diarrhoea and vomit; in immunocompromised patients, it may act as an opportunistic agent, leading to chronic wasting disease and even death (Koehler *et al.*, 2022; Li *et al.*, 2022; Santin-Duràn, 2015). This species, therefore, poses a risk for immunocompromised individuals, such as people with AIDS or chemotherapy patients, and it is recognised as having the potential to cause waterborne or foodborne outbreaks (Nourisson *et al.*, 2024). Reports of outbreaks involving *E. bieneusi* have been described in Sweden (2009), Denmark (2020) and France (2018), although the actual extent of its epidemiological impact is underestimated. The first and largest published outbreak occurred in the 1990s in Lyon (France) and involved at least 200 people (Nourisson *et al.*, 2024).

Despite its remarkable clinical significance, the parasite remained undiscovered until the HIV/AIDS pandemic in the 1980s. Based on electron microscopic morphology, Desportes *et al.* (1985) identified a new genus of microsporidian parasite in the enterocytes of a Haitian AIDS patient and chose the species name *Enterocytozoon bieneusi* referring to the microsporidian's localization within the host and in memory of the patient, Bieneus (Moniot *et al.*, 2021).

Nowadays, nearly four decades later, *Enterocytozoon bieneusi* stands as the main agent of human microsporidiosis and is widely identified both in domesticated and wild animals (Li *et al.*, 2022; Nourisson *et al.*, 2024).

1.3.2. Taxonomy and life cycle

Microsporidia are a eukaryotic phylum of obligate intracellular parasites classified within the superphylum Opisthosporidia, clade Opisthokonta (Bojko *et al.*, 2022). The current taxonomy, based on phylogenetic analyses of various genes, classifies Microsporidia as a fungal taxon or, at least, as a sister group to Fungi (Santin and Fayer, 2010; Zhang *et al.*, 2021).

Microsporidia are characterised by the production of resistant spores, which represent the infectious

stage of their life cycle and contain a polar tube, a unique infection organelle that acts as a conduit for transferring the spore contents into host cells (Chen *et al.*, 2023). Microsporidia organisms are also characterised by a marked reduction in their genomes and proteomes, which results in complete host metabolic dependence (Bojko *et al.*, 2022).

Despite ongoing complex taxonomic revisions, the phylum is divided into five clades – 1, 3, 4a, 4b, 5 – and comprises more than 200 genera and 1600 species that infect a wide range of hosts, mainly insects, crustaceans, molluscs, and fishes (Nourisson *et al.*, 2024; Bojko *et al.*, 2022; Santin-Duràn, 2015). Among these, there are only 17 species belonging to various genera reported to infect humans, with the most common being *Enterocytozoon bieneusi* and three species of the genus *Encephalitozoon* (*E. cuniculi*, *E. intestinalis*, *E. hellem*) (Bojko *et al.*, 2022). *Enterocytozoon bieneusi*, however, is responsible for more than 90% of documented cases of human infection, being the major agent of human microsporidiosis (Santin and Fayer, 2010 ; Li *et al.*, 2022; Allievi *et al.*, 2026). This species is the sole representative of the genus *Enterocytozoon*, which belongs to the family Enterocytozoonidae, order Chytridiopsida, and class Microsporea (Li *et al.*, 2022).

Although many aspects of the epidemiology of *E. bieneusi* remain elusive, the main transmission route is thought to be faecal-oral (Nourisson *et al.*, 2024; Li *et al.*, 2019). The life cycle of the parasite is monoxenous and takes place within the cytoplasm of a single cell type, the enterocytes of the host (Cali and Owen, 1990). However, host-parasite interactions are not yet fully understood because of the lack of suitable laboratory study models (Nourisson *et al.*, 2024).

As an obligate parasite lacking host-independent metabolic processes, none of the life stages of *Enterocytozoon bieneusi* can survive outside the host, with the exception of the spore, which represents the infective stage.

The spores are oval, measuring 0.70–0.98 x 1.08–1.64 µm (Cali and Owen, 1990), and are covered by a thick wall that confers them resistance and long-term viability in the environment (Li *et al.*, 2019). The spore wall consists of three layers: the outer, electron-dense layer called the exospore, composed of glycoproteins; the electron-lucent chitinous layer, called the endospore; and the inner plasma membrane, which lines the wall and covers the sporoplasm (Nourisson *et al.*, 2024). Inside the spore wall lies the sporoplasm, which is the infectious material; it consists of a single nucleus (*monokaryon*) and a cytoplasm containing primitive ribosomes (Li *et al.*, 2019; Cali and Owen, 1990). As shown in **Figure 10**, the sporoplasm also contains: the posterior vacuole, involved in generating the osmotic pressure necessary for the extrusion of the polar tube; the membrane-bound lamellar polaroplast, a type of atypical Golgi apparatus that occupies one-third of the anterior portion of the spore; and the anchoring disk, an umbrella-shaped structure in direct contact with the spore wall, from which the polar tube originates (Li *et al.*, 2019; Nourisson *et al.*, 2024;). The latter

extends down through the centre of the spore, forming a curve before winding into five to seven coils in a double row around the sporoplasm in the mid-to-posterior region of the spore (Nourrisson *et al.*, 2024; Santin and Fayer, 2010).

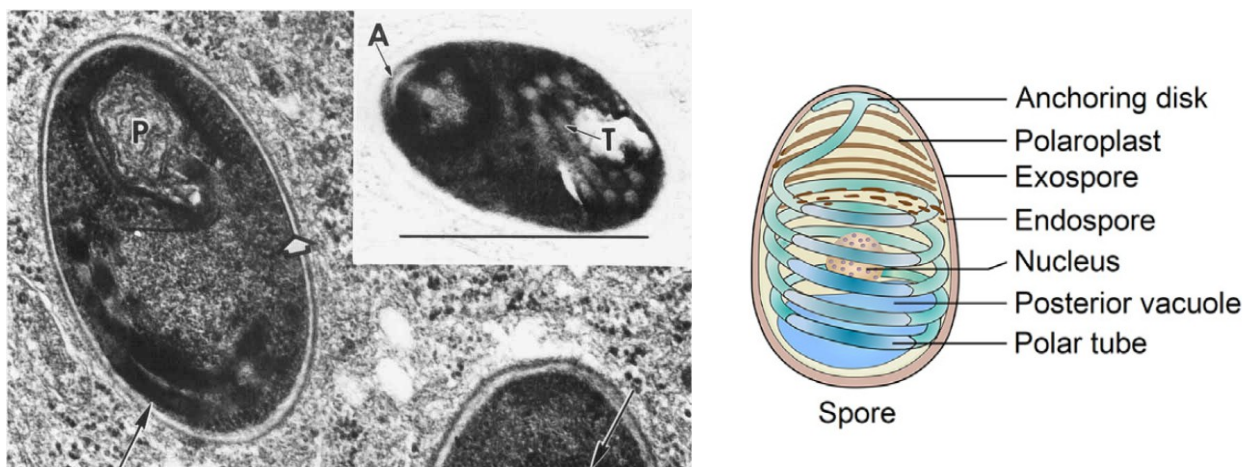


Figure 10, left. Transmission electron micrograph (TEM) of mature spores in the cytoplasm of an enterocytes. (P) indicates the vesicular polaroplast, (A) the anchoring disc, (T) portions of the coiled polar tube; the arrow points longitudinal arcs formed by the polar tube, while the arrowhead the spore wall, with an electron lucent endospore and an outer electron dense exospore (Cali and Owen, 1990)

Figure 10, right. Schematic representation of an *Enterocytozoon bieneusi* spore (Li *et al.*, 2022)

The life cycle of *Enterocytozoon bieneusi* begins after the ingestion of spores by a susceptible host. In response to environmental stimuli, such as the alkaline pH of the gut, the spore germinates and invades the target cells: primarily enterocytes and, more rarely, hepatobiliary cells (Nourrisson *et al.*, 2024; Chen *et al.*, 2023).

Cell invasion consists of the extrusion of the polar filament, which is everted, similarly to the reversed finger of a glove, to form a hollow polar tube through which the sporoplasm is injected into the host cell (Santin-Duràn, 2015; Chen *et al.*, 2023). The ejection process of the polar tube is extremely rapid, allowing invasion to be completed in less than 2 or 3 seconds (Chen *et al.*, 2023). The mechanism by which the polar tube mediates the sporoplasm entry into the cell is still unclear. Observations on several microsporidian species include both the penetration of the polar tube into the host cell, delivering the infectious sporoplasm directly inside, and the fusion of the polar tube with the host cell membrane. This fusion forms a synapse-like structure that protects the sporoplasm, which is subsequently internalized via endocytosis (Chen *et al.*, 2023).

The parasite is located in the cytoplasm of the enterocytes, between the nucleus and the luminal surface (Nourrisson *et al.*, 2024). Its entire development occurs in direct contact with the host cytoplasm, without an envelope or a parasitophorous vacuole like in *Encephalitozoon* spp., the second most common agent of human microsporidiosis (Cali and Owen, 1990).

After the infective stage, *Enterocytozoon bieneusi* is referred to as a meront and undergoes a proliferative phase of growth and division (Nourisson *et al.*, 2024). The meront nucleus divides several times without subsequent cytokinesis, resulting in a multinucleated plasmodium containing typical elongated nuclei and electron-dense inclusions (Cali and Owen, 1990; Santin-Duràn, 2015). After maturation the plasmodium is referred to as sporogonial plasmodium, rich in electron-dense disks stacked or fused in arcs, which represent the early stages of polar tube formation (Santin and Fayer, 2010). It then undergoes sporogony, during which individual nuclei segregate with their own polar tube complexes, maturing into separate sporoblasts. Finally, the sporoblasts complete the development of their spore organelles to form mature spores. These accumulate within the host cell until the cell membrane ruptures, releasing the spores into the faeces and completing the life cycle (Santin-Duràn, 2015; Nourisson *et al.*, 2024).

Interestingly, studies conducted by Desportes *et al.* (1985) and Cali and Owen (1990) show that each enterocyte can contain multiple parasites at different stages of development.

The *E. bieneusi* life cycle is schematically summarised in **Figure 11**.

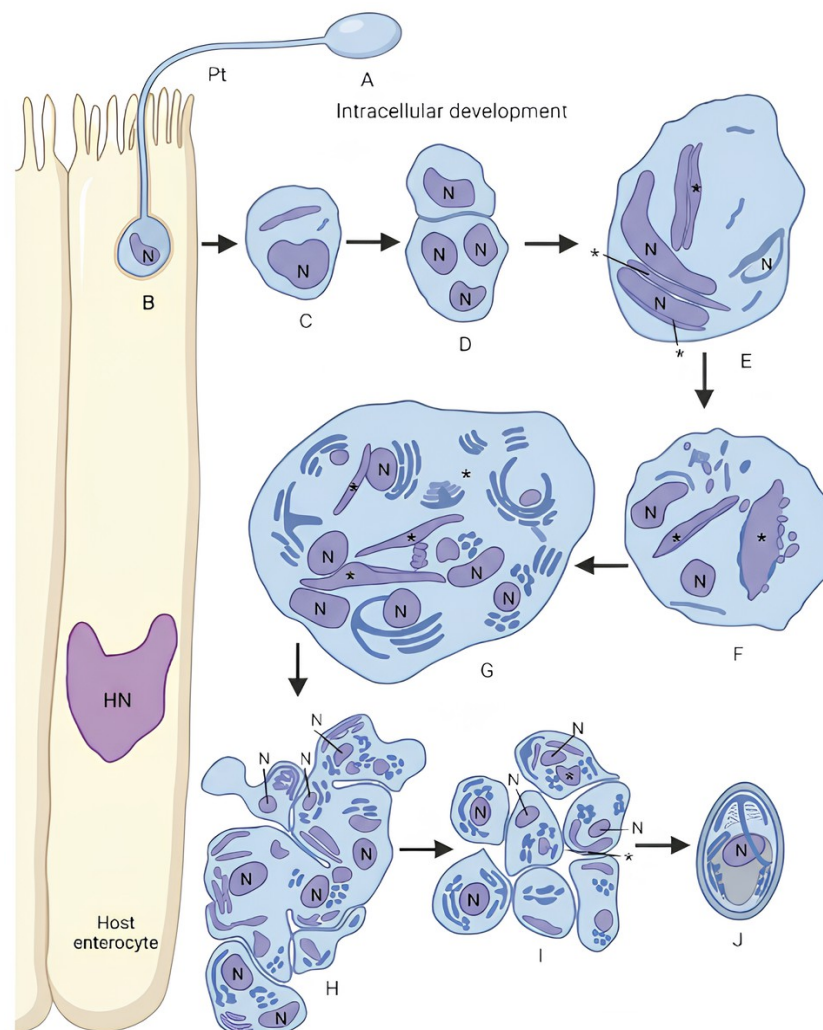


Figure 11. *Enterocytozoon bieneusi* life cycle. A-B) Invasion of the host cell. C-F) Formation of multinucleated plasmodium, containing multiple elongated nuclei (N) and electron-lucent inclusions (*).

F-G) Maturation of sporogonial plasmodium, filled with electron-dense disks. H) Sporogony. I-J) Formation of sporoblast cells and mature spores, which will cause the rupture of the host cell membrane.

(Modified from Cali and Takvorian, 2014).

1.3.3. Genome

The *Enterocytozoon bieneusi* genome has an estimated size of only 6 Mb, with 3,804 predicted genes, and consists of 6 chromosomes (Keeling *et al.*, 2010; Li *et al.*, 2019). Such a small genome is not unusual among microsporidia, which possess some of the most compact and highly reduced genomes among eukaryotes, in some cases even smaller than those of many bacteria (Li and Xiao, 2021; Keeling *et al.*, 2010). As highly specialised obligate intracellular parasites, microsporidia depend on their hosts for many essential metabolites. Consequently, progressive gene loss and the resulting genome reduction have been evolutionary tolerated, as there has been little or no selective pressure to retain genes encoding metabolic pathways that are supplied by the host (Li and Xiao, 2021).

This process has reached its extreme in *E. bieneusi*, which exhibits a degree of host dependence unmatched by any other known parasite. Whereas all other microsporidian genomes retain complete pathways for glycolysis, the pentose phosphate pathway, and trehalose and lipid metabolism, *E. bieneusi* has undergone a dramatic reduction in genes involved in energy production and carbon metabolism, to the extent that these metabolic functions are considered to have been completely lost (Keeling *et al.*, 2010). In addition to this extensive reduction of core carbon metabolism, *E. bieneusi* also lacks all genes encoding the spliceosome (Keeling *et al.*, 2010).

1.3.4. Clinical significance

Enterocytozoon bieneusi infection, in most cases, is limited to the enterocytes of the small intestine, causing non-specific digestive symptoms whose severity is directly related to the host's immune status. In immunocompetent hosts, the infection is often asymptomatic or presents as self-limiting diarrhoea and vomiting lasting up to one month. Conversely, in immunocompromised individuals, the infection may become chronic, causing persistent, watery diarrhoea, abdominal pain, nausea, anorexia, malabsorption, and weight loss, potentially becoming a life-threatening wasting disease (Wang *et al.*, 2024; Zhang *et al.*, 2021; Nourisson *et al.*, 2024). The diarrhoea induced by *E. bieneusi* may result from the aggregation of spores at the extremity of infected villi and the subsequent rupture of the host cells (Zhang *et al.*, 2021).

Sporadic involvement of the respiratory tract may occur in immunocompromised patients, leading to the development of persistent cough, dyspnoea and wheezing (Zhang *et al.*, 2021). In addition, there are reports of cholecystitis and cholangitis associated with *E. bieneusi* infections of the bile ducts (Nourisson *et al.*, 2024).

In light of these clinic aspects, the primary population at risk is represented by

immunocompromised patients, particularly HIV/AIDS patients or solid organ transplant (Nourisson *et al.*, 2024; Ruan *et al.*, 2021). However, young and advanced age, living in rural areas, and poor sanitation are all considered risk factors for intestinal microsporidiosis caused by *E. bieneusi*. This species is also a possible cause of diarrhoea in travellers returning from tropical countries (Nourisson *et al.*, 2024).

Clinical manifestations of *E. bieneusi* infection in animals are influenced by host species and immune status. Regarding suids, *E. bieneusi* infection is not consistently associated with clinical gastrointestinal disease, although the prevalence rates in domestic pigs and wild boars are relatively high (Allievi *et al.*, 2026; Li *et al.*, 2019).

The treatment of *E. bieneusi* infection, especially in immunocompromised patients, can be challenging due to the lack of effective drugs with tolerable side effects.

Although symptomatic treatment is generally effective in immunocompetent patients, restoration of immune function is essential for the control and eradication of *E. bieneusi* infection in immunocompromised individuals. Whenever possible, this is achieved through immune reconstitution by means of highly active antiretroviral therapy (HAART) in people living with HIV or by reducing or discontinuing immunosuppressive therapy in transplant recipients or other immunosuppressed patients (Santín-Durán, 2015; Nourisson *et al.*, 2024).

Therapeutic options targeting *E. bieneusi* are limited to three drugs, with different efficacy and side effects. The highest effectiveness is associated with fumagillin, an antibiotic isolated from *Aspergillus fumigatus* that acts as inhibitor of methionine aminopeptidase type 2, thereby blocking microsporidian replication (Zhang *et al.*, 2021; Santín-Durán, 2015). Despite its effectiveness in immunocompromised patients, fumagillin can induce haematological toxicity manifesting as thrombocytopenia and neutropenia (Zhang *et al.*, 2021).

In this context, albendazole and nitazoxanide gain greater clinical importance. Albendazole, a benzimidazole derivative, is a widely used antiparasitic drug that is effective against microsporidian species other than *E. bieneusi*. It appears to have a parasitostatic effect on *E. bieneusi* and alleviate clinical signs of infection, although it cannot eradicate the infection (Zhang *et al.*, 2021; Santín-Durán, 2015). Nitazoxanide, a broad-spectrum antiparasitic agent active against both protozoan and helminth parasites, is used when albendazole is ineffective (Zhang *et al.*, 2021).

1.3.5. Epidemiology

The life cycle of *Enterocytozoon bieneusi* is thought to involve faecal-oral transmission through the ingestion of environmentally resistant spores (Nourisson *et al.*, 2024). Contaminated water or food

and direct contact with infected individuals (person-to-person, animals-to-person, person-to-animal) thus represent the major transmission routes. In particular, water may play a crucial role in the epidemiology of the parasite, as it is frequently found in surface water, groundwater, agricultural effluent, recreational water and wastewater (Santín-Durán, 2015; Zhang *et al.*, 2021; Nourisson *et al.*, 2024). For example, *E. bienewsi* has been detected in 64.5% of water samples in China (Ruan *et al.*, 2021). The presence of *E. bienewsi* in surface water also supports the hypothesis that domestic and wild animals can serve as direct sources of contamination, as spores have been identified in the faeces of animals that are closely associated with surface water, such as raccoons, muskrats, beavers, foxes, and otters (Didier *et al.*, 2004).

Furthermore, contamination of irrigation water by sewage and agricultural run-off poses a risk of contaminating fruits and vegetables, thereby presenting a threat to human consumers (Santín-Durán, 2015). As a result of globalisation of food supply chains and faster transport times, microsporidia are raising increasing concerns as foodborne parasites (Didier *et al.*, 2004), and *E. bienewsi* is no exception. Spores of the parasite have been detected in raspberries and curly lettuce sold at market stalls and in bean sprouts from a supermarket in Poland (Jedrzejewski *et al.*, 2007), in fresh fruits and vegetables in China (Li *et al.*, 2019) and even in unpasteurized milk from sheep and cows (Nourisson *et al.*, 2024).

These findings prompted the U.S. Environmental Protection Agency (EPA) to include *Enterocytozoon bienewsi* in its Contaminant Candidate List (CCL) of microbial water contaminants (EPA). In addition, the U.S. National Institute of Allergy and Infectious Diseases (NIAID) has classified *E. bienewsi* as a Category B priority pathogen because of its potential for waterborne transmission (NIAID).

The risk posed by *E. bienewsi* is directly linked to the environmental resistance of the spores: although specific data regarding this species are lacking, studies on related microsporidian spores indicate that they are highly resistant in the environment and can survive for months or years under favourable conditions (Zhang *et al.*, 2021; Nourisson *et al.*, 2024). The impact of solar radiation, temperature, and humidity, as well as the effectiveness of UV light inactivation and chlorination on the viability of *E. bienewsi* spores, remains unknown (Zhang *et al.*, 2021; Nourisson *et al.*, 2024).

According to recent meta-analyses (**Figure 12**), the overall estimated prevalence of *E. bienewsi* infection in humans ranges between 6.6% and 7.9%, with considerable variation depending on previous illnesses, immunocompetence, and the presence or absence of digestive symptoms (Ruan *et al.*, 2021; Wang *et al.*, 2024). In HIV-positive patients, infection rates usually range between 1.3% and 11.6%, although higher prevalences have been reported in Nigeria (16.6%), Iran (28.4%), and Australia (85.2%) (Li *et al.*, 2019). Nearly 10% of children with diarrhoea in low-income

countries test positive for *E. bieneusi* (Nourisson *et al.*, 2024) and the prevalence of infection is higher among people living in rural areas than among those living in urban settings (Wang *et al.*, 2024). Interestingly, Wang *et al.* (2024) reported high infection rates in several European countries. This may reflect the greater resources allocated to parasite surveillance and diagnostic activities; however, temperate climates may also provide more favourable conditions for the survival and transmission of *E. bieneusi* (Wang *et al.*, 2024).

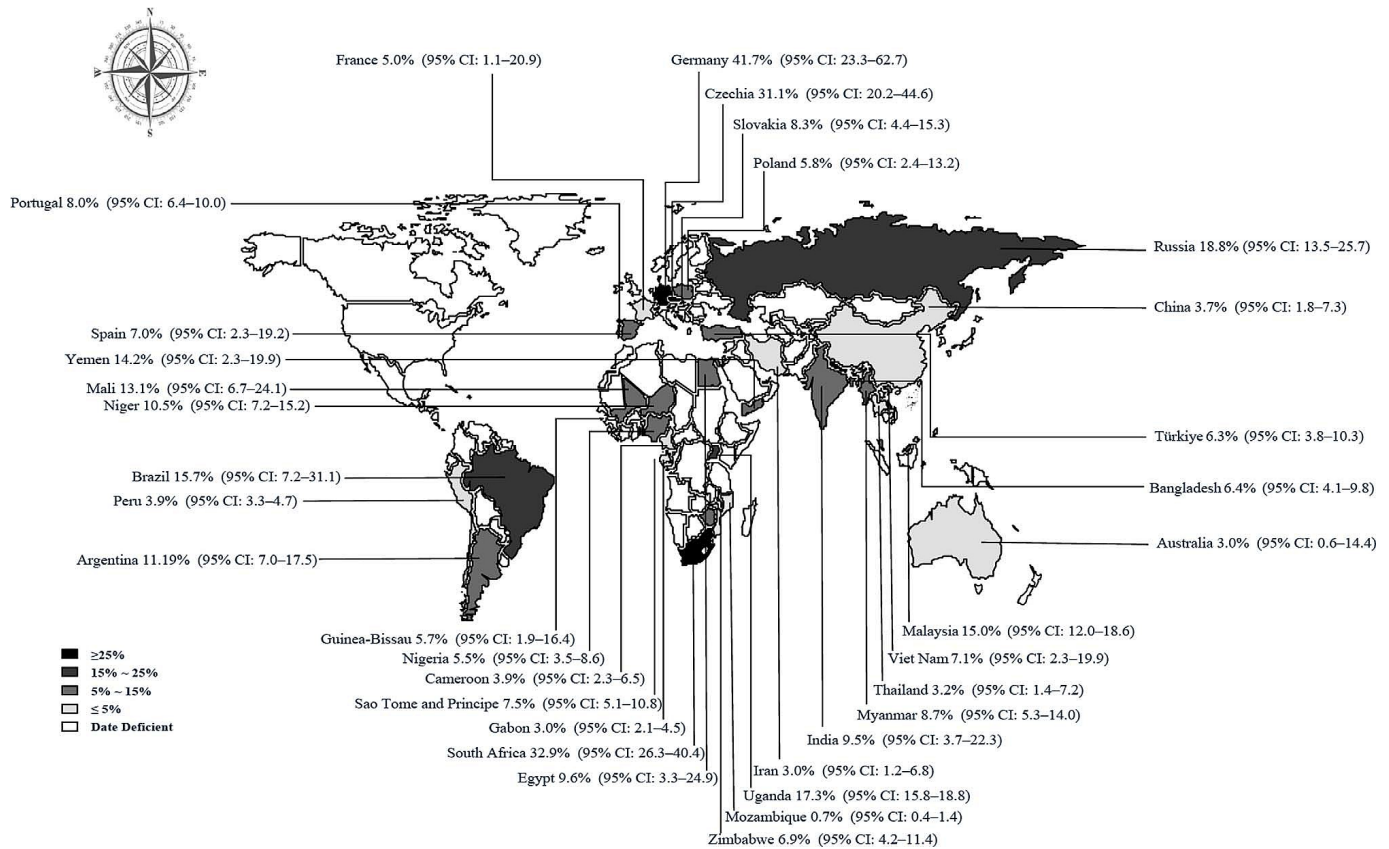


Figure 12. Worldwide prevalence of *Enterocytozoon bieneusi* in humans (Wang *et al.*, 2024).

Enterocytozoon bieneusi infects a wide range of hosts. It has been reported in over 40 countries worldwide in approximately 170 animal species, including mammals, birds, reptiles and some invertebrates, such as Diptera (Zhang *et al.*, 2021; Santin-Durán, 2015). To date, the only vertebrate groups in which *E. bieneusi* has not been reported are fish and amphibians (Zhang *et al.*, 2021). The prevalence of *E. bieneusi* infection varies considerably among animal species and is influenced by host species and immune status. Although the overall prevalence in ruminants has been estimated at approximately 17%, the highest mean prevalence (32.9–39.3%) has been documented in domestic and feral suids (Ruan *et al.*, 2021). *Enterocytozoon bieneusi* has also been reported in companion animals, particularly dogs and cats, with a mean prevalence of 8% (Nourisson *et al.*, 2021; Ruan *et al.*, 2021, Santin-Durán, 2015; **Figure 13**).

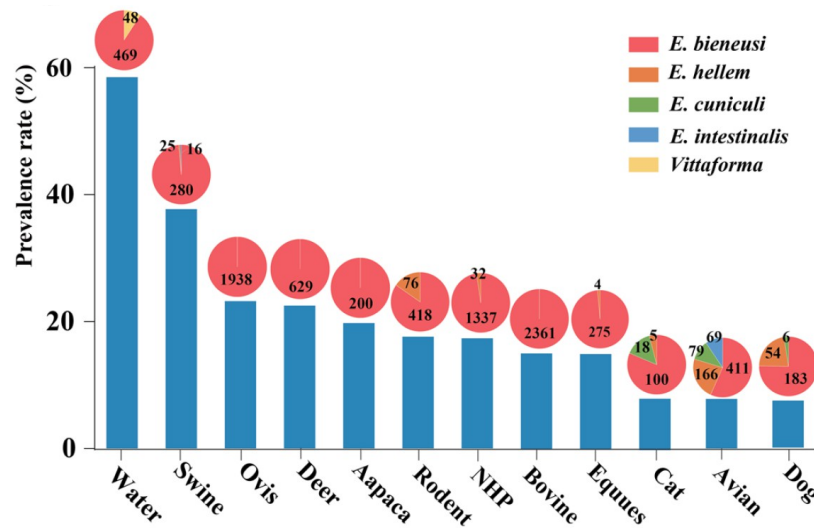


Figure 13. Overall prevalence of microsporidia in animal hosts and water, and species composition according to Ruan *et al.* (2021). NHP: non-human primates. *Enterocytozoon bienewsi* is the predominant microsporidian species identified in the pie chart.

1.3.6. Genetic variability and zoonotic potential

The intraspecific genetic diversity of *Enterocytozoon bienewsi* has been widely studied through ITS genotyping, a typing approach based on the analysis of polymorphisms within the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) gene (Li *et al.*, 2019; Koheler *et al.*, 2022; Allievi *et al.*, 2026). The ITS region (approximately 243 bp) is part of a non-functional RNA located between ribosomal RNAs and presents a high degree of polymorphism, thus serving as an ideal marker for genotyping studies (Li *et al.*, 2019). Three sets of nested PCR primer pairs covering the entire ITS region (MSP1/MSP2B and MSP3/MSP4B, EBITS3/EBITS4 and EBITS1/EBITS2.4, AL4037/AL4039 and AL4038/ALA040) have been used in over 95% of the published genotyping studies (Li and Xiao, 2021; Li *et al.*, 2019).

To date, over 500 genotypes have been reported and validated globally (Nourisson *et al.*, 2024; Li *et al.*, 2019; Li and Xiao, 2021). Due to the lack of a standardised naming system and the frequent incorrect inclusion of polymorphisms in the flanking regions of the ITS sequence, several identical ITS genotypes have received multiple names over time. The large number of synonymous genotype names generated considerable confusion regarding the true intraspecific diversity of *E. bienewsi* until Santin and Fayer (2009) proposed a standardised nomenclature system for ITS genotypes, thereby eliminating redundant names from literature.

Enterocytozoon bienewsi genotypes are distributed across 11 phylogenetic groups displaying varying degrees of host specificity and zoonotic potential (Li *et al.*, 2019; Li and Xiao, 2021; **Figure 14**).

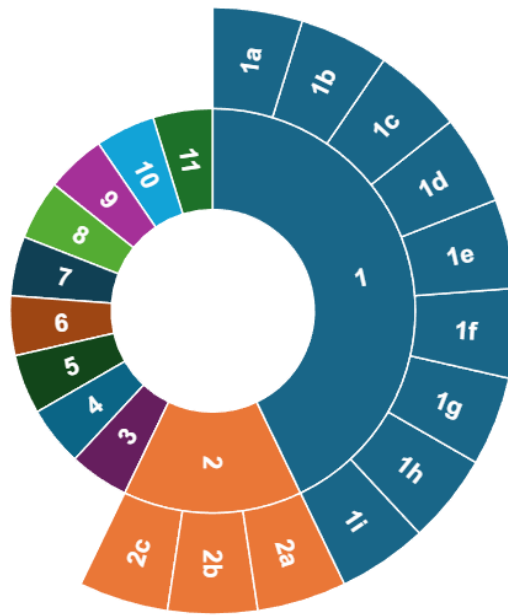


Figure 14. Phylogenetic groups and subgroups of *Enterocytozoon bieneusi* genotypes (modified from Nourisson *et al.*, 2024).

Group 1 is the largest, containing over 300 genotypes, and is divided into nine subgroups (1a to 1i) (Li *et al.*, 2019; Nourisson *et al.*, 2024). The genotypes within this group are the major responsible for human and animal infections and show low host specificity, as they have been detected in both humans and animals worldwide, suggesting a high zoonotic potential and cross-species transmission (Li *et al.*, 2019; Li and Xiao, 2021; Santin and Fayer, 2010). Among them, genotypes D, EbpC, and type IV are those most frequently isolated from humans and animals (Li *et al.*, 2019).

This group also includes some genotypes considered to be more host-adapted, as they have been identified only in a single host, such as the human-specific genotypes B and C (Nourisson *et al.*, 2024; Li *et al.*, 2019; Li and Xiao, 2021). However, the actual extent of host specificity for these genotypes is difficult to determine because of the limited number of studies and sampled animal species (Li *et al.*, 2019).

Because of the widespread distribution of its zoonotic genotypes, Group 1 is considered the most important group from a public health perspective (Li *et al.*, 2019).

Group 2, the second largest group, is composed of three subgroups (2a, 2b and 2c). Previously considered adapted to ruminants, this group is raising increasing zoonotic concerns for the recently established zoonotic potential of several genotypes, including BEB4, identified in cattle, yaks, humans, and pigs, and J, detected in alpacas, cattle, deer, goats, sheep, yaks, humans, bears, birds, donkeys, meerkats, zebras, and non-human primates.

Taken together, genotypes of Groups 1 and 2 alone account for over 90% of human infections (Allievi *et al.*, 2026), and the host range of several genotypes has expanded in recent years (Li and

Xiao, 2021). Notably, the ITS sequence divergence between genotypes belonging to Group 1 and Group 2 is minimal, corresponding to approximately 20 single nucleotide substitutions (Li and Xiao, 2021).

Groups 3 to 11 contain fewer genotypes and appear to be more host-specific, with limited zoonotic potential, although scarce information is currently available regarding these groups (Li *et al.*, 2019; Nourisson *et al.*, 2024).

Although the ITS region has proven to be a useful marker, it represents only a small portion of the genome, and sequence identity or genetic relatedness within this region may not accurately reflect the overall genetic or phenotypic similarity between two isolates (Li and Xiao, 2021). To overcome this limitation, a multilocus sequence typing (MLST) scheme was developed based on the simultaneous analysis of one minisatellite locus (MS-4) and three microsatellite markers (MS-1, MS-3, and MS-7). When combined with ITS sequencing, this approach enhances the resolution of the intraspecific genetic structure of *E. bieneusi* and provides a robust framework for elucidating transmission patterns, host adaptation mechanisms, and population structure, including the identification of distinct subpopulations (Li *et al.*, 2019; Li and Xiao, 2021).

Although animals can act as reservoirs of genotypes with a broad host range, thereby posing a zoonotic risk to humans, the reverse is also true. The genotypes most frequently identified in humans (D, EbpC, Peru8, and Type IV), all belonging to Group 1, are also commonly detected in domestic and wild animals, as well as in water sources, suggesting frequent interspecies and waterborne transmission (Li *et al.*, 2019; Li and Xiao, 2021). In contrast, the Group 1 genotype A is relatively common in humans but appears to exhibit greater host specificity than the other major zoonotic genotypes (Li *et al.*, 2019).

A marked predominance of Group 1 is also observed in domestic pigs and wild boars, in which genotypes D, EbpA, EbpC, G, H, and O are the most frequently identified, whereas the most common Group 2 genotypes are BEB4, I, CHN9, and CHN10. Some genotypes appear to be pig-specific, such as EbpB, PigEBITS1, PigEBITS2, PigEBITS3, PigEBITS6, CHN9 and CHN10 (Li *et al.*, 2019; Taghipour *et al.*, 2022; Li and Xiao; 2021). However, most of the genotypes found in pigs and wild boars (including CS-4, D, EbpA, EbpC, G, H, Henan-IV, PigEBITS5, PigEBITS7, BEB4, and I) have also been detected in humans, highlighting the potential role of suids as reservoirs of zoonotic *E. bieneusi* genotypes (Dashti *et al.*, 2020). Slurry from intensive pig farms should therefore be considered a major source of environmental contamination, posing a potential risk to surface waters, vegetables, farmers, veterinarians, and other individuals in close contact with pigs (Li and Xiao, 2021).

1.3.7. Diagnosis

The diagnosis of *Enterocytozoon bieneusi* infection is based on the detection of spores in faecal samples, urine, duodenal aspirates, or biopsy specimens (García, 2002).

Light microscopy combined with conventional staining methods represents a rapid and widely accessible diagnostic approach. However, its diagnostic performance is limited by relatively low sensitivity (the limit of detection is $\sim 5 \times 10^4$ spores/mL) and specificity, as food particles, bacteria, fungi, and other microsporidian species may also be stained (Santín-Durán, 2015; Li and Xiao, 2021; Nourisson *et al.*, 2024). In addition, the small size of *E. bieneusi* spores (approximately $0.5 \times 1.5 \mu\text{m}$) makes diagnosis highly dependent on the examiner's expertise and requires the use of oil immersion microscopy at high magnification ($\times 1,000$) (Santín-Durán, 2015; García, 2002). Neither bright-field nor fluorescence microscopy alone is sufficient for species-level identification of microsporidia (Nourisson *et al.*, 2024; Santín and Fayer, 2011).

The most commonly used staining techniques for faecal samples and other clinical specimens are modified trichrome stains, which require high dye concentrations and relatively long processing times (approximately 90 minutes), as penetration of the chitin-rich spore wall is particularly challenging (Santín-Durán, 2015). Following staining, spores typically appear as oval structures with a pink-to-red spore wall surrounding a clear interior and may occasionally exhibit a transverse or diagonal band corresponding to the polar tube. In contrast, the background appears green or blue, depending on the staining protocol employed (**Figure 15**).

The most common modified trichrome stains are the Weber-Green and the Ryan Blue. Several modifications of the original chromotrope staining solution have been developed, such as the rapid hot Gram-chromotrope staining method, with a staining time of only 5 min, and the acid-fast trichrome stain, which can detect both cryptosporidian oocysts and microsporidian spores in the same smear (García, 2002; Santín-Durán, 2015).

Another common staining technique employs fluorescent brightener agents, such as Calcofluor white and Uvitex 2B, which bind to chitin in the endospore layer of the spore wall (Nourisson *et al.*, 2024; García, 2002). A fluorescent microscope is therefore required, and depending on the agent used as well as the excitation wavelength, the spores wall emits fluorescence, appearing as white or turquoise ovals against a black background (García, 2002; Santín-Durán, 2015; **Figure 15**).

Both staining techniques lack specificity, as artefacts, small fungal cells, and sample debris can lead to false positives. Nevertheless, clinical specimens other than faecal samples, such as body fluid cytology preparations or intestinal biopsy specimens, contain fewer artefacts and less debris, making them easier to examine (García, 2002).

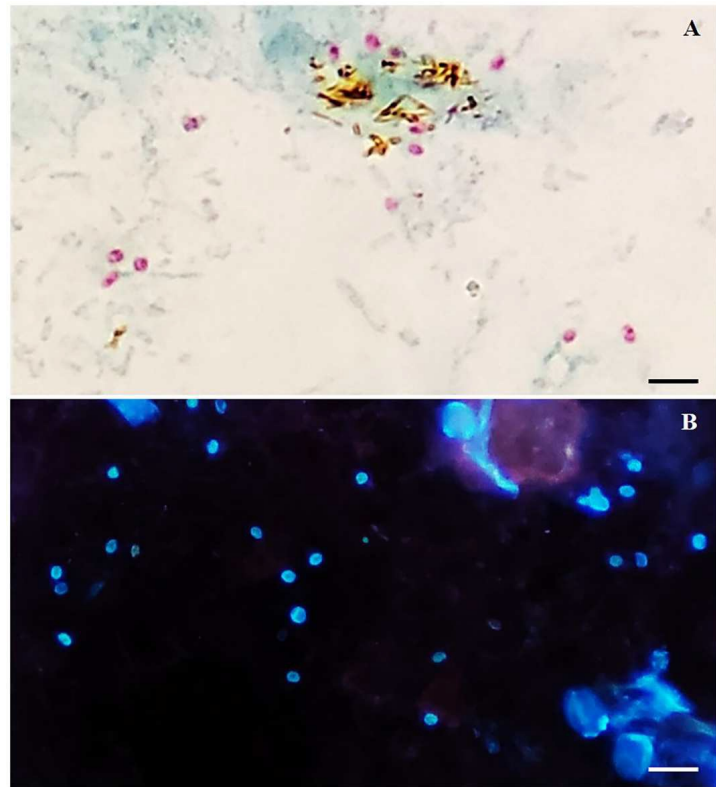


Figure 15. Different stains of *Enterocytozoon bieneusi* spores in faecal smears (scale bar 5 μm , magnification 1,000 $\times$). (A) Weber's modified trichrome stain: spores appear pink on a green background. (B) Calcofluor-white stain: spores appear under blue fluorescence (Nourisson *et al.*, 2024).

Transmission electron microscopy (TEM) has long been considered the gold standard for the diagnosis of microsporidiosis and the identification of the causative species, owing to its ability to reveal the polar tube and other species-specific ultrastructural features. However, TEM is unsuitable for routine diagnostic laboratories because it is expensive, time-consuming, and requires specialised equipment and highly trained personnel (Santín and Fayer, 2011; Santín-Durán, 2015).

In fluorescence microscopy, species identification can be achieved using species-specific monoclonal antibodies in immunofluorescence assays (IFAs), which represent a powerful tool for large-scale epidemiological studies (Nourisson *et al.*, 2024; Li and Xiao, 2021). However, these assays do not provide genotyping information (Santín-Durán, 2015; Santín and Fayer, 2011).

Thanks to the advent of molecular techniques, *E. bieneusi* is now identified primarily by conventional or nested PCR targeting the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (Li and Xiao, 2021; Nourisson *et al.*, 2024; Santín-Durán, 2015). Molecular methods enable both identification and genetic characterisation of *E. bieneusi* isolates, providing at the same time higher sensitivity (1-10 copies/ μL) and specificity ($\geq 99\%$) than microscopic and immunological assays (Nourisson *et al.*, 2024; Santín and Fayer, 2011).

Although PCR-based assays are not routinely available in clinical diagnostic laboratories, because

of their cost and time requirement, they are widely employed in research and epidemiological studies (Santìn and Fayer, 2011).

1.3.8. *In vivo* animal models and *in vitro* culture systems

The lack of suitable animal models and continuous *in vitro* culture systems has made *Enterocytozoon bieneusi* one of the most challenging microsporidian species to study (Snowden *et al.*, 1998; Nourisson *et al.*, 2024).

A major step forward in the development of an animal model was made by Tzipori *et al.* (1997), who highlighted the occurrence of *E. bieneusi* in immunodeficient macaques. Spores obtained from these infected monkeys were administered to immunosuppressed gnotobiotic piglets to investigate the potential transmission of the microsporidian. As the pigs developed the infection, in some cases symptomatically, both immunodeficient macaques and immunosuppressed pigs appeared to be potential models for *E. bieneusi* infections (Snowden *et al.*, 1998).

The only successful short-term culture of *E. bieneusi* isolated from AIDS patients was achieved by Visvesvara *et al.* (1995). Duodenal aspirate and macerated biopsy specimens from the same patient were inoculated into E6 (monkey kidney cells) / HLF (human lung fibroblast) cell lines, maintaining a viable culture for up to six months (Visvesvara *et al.*, 2002). Eventually, the cultures were destroyed by the outgrowth of a contaminant adenovirus (Visvesvara *et al.*, 2002).

2. AIM OF THE WORK

The work described in this thesis was carried out at the Department of Veterinary Medicine and Animal Sciences of Università degli Studi di Milano, located in Lombardy, Italy.

Cryptosporidium spp. and *Enterocytozoon bieneusi* have attracted increasing attention among foodborne and waterborne parasites because of their remarkable ability to persist and spread in the environment. Both pathogens have been detected in drinking water sources, wastewater, soil, and food products, representing an important source of infection for both immunocompetent and immunocompromised individuals. Furthermore, an increasing number of studies has highlighted their circulation in companion animals, wildlife, and livestock, including the presence of species and genotypes with clear zoonotic potential. Combined with their faecal-oral transmission, these characteristics have contributed to their global distribution.

Understanding the role of wild animals in their life cycle, spread and maintenance is crucial in the context of ongoing environmental changes driven by climate change, human activities, and pollution, which are enhancing interactions and conflicts between wildlife and human populations. In particular, wildlife may act as active reservoir of a wide range of pathogens, contributing to environmental contamination with infective parasitic elements and posing a risk to livestock and humans.

Due to its relatively recent and rapid expansion across many regions of the world, the wild boar is one the most relevant wildlife species at the human-livestock-wildlife interface. Nevertheless, data regarding the prevalence of *Cryptosporidium* spp. and *Enterocytozoon bieneusi* in wild boar populations as well as on their role in the epidemiology and transmission of these parasites remain limited, especially in Italy.

The main aim of this thesis was to detect and molecularly characterize *Cryptosporidium* spp. and *Enterocytozoon bieneusi* in wild boars hunted in a highly anthropised area of the province of Cremona (Lombardy region). Molecular characterization was performed to identify species or genotypes with zoonotic potential and to assess the ecological impact of wild boars as reservoirs of zoonotic parasites affecting both humans and livestock. Given the high density of livestock farms and human settlements, together with the recent expansion of the wild boar population, the study area provides an ideal setting for investigating interactions between domestic and wild animals and studying the potential transmission of pathogens.

To provide a preliminary assessment of the circulation dynamics between domestic and wild

animals – specifically between domestic pigs and wild boars – the molecular data obtained from the sampled wild boars were integrated with prevalence data for *Cryptosporidium* spp. in 22 fattening pig farms from northern Italy. The two datasets were then analysed and discussed together. Finally, the findings on *Enterocytozoon bieneusi* in the wild boar population were compared with those reported in a study published by Allievi *et al.* (2026), who investigated the circulation of *E. bieneusi* in domestic pigs from the same fattening pig farms.

3. MATERIALS AND METHODS

3.1. Wild boar sampling

3.1.1. Study area

The wild boar faecal samples analysed in this thesis were collected as part of an ongoing epidemiological survey investigating gastrointestinal nematodes and protozoa using copromicroscopical techniques. The examined wild boars were hunted in fifteen municipalities in the province of Cremona (**Table 1** and **Figure 16**). The study area is a flat lowland located in the centre of the Po valley, characterised by the absence of mountainous reliefs and bordered by three major rivers: the Po River to the south, the Oglio River to the north and the Adda River to the west. In addition to these major waterways, an extensive network of irrigation canals further enriches the area's hydrographic system.

The Province of Cremona has a continental climate, with marked seasonal temperature fluctuations between hot summers (mean maximum temperatures ranging from 28 to 35 °C) and cold, foggy winters (mean minimum temperature of approximately –2 °C). Relative humidity remains high throughout the year, ranging from 70% to 100%, while precipitation occurs year-round, with peaks in October and November. Fog formation, particularly near waterways during the winter months, is another characteristic feature of the area.

The area is highly anthropised, with a population density of 202.9 inhabitants/km². Agricultural land covers 982.7 km², representing approximately 56% of the territory, and is primarily devoted to arable crops (*Territorial Plan of Provincial Coordination* – PTCT, 2005). The area is also characterised by intensive livestock farming, particularly dairy cattle and pigs. According to the Italian National Zootechnical Database, there are 1,191 cattle farms (339 beef, 778 dairy, and 74 mixed) with a total of 312,907 animals, and 418 pig farms rearing 851,191 animals (<https://www.vetinfo.it>).

The presence of wild boars in the study area is relatively recent. According to the *Pluriannual Wild Boar Control Project 2025-2029*, the first sightings of isolated individuals date back to 2005, while stable groups were first reported in 2009. Wild boar colonisation most likely originated from populations in the Apennines, which gradually expanded by exploiting river corridors, floodplains and abandoned branches of the Po. Initially confined to protected riparian areas, wild boars subsequently spread throughout the entire province (*Pluriannual Wild Boar Control Project* – PPCG 2025-2029).

Table 1. Number of sampled wild boars by municipality.

Municipality	Sampled animals
Azzanello	6
Casalmaggiore	6
Casteldidone	11
Cella Dati	1
Cremona	1
Genivolta	3
Gussola	47
Martignana di Po	7
Motta Baluffi	40
S. Daniele Po	4
S. Giovanni in Croce	1
Scandolara-Ravana	4
Spinadesco	7
Stagno Lombardo	2
Torricella del Pizzo	51

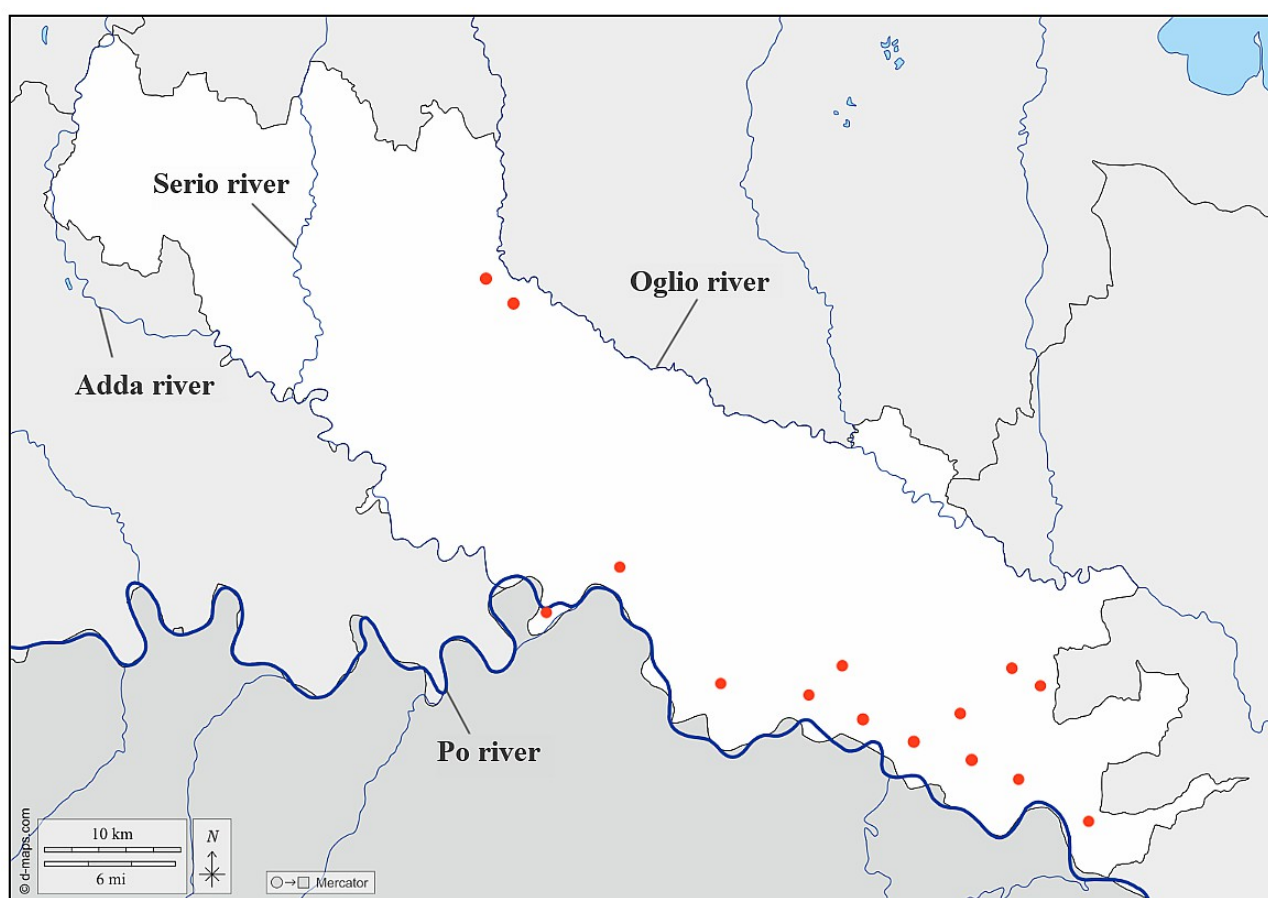


Figure 16. Distribution of hunting municipalities (red dots) within the province of Cremona (Lombardy region).

3.1.2. Faecal sampling

Between April 2024 and May 2025, 191 wild boars intended for human consumption were hunted as part of the regional wildlife surveillance program. Fresh faecal samples were collected directly from the rectum of each animal during the mandatory post-mortem inspection of the carcasses, in collaboration with the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini" (Cremona section, Italy).

The samples were placed in plastic containers, appropriately labelled with an identification number, and transported to the laboratory at + 4 °C. Within two to three days of arrival, an aliquot of each faecal sample was transferred into a 1.5 ml Eppendorf tube to minimise chemical and biochemical degradation. The tubes were stored at – 20 °C until DNA extraction.

For each animal epidemiological data regarding age, sex, sampling date and hunting location were recorded. The animals were divided into three age classes: young (<1 year), subadult (1–2 years) and adult (>2 years). Age was determined based on the association between tooth eruption and age, according to the dental tables described by Boitani and Mattei (1991).

3.2. Fattening pigs sampling

3.2.1 Faecal sampling

The epidemiological data on pig faecal samples presented in this thesis were obtained from a sampling campaign conducted between 2023 and 2024 as part of previous parasitological study by Allievi *et al.* (2024), which investigated the prevalence of gastrointestinal parasites in fattening pigs in northern Italy.

Sampling was carried out on 22 intensive pig farms located in Lombardy, Piedmont, and Emilia-Romagna (**Figure 17**). All the farms included in the study were intensive fattening systems, where pigs were introduced at approximately 3 months of age, weighing 35-50 kg, and slaughtered at around 9 months of age, when they reached a body weight of 160-180 kg.

Twenty pigs were sampled from each farm: 10 at the beginning of the fattening period and 10 immediately before slaughter, at the end of the production cycle. Overall, 440 faecal samples were collected, including 220 at the beginning and 220 at the end of the fattening cycle. At the time of sampling, none of the pigs showed clinical signs of gastrointestinal disease.

Faecal samples were collected manually from the rectal ampulla using a gloved hand, placed in labelled plastic containers, and transported to the laboratory at + 4 °C. Aliquots of each sample were transferred into 1.5 ml Eppendorf tubes and stored at –20 °C until molecular analysis.

For each animal, data on age (≤ 5 months, > 5 months), sampling season (spring, summer, autumn, winter), and faecal soiling score (0, 1, or 2) were recorded. The faecal soiling score is a cleanliness indicator based on the ClassyFarm system (<https://www.classyfarm.it>), a risk categorization system funded by the Ministry of Health and implemented by the Istituto Zooprofilattico Sperimentale of Lombardy and Emilia-Romagna.

Information on farm management practices was also collected, including farm size ($< 1,900$ animals or $\geq 1,900$ animals) and the use of the all-in/all-out production system (yes/no).

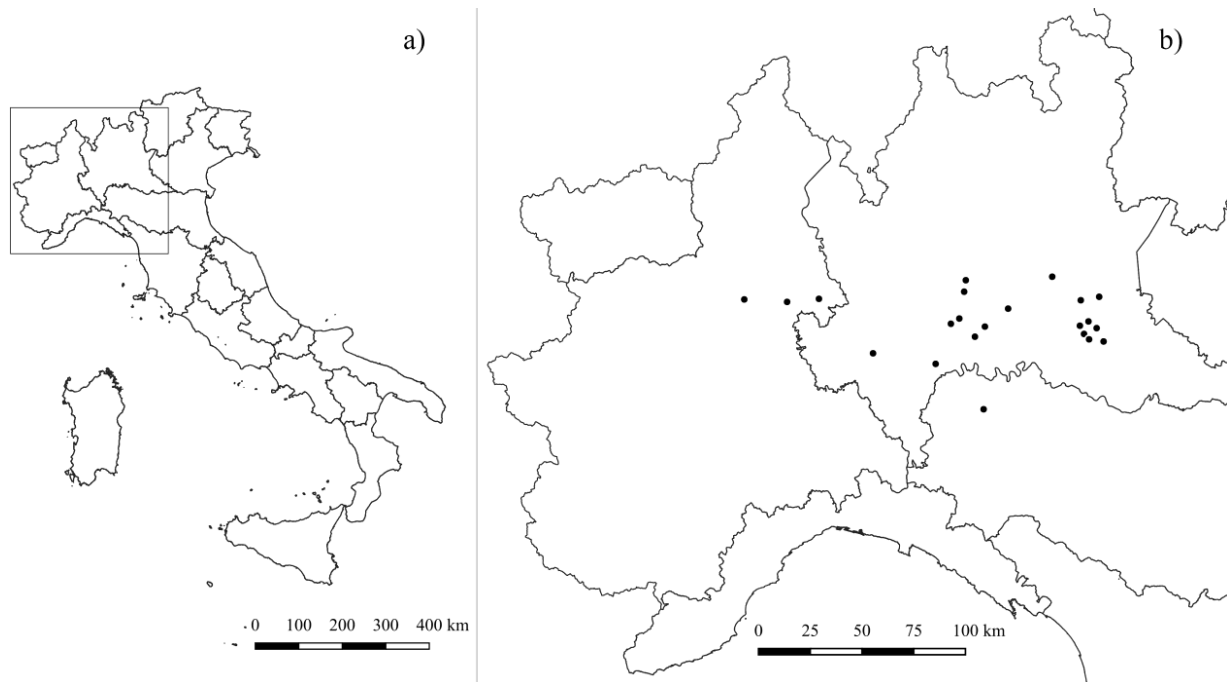


Figure 17. Selected fattening pig farms (Allievi *et al.*, 2024).

3.3. DNA extraction and quantification

DNA extraction from the faecal aliquots was performed using the kit QIAamp[®] Fast DNA Stool Mini Kit (Qiagen). This kit is specifically designed for the purification and extraction of DNA from fresh or frozen stool samples, as faeces represent a complex biological matrix rich in PCR inhibitors, such as polysaccharides, bile salts, and urate. The procedure includes the use of an inhibitor removal buffer that efficiently eliminates substances known to interfere with PCR amplification (Qiagen, www.qiagen.com).

Briefly, 180-220 mg of faeces were transferred into a 2 ml tube and mixed with 1 ml of inhibition buffer. After vortexing for 1 min to homogenise the sample, the tubes were incubated at 95°C for 5 min to enhance cell lysis. The sample were centrifuged at $20.000 \times g$ for 1 min, and the supernatant was transferred to a new tube containing 15 μ l of Proteinase K and 200 μ l of lysis buffer. Following

incubation at 70°C for 10 min to complete cell lysis and inactivate nucleases, 200 µl of ethanol 96–100% were added to promote DNA binding the silica membrane. The lysate was subsequently transferred to a spin column, where DNA was selectively retained on the silica membrane while contaminants were removed through successive washing steps using the buffers provided by the kit. Finally, DNA was eluted with the elution buffer according to the manufacturer's instruction, yielding a final volume of 100 µl.

DNA concentration and purity were assessed using a NanoRead spectrophotometer (Lifereal Biotechnology Co., Ltd., Hangzhou, China). DNA concentration was determined by measuring absorbance at 260 nm and expressed as ng/µl. The instrument also provides the A260/A280 ratio, used to evaluate protein contamination, and the A260/A230 ratio, indicative of contamination by extraction reagents or other organic compounds. Optimal values range from 1.8 to 2.1 for the A260/A280 ratio and from 2.0 to 2.2 for the A260/A230 ratio. However, lower values are commonly observed in faecal samples because of the complexity of the starting material.

The extracted DNA samples were stored at – 20°C until further analysis.

3.4. Nested PCR (nPCR)

The polymerase chain reaction (PCR) is a molecular biology technique developed in 1983 by the biochemist Kary Mullis. The technique mimics the natural process of DNA replication by selectively amplifying a target DNA sequence, generating a large number of copies *in vitro*. This allows the target sequence to be detected and subsequently analysed using a range of molecular techniques.

A PCR reaction requires the following components:

- *Taq* DNA polymerase, a thermostable enzyme originally isolated from the bacterium *Thermus aquaticus*, which retains its activity at the high temperatures required during the PCR cycling;
- equimolar concentrations of deoxyribonucleotide triphosphates (dNTPs), which serve as substrates for DNA synthesis;
- a pair of primers, consisting of short DNA oligonucleotides complementary to the regions flanking the target sequence. The forward primer anneals to the 3'-5' template strand and is synthesised in the 5'-3' direction, whereas the reverse primer anneals to the opposite strand;
- a reaction buffer containing MgCl₂, which maintains optimal pH conditions and provides magnesium ions, an essential cofactor for DNA polymerase activity.

PCR consists of repeated amplification cycles, each comprising three main steps (**Figure 18**): 1) denaturation (92-95°C), during which the double-stranded DNA template separates into two single strands; 2) annealing (typically 50-60°C), during which the primers hybridise to their complementary target sequences; and 3) extension (70-72°C), during which *Taq* DNA polymerase synthesises the new complementary DNA strand starting from the primer. The temperatures and duration of each step are critical parameters that vary according to the target sequence and the PCR protocol employed.

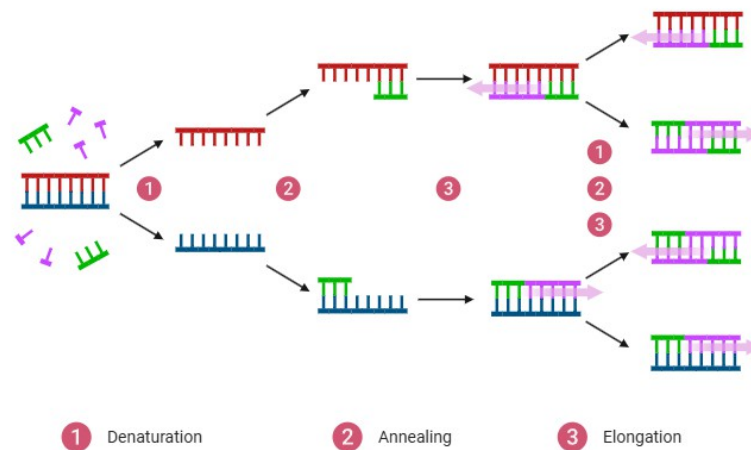


Figure 18. Polymerase chain reaction.

Although the amplification process could theoretically continue indefinitely, a conventional PCR typically consists of 30-35 cycles. During the initial phase, the amount of amplified DNA increases exponentially, approximately doubling with each cycle. However, amplification eventually reaches a plateau as PCR products accumulate and primers become limiting. As a result, the single-stranded DNA molecules are more likely to re-anneal to one another than to the primers, preventing further exponential amplification. Once this plateau phase is reached, no significant increase in PCR product is observed.

PCR is performed using a thermocycler, an instrument that automatically changes the temperature according to the programmed amplification protocol. Owing to its versatility, sensitivity, and relative simplicity, PCR has become one of the most widely used techniques in molecular biology and has a broad range of applications, including pathogen detection, genotyping, comparative genomics, and oncogene research.

Since the development of conventional PCR, several modified techniques have been introduced. Among these, nested PCR consists of two successive amplification reactions, in which the product of the first PCR serves as the template for the second. The two reactions employ different pairs of primers. The first amplification uses a pair of external primers that flank a relatively large DNA

fragment containing the target sequence, whereas the second amplification uses a pair of internal primers that anneal to sequences located within the first amplicon (**Figure 19**).

Although nested PCR requires more time and reagents, as it involves the preparation of two reaction mixtures and two successive amplification reactions, it offers greater sensitivity and specificity than conventional PCR. The use of two pairs of primers allows a higher overall number of amplification cycles, thereby increasing the sensitivity of the assay. In addition, the requirement for two independent primer pairs to anneal to the target sequence greatly reduces non-specific amplification, resulting in improved specificity. Therefore, nested PCR is particularly useful when greater sensitivity and specificity are required, such as when the target DNA is present at low abundance or when analysing complex samples that may contain PCR inhibitors.

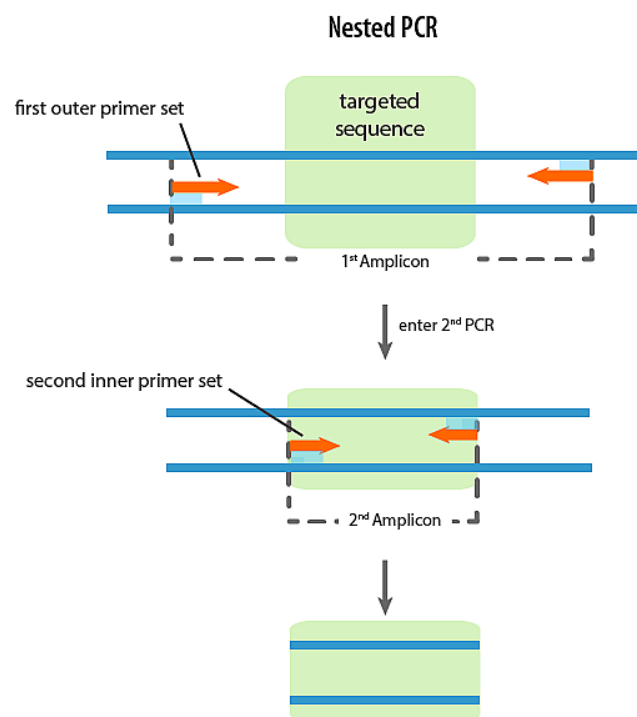


Figure 19. Schematic reaction of nested PCR (source: Applied Biological Materials Inc.)

The main limitation of PCR and nested PCR is the high risk of contamination, particularly during the handling of the products generated in the first amplification reaction. Additional limitations include the cost of reagents and equipment, the complexity of primer design, and the fact that only previously characterised DNA sequences can be specifically amplified.

In this study, all wild boar faecal samples were analysed using two nested PCR (nPCR) protocols for the detection of *Cryptosporidium* spp. and *Enterocytozoon bieneusi*, whereas pig faecal samples were tested only for the detection of *Cryptosporidium* spp.

Samples with a DNA concentration exceeding 100 ng/μl were analysed both undiluted and after a 1:10 dilution to ensure that excessive DNA concentrations did not inhibit the amplification reaction.

3.4.1. nPCR protocol for *Cryptosporidium* spp.

The presence of *Cryptosporidium* spp. in the faecal samples was investigated by a nested PCR targeting a fragment of the small subunit ribosomal RNA (SSU rRNA) gene, following the protocol described by Xiao *et al.* (1999, 2000). Each PCR run included both a positive and a negative control. The positive control consisted of a porcine sample from a previous study, that had been confirmed as positive for *Cryptosporidium* spp. by both nested PCR and DNA sequencing, whereas nuclease-free water was used as the negative control in place of template DNA.

The primer pairs used in the two amplification rounds are listed in **Table 2**.

Table 2. Primers used for nested PCR amplification of *Cryptosporidium* spp. **F**, forward primer; **R**, reverse primer; **e**, external primer; **i**, internal primer.

Primers	Sequence	Melting temperature	Amplicon size
F _e (18SExtF)	5'-TTCTAGAGCTAATACATGCG-3'	54°C	1325 bp
R _e (18SExtR)	5'-CCCATTTCCTTCGAAACAGGA-3'	58°C	
F _i (18SIntF)	5'-GGAAGGGTTGTATTATTAGATAAAG-3'	57°C	826-864 bp
R _i (18SIntR)	5'-AAGGAGTAAGGAACAACCTCCA-3'	59°C	

The final reaction volume was 20 µl for both amplification rounds. In the primary PCR, 2 µl of template DNA were added to the reaction, whereas in the secondary PCR, 1 µl of the primary PCR product was used as the template. The same thermal cycling conditions were applied in both amplification rounds. The composition of the reactions and the thermal cycling conditions are reported in **Table 3**, **4** and **5**.

Table 3. Reagents used in the primary PCR (1,325 bp amplicon)

Reagents	Initial concentration	Final concentration	Final volume
DreamTaq Buffer	10X	1X	2 µl
dNTPs	2 mM	0,2 mM	2 µl
18SExtF	10 µM	1 µM	2 µl
18SExtR	10 µM	1 µM	2 µl
DreamTaq	5 U/µl	1 U / 20 µl	0,2 µl
DNA	—	—	2 µl
H ₂ O	—	—	9,8 µl
Total			20 µl

Table 4. Reagents used in the secondary PCR (826–864 bp amplicon).

Reagents	Initial concentration	Final concentration	Final volume
DreamTaq Buffer	10X	1X	2 µl
dNTPs	2 mM	0,2 mM	2 µl
18SExtF	10 µM	1 µM	2 µl
18SExtR	10 µM	1 µM	2 µl
DreamTaq	5 U/µl	1 U / 20 µl	0,2 µl
PCR product	–	–	1 µl
H ₂ O	–	–	10,8 µl
Total			20 µl

Table 5. Thermal cycling conditions for the primary and secondary PCR amplifications.

Phase	Temperature	Time	Cycles
Initial denaturation	94°C	3 minutes	1x
Denaturation	94°C	45 seconds	35x
Annealing	55°C	45 seconds	
Elongation	72°C	1 minute	
Final elongation	72°C	7 minutes	1x

3.4.2. nPCR protocol for *Enterocytozoon bieneusi*

The presence of *Enterocytozoon bieneusi* in the faecal samples was investigated by a nested PCR targeting the internal transcribed spacer (ITS) region, following the protocol described by Buckholt *et al.* (2002). As for the *Cryptosporidium* assay, each PCR run included both a positive and a negative control. The positive control consisted of a pig faecal sample from a different study that had been confirmed as positive for *E. bieneusi* by DNA sequencing, whereas nuclease-free water was used as the negative control in place of template DNA.

The primer pairs used for the nested PCR are listed in **Table 6**.

Table 6. Primers used for the nested PCR detection of *Enterocytozoon bieneusi*. F, forward primer; R, reverse primer; e, external primer; i, internal primer.

Primers	Sequence	Melting temperature	Amplicon size
F _e (EBIT S3)	5'-GGTCATAGGGATGAAGAG-3'	54 °C	435 bp
R _e (EBIT S4)	5'-TTCGAGTTCTTTCGCGCTC-3'	57°C	
F _i (EBIT S1)	5'-GCTCTGAATATCTATGGCT-3'	53°C	240-390 bp
R _i (EBIT S2.4)	5'-ATCGCCGACGGATCCAAGTG-3'	61°C	

The primary PCR was performed in a final reaction volume of 20 µl, including 3 µl of template DNA. The composition of the reaction mixture and the thermal cycling conditions are reported in **Tables 7 and 8**.

The secondary PCR was performed in a final reaction volume of 20 µl, using 1 µl of the primary PCR product as the template. The composition of the reaction mixture and the thermal cycling conditions are reported in **Tables 9 and 10**.

Table 7. Reagents used in the primary PCR (435 bp amplicon).

Reagents	Initial concentration	Final concentration	Final volume
DreamTaq Buffer	10X	1X	2 µl
dNTPs	2 mM	0,2 mM	2 µl
EBIT S3	10 µM	1 µM	2 µl
EBIT S4	10 µM	1 µM	2 µl
DreamTaq	5 U/µl	1 U / 20 µl	0,2 µl
DNA	–	–	3 µl
H ₂ O	–	–	8,8 µl
Total			20 µl

Table 8. Thermal cycling conditions for the primary PCR amplification.

Phase	Temperature	Time	Cycles
Initial denaturation	94°C	5 minutes	1x
Denaturation	94°C	45 seconds	35x
Annealing	57°C	45 seconds	
Elongation	72°C	1 minute	
Final elongation	72°C	10 minutes	1x

Table 9. Reagents used in the secondary PCR (390 bp amplicon).

Reagents	Initial concentration	Final concentration	Final volume
DreamTaq Buffer	10X	1X	2 µl
dNTPs	2 mM	0,2 mM	2 µl
EBIT S3	10 µM	1 µM	2 µl
EBIT S4	10 µM	1 µM	2 µl
DreamTaq	5 U/µl	1 U / 20 µl	0,2 µl
PCR product	–	–	1 µl
H ₂ O	–	–	10,8 µl
Total			20 µl

Table 10. Thermal cycling conditions for the secondary PCR amplification.

Phase	Temperature	Time	Cycles
Initial denaturation	94°C	5 minutes	1x
Denaturation	94°C	45 seconds	30x
Annealing	55°C	45 seconds	
Elongation	72°C	1 minute	
Final elongation	72°C	10 minutes	1x

3.5. Agarose gel electrophoresis

Gel electrophoresis is one of the most widely used techniques in molecular biology for the separation of nucleic acids and proteins. The technique separates molecules primarily according to their size, allowing the qualitative and semi-quantitative analysis of DNA, RNA, or proteins.

Electrophoresis is performed using a gel matrix composed of agarose or polyacrylamide. These two matrices differ mainly in pore size: polyacrylamide forms smaller pores and therefore provides higher resolution than agarose. However, because of its toxicity and more demanding preparation, polyacrylamide is less commonly used for routine applications. The pore size of the gel depends on the concentration of agarose or polyacrylamide used.

Samples are loaded into wells formed in the gel, after which an electric field is generated by applying a voltage across the gel. Since DNA molecules carry a net negative charge due to their phosphate backbone, they migrate from the negative electrode (cathode) towards the positive electrode (anode). Smaller DNA fragments migrate more rapidly than larger ones because they pass more easily through the pores of the gel matrix. Consequently, DNA fragments are separated according to their size (**Figure 20**).

Migration rate is also influenced by the applied voltage. Higher voltages accelerate DNA migration and may improve separation over short distances; however, excessive voltage generates heat, which can damage both the gel matrix and the DNA. For this reason, electrophoresis is commonly performed at 70-90 V.

To ensure proper electrophoretic separation, the gel is immersed in a running buffer, such as TAE (Tris-acetate-EDTA) or TBE (Tris-borate-EDTA), which maintains electrical conductivity and a stable pH throughout the run.

For the analyses performed in this study, agarose gel electrophoresis was carried out using gels prepared in 100 ml of 1× TAE buffer with different agarose concentrations (Agarose E®, LLG-Labware, Meckenheim, Germany), depending on the expected size of the PCR products. A 1.5%

agarose gel was used for the analysis of the *Cryptosporidium* spp. nested PCR products, whereas a 2% agarose gel was used for the *Enterocytozoon bienersi* nested PCR products. The higher agarose concentration provided smaller pore sizes, allowing better resolution of the smaller expected amplicons.

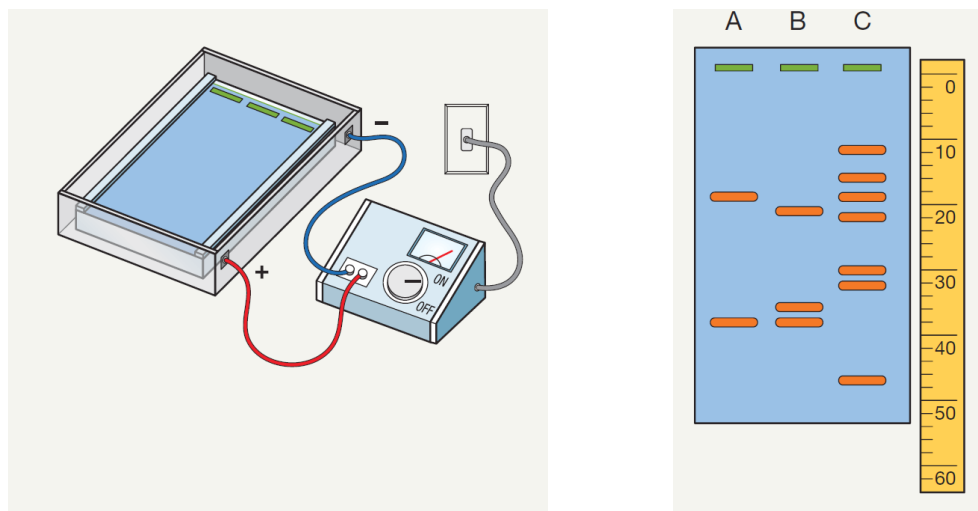


Figure 20. On the left, the electrophoresis cell and tension generator. On the right, the result of a running: the smallest fragments have a higher migration speed and move farther from the loading wells (Amaldi *et al.*, 2022).

After the agarose solution had been heated until completely dissolved, 10 µl of ethidium bromide (final concentration: 1 mg/ml) were added. Ethidium bromide is a DNA-intercalating agent that fluoresces under ultraviolet (UV) light. When incorporated into the gel matrix, it binds to DNA fragments, enabling their visualisation on a UV transilluminator following electrophoresis.

A GelPilot® DNA Molecular Weight Marker (Qiagen) was included in each electrophoresis run. This DNA ladder consists of fragments of known sizes and concentrations and was used as a reference to estimate both the size and the approximate concentration of the PCR products.

Following electrophoresis, the gels were visualised using a ClearView® UV transilluminator (Cleaver Scientific, Rugby, UK). Positive samples were identified by comparing the migration of the amplified bands with both the DNA ladder and the positive control. The corresponding DNA bands were then excised from the gel using a sterile scalpel and stored in 1.5 ml Eppendorf tubes at – 20 °C until purification and sequencing.

3.6. Purification and sequencing of the nPCR products

PCR products were purified from agarose gels using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) to recover the amplified DNA. Briefly, the excised gel fragments containing the DNA bands were incubated at 50 °C with an equal volume of binding buffer to dissolve the agarose

and promote DNA binding to the silica membrane. The resulting solution was then transferred to a spin column containing a silica membrane. Following two washing steps to remove contaminants and residual reagents, the purified DNA was eluted in 30 µl of RNase-free water.

The concentration and purity of the purified DNA were assessed using a NanoRead spectrophotometer (Lifereal Biotechnology Co., Ltd., Hangzhou, China).

3.7. Sequencing

Purified PCR products were sent to a commercial laboratory (Eurofins Genomics, Cologne, Germany) to be sequenced using the Sanger method, also known as chain-termination sequencing. Modern Sanger sequencing is based on a thermal cycling approach that resembles PCR but differs in two important aspects: (1) only a single primer, typically the forward primer, is used, and (2) the reaction mixture contains both deoxyribonucleotide triphosphates (dNTPs) and a small proportion of fluorescently labelled dideoxyribonucleotide triphosphates (ddNTPs).

Unlike dNTPs, ddNTPs lack the 3'-hydroxyl group required for the formation of a phosphodiester bond with the next nucleotide. Consequently, when DNA polymerase incorporates a ddNTP, DNA strand elongation is terminated. Because the incorporation of dNTPs and ddNTPs occurs randomly, the sequencing reaction generates a collection of DNA fragments of different lengths, each terminating with a fluorescently labelled ddNTP. The identity of the terminal ddNTP indicates the nucleotide (A, C, G, or T) present at the corresponding position in the template DNA.

The sequencing products are then separated by capillary electrophoresis, which resolves DNA fragments differing by a single nucleotide. As the fragments migrate through the capillary, a laser excites the fluorescent labels attached to the terminal ddNTPs, and the emitted fluorescence is detected and recorded. The resulting data are displayed as an electropherogram, consisting of coloured peaks, each corresponding to a specific nucleotide. The DNA sequence is reconstructed by analysing the order of these fluorescent peaks (**Figure 21**).

In general, Sanger sequencing produces reads of up to approximately 900 base pairs. However, the first portion of the electropherogram is often difficult to interpret because of background noise, while sequence quality progressively declines after approximately 700 bases owing to signal broadening and reduced resolution during capillary electrophoresis. Although this limited read length makes Sanger sequencing unsuitable for whole-genome sequencing projects, it remains the method of choice for sequencing relatively short DNA fragments, such as those analysed in the present study.

Electropherograms can be visualised and analysed using online tools or dedicated software, such as

MEGA or BioEdit. In addition to assessing sequence quality, the obtained sequences can be compared with reference sequences deposited in public databases using the Basic Local Alignment Search Tool (BLAST; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

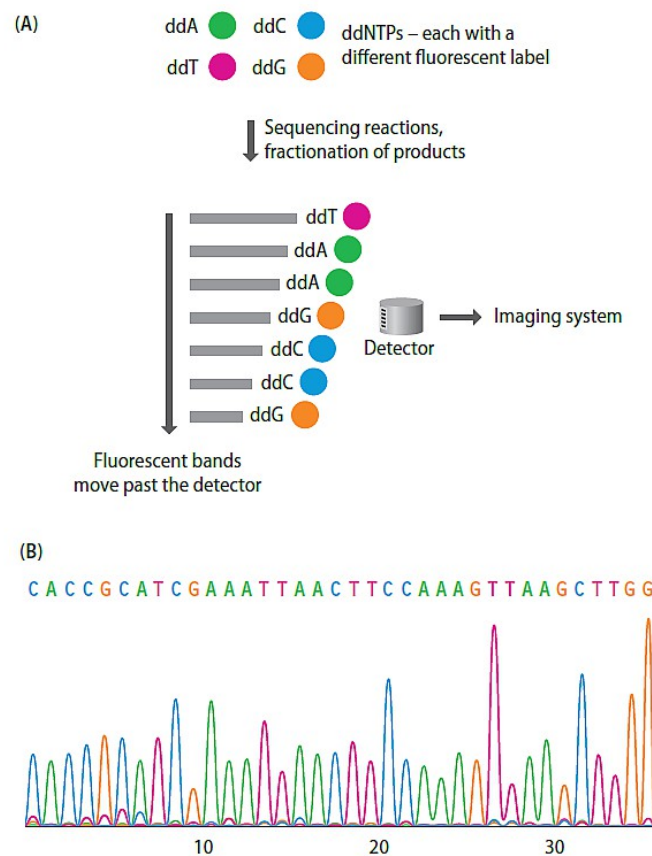


Figure 21. Chain-termination DNA sequencing and electropherogram (Brown, 2023).

3.8. Molecular identification and phylogenetic analysis

Electropherograms obtained from sequencing were analysed and consensus sequences were manually assembled using BioEdit software. The resulting sequences were compared with those deposited in the GenBank database using the BLASTn algorithm. As regards the analysis of *Cryptosporidium* sequences, molecular identification was carried out using the *C. scrofarum* sequence deposited in GenBank (accession number PQ856493) as a reference, obtaining 99.85–100% sequence identity. Conversely, *Enterocytozoon bieneusi* sequences were compared with those available in GenBank using the sequence with accession number AF076040 as a reference, obtaining 94.65–100% sequence identity.

For the phylogenetic analysis, the *Cryptosporidium* sequences obtained in the present study were aligned with a representative selection of SSU rRNA gene sequences belonging to different

Cryptosporidium species. Similarly, the *E. bienersi* sequences aligned with *E. bienersi* genotypes from the main phylogenetic groups.

Multiple alignment was then performed using the MUSCLE algorithm, and subsequently refined using Gblocks, a programme that removes poorly aligned regions. The optimal model of nucleotide substitution was selected using the jModelTest tool and the Bayesian Information Criterion (BIC).

Phylogenetic reconstruction was inferred using the Maximum Likelihood (ML) method, employing the online phylogenetic software MEGAX. The robustness of the phylogeny was tested with 1000 bootstrap pseudo-replicates both for *Cryptosporidium* and *E. bienersi* sequences. The resulting phylogenetic tree was visualised and edited using the online tool iTOL (Interactive Tree Of Life, <https://itol.embl.de>).

3.9. Statistical analyses

Prevalence in pigs and wild boars was calculated as the percentage of infected animals among the total number of animals examined and is reported with 95% confidence intervals (95% CI) both for *Cryptosporidium* spp. and *Enterocytozoon bienersi*. For wild boar samples, associations between infection status and the variables sex (male/female), age class (young, subadult, adult), and hunting season (spring, summer, autumn, winter) were assessed using Pearson's chi-square test. The presence of gastrointestinal nematodes and protozoa (*Ascaris suum*, *Metastrongylus* spp., gastrointestinal strongyles, *Trichuris suis*, coccidia, and *Balantiodides coli*), previously determined by copromicroscopic examination of the same faecal samples, was also evaluated as a potential risk factor.

For the pig samples, positivity to *Cryptosporidium* spp. was studied in relation to the variables sex, age, sampling season, farm size, and application of the all-in/all-out system. Similarly, the presence of the nematodes *Ascaris suum* and *Trichuris suis* and the protozoan *Balantiodides coli*, previously investigated by Allievi *et al.* (2024), was included as a risk factor.

In the univariable analyses, the nested PCR result (positive/negative) was considered the dependent variable, whereas the epidemiological variables and the presence of the above-mentioned parasites were considered independent variables. Variables with a p-value < 0.1 in the univariable analyses were included in multivariable logistic regression models. Model selection was performed using a backward stepwise procedure based on the Akaike Information Criterion (AIC), and variables with $p < 0.05$ were retained in the final models.

All statistical analyses were performed using R software (version 4.4.2; R Foundation for Statistical Computing, Vienna, Austria).

4. RESULTS

4.1. Results of *Cryptosporidium* spp. analyses

4.1.1. Epidemiological variables and prevalence of *Cryptosporidium* spp. in wild boars

A total of 191 wild boars were included in this thesis. The epidemiological data regarding the sampled population – hunting season, age and sex – are summarised in **Table 11**.

Previous copromicroscopic investigation of gastrointestinal nematodes and protozoa revealed a high overall positivity, with 179 out of 191 wild boars testing positive for at least one parasitic taxon (overall prevalence: 93.7%, 95% CI: 89.28 – 96.71). Among nematodes, *Metastrongylus* spp. showed the highest prevalence, followed by strongyle nematodes. Among protozoa, coccidia and *Balantoides coli* were the most frequently detected. For each parasite, prevalence was calculated as the proportion of positive animals, whereas egg or oocyst excretion was expressed as eggs or oocysts per gram of faeces (EPG/OPG). Quantitative copromicroscopic results were described using mean abundance and the minimum-maximum range of EPG/OPG values. The detected parasites, their prevalence, and quantitative copromicroscopic results are summarised in **Table 12**.

Table 11. Epidemiological data of the wild boars included in this study.

Variables	Category	Number of samples
Hunting season	Spring	80
	Summer	29
	Fall	57
	Winter	25
Age	Young	38
	Subadult	81
	Adult	72
Sex	Male	110
	Female	81

Table 12. Prevalence and quantitative copromicroscopic findings of the detected parasites in wild boars hunted in northern Italy. P%: prevalence; CI: Confidence Interval; SD: Standard deviation; EPG: eggs per gram of faeces; OPG: oocysts per gram of faeces; Min-Max: minimum and maximum excretion.

Parasites	Overall (N=191)		EPG/ OPG	
	N° of positives samples	P% (95% CI)	Abundance (SD)	Min-Max
<i>Ascaris suum</i>	13	6.8 (3.7 – 1.3)	31.1 (203.9)	4 – 2274

<i>Ascarops strongylina</i>	1	0.5 (0.01 – 2.8)	0.0 (0.3)	4
<i>Balantioides coli</i>	74	38.7 (31.8 – 46.1)	ND	ND
<i>Capillaria</i> spp.	32	16.7 (11.7 – 22.8)	0.8 (2.2)	2 – 16
Coccidia	116	60.7 (53.4 – 67.7)	832.3 (4198.1)	6 – 49488
<i>Metastrongylus</i> spp.	84	44 (36.8 – 51.3)	24.9 (101.9)	2 – 1168
Strongylid nematodes	71	37.2 (30.3 – 44.4)	22.5 (71.9)	2 – 592
<i>Strongyloides ransomi</i>	17	8.9 (5.3 – 13.9)	1.5 (8.7)	0 – 90
<i>Trichuris suis</i>	30	15.7 (10.9 – 21.7)	2.3 (10.8)	2 – 110

Using a nested PCR protocol, *Cryptosporidium* was detected in 11 out of the 191 samples tested, corresponding to an overall prevalence of 5.8% (95% CI: 2.9 – 10.0).

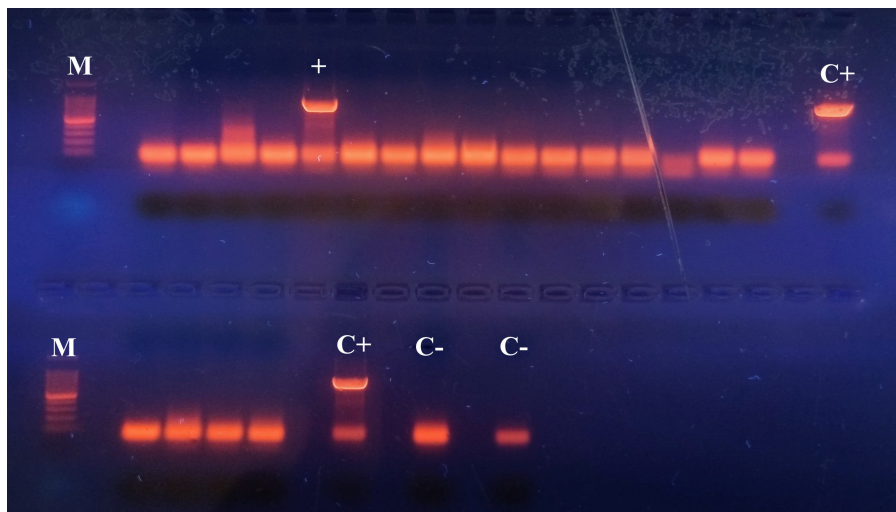


Figure 16. Nested PCR analysis targeting SSU rRNA gene of *Cryptosporidium* spp. **M:** molecular weight marker; **(+):** positive sample with amplification band of the expected size (826–864 bp); **C+:** positive control; **C-:** negative control of the first and second PCR.

4.1.2. Statistical analysis for *Cryptosporidium* spp. in wild boars

Possible associations between *Cryptosporidium* spp. positivity and the included variables were evaluated using Pearson's Chi-squared test, with a p-value <0.05 considered statistically significant. Statistical analysis revealed no significant associations between *Cryptosporidium* spp. positivity and any of the evaluated variables.

The distribution of *Cryptosporidium* spp. positivity according to the variables and the corresponding Chi-squared test results are presented in **Table 13**.

Table 13. Distribution of *Cryptosporidium* spp. positivity in wild boars in relation to the variables considered.

Variables	Categories	N° positives/tested	P% (95% CI)	X ²	P-value
Hunting season	Spring	2/80	2.5 (0.3 – 8.7)	3.10	0.38
	Summer	3/29	10.3 (2.2 – 27.4)		
	Fall	4/57	7.0 (1.9 – 17.0)		
	Winter	2/25	8.0 (1.0 – 26.0)		
Age	Young	2/38	5.3 (0.6 – 17.7)	0.05	0.98
	Subadult	5/81	6.2 (2.0 – 13.8)		
	Adult	4/72	5.6 (1.5 – 13.6)		
Sex	Male	10/110	9.1 (4.5 – 16.1)	3.96	0.05
	Female	1/81	1.2 (0,03 – 6,69)		
<i>Ascaris suum</i>	Present	0/13	0.0	0.09	0.76
	Absent	11/178	6.2 (3.1 – 10.8)		
<i>Ascarops strongylina</i>	Present	0/1	0.0	6.88 ⁻²⁸	1
	Absent	11/190	5.8 (2.9 – 10.1)		
<i>Balantioides coli</i>	Present	4/74	5.4 (1.5 – 13.3)	2.95 ⁻³¹	1
	Absent	7/117	6.0 (2.4 – 11.9)		
<i>Capillaria</i> spp.	Present	3/32	9.4 (1.9 – 25.0)	0.30	0.58
	Absent	8/159	5.0 (2.2 – 9.7)		
Coccidia	Present	6/116	5.2 (1.9 – 10.9)	0.01	0.91
	Absent	5/75	6.7 (2.2 – 14.9)		

<i>Metastrongylus</i> spp.	Present	2/84	2.4 (0.3 – 8.3)	2.14	0.14
	Absent	9/107	8.4 (3.9 – 15.4)		
Strongylid nematodes	Present	4/71	5.6 (1.6 – 13.8)	1.96 ⁻²⁹	1
	Absent	7/120	5.8 (2.4 – 11.6)		
<i>Strongyloides ransomi</i>	Present	1/17	5.9 (0.2 – 28.7)	1.20 ⁻²⁸	1
	Absent	10/174	5.8 (2.8 – 10.3)		
<i>Trichuris suis</i>	Present	2/30	6.7 (0.8 – 22.1)	3.50 ⁻³⁰	1
	Absent	9/161	5.6 (2.6 – 10.3)		

4.1.3. Molecular characterisation of *Cryptosporidium* spp. in wild boars

PCR products from all positive samples were purified and submitted to a commercial laboratory (Eurofins Genomics, Cologne, Germany) for SSU rRNA gene sequencing to identify the *Cryptosporidium* species present. The resulting 11 sequences were analysed using BioEdit Sequence Alignment Editor (version 7.2.5).

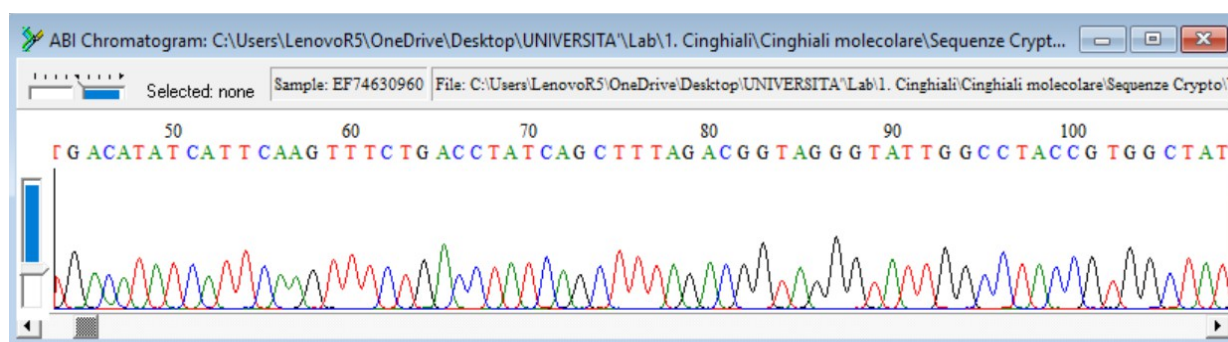


Figure 17. Electropherogram portion obtained by sequencing a SSU rRNA gene fragment of *Cryptosporidium* spp., visualised using BioEdit software.

BLAST analyses revealed that all samples belonged to *Cryptosporidium scrofarum*, exhibiting 99.85-100% sequence identity and query coverage with the *C. scrofarum* sequence deposited in GenBank (accession number PQ856493). The obtained sequences showed limited intraspecific variability, which nevertheless allowed the identification of two distinct isolates sharing 99.87% sequence identity. Nine sequences were assigned to the isolate designated "wild boar isolate 2", while the remaining two were assigned to "wild boar isolate 1".

4.1.4. Epidemiological variables and prevalence of *Cryptosporidium* spp. in pigs

The presence of *Cryptosporidium* was investigated in 440 pig faecal samples. Epidemiological data regarding age, sampling season, farm size, and application of the all-in/all-out system are summarised in **Table 14**.

Table 14. Epidemiological data regarding the pigs examined in this study.

Variables	Category	Number of samples
Age	≤ 5 months	210
	> 5 months	230
Sampling season	Spring	170
	Summer	100
	Autumn	100
	Winter	70
Faecal soiling score	0	180
	1	120
	2	140
Farm size	< 1,900 animals	280
	≥ 1,900 animals	160
All-in/all-out production system	Present	320
	Absent	110

Previous copromicroscopic data indicated the presence of *Ascaris suum*, *Trichuris suis*, and *Balantoides coli* at varying prevalences, as summarised in **Table 15**.

Table 15. Prevalence and quantitative copromicroscopic findings of the detected parasites in fattening pigs sampled in northern Italy. P%: prevalence; CI: Confidence Interval; SD: Standard deviation; EPG: eggs per gram of faeces; OPG: oocysts per gram of faeces; Min-Max: minimum and maximum excretion.

Parasites	Overall (N=440)		EPG/ OPG	
	N° of positives samples	P% (95% CI)	Abundance (SD)	Min-Max
<i>Ascaris suum</i>	32	7.3 (5.1 – 10.1)	42.1 (348.8)	2 – 120
<i>Balantoides coli</i>	399	90.7 (87.6 – 93.2)	ND	ND
<i>Trichuris suis</i>	9	2.0 (0.9 – 3.9)	0.50 (8.89)	2 – 186

Cryptosporidium spp. was detected in 109 of the 440 samples, corresponding to an overall prevalence of 24.8% (95% CI: 20.8 – 29.1).

4.1.5. Statistical analyses for *Cryptosporidium* spp. in pigs

Table 16 summarises the distribution of *Cryptosporidium* spp. positivity according to the epidemiological and copromicroscopic variables evaluated, along with the results of Pearson's Chi-squared test.

Table 16. Distribution of *Cryptosporidium* spp. positivity in pigs in relation to the variables considered in this thesis. Variables showing a p-value < 0.05 were considered significant and significant values were marked in bold.

Variables	Categories	N° positives/tested	P% (95% CI)	X ²	P-value
Age	≤ 5 months	90/210	42.9 (36.1 – 49.8)	68.66	< 2.2 ⁻¹⁶
	> 5 months	19/230	8.3 (5.1 – 12.6)		
Sampling season	Spring	61/170	35.9 (28.7 – 43.6)	29.17	2.06 ⁻⁰⁶
	Summer	29/100	29.0 (20.4 – 38.9)		
	Fall	12/100	12.0 (6.4 – 20.0)		
	Winter	7/70	10.0 (4.2 – 19.5)		
Faecal soiling score	0	49/180	27.2 (20.9 – 34.3)	1.89	0.39
	1	31/120	25.8 (18.3 – 34.6)		
	2	29/140	20.71 (14.3 – 28.4)		
Farm size	< 1,900 animals	58/280	20.7 (16.1 – 25.9)	6.22	0.01
	≥ 1,900 animals	51/160	31.9 (24.7 – 39.7)		
All-in/all-out production system	Present	82/320	25.6 (20.9 – 30.8)	0.30	0.58
	Absent	27/110	24.5 (16.8 – 33.7)		
<i>Ascaris suum</i>	Present	9/32	28.1 (13.8 – 46.8)	0.06	0.81
	Absent	100/408	24.5 (20.4 – 28.9)		
<i>Balantiodides coli</i>	Present	92/399	23.1 (19.1 – 27.5)	5.81	0.01
	Absent	17/41	41.5 (26.3 – 57.9)		

<i>Trichuris suis</i>	Present	5/9	23.1 (19.1 – 27.5)	3.14	0.04
	Absent	104/431	24.1 (20.2 – 28.4)		

Univariate analysis using Pearson's Chi-Squared test identified significant associations between *Cryptosporidium* spp. positivity and animal age, farm size, and co-infection with *Balantiodides coli* and *Trichuris suis*. Specifically, risk factors analysis showed that younger pigs were significantly more likely to be infected than older animals (OR = 8.33; $p < 0.001$), whereas pigs from smaller farms had a lower likelihood of infection compared to those from larger farms (OR = 0.56; $p = 0.010$). *Trichuris suis* co-infection was identified as a risk factor for *Cryptosporidium* spp. infection (OR = 3.93; $p = 0.044$), whereas *Balantiodides coli* infection appeared to be a protective factor, being associated with a lower likelihood of *Cryptosporidium* spp. infection (OR = 0.42; $p = 0.011$).

Variables with a p -value < 0.05 were included in the multivariate logistic regression model. The analysis confirmed that *Cryptosporidium* spp. positivity remained significantly associated with farm size (OR = 0.50; $p = 0.007$), age (OR = 8.69; $p < 0.001$), and *Trichuris suis* co-infection (OR = 8.4; $p = 0.007$), whereas the association with *Balantiodides coli* was no longer statistically significant.

4.1.6. Molecular characterisation of *Cryptosporidium* spp. in pigs

Among the 109 pig samples that tested positive for *Cryptosporidium* spp., a representative subset of 30 samples from different farms was selected for sequencing.

Thirty sequences were obtained and analysed using BLAST, revealing 99%-100% sequence identity and 100% query coverage with the *C. scrofarum* sequence deposited in GenBank (accession number PQ856493). Three distinct isolates were identified: twenty-eight sequences were assigned to the isolate designated "pig isolate 1", while the remaining two corresponded to "pig isolate 2" and "pig isolate 3", respectively. Sequence identity among the 18S rRNA gene sequences of the three isolates ranged from 99.71% to 99.87%.

4.1.7. Phylogenetic analysis of *Cryptosporidium* spp. sequences from wild boars and pigs

Based on 18S rRNA gene sequences, phylogenetic analysis confirmed that all isolates belonged to *Cryptosporidium scrofarum* (**Figure 18**). Isolates from both pigs and wild boars clustered together with *C. scrofarum* reference sequences retrieved from GenBank (accession numbers PQ615455, PQ856493, and PQ856498). The low genetic distance between pig and wild boar isolates was

consistent with the high sequence identity observed by BLAST analysis, further supporting the limited intraspecific variability of the 18S rRNA gene in *C. scrofarum*.

The isolates designated “wild boar isolate 1” and “wild boar isolate 2” clustered within the same clade as the pig isolates (“pig isolate 1”, “pig isolate 2” and “pig isolate 3”), with no evident phylogenetic segregation according to host species.

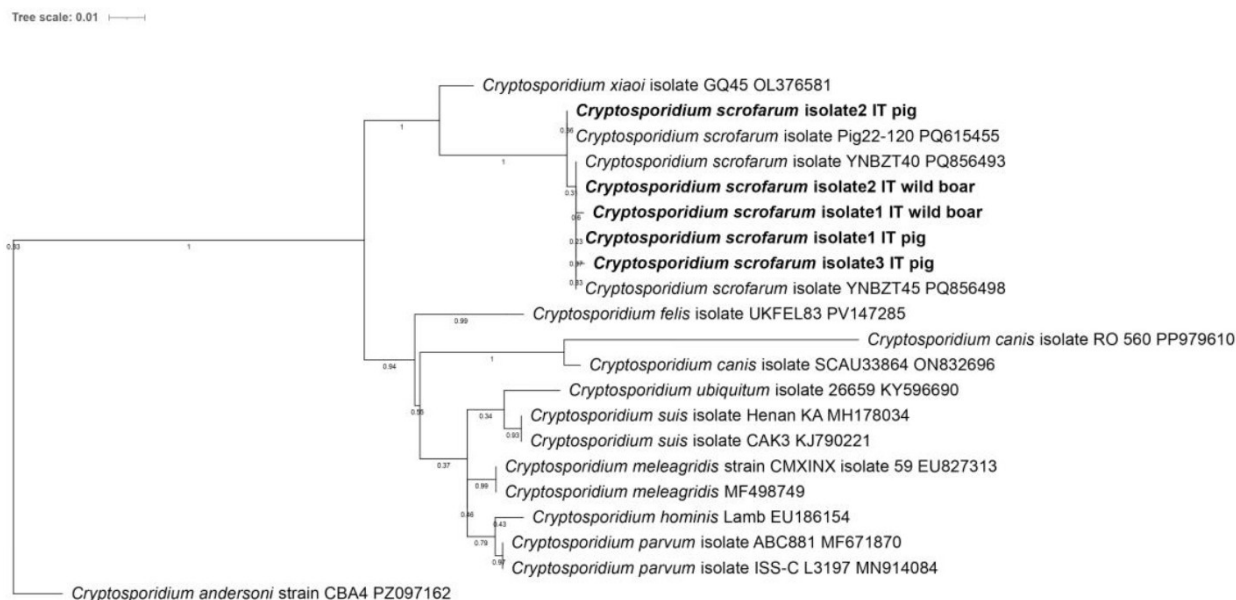


Figure 18. Phylogenetic relationship among *Cryptosporidium scrofarum* isolates obtained from wild boars and pigs (marked in bold) and reference sequences retrieved from GenBank (accession numbers follow the species name). The TN93 model with gamma distribution (G) was selected as the best-fit nucleotide substitution model. The phylogram was reconstructed using the Maximum Likelihood method and visualised using the online tool iTOL (Interactive Tree Of Life, <https://itol.embl.de>). Support bootstrap values below 0.50 were omitted. The scale bar stands for proportional sequence divergence. Numbers on branches report bootstrap supports with 1000 pseudo-replicates.

4.2. Results of *Enterocytozoon bieneusi* analysis

4.2.1. Prevalence of *Enterocytozoon bieneusi* in wild boars

A total of 191 wild boar samples were tested using a nested PCR protocol to investigate the presence of *Enterocytozoon bieneusi*. The parasite was detected in 11 samples, with an overall prevalence of 5.8% (95% CI: 2.9 – 10.1).

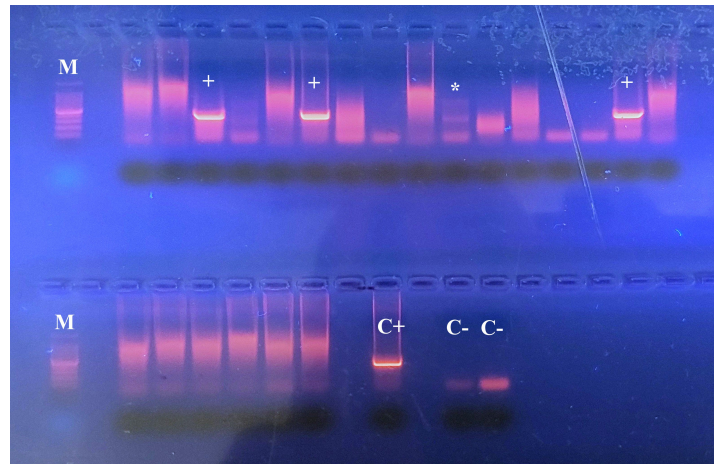


Figure 19. Nested PCR analysis targeting ITS region of *E. bieneusi* spp. **M**: molecular weight marker; **+**: positive sample with amplification band of the expected size (240–390 bp); **C+**: positive control; **C-**: negative control of the first and second PCR.

4.2.2. Statistical analyses for *Enterocytozoon bieneusi* in wild boars

Associations with *Enterocytozoon bieneusi* positivity and the same epidemiological and copromicroscopic variables evaluated for *Cryptosporidium* spp. were assessed using Pearson's Chi-Squared test. The results are summarised in **Table 17**.

Univariate analysis identified significant associations between *E. bieneusi* positivity and both age and *Ascaris suum* co-infection. Wild boars younger than one year were significantly more likely to be infected than older animals (OR = 13.3, p-value = 0.02). In addition, *A. suum* co-infection was identified as a significant risk factor for *E. bieneusi* positivity (OR = 6.4, p-value = 0.03).

Only variables with p-value < 0.05 were included in the multivariate logistic regression model. The analysis showed that *E. bieneusi* positivity remained marginally associated only with age, as older animals were less likely to be infected than younger animals (OR = 0.09; p = 0.03), whereas the association with *A. suum* was no longer statistically significant.

Table 17. Distribution of *Enterocytozoon bieneusi* positivity in wild boars in relation to the variables considered in this thesis. Variables showing a p-value <0.05 were considered significant and significant values were marked in bold.

Variables	Categories	N° positives/tested	P% (95% CI)	X ²	P-value
Hunting season	Spring	6/80	7.5 (2.8 – 15.61)	7.10	0.068
	Summer	4/29	13.8 (3.9 – 31.7)		
	Fall	1/57	1.7 (0.4 – 9.4)		
	Winter	0/25	0.0		
Age	Young	6/38	15.8 (6.0 – 31.3)	9.68	0.007
	Subadult	4/81	4.9 (1.4 – 12.2)		
	Adult	1/72	1.4 (0.03 – 7.5)		
Sex	Male	8/110	7.3 (3.2 – 13.8)	0.53	0.46
	Female	3/81	3.7 (0.8 – 10.4)		
<i>Ascaris suum</i>	Present	3/13	23.1 (5.0 – 53.8)	4.66	0.03
	Absent	8/178	4.5 (2.0 – 8.7)		
<i>Ascarops strongylina</i>	Present	1/1	100	3.62	0.06
	Absent	0/190	0.0		
<i>Balantiodides coli</i>	Present	7/74	9.5 (3.9 – 18.5)	2.04	0.15
	Absent	4/117	3.4 (0.9 – 8.5)		
<i>Capillaria</i> spp.	Present	1/32	3.1 (0.08 – 16.2)	0.08	0.76
	Absent	10/159	10.1 (5.9 – 15.8)		
Coccidia	Present	5/116	4.3 (1.4 – 9.8)	0.56	0.45
	Absent	6/75	8.0 (3.0 – 16.6)		
<i>Metastrongylus</i> spp.	Present	4/84	4.8 (1.3 – 11.7)	0.04	0.83
	Absent	7/107	6.5 (2.7 – 13.0)		

Strongylid nematodes	Present	3/71	4.2 (0.9 – 11.9)	0.14	0.70
	Absent	8/120	6.7 (2.9 – 12.7)		
<i>Strongyloides ransomi</i>	Present	0/17	0.0	0.27	0.60
	Absent	11/174	6.3 (3.2 – 11.0)		
<i>Trichuris suis</i>	Present	1/30	3.3 (0.1 – 17.2)	0.04	0.84
	Absent	10/161	6.2 (3.0 – 11.1)		

4.2.3. Molecular characterisation of *Enterocytozoon bieneusi* in wild boars

Positive samples were subjected to ITS region sequencing to confirm the presence of *Enterocytozoon bieneusi*. The resulting 11 sequences were analysed using BioEdit Sequence Alignment Editor (version 7.2.5).

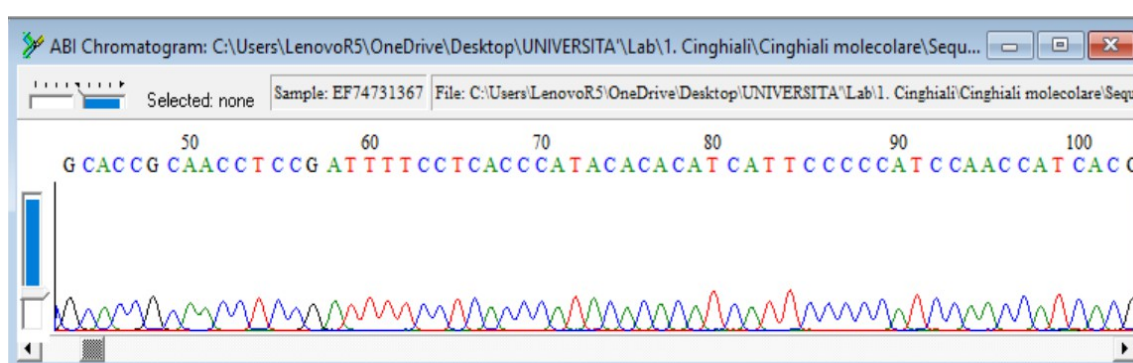


Figure 20. Electropherogram portion obtained by sequencing the ITS region of *Enterocytozoon bieneusi*, visualised using BioEdit

The sequences showed 94.65–100% sequence identity and 100% query coverage with *E. bieneusi* reference sequences deposited in GenBank (accession number AF076040).

Seven distinct isolates were identified: three sequences were assigned to "isolate 1", two to "isolate 2", and two to "isolate 6", while the remaining four were assigned to "isolate 3", "isolate 4", "isolate 5" and "isolate 7", respectively.

4.2.4. Phylogenetic analysis of *Enterocytozoon bieneusi* sequences from wild boars

Phylogenetic analysis of the ITS region confirmed that all *Enterocytozoon bieneusi* isolates belonged to the zoonotic Group 1 (**Figure 21**).

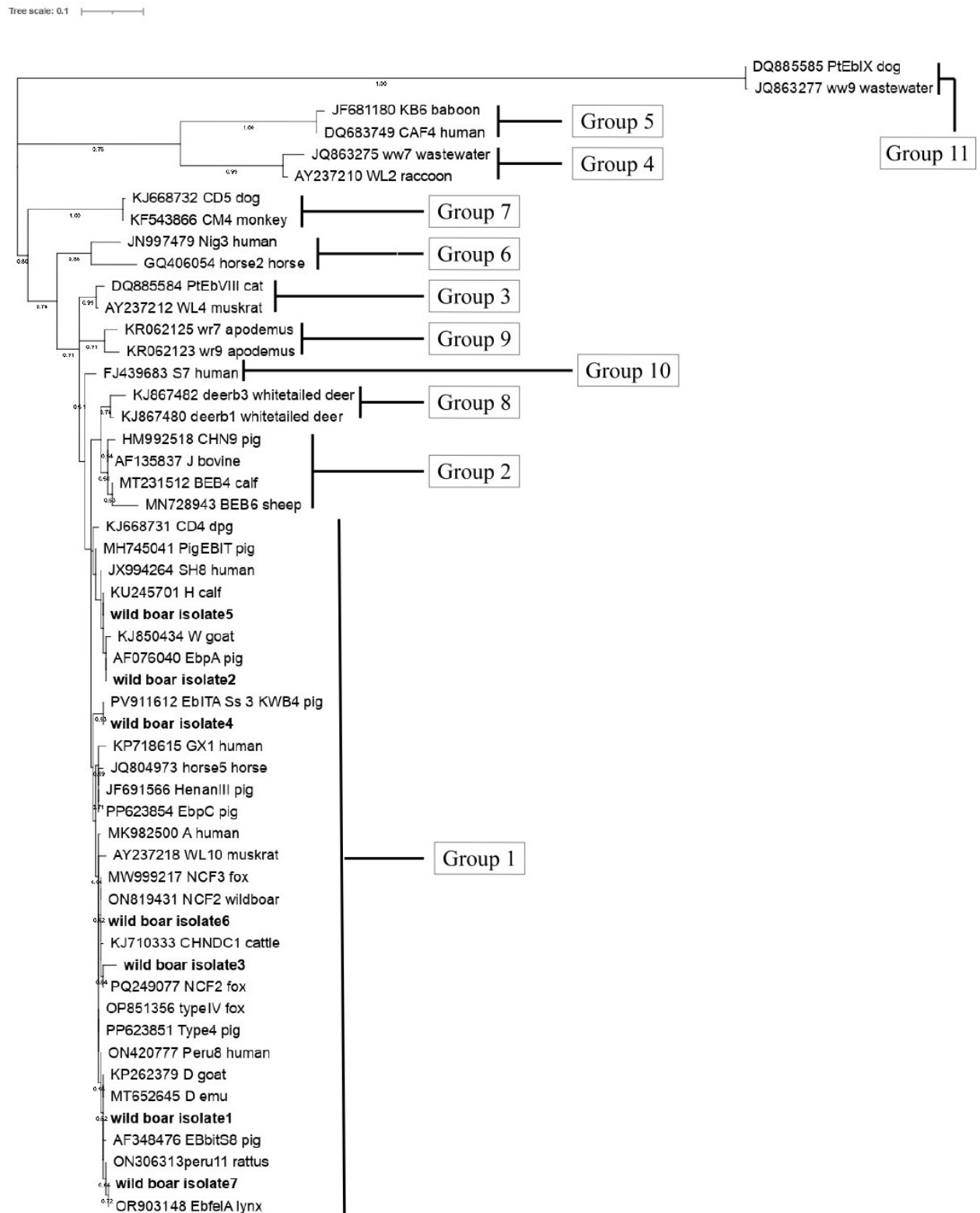


Figure 21. Phylogenetic relationship among *Enterocytozoon bieneusi* isolates obtained from wild boars (marked in bold) and reference sequences retrieved from GenBank (accession numbers follow the species name). The HKY85 model with gamma distribution (G) was selected as the best-fit nucleotide substitution model. The phylogram was reconstructed using the Maximum Likelihood method and visualised using the online tool iTOL (Interactive Tree Of Life, <https://itol.embl.de>). Support bootstrap values below 0.50 were omitted. The scale bar stands for proportional sequence divergence. Numbers on branches report bootstrap supports with 1000 pseudo-replicates.

"Isolate 1" sequences clustered within genotype D (accession numbers KP262379 and MT652645). "Isolate 2" belonged to the genotype EbpA clade (accession number AF076040), whereas "isolate 3" and "isolate 6" exhibited low genetic distance and were assigned to genotypes NCF2/NCF3 (sequences with accession number ON819431 and KJ710333), which are considered synonyms of WildBoar3 genotype (Li *et al.*, 2019). "Isolate 5" clustered within genotype H (accession number KU245701) and "isolate 7" within the EbfelA clade (accession number OR903148). Finally, "isolate 4" showed 100% sequence identity with two sequences belonging to genotype KWB4 (accession number MT984295 and PV911612). The latter sequence was isolated from fattening pigs and deposited in GenBank by Allievi *et al.* (2026).

5. DISCUSSION

5.1. Discussion of the results concerning *Cryptosporidium* spp.

5.1.1. Prevalence of *Cryptosporidium* spp. in wild boars

The overall prevalence of *Cryptosporidium* spp. in the examined wild boars was 5.8%. This result aligns with the wide variability in prevalence reported across various European studies. Regarding the Iberian Peninsula, Santos-Silva *et al.* (2023) observed a prevalence of 1.4% in Portugal, whereas García-Presedo *et al.* (2013) and Rivero-Juarez *et al.* (2020) reported values of 16.7% in northwestern Spain and 8.2% in southern Spain, respectively. Meanwhile, Němejc *et al.* (2013) documented prevalences of 18.2% in Austria, 5.4% in Slovakia, 8.4% in Poland and 16.9% in the Czech Republic. This high variability is likely attributable to differences in the geographical characteristics of the study areas.

Based on these studies, the mean prevalence of *Cryptosporidium* spp. in European wild boars is approximately 10%. The prevalence observed in the present study is therefore slightly lower than the European average, suggesting a limited but significant circulation of the parasite within the sampled population.

Statistical analysis revealed no significant associations between the considered variables and *Cryptosporidium* spp. positivity. This finding may have been influenced by the low number of positive samples (11/191), which likely reduced the statistical power of the analyses. The results of this study, in particular, showed no relationship between age and prevalence, although previous studies identified age as a relevant risk factor both in wild boars and pigs, as *Cryptosporidium* spp. prevalences tend to decrease with age (García-Presedo *et al.*, 2013; Martí-Marco *et al.*, 2023).

Sequencing of all positive samples identified a single *Cryptosporidium* species, *C. scrofarum*. This species, together with *C. suis*, is considered one of the predominant species infecting suids (Ryan *et al.*, 2021b) and has been reported in both domestic pigs and wild boars across several countries (Němejc *et al.*, 2013; García-Presedo *et al.* 2013; Pettersson *et al.*, 2020; Chen *et al.*, 2023; Li *et al.*, 2017).

5.1.2. Prevalence of *Cryptosporidium* spp. in pigs

The prevalence of *Cryptosporidium* spp. in the sampled fattening pigs was 24.8%. This result is consistent with previous studies by Giangaspero *et al.* (2007) and Trotti *et al.* (1984a), which documented prevalences up to 19.8% in farmed pigs from northern Italy, supporting the widespread

circulation of the parasite within the Italian swine industry.

According to Chen *et al.* (2023), the estimated global prevalence of *Cryptosporidium* spp. in pigs is 16.3%, increasing to 18.3% in Europe. Nevertheless, prevalence values reported by individual studies vary widely owing to differences in diagnostic methods, farming systems, and geographical areas, ranging from 1.4% in Germany (Wieler *et al.*, 2001) to 40.9% in Denmark (Petersen *et al.*, 2015).

Multivariate statistical analysis identified significant associations between *Cryptosporidium* spp. positivity, animal age, farm size, and *Trichuris suis* coinfection. Younger age emerged as a major risk factor, with pigs aged five months or less being around nine times more likely to test positive than older animals. Piglets in this age group are in the post-weaning stage, a particularly delicate phase of growth characterised by reduced immunity due to the loss of maternally derived immunity and the stress associated with relocation to the fattening unit. These marked immunological and environmental changes may increase susceptibility to enteric pathogens, thereby contributing to the higher prevalence of *Cryptosporidium* spp. infection typically observed in this production category (Chen *et al.*, 2023; Pettersson *et al.*, 2020). Subsequently, the progressive acquisition of immune competence may contribute to a lower circulation of *Cryptosporidium* in older animals (Pettersson *et al.*, 2020).

Farm size was significantly associated with *Cryptosporidium* spp. positivity, with pigs raised on farms housing more than 1,900 animals showing a higher risk of infection than those from smaller farms. Larger farms are generally characterised by higher animal densities and shared spaces that may facilitate the transmission of *Cryptosporidium* spp., thereby supporting the persistence and environmental dissemination of oocysts (Pettersson *et al.*, 2020).

Co-infection with *Trichuris suis* was significantly associated with *Cryptosporidium* spp. positivity. Given the limited number of animals positive to *T. suis* (9/440), this finding is more likely explained by environmental conditions favouring faecal-oral transmission than by a true biological interaction between the two parasites. *Trichuris suis* eggs exhibits high environmental resistance, similarly to *Cryptosporidium* spp. oocysts. Consequently, co-infection is likely the result of persistent environmental contamination and the prolonged survival of the infesting stages in farms. However, further studies with larger sample sizes are needed to clarify this possible association.

All sequenced samples were identified as *C. scrofarum*. In agreement with previous studies, *C. scrofarum* is the predominant species in pigs older than five weeks, whereas *C. suis* is more frequently detected in suckling piglets and during the early post-weaning period (Petersen *et al.*, 2015; Chen *et al.*, 2023; Pettersson *et al.*, 2020). Therefore, the exclusive identification of *C. scrofarum* in the present work can be attributed to the sampled production category, which consisted

exclusively of fattening pigs.

5.1.3. Comparison between epidemiological data in wild boars and pigs

The comparison of *Cryptosporidium* spp. prevalence in the examined wild boars and pigs highlights a clear difference in parasite circulation between wild and domestic populations. Indeed, the prevalence in wild boars was approximately four times lower than that observed in farmed animals. This finding is consistent with most previous studies, which have reported a prevalence of *Cryptosporidium* spp. below 15% in wild boars (Němejc *et al.*, 2013; García-Presedo *et al.*, 2013). The main exception is the study by Martí-Marco *et al.* (2023), which documented a prevalence of 21.7% in wild boars from eastern Spain, a value comparable to or even higher than that observed in domestic pigs.

The results obtained suggests that the conditions typical of intensive farming systems may facilitate parasite transmission, whereas the lower animal density and greater dispersion of faeces in natural environment may reduce the efficiency of faecal-oral transmission (Němejc *et al.*, 2013). Nevertheless, the detection of *Cryptosporidium* spp. in wild boars, even at a low prevalence, indicates that these animals may contribute to the maintenance of the parasite, potentially acting as natural reservoirs (Němejc *et al.*, 2013; García-Presedo *et al.*, 2013; Li *et al.*, 2017).

No association with diarrhoea was observed in either wild boars or pigs, supporting the absence of clinical signs in the examined animals. These observations align with previous literature reporting the predominantly asymptomatic nature of *Cryptosporidium* spp. infections in both wild and domestic suids (Ryan *et al.*, 2021b; Chen *et al.*, 2023).

In pig farming, the frequent absence of clinical signs is particularly relevant, as it can lead to an underestimation of infection prevalence, while facilitating parasite persistence within farms through apparently healthy animals (Pettersson *et al.*, 2020). In addition, asymptomatic pigs and wild boars may act as carriers of *Cryptosporidium* spp. and contribute to parasite transmission to other susceptible hosts (García-Presedo *et al.*, 2013).

The same species, *C. scrofarum*, was identified in both the wild boar population and the examined fattening pigs. Moreover, the obtained sequences showed low genetic distance and no clear phylogenetic segregation according to host species.

5.2. Discussion of the results concerning *Enterocytozoon bieneusi*

5.2.1. Prevalence of *Enterocytozoon bieneusi* in wild boars

The most recent global estimate of *Enterocytozoon bieneusi* prevalence in wild boars is approximately 10.1% (Taghipour *et al.*, 2022). This value should be interpreted with caution, as current data on the circulation of *E. bieneusi* in wild boars remain limited. To date, only six studies have been carried out: four of them focused on free-ranging animals in Central Europe, Iberian Peninsula, and Korea (Němejc *et al.*, 2014; Dashti *et al.*, 2020; Figueiredo *et al.*, 2023; Li *et al.*, 2021), while the remaining two investigated its circulation in farmed wild boars in China (Li *et al.*, 2017; Feng *et al.*, 2020). These studies reported variable prevalence rates: 1.9 – 4.0% in the Iberian Peninsula, 3.6 – 13.6% in Central Europe, 2.6% in Korea, and 41.2 – 42.0% in farmed wild boars in China. Such limited data, derived from highly diverse geographical and ecological contexts (free-ranging vs. farmed animals), hinder a clear assessment of *E. bieneusi* epidemiology in wild boars. The overall prevalence observed in the present study was 5.8%, consistent with previously reported European data. Furthermore, the absence of diarrhoea in positive animals suggests that infected wild boars were asymptomatic carriers.

Multivariate statistical analysis highlighted a significant association between *E. bieneusi* positivity and animal age, with adult animals being less likely to be infected. This trend is frequently reported in pigs, which can acquire *E. bieneusi* infection from sows just after birth (Sak *et al.*, 2008; Allievi *et al.*, 2026; Wan *et al.*, 2015). Moreover, the transient immunosuppression that may follow weaning can increase piglets' susceptibility to infection (Allievi *et al.*, 2026). However, the only study that observed a correlation between infection and age in wild boars was Feng *et al.* (2020).

This study identified six distinct *E. bieneusi* genotypes (D, H, EbpA, KWB4, WildBoar3, and EbfelA), all belonging to the zoonotic Group 1.

EbpA (subgroup 1e) and D (subgroup 1a) are among the most frequently reported of the 27 genotypes currently identified in wild boars (Dashti *et al.*, 2020; Taghipour *et al.*, 2022). Both genotypes show low host specificity, having been detected in domestic pigs, wild boars, humans, primates, livestock, cats, dogs, horses, and wild animals (Li *et al.*, 2019).

In contrast, WildBoar3 genotype has been reported exclusively in European wild boars from the Czech Republic and Poland (Němejc *et al.*, 2014; Dashti *et al.*, 2020). This genotype belongs to the ITS subgroup 1b/c and is predominantly associated with wildlife species (foxes, raccoons, raccoon dogs, martens, badgers and wild boars) (Li *et al.*, 2019).

This study provides the first molecular evidence of genotypes H, EbfelA, and KWB4 in wild boars.

Genotype H (subgroup 1e) is frequently found in pigs, suggesting a certain degree of host specificity. However, its host range remains to be fully clarified, as sporadic cases have also been reported in humans, dogs, and cats (Li *et al.*, 2019; Allievi *et al.*, 2026). The EbfelA genotype (subgroup 1a) has previously been reported only in captive non-human primates, including orangutans and macaques, as well as in cats and lynxes (Ávalos *et al.*, 2024). Therefore, the present study expands the known host range of this genotype to include wild boars. Finally, the KWB4 genotype (subgroup 1e) was first reported in Korean wild boars by Li *et al.* (2021) and was subsequently identified in fattening pigs in northern Italy (Allievi *et al.*, 2026).

5.1.2. Current knowledge on the epidemiology of *Enterocytozoon bieneusi* in pigs and comparison with findings in wild boars

Epidemiological studies in pigs have reported variable prevalence rates of *Enterocytozoon bieneusi*, ranging from 10% to 94% (Němejč *et al.*, 2014; Allievi *et al.*, 2026; Li *et al.*, 2024). According to recent meta-analyses by Li *et al.* (2024) and Taghipour *et al.* (2022), the overall prevalence of *E. bieneusi* in pigs is estimated at approximately 35–37%. The main factors contributing to this variability include geographic location, diagnostic methods, production category, farm type, stocking density, and animal age. To date, more than 90 genotypes have been identified in pigs, with EbpA, EbpC, H, and D being the most frequently found. Several of these genotypes – including CS-4, D, EbpA, EbpC, G, H, Henan-IV, PigEBITS5, PigEBITS7, BEB4, and I – have been detected in both pigs and humans, demonstrating their zoonotic potential (Dashti *et al.*, 2020; Taghipour *et al.*, 2022).

The study by Allievi *et al.* (2026) provided the first molecular detection of *E. bieneusi* in Italian pigs, using the same farms and samples analysed in the present thesis. That study reported an overall prevalence of 63.4% (279/440), with higher values observed in younger animals. Furthermore, it identified a significant association between body faecal soiling score and infection status. Finally, seven genetic isolates were characterised: five correspond to known genotypes (EbpA, H, KWB4, and CC-1/CHPR2), all belonging to the zoonotic Group 1, while two isolates remained unassigned (**Table 18**).

Table 18. *Enterocytozoon bieneusi* isolates from 22 fattening pig farms of northern Italy (Allievi *et al.*, 2026).

Isolate	Assigned genotype	Phylogenetic group	N° of samples per isolate
EbITA Ss 1	EbpA	1	14
EbITA Ss 2	unassigned	1	1

EbITA Ss 3	KWB4	1	2
EbITA Ss 4	H	1	1
EbITA Ss 5	H	1	1
EbITA Ss 6	CC-1/CHPR2	1	2
EbITA Ss 7	EbpA	1	1

The prevalence of *E. bieneusi* in the wild boars examined in this thesis was lower than that reported in pig farms. Specifically, the overall prevalence observed by Allievi *et al.* (2026) in fattening pigs from northern Italy was approximately ten times higher than that found in wild boars sampled in the province of Cremona. As observed for *Cryptosporidium* spp., the markedly higher prevalence of *E. bieneusi* in domestic pigs may be associated with the higher stocking density and closer animal contact typical of intensive farming systems, compared with the natural conditions in which wild boars live (Němejc *et al.*, 2014).

None of the pigs examined by Allievi *et al.* (2026) exhibited gastrointestinal symptoms, and no significant association with diarrhoea was observed in the wild boars analysed in this thesis. Although numerous studies have documented *E. bieneusi* infections in clinically healthy animals, infections have also been reported in pigs presenting gastrointestinal symptoms (Dashti *et al.*, 2020). Consequently, the pathogenic role of *E. bieneusi* in pigs remains unclear and requires further investigations.

5.3. Potential circulation of *Cryptosporidium scrofarum* and *Enterocytozoon bieneusi* at the wild-domestic interface

The results of this thesis led to the identification of the same *Cryptosporidium* species, *C. scrofarum*, in both wild boars sampled in the province of Cremona and fattening pigs from northern Italy. Phylogenetic analysis revealed a low genetic distance between the sequences obtained from pigs and wild boars, showing no distinct phylogenetic segregation based on host species.

At the same time, several *E. bieneusi* genotypes were identified within the sampled wild boar population. Some of these, such as WildBoar3 and EbfelA, are primarily associated with wildlife hosts, whereas genotypes H and EbpA are typically detected in domestic pigs. A comparison with the epidemiological data on *E. bieneusi* reported by Allievi *et al.* (2026) for the same population of fattening pigs revealed that three genotypes (EbpA, H, and KWB4) were shared between wild boars and domestic pigs.

Given the distinct geographic areas of the sampled populations, the present study does not

demonstrate direct transmission between pigs and wild boars. Nevertheless, these findings suggest the potential circulation of parasites between wild and domestic populations, likely mediated by the environment. Indeed, *Cryptosporidium* spp. oocysts and *E. bieneusi* spores can persist for extended periods in manure, livestock slurry, waterways, and contaminated soil (Pinto *et al.*, 2022; Nourisson *et al.*, 2024).

The transmission of *E. bieneusi* between domestic and wild populations has been documented in a sympatric setting in southern Spain, where Iberian pigs are raised under semi-free-range conditions in open areas frequented by wild boars (Dashti *et al.*, 2020). Although this extensive farming system differs substantially from the intensive system described in this thesis, the findings of Dashti *et al.*, (2020) emphasises the role of the environment in parasite transmission, particularly through shared water and food sources.

The province of Cremona hosts a high number of intensive pig farms and has recently experienced an expansion of the wild boar population. These factors have led to an increasing overlap between natural habitats, agricultural lands, and intensive farming systems, potentially creating shared environmental sources of infection, that may play an important role in parasite circulation at the wildlife-livestock interface.

5.4. Impact of management practices on parasite transmission in pig farming

Correct identification of the risk factors associated with the spread and persistence of gastrointestinal pathogens, such as *Cryptosporidium* spp. and *Enterocytozoon bieneusi*, at the farm level is essential for implementing effective hygiene and biosecurity measures.

Outdoor access, as occurs in extensive farming systems or systems using outdoor paddocks, is a major factor contributing to parasite circulation (Kochanowski *et al.*, 2017). Indeed, it may increase the likelihood of farmed pigs coming into contact with wild animals, including rodents, as well as with potentially contaminated environmental sources, thereby increasing the risk of infection (Taghipour *et al.*, 2022). In intensive farming systems, such as those commonly used in Italy, outdoor access is usually limited (Allievi *et al.*, 2026). Intensive pig farms are characterised by high stocking densities, enclosed housing, and segregation of animals according to age and production category (Sak *et al.*, 2008). The close contact among animals under these conditions may facilitate the transmission of enteric pathogens via the faecal-oral route, including *Cryptosporidium* and *E. bieneusi* (Allievi *et al.*, 2024). Given the remarkable environmental resistance of *Cryptosporidium*

oocysts and *E. bieneusi* spores, the application of adequate biosecurity and hygiene measures is essential to prevent and limit pathogen transmission among pigs. The most important biosecurity practices include:

- daily removal of faeces;
- use of effective disinfectants according to the manufacturer's instructions regarding concentrations and contact time;
- implementation of effective rodent control programmes, as rodents can serve as reservoirs of *Cryptosporidium* and microsporidian species (Pettersson *et al.*, 2020; Li *et al.*, 2019);
- systematic application of the all-in/all-out system, in which all animals are removed from a unit simultaneously to allow thorough cleaning, disinfection, and drying before the introduction of the next batch (Kochanowski *et al.*, 2017; Allievi *et al.*, 2024);
- strict control of water and feed sources;
- appropriate management and treatment of manure.

5.5. Assessment of the zoonotic risk associated with *Cryptosporidium scrofarum* and *Enterocytozoon bieneusi*

Cryptosporidium scrofarum is considered a host-adapted species in suids; however, its actual zoonotic potential remains unclear due the lack of a *gp60* subtyping tool and requires further molecular investigation. To date, only one human case of *C. scrofarum* infection has been documented, involving a symptomatic immunocompetent male co-infected with *Giardia duodenalis* in the Czech Republic (Ryan *et al.*, 2021b). This finding indicates that *C. scrofarum* has the capacity, albeit apparently limited, to cross the host barrier (Ryan *et al.*, 2021b).

The results of the present study suggest that wild boars, like domestic pigs, may represent natural reservoirs of zoonotic *E. bieneusi* genotypes. Although the role of wildlife in the epidemiology of *E. bieneusi* remains to be fully clarified, the identification of zoonotic genotypes supports their potential involvement in the maintenance of the sylvatic cycle through the environmental dispersal of spores (Figueiredo *et al.*, 2023). In particular, genotypes D and EbpA may have important implications for public health, having been frequently reported in humans, wild and domestic mammals, and birds (Li *et al.*, 2019; Dashti *et al.*, 2020).

Given the widespread occurrence of *C. scrofarum* and zoonotic *E. bieneusi* genotypes in pigs and their detection in wild boars, further research is needed to clarify the role of domestic and wild suids

in the epidemiology of these parasites at the wild-domestic interface within a One Health framework.

Moreover, particular attention should be paid to the appropriate management and disposal of faeces and wastewater on farms to prevent contamination of the surrounding environment and water sources, which may contribute to the wider dissemination of infective spores and oocysts.

Finally, the potential implications for human health should also be considered, particularly for individuals with frequent occupational or recreational exposure to domestic pigs, wild boars, or contaminated environments. Groups at higher risk include pig farm workers, slaughterhouse personnel, and hunters. Proper training on occupational zoonotic risks and appropriate hygiene practices is therefore essential to minimise the risk of zoonotic transmission.

6. CONCLUSIONS

This study provides an updated analysis of the prevalence and molecular characterisation of *Cryptosporidium scrofarum* and *Enterocytozoon bieneusi* in wild boars and pigs from northern Italy. In wild boars, both *C. scrofarum* and *E. bieneusi* were detected, each with a prevalence of 5.8%. In contrast, higher prevalence were observed in pigs, indicating their widespread circulation in intensive pig farms. Specifically, *C. scrofarum* was detected in 24.8% of the analysed pig samples, while a previous study by Allievi *et al.* (2026) observed an *E. bieneusi* prevalence of 63.4% in the same sampled population. These markedly higher values could be attributed to the high stocking density and close contact among animals typical of intensive farming systems. However, the detection of both parasites in wild boars confirmed their circulation also in wild populations within the same study area.

Risk factor analysis in fattening pigs highlighted the important role of age in infections with both parasites, with younger pigs being more likely to be infected than older animals. Furthermore, the risk of infection with both *C. scrofarum* and *E. bieneusi* was significantly higher in larger farms, possibly because of higher animal densities. These findings emphasise the importance of appropriate hygiene and management practices, including the application of the all-in/all-out system to reduce parasite persistence within farms.

The identification of the same *Cryptosporidium* species (*C. scrofarum*) and three *E. bieneusi* genotypes (EbpA, H, and KWB4) both in wild boars and pigs suggests a potential epidemiological connection between the two populations, likely mediated by environmental contamination. However, determining whether transmission actually occurs between wild and domestic suids, as well as its possible directionality, requires further investigations.

Given the predominantly asymptomatic nature of these infections, pigs and wild boars may act as reservoirs of *C. scrofarum*, whose zoonotic potential remains unclear, as well as of zoonotic *E. bieneusi* genotypes, including D, H, and EbpA. Their widespread occurrence in domestic pigs and detection in wild boars highlight the need for robust epidemiological surveillance programmes that include both *Cryptosporidium* spp. and *E. bieneusi*. Future research involving environmental sampling, particularly of water sources used by livestock or accessible to wildlife, is needed to elucidate the role of wild boars in the epidemiology of these parasites at the wildlife-livestock interface within a One Health framework. In addition, studies including pre-weaning and weaning piglets from a larger number of farms across Italy will be necessary to clarify the epidemiology and geographical distribution of *C. scrofarum* and *E. bieneusi* within the Italian pig farming system.

7. BIBLIOGRAPHY

- Agyabeng-Dadzie F et al. *Cryptosporidium* Genomics - Current Understanding, Advances, and Applications. *Curr. Trop. Med. Rep.* 2024; 11: 92-103.
- Allievi C et al. Epidemiology and distribution of gastrointestinal parasites in fattening pig farms in northern Italy. *Parasitol. Res.* 2024; 123: 307.
- Allievi C et al. First molecular detection and genotyping of *Enterocytozoon bieneusi* (Microsporidia) in fattening pigs from Italy. *BMC Vet. Res.* 2026; 22: 145.
- Amaldi F, Benedetti P, Pesole G, Plevani P. *Biologia molecolare*. 3^a edizione. Casa Editrice Ambrosiana, 2022.
- Ávalos G et al. Detection and genotyping of zoonotic microsporidia in the endangered Iberian lynx (*Lynx pardinus*). *Med. Mycol.* 2024; 62: myad027.
- Barrios-Garcia MN, Ballari SA. Impact of wild boar (*Sus scrofa*) in its introduced and native range: a review. *Biol. Invasions.* 2012; 14: 2283-2300.
- Blasi C et al. Impatto degli ungulati sulla biodiversità. Università “La Sapienza” di Roma. 2014.
- Boitani L, Mattei L. Aging wild boar by tooth eruption. *Ungulates.* 1991; 91: 419-421.
- Bojko J et al. Microsporidia: a new taxonomic, evolutionary, and ecological synthesis. *Trends Parasitol.* 2022; 38: 642-659.
- Buchanan R et al. Global prevalence of *Cryptosporidium* infections in cattle: A meta-analysis. *Curr. Res. Parasitol. Vector-Borne Dis.* 2025; 7: 100264.
- Buckholt MA et al. Prevalence of *Enterocytozoon bieneusi* in Swine: an 18-Month Survey at a Slaughterhouse in Massachusetts. *Appl. Environ. Microbiol.* 2002; 68: 2595–2599.
- Cali A, Owen RL. Intracellular development of *Enterocytozoon*, a unique microsporidian found in the intestine of AIDS patients. *J. Protozool.* 1990; 37: 145-155.
- Cali A, Takvorian PM. 2014. Developmental morphology and life cycles of the Microsporidia. In: *Microsporidia: Pathogens of Opportunity* (Weiss LM, Becnel JJ). 1st Edition. Chichester: John Wiley & Sons, Inc., 71-133.
- Carnevali L et al. Banca Dati Ungulati: Status, distribuzione, consistenza, gestione e prelievo venatorio delle popolazioni di Ungulati in Italia. Rapporto 2001-2005. *Biol. Cons. Fauna.* 2009; 117:1-168.
- Castillo-Contreras R et al. Wild boar in the city: Phenotypic responses to urbanisation. *Sci. Total. Environ.* 2021; 773: 145593.
- Certad G et al. Pathogenic Mechanisms of *Cryptosporidium* and *Giardia*. *Trends Parasitol.* 2017; 33: 561-576.
- Chen Y et al. Global prevalence of *Cryptosporidium* spp. in pigs: a systematic review and meta-analysis. *Parasitology.* 2023; 150: 531-544.
- Chen Y et al. The microsporidian polar tube: origin, structure, composition, function, and application. *Parasit. Vectors.* 2023; 16: 305.
- Courchamp F et al. Mammal invaders on islands: impact, control and control impact. *Biol. Rev.* 2003; 78: 347-383.

- Dashti A et al. *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. *Transbound Emerg Dis.* 2020; 67: 2869-2880.
- Desportes I et al. Occurrence of a New Microsporidan: *Enterocytozoon bieneusi* n. g., n. sp., in the Enterocytes of a Human Patient with AIDS. *J. Protozool.* 1985; 32: 250-254.
- Didier ES et al. Epidemiology of microsporidiosis: sources and modes of transmission. *Vet. Parasitol.* 2004; 126: 145-166.
- Dong S et al. Prevalence of *Cryptosporidium* infection in the global population: a systematic review and meta-analysis. *Acta Parasitol.* 2020; 65: 882-889.
- Fayer R, Xiao L. *Cryptosporidium* and cryptosporidiosis. 2nd ed. Boca Raton: Taylor & Francis Group, 2008.
- Feng S et al. Identification of *Enterocytozoon bieneusi* and *Cryptosporidium* spp. in farmed wild boars (*Sus scrofa*) in Beijing, China. *Infect. Genet. Evol.* 2020; 80: 104231.
- Figueiredo AM et al. Endoparasite diversity of the main wild ungulates in Portugal. *Wildl. Biol.* 2020; 2020: 1-7.
- Figueiredo AM et al. Occurrence and molecular characterization of *Enterocytozoon bieneusi* in wild and domestic animal species in Portugal. *Med. Mycol.* 2023; 61: myad018.
- Garcia LS. Laboratory Identification of the Microsporidia. *J. Clin. Microbiol.* 2002; 40: 1892-1901.
- García-Preseido I et al. Presence of *Cryptosporidium scrofarum*, *C. suis* and *C. parvum* subtypes IlaA16G2R1 and IlaA13G1R1 in Eurasian wild boars (*Sus scrofa*). *Vet. Parasitol.* 2013; 196: 497-502.
- Giangaspero A et al. *Giardia* and *Cryptosporidium* and public health: the epidemiological scenario from the Italian perspective. *Parasitol. Res.* 2007; 101: 1169-1182.
- Guérin A, Striepen B. The Biology of the intestinal intracellular parasite *Cryptosporidium*. *Cell Host Microbe.* 2020; 28: 509-515.
- Hatam-Nahavandi K et al. *Cryptosporidium* infections in terrestrial ungulates with focus on livestock: a systematic review and meta-analysis. *Parasit. Vectors.* 2019; 12: 453.
- Howe TD et al. Forage relationships of European wild boar invading northern hardwood forest. *J Wildl. Manage.* 1981; 45: 748-754.
- ISPRA, <https://www.isprambiente.gov.it>
- IUCN, <https://iucn.org>.
- Jones KE et al. Global trends in emerging infectious diseases. *Nature.* 2008; 451: 990-993.
- Jota Baptista C et al. Wild boar (*Sus scrofa*) as a potential reservoir of infectious agents in Portugal: a review of two decades (2001–2021). *Eur. J. Wildl. Res.* 2023; 69:101.
- Keeling PJ et al. The reduced genome of the parasitic microsporidian *Enterocytozoon bieneusi* lacks genes for core carbon metabolism. *Genome Biol. Evol.* 2010; 2: 304-309.
- Kochanowski M et al. Occurrence of intestinal parasites in pigs in Poland - the influence of factors related to the production system. *J. Vet. Res.* 2017; 61: 459-466.
- Koehler AV et al. A perspective on the molecular identification, classification, and epidemiology

of *Enterocytozoon bieneusi* of animals. In: *Microsporidia. Current Advances in Biology* (Weiss LM, Reinke AW). 1st Edition. Springer Cham, 389-415.

- Koh W et al. Multiplication of the waterborne pathogen *Cryptosporidium parvum* in an aquatic biofilm system. *Parasit. Vectors*. 2013; 6: 270.
- Khwarahm NR et al. Spatial distribution modeling of the wild boar (*Sus scrofa*) under current and future climate conditions in Iraq. *Biologia*. 2022; 77: 369-383.
- Larson G et al. Worldwide Phylogeography of Wild Boar Reveals Multiple Centers of Pig Domestication. *Science*. 2005; 307: 1618-1621.
- Leitch GJ, He Q. Cryptosporidiosis – an overview. *J. Biomed. Res*. 2011; 25: 1-16.
- Li W et al. *Enterocytozoon bieneusi*. *Trends Parasitol*. 2022; 38: 95-96.
- Li W et al. Host specificity of *Enterocytozoon bieneusi* and public health implications. *Trends Parasitol*. 2019; 35: 436-451.
- Li W et al. Presence of zoonotic *Cryptosporidium scrofarum*, *Giardia duodenalis* assemblage A and *Enterocytozoon bieneusi* genotypes in captive Eurasian wild boars (*Sus scrofa*) in China: potential for zoonotic transmission. *Parasit. Vectors*. 2017; 10:10.
- Li W, Xiao L. Ecological and public health significance of *Enterocytozoon bieneusi*. *One Health*. 2021; 12: 100209.
- Li W, Xiao L. Multilocus Sequence Typing and population genetic analysis of *Enterocytozoon bieneusi*: host specificity and its impacts on public health. *Front. Genet*. 2019; 10: 307.
- Li XM et al. Meta-analysis of the global prevalence and risk factors of *Enterocytozoon bieneusi* infection in pigs from 1999 to 2021. *Prev. Vet. Med*. 2024; 225: 106159.
- Lombardini M et al. Factors influencing wild boar damage to agricultural crops in Sardinia (Italy). *Curr. Zool*. 2017; 63: 507-514.
- Markov N et al. The wild boar *Sus scrofa* in northern Eurasia: a review of range expansion history, current distribution, factors affecting the northern distributional limit, and management strategies. *Mammal Rev*. 2022; 52: 519-537.
- Martí-Marco A et al. Molecular detection and epidemiology of potentially zoonotic *Cryptosporidium* spp. and *Giardia duodenalis* in wild boar (*Sus scrofa*) from Eastern Spain. *Animals*. 2023; 13: 2501.
- Massei G, Genov PV. The environmental impact of wild boar. *Galemys: Boletín Informativo de La Sociedad Española Para La Conservación y Estudio de Los Mamíferos*. 2004. 16: 135-145.
- Matos O et al. Epidemiology of *Enterocytozoon bieneusi* infection in humans. *J. Parasitol. Res*. 2012; 2012: 981424.
- Miller CN et al. A cell culture platform for *Cryptosporidium* that enables long-term cultivation and new tools for the systematic investigation of its biology. *Int. J. Parasitol*. 2018; 48: 197-201.
- Monaco A et al. 2007. Il Cinghiale sull'arco alpino: status e gestione delle popolazioni. In: *Il Cinghiale sull'arco alpino: status e gestione* (Hauffe HC, Crestanello B, Monaco A), Report N. 38, Centro di Ecologia Alpina.
- Monaco A, Carnevali L, Toso S. *Linee guida per la gestione del Cinghiale (Sus scrofa) nelle aree protette*. Quad. Cons. Natura, 34. 2nd ed. Min. Ambiente – ISPRA: 2010.

- Moniot M et al. Etymologia: *Enterocytozoon bieneusi*. Emerg. Infect. Dis. 2021; 27: 1587.
- Němejc K et al. *Cryptosporidium suis* and *Cryptosporidium scrofarum* in Eurasian wild boars (*Sus scrofa*) in Central Europe. Vet. Parasitol. 2013; 197: 504-508.
- Němejc K et al. Prevalence and diversity of *Encephalitozoon* spp. and *Enterocytozoon bieneusi* in wild boars (*Sus scrofa*) in Central Europe. Parasitol. Res. 2014; 113: 761-767.
- Nourrisson C et al. *Enterocytozoon bieneusi*, a human pathogen. Emerg. Microbes Infect. 2024; 13: 1, 2406276.
- O’Leary JK et al. *Cryptosporidium* spp. diagnosis and research in the 21st century. Food Waterborne Parasitol. 2021; 24.
- Pavlov PM et al. Predation by feral pigs on Merino lambs at Nyngan, New South Wales. Aust. J. Exp. Agric. Anim. Husb. 1987; 21: 570-574.
- Petersen HH et al. *Cryptosporidium* and *Giardia* in Danish organic pig farms: Seasonal and age-related variation in prevalence, infection intensity and species/genotypes. Vet. Parasit. 2015; 214: 29-39.
- Pettersson E et al. Detection and molecular characterisation of *Cryptosporidium* spp. in Swedish pigs. Acta Vet. Scand. 2020; 62: 40.
- Pilarczyk B et al. A Comparison of the prevalence of gastrointestinal parasites in wild boar (*Sus scrofa* L.) foraging in urban and suburban areas. Animals. 2024. 14: 408.
- Pinto DJ, Vinayak S. *Cryptosporidium*: Host-Parasite interactions and pathogenesis. Curr. Clin. Microbiol. Rep. 2021; 8: 62-67.
- Pinto P et al. 2022. *Cryptosporidium*. In: *Lifecycles of Pathogenic Protists in Humans* (de Souza W). 1st Edition. Springer Cham, 331-389.
- Poglayen et al. Lung parasites of the genus *Metastrongylus* Molin, 1861 (Nematoda: Metastrongilidae) in wild boar (*Sus scrofa* L., 1758) in Central-Italy: An eco-epidemiological study. Vet. Parasitol. 2016; 217: 45–52.
- Provincia di Cremona, *Piano Territoriale di Coordinamento Provinciale* (PTCT, 2005).
- Provincia di Cremona, *Progetto Pluriennale di Controllo del Cinghiale* (*Sus scrofa*) 2025-2029 (PPCG 2025-2029).
- Qiagen, www.qiagen.com.
- Ramanzin M et al. Meat from wild ungulates: ensuring quality and hygiene of an increasing resource. Ital. J. Anim. Sci. 2010; 9: 318-331.
- Ramirez NE et al. A review of the biology and epidemiology of cryptosporidiosis in humans and animals. Microbes Infect. 2004; 6: 773-785.
- Riga F et al. Impatto degli Ungulati sulle colture agricole e forestali: proposta per linee guida nazionali. Manuali e linee guida ISPRA. 2011; 68: 1-251.
- Rivero-Juarez A et al. Protist enteroparasites in wild boar (*Sus scrofa ferus*) and black Iberian pig (*Sus scrofa domesticus*) in southern Spain: a protective effect on hepatitis E acquisition? Parasit. Vectors. 2020; 13: 281.
- Ruan Y et al. The largest meta-analysis on the global prevalence of microsporidia in mammals, avian and water provides insights into the epidemic features of these ubiquitous pathogens.

Parasit. Vectors. 2021; 14: 186.

- Ruiz-Fons F et al. A review of viral diseases of the European wild boar: Effects of population dynamics and reservoir role. TVJ. 2008; 176: 158-169.
- Ruiz-Fons F. A Review of the Current Status of Relevant Zoonotic Pathogens in Wild Swine (*Sus scrofa*) Populations: Changes Modulating the Risk of Transmission to Humans. Transbound. Emerg. Dis. 2015; 64: 68-88.
- Ryan UM et al. An update on zoonotic *Cryptosporidium* species and genotypes in humans. Animals. 2021; 11: 3307.
- Ryan UM et al. Taxonomy and molecular epidemiology of *Cryptosporidium* and *Giardia* – a 50 year perspective (1971–2021). Int. J. Parasitol. 2021A; 51: 1099-1119.
- Sak B et al. First report of *Enterocytozoon bieneusi* infection on a pig farm in the Czech Republic. Vet. Parasitol. 2008. 153: 220-224.
- Sandom CJ et al. Rewilding the Scottish Highlands: Do Wild Boar, *Sus scrofa*, use a suitable foraging strategy to be effective ecosystem engineers? Restor. Ecol. 2012; 21: 336-343.
- Santín M, Fayer R. *Enterocytozoon bieneusi* genotype nomenclature based on the Internal Transcribed Spacer Sequence: a consensus. J. Eukaryot. Microbiol. 2009; 56: 34-38.
- Santín M, Fayer R. Microsporidiosis: *Enterocytozoon bieneusi* in domesticated and wild animals. Res. Vet. Sci. 2011; 90: 363-371.
- Santín-Durán M. 2015. *Enterocytozoon bieneusi*. In: *Biology of Foodborne Parasites* (Xiao L, Ryan U, Feng Y). 1st Edition. Chichester: CRC Press, 149-174.
- Santos-Silva S et al. Survey of Zoonotic Diarrheagenic Protist and Hepatitis E Virus in Wild Boar (*Sus scrofa*) of Portugal. Animals. 2023; 13: 256.
- Sateriale A et al. A Genetically Tractable, Natural Mouse Model of Cryptosporidiosis Offers Insights into Host Protective Immunity. Cell Host Microbe. 2019; 26: 135-146.
- Singer FJ et al. Home ranges, movements, and habitat use of European Wild Boar in Tennessee. J. Wildl. Manag. 1981; 45: 343-353.
- Smith GC et al. Mapping wild boar density across Europe: combining spatial models and density estimates. Eur. J. Wildl. Res. 2026; 72: 31.
- Snowden KF et al. Animal Models of Human Microsporidial Infections. Lab. Anim. Sci. 1998; 48: 589-592.
- Taghipour A et al. Global molecular epidemiology of microsporidia in pigs and wild boars with emphasis on *Enterocytozoon bieneusi*: A systematic review and meta-analysis. Vet. Med. Sci. 2022; 8: 1126-1136.
- Timmermans M et al. The first commercially approved efficacious cryptosporidium vaccine protecting New-Born calves from severe diarrhea. Vet. Vaccine. 2024; 100054.
- Tomazic ML et al. 2018. *Cryptosporidium*. In: *Parasitic Protozoa of Farm Animals and Pets* (Florin-Christensen M, Schnittger L). 1st Edition. Springer Cham, 11-54.
- Toso S, Pedrotti L. *Linee guida per la gestione del Cinghiale (Sus scrofa) nelle aree protette*. Quad. Cons. Natura, 2. 1st ed. Min. Ambiente – Ist. Naz. Fauna Selvatica: 2001.
- Trotti GC et al. *Cryptosporidium* spp. and *Isospora suis* in pigs in Italy. Parassitologia. 1984;

26: 299-304.

- Tzipori S, Widmer G. A hundred-year retrospective on cryptosporidiosis. *Trends Parasitol.* 2008; 24: 184-189.
- Villa L et al. Serological prevalence of *Toxoplasma gondii*, *Neospora caninum*, and *Sarcoptes scabiei* var. *suis* in Wild Boars (*Sus scrofa*) hunted in a highly anthropized area in Italy. *Animals*; 2023; 13, 1730.
- Visvesvara GS. In Vitro Cultivation of Microsporidia of Clinical Importance. *Clin. Microbiol. Rev.* 2002; 15: 401-413.
- Wang Y et al. *Cryptosporidium* and cryptosporidiosis in wild birds: A One Health perspective. *Parasitol. Res.* 2021; 120: 3035-3044.
- Wang Y et al. Global prevalence and risk factors of *Enterocytozoon bieneusi* infection in humans: a systematic review and meta-analysis. *Parasite.* 2024; 31: 9.
- Weidner E. Ultrastructural study of Microsporidian invasion into cells. *Z. Parasitenk.* 1972; 40: 227-242.
- Wieler LH et al. Prevalence of Enteropathogens in Suckling and Weaned Piglets with Diarrhoea in Southern Germany. *J. Vet. Med.* 2001; 48: 151-159.
- Wilcox JT, Van Vuren DH. Wild pigs as predators in oak woodlands of California. *J. Mammal.* 2009; 90:114-118.
- Xiao L et al. Identification of Species and Sources of *Cryptosporidium* Oocysts in Storm Waters with a Small-Subunit rRNA-Based Diagnostic and Genotyping Tool. *Appl. Environ. Microbiol.* 2000; 66: 5492-5498.
- Xiao L. Molecular epidemiology of cryptosporidiosis: An update. *Exp. Parasitol.* 2010; 124: 80-89.
- Xu P et al. The genome of *Cryptosporidium hominis*. *Nature.* 2004; 431: 1107-1112.
- Zhang Y et al. *Enterocytozoon bieneusi* of animals - With an 'Australian twist'. *Adv. Parasitol.* 2021; 111: 1-73.