U.PORTO

MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

Detection of *Enterocytozoon bieneusi* In Non-Human Primates In Portuguese Zoos

Guilherme Ramos Moreira

MI 2024

Mestrado Integrado em Medicina Veterinária
Instituto de Ciências Biomédicas de Abel Salazar
Universidade do Porto

Guilherme Ramos Moreira

Detection of *Enterocytozoon bieneusi*
in non-human primates in Portuguese Zoos

Área científica: Medicine and/or surgery of exotic and wild animals
Pathology and Clinical Laboratory

Orientador: Professor Doutor Augusto Faustino

Co-orientador: Dr.ª Vanessa Soeiro

Abstract

Enterocytozoon bieneusi, an intracellular eukaryotic pathogen closely related to fungi, poses a significant health risk, particularly to immunocompromised individuals. While fecal-oral transmission is the primary mode of spread, other transmission routes remain unclear. The pathogen's wide host range underscores its zoonotic potential, despite limited direct evidence of animal-to-human transmission. Genotyping based on the internal transcribed spacer (ITS) region is crucial for delineating genetic diversity, with zoonotic genotypes predominantly associated with Groups 1 and 2. Research on microsporidian infections, particularly *E. bieneusi*, in zoo animals remains limited despite the broad spectrum of susceptible hosts.

This study aimed to evaluate the occurrence of *E. bieneusi* infection in diverse captive animals within zoo settings in Portugal. A total of 127 fecal samples were collected from various animal species, and molecular detection of *E. bieneusi* was conducted using nested PCR targeting the ITS region. The results revealed that 1.57% (95% CI: 0.19−5.57) of the samples tested positive for *E. bieneusi*, with a notably higher occurrence of 18.18% (95% CI: 2.28−51.78) among non-human primates (NHPs). Phylogenetic analysis indicated that the detected strains clustered within Group 2 genotypes, highlighting potential zoonotic implications.

The findings underscore the need for further research to better understand the epidemiology of *E. bieneusi* in zoo environments and its potential transmission pathways to humans. Given the close interaction between zoo animals and human caretakers, visitors, and researchers, understanding the genetic diversity and transmission dynamics of *E. bieneusi* is essential for mitigating the risk of zoonotic transmission. Enhanced molecular diagnostic techniques and comprehensive monitoring programs are recommended to identify and control microsporidian infections. This integrated approach will contribute to protecting both animal and human health, ensuring safer interactions in shared environments.

Keywords: *Enterocytozoon bieneusi*, Microsporidia, Zoo, non-human primates, Portugal

Resumo

Enterocytozoon bieneusi, um organismo patogénico eucariota intracelular intimamente relacionado com os fungos, representa um risco significativo para a saúde particularmente no caso de indivíduos imunodeprimidos. Embora a transmissão fecal-oral seja o principal modo de disseminação, a possibilidade de outras vias de transmissão continua incerta. A ampla gama de hospedeiros do parasita realça o seu potencial zoonótico, apesar de evidências diretas limitadas de transmissão de animais para humanos. A genotipagem baseada na região do espaçador interno transcrito (ITS) é crucial para delinear a diversidade genética, com genótipos zoonóticos predominantemente associados aos Grupos 1 e 2. A investigação sobre infeções por microsporidia, particularmente *E.bieneusi,* em animais de jardim zoológico continua limitada, apesar da ampla gama de espécies suscetíveis.

Este estudo teve como objetivo avaliar a ocorrência de infeção por *E.bieneusi* em diversos animais em cativeiro em jardins zoológicos de Portugal. Foram recolhidas 127 amostras fecais de várias espécies de animais, e a deteção molecular de *E.bieneusi* foi realizada através de *Nested PCR* direcionado para a região ITS. Os resultados revelaram que 1,57% (IC 95%: 0,19−5,57) das amostras testaram positivo para *E. bieneusi*, com uma ocorrência notavelmente mais alta de 18,18% (IC 95%: 2,28−51,78) entre primatas não humanos. A análise filogenética revelou que as estirpes detetadas se agruparam dentro de genótipos do Grupo 2, destacando potenciais implicações zoonóticas.

Os resultados realçam a necessidade de mais investigação para melhor compreender a epidemiologia de *E.bieneusi* em ambientes de jardim zoológico e as suas potencias vias de transmissão para humanos. Dada a estreita interação entre animais de jardim zoológico e cuidadores, visitantes e investigadores, compreender a diversidade genética e as dinâmicas de transmissão de *E.bieneusi* é essencial para mitigar o risco de transmissão zoonótica. Técnicas avançadas de diagnóstico molecular e programas abrangentes de monotorização são recomendados para identificar e controlar infeções por microsporidia. Esta abordagem integrada contribuirá para proteger tanto a saúde animal como humana, garantindo interações mais seguras em ambientes de coexistência.

Keywords: *Enterocytozoon bieneusi*, Microsporidia, Zoo, primatas não humanos, Portugal

Case registry and activities developed during the internship

During the 4 months of curricular internship, I had the opportunity of being part of two very distinct environments: Centro de Recuperação de Fauna do Parque Biológico de Gaia (CRF-PBG) and ICBAS' Microbiology and Infectious Diseases laboratory (MIDlab).

Before the curricular internship, I had the opportunity to join MIDlab's research team for 4 months, under the guidance of Professor João Mesquita, where I learned more about the molecular biology techniques used in their research, including primer design, DNA extraction from various sources using different methodologies, DNA purification, several PCR techniques (conventional, nested and semi-nested, qPCR, RT-PCR and RTq-PCR, multiplexPCR, touchdownPCR, among others), electrophoresis gel preparation and interpretation, DNA sequencing, and gained experience using an abundance of bioinformatics software. This time was spent working on several research projects in the parasitology and virology areas. I soon gained proficiency in the lab's protocols and methodologies, and I was trusted with more autonomy. With their guidance and support, I seized the opportunity to propose and develop my own study, leveraging the skills and insights acquired during my internship. This project was developed over the following months and turned out to be the main theme of this dissertation, tackling the presence of *Enterocytozoon bieneusi* in zoo animals. The findings originating from this study were presented by me in an oral communication at the IJUP 2024 congress (Encontro de Investigação jovem da Universidade do Porto 2024). These projects gave me insights on how scientific studies are planned, the logistics behind them, and allowed me to gain knowledge on systematic literature review, microbiology methodologies, interpretation and communication of results, data scientific writing, data management, statistical analysis.

CRF-PBG receives, rehabilitates, and returns wild animals to nature, and additionally has an important role in citizen education and awareness in relation to wildlife and biological diversity. During my time there, I had the opportunity of being in contact with a plentitude of wild species with which I had not had the opportunity to interact with during my Veterinary Medicine degree. In this way, I sought to fill this gap in my education.

During these 4 months, I was able to accompany the day-to-day activities of the clinic, treatments, and other procedures in the animal enclosures, and perform necropsies on several wildlife species. Some of these activities included feeding of young animals, administration of medications using different routes (oral, intramuscular, intravenous, intracelomic, ocular, inhalation, etc.) with all the challenges synonymous with wildlife species. I performed X-ray image acquisition and interpretation, multiple immobilization techniques, gavage, and preparation of animal enclosures that satisfy each species particular needs, among a multitude of other procedures (Annex B). During my stay, I was part

of the introduction of endoscopic technology into the clinic who had yet to incorporate it into their diagnostic toolkit. The endoscope revealed itself an essential instrument as it allowed us to better understand and diagnose different conditions in a relatively non-invasive manner.

A relevant portion of my time was spent diagnosing parasitosis by coprology, using direct and fecal flotation techniques, among others. The identification of coccidian oocysts, nematodes, nematode eggs and other organisms was challenging and yet very interesting. Moreover, the diversity and frequency of the parasites found differed from what was expected from the available literature. Consequently, I led an effort to create a comprehensive resource for parasite identification and host-species correlation. This was achieved by the establishment of a Google Drive "Atlas", categorized based on host families and classes, facilitating easy navigation and reference. By systematically documenting our findings, we not only enhanced our understanding of parasitic diversity but also provided a valuable tool for future reference and practical application.

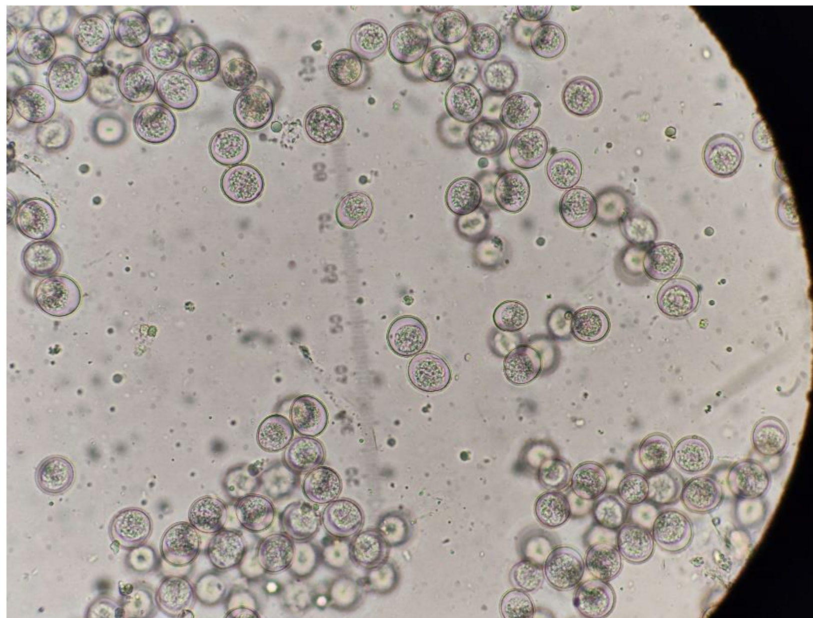


Figure 1 - Coccidia found in *Corvus corone* fecal sample. Found in Atlas Parasitas PBG > Aves > Corvidae > Coccidia. This resource can be freely accessed through the following link:https://drive.google.com/drive/folders/1-diCycBT9YXkX7-puKKNWMFi6mgAyzaW?usp=sharing

At CRF-PBG, I also performed numerous pathological diagnostic techniques such as skin scrapings, histological cuts and cytological preparations, in addition to molecular identification of some parasites with the help of the resources made available to me at MIDlab, I performed basic stain techniques and re-introduced the use of Gram stain for better identification of prokaryotic and eukaryotic microorganisms. More than 100 fecal samples belonging to various wildlife species at PBG-CRF were collected which will be used for research purposes. Additionally, I took part in wild animal release programs aimed at educating young school students and the general population.

During these 4 months, I also dedicated some of time to continuing my research endeavors at the MIDlab, working on multiple projects.

Finally, over the course of these months, I also participated and successfully completed a course on bacterial genome (GENBAC – Estudo de Genomas Bacterianos: do controlo da infeção à pesquisa de novas moléculas), hosted by Faculdade de Farmácia da Universidade de Porto, worth 3 ECTS, with the final classification of 18/20.

Agradecimentos

Ao professor Augusto Faustino, pela orientação, conselhos e paciência durante todo o estágio e durante o processo de desenvolvimento deste relatório.

Ao ICBAS, que me deu a conhecer alguns dos meus animais preferidos, de quem falarei mais à frente. Aos muitos professores que me guiaram neste percurso e que me ajudaram a ser a pessoa que sou, e aos muito poucos que mostraram precisamente quem não quero ser.

À Dra. Vanessa Soeiro, que me fez sentir bem-vindo desde o meu primeiro dia no PBG. Obrigado por toda a disponibilidade, flexibilidade e orientação. À Sofia e à Sara, por toda a ajuda e conhecimento que transmitiram. Por tolerar todas as 400.000 interrupções para as minhas perguntas, comentários, pedidos de fotografias e o ocasional achado de necrópsia particularmente nojento. Demonstra alguma superioridade genética. Aos engenheiros zootécnicos, Ana e Nuno, e às enfermeiras Jéssica e Sofia pelas mesmas razões. A todo o resto da equipa do parque, e ao grupo da hora de almoço pela boa disposição, gomos de laranja e pela oportunidade de ver gente com mais que idade para ter juízo a não o ter, que é algo que quero levar comigo para o meu futuro. À pyrrhura que me deu cabo dos tímpanos, à gralha "Força Porto!!" e a todos os outros animais que me deram tantas histórias para contar e tantas fezes para analisar.

Obrigado por terem tornado a minha estadia tão fácil (e rápida) e a minha despedida tão difícil.

Ao incansável Professor Mesquita, por toda a paciência, tolerância por pipetas partidas e contaminações causadas, pedidos de ajuda e de opinião que tendem para infinito, faltas de itálicos em nomes científicos e outras inúmeras palermices, e ainda por todas as oportunidades que me deu e procurou arranjar. Independentemente do que se passar a partir de agora, fico a dever muito. Talvez um dia pague um café para ficarmos quites. Da mesma forma, muito obrigado ao resto do pessoal do laboratório. Ao Mega, estimado co-autor. À Ana Luísa, responsável por tantas viagens com parasitas na mochila. Ao Luís, com a promessa um dia finalmente conseguir ir ver um jogo. À Patrícia Barradas, que vê o valor em chatear a Rafaela. À Mahi que tanto me insulta, sempre de um lugar de grande carinho e ternura, e à potentíssima ainda que diminuta Sara, obrigado por me ensinarem as bases de tudo o que tenho feito estes últimos meses. Ao Sérgio que talvez um dia seja tão brilhante como a sua careca, mas que já não está assim tão longe. À Andreia por ser das poucas pessoas com a cabeça no sítio, e ao Pepe pelas suas aparições que levantam a moral de todo o corredor. *Thank you Sia for all the coffee breaks and masterclasses in procrastination. Sometimes they are needed. To Pedro, Violetta and everyone else that passed through the lab and was a part of my journey*. À Rafaela por toda a

organização (e por inspirar medo nos corações de quem desorganiza) e ao Inspetor Mário por todos os side projects.

Aos caríssimos colegas, os melhores animais do curso, ao lado dos quais tenho a honra de ter sobrevivido a este local frio, complicado e cheio de fugas que é o ICBAS. À Kató, Sofia, Pedrosa, Cátia, Lia, Inês, Lita, Mário e Rita por serem o principal apoio, as principais vítimas de inúmeras instâncias de bullying e simultaneamente os melhores amigos que já tive. Por nunca serem a razão de um dia mau e por serem sempre a de um dia bom. Ao João e à Rafaela por tudo isso e muito mais, e por me aturarem desde o início.

Ao Gatafunho, pelas inúmeras horas a estudar comigo. Ao Pablo, Tobias e ao Loki por me terem empurrado nesta direção. A todos os outros animais que tentei ajudar, independentemente do resultado.

À (Exma Dra.) Xana, por ter tornado este parágrafo tão difícil de escrever e necessariamente incompleto. Por toda a ajuda, por todo o apoio, por me dares um empurrão quando preciso e por me pores travões quando exagero. Por aceitares recolher centenas de tubos de fezes de vaca só porque pedi. Por todas as revisões científicas. Por todas as outras coisas que não vou conseguir enumerar. São muitas. Múltiplas. Inúmeras. Ao Pupi, o melhor assistente laboratorial.

Ao Miguel, por toda a companhia e amizade durante muito literalmente a sua vida inteira, e por me fazer querer ser um melhor exemplo. Por me ajudar a lidar com todas as aventuras e desventuras no Porto.

Aos meus avós, por serem os meus melhores amigos, por todo o interesse, todo o suporte e por realçarem que sempre que eu precisar de ajuda, estão a um telefonema de distância. Sempre.

Aos meus pais por me terem sempre apoiado incondicionalmente. Não teria conseguido sem vocês. Por toda a educação, conselhos, ajuda, por todas as ferramentas que me deram. Por fazerem tudo o que está ao vosso alcance, e às vezes mais ainda. Por me terem tornado quem sou.

Até correu bem.

List of abbreviations

AIDS	Acquired Immunodeficiency Syndrome
ATP	Adenosine triphosphate
bp	Base pairs
CI	Confidence Interval
CIA	Chemiluminescent immunoassay
CRF-PBG	Centro de Recuperação de Fauna do Parque Biológico de Gaia
DNA	Deoxyribonucleic acid
E.bieneusi	*Enterocytozoon bieneusi*
ELISA	Enzyme-linked immunosorbent assay
HIV	Human Immunodeficiency Virus
IFAT	Immunofluorescence Antibody Test
ITS	Internal transcribed spacer
LSU	Large subunit
Mb	Megabyte
MetAp2	Methionine aminopeptidase type 2
NHP	Non human primate
PCR	Polymerase chain reaction
pH	potential of Hydrogen
qPCR	Quantitative polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
RTq-PCR	Quantitative reverse transcription polymerase chain reaction
SSU	Small subunit
TEM	Transmission electron microscopy
UV	Ultraviolet
WB	Western Blot

Index

Abstract III

Resumo IV

Case registry and activities developed during the internship V

Agradecimentos VIII

List of abbreviations X

1. Introduction 1

   1.1. Coprology and enteric parasites 1
   1.2. Context: 3
   1.3. Motivation: 3
   1.4. Objectives: 3
   1.5. Organization 4

2. *Enterocytozoon bieneusi* 4

   2.1. Historical Background 4
   2.2. Microsporidia 5
   2.3. The Enterocytozoon spore: Infectious Unit 6
   2.4. The life cycle of Microsporidia 7
   2.5. Survival 8
   2.6. Pathogenesis 8
   2.7. Microsporidian genome structure 9
   2.8. Detection and identification of microsporidia 9
   2.9. Molecular epidemiology of *E. bieneusi* 10
      2.9.1. *E. bieneusi* infection in humans 11
      2.9.2. *E. bieneusi* infection in Animals 11
   2.10. Treatment 13

3. Practical Work 13

4. Conclusion 14

References 15

Annex A - Detection of Enterocytozoon bineusi in non-human primates in Portuguese Zoos 22

Annex B – activities performed at PBG-CRF 40

1. Introduction

1.1. Coprology and enteric parasites

During my tenure in CRF-11PBG, a considerable amount of my effort was spent diagnosing parasitic infections through coprological examination. This detailed process required the use of various techniques, such as fecal smear and fecal flotation. A plethora of parasites from hugely different families were identified: coccidian oocysts (mostly belonging to Eimeria and Isospora genera); adult helminths (*Dipylidum caninum, Capillaria spp., Crenosoma sp, among various others); helminth eggs (Ascaridae, Capillaria spp, Eustrongylides spp., Taenia spp., Trichuris spp., Ancylostoma spp*., among various others); as well as a variety of other microorganisms – flagellate microorganisms, and the occasional fungi or bacteria as an example. Each diagnosis presented unique challenges but was inherently fascinating due to the complexity and diversity of the parasites encountered.

Coprologies revealed themselves to be an indispensable tool in wildlife management. Their importance cannot be overstated, as it allows for the identification and management of parasitic infections that can significantly impact the health and recovery of wildlife. The detection of parasites that would otherwise go unnoticed enables timely and appropriate treatment interventions, aiding in the recovery of animals and helping to prevent the spread of parasitic infections within wild populations, which is crucial for the conservation of species and maintenance of biodiversity. During my time at the clinic the high occurrence of antiparasitic drug resistance was also noticed, with a particular case of highly fenbendazole refractory *Crenosoma spp*. in an European hedgehog (Erinaceus europaeus) being especially eye-opening.

The unexpected diversity and frequency of parasites discovered in wildlife during coprological examinations also highlights the gaps existing in our knowledge and the need for ongoing research. The literature often provides a baseline understanding, but field studies reveal the dynamic nature of parasite-host interactions and the influence of environmental factors. Field-based research and continuous surveillance in wildlife rehabilitation centers do have and will continue to have an invaluable role in keeping up with parasitic threats.

My interest and experience in enteric parasites developed in the clinic was luckily paired with the focus on enteric microorganisms MIDlab, which allowed me to better study and understand this area, having built a strong foundation on morphological identification and pathology of a wide array of species. In a molecular biology setting, I was able to leverage this knowledge to delve into the study of some of the parasites observed from an evolutionary and genetic perspective, allowing for a more complete understanding of their life cycles, host interactions and resistance mechanisms.

The microsporidian *Enterocytozoon bieneusi* is characterized by unique diagnostic challenges. It is responsible for causing severe enteric diseases that compromise the health and survival of various animal species. Common symptoms often include diarrhea, weight loss, dehydration, and malabsorption of nutrients. This can, in turn, lead to malnutrition and a weakened immune system, making the affected wildlife more susceptible to secondary infections and other health complications. *E. bieneusi* can have a substantial impact on populations, particularly those that are already vulnerable to habitat loss, climate change, or other environmental pressures. For instance, chronic infections can reduce an animal's ability to forage effectively, evade predators, and reproduce successfully, leading to decline in population. Wild animal populations can also be responsible for the spread of the parasite to numerous other animals, including humans, as the spores are shed in feces and can contaminate water sources and food supplies. Monitoring and managing *E. bieneusi* infections in wildlife is therefore crucial not only for the health of individual animals but also for the preservation of entire species and ecosystems. This organism cannot be, however, reliably diagnosed through conventional microscopy due to its small size and lack of distinctive morphological features. Through molecular diagnostics, such as Polymerase Chain Reaction (PCR) and sequencing, we can precisely identify *E. bieneusi* at a molecular level.

In spite of its substantial impact, *E. bieneusi* remains relatively unknown. Routine diagnosis and recognition have been historically impeded in both clinical and wildlife settings. Secondly, the symptoms of *E. bieneusi* infection are often nonspecific and can be attributed to a wide range of other gastrointestinal pathogens, leading to potential misdiagnosis or underreporting. Microsporidia have only gained more attention in the scientific community recently. They were once thought to be protozoans but are now fungi-related, necessitating a shift in diagnostic and research approaches. This taxonomic reclassification and the specialized techniques required for its study have limited the amount of research developed around this organism. Furthermore, much of the research is focused on human health, overshadowing its importance in wildlife and broader ecological contexts, contributing to its relative obscurity outside specialized parasitological fields. The zoonotic potential of *E. bieneusi* is increasingly recognized, but a comprehensive understanding of its prevalence, transmission dynamics and impact in wildlife is still emerging.

At MIDlab, I was able to research this parasite, focusing on prevalence, transmission dynamics, and impact on both wildlife and human health. By integrating field data with laboratory analyses, we are contributing to a broader knowledge base that supports better disease management and conservation strategies. This work not only addresses the gaps in current parasitological research but also underscores the importance of a One Health approach, which considers the interconnectedness of human, animal and environmental health.

1.2. Context:

Microsporidia are intracellular eukaryotic organisms closely related to fungi, infecting a wide range of hosts, including both invertebrates and vertebrates. Among the 220 genera and over 1,700 species catalogued, *Enterocytozoon bieneusi* is particularly notable for its pathogenic impact on humans, especially those who are immunocompromised. Transmission primarily occurs via the fecal-oral route, although precise modes remain incompletely described. The pathogen infects various domestic and wild animals, indicating its zoonotic potential, yet direct evidence of animal-to-human transmission is limited. Genotyping of *E. bieneusi*, based on internal transcribed spacer (ITS) polymorphisms, has identified over 600 genotypes, with Groups 1 and 2 being predominantly associated with zoonotic transmission.

1.3. Motivation:

Despite the broad host range, research on microsporidian infections in zoo animals remains scarce, highlighting the need for studies evaluating the occurrence and zoonotic potential of *E. bieneusi* in these settings. Understanding the epidemiology of this pathogen in zoo animals is essential for maintaining the health and well-being of captive animals and for developing strategies to reduce pathogen spread. Moreover, enhancing our understanding of pathogen dynamics in diverse animal populations contributes to ensuring the health of wild and endangered animals in rehabilitation.

1.4. Objectives:

This study aims to investigate *E. bieneusi* infections across diverse captive animal species in zoos, elucidating the occurrence and zoonotic implications of circulating genotypes, as well as the prevalence of *E. bieneusi* in different animal species. The identification and characterization of this species in zoo animals can inform public health strategies to prevent and control human infections, particularly in immunocompromised individuals. This study focuses on evaluating the occurrence and genetic diversity of *Enterocytozoon bieneusi* in various captive animal species within zoos located in Portugal, including mammals, birds, reptiles, amphibians, and arthropods. By targeting a broad spectrum of animal hosts, the research aims to provide a comprehensive overview of *E. bieneusi* infection across different taxa within zoo environments. The selection of zoos in Portugal provides a geographically constrained yet diverse range of habitats and management practices, which are representative of typical zoo settings in similar climates and regions.

1.5. Organization

The following chapters are structured to provide a comprehensive examination of these themes:

Chapter 1 – Introduction : This chapter provides an overview of the study, detailing the background of *Enterocytozoon bieneusi*, the rationale for investigating its presence in zoo environments, the research objectives, and the overall scope of the study.

Chapter 2 – Literature Review: This chapter reviews the existing literature on microsporidia, with a particular focus on *E. bieneusi*. It discusses the biology, transmission dynamics, zoonotic potential of the pathogen, and summarizes previous research conducted in various settings.

Chapter 3 – Practical Work : The practical work undertaken in this study is comprehensively detailed in **Annex A**. It is presented as a manuscript submitted to the journal *Animals*, ensuring thorough documentation and peer-reviewed validation of the methodologies and findings. This document includes an **introduction**, **materials and methods**, **results**, **discussion**, and the relevant bibliography, that substitutes the corresponding chapters of this work.

Chapter 4 – Conclusion : This chapter summarizes the main findings of the study, discusses their significance, and provides recommendations for mitigating the zoonotic risks associated with *E. bieneusi* in zoo environments. It also points pertinent avenues for future work.

2. Enterocytozoon bieneusi

2.1. Historical background

Enterocytozoon bieneusi was first identified and described in 1985. The discovery came about through the investigation of a Haitian patient with AIDS suffering from chronic diarrhea. Intestinal biopsy specimens were collected and examined using electron microscopy. Unusual structures were observed within the intestinal cells of the patient. These structures appeared as minute spore-like bodies enclosed in the host's enterocytes. Further examination revealed that these structures closely resembled characteristics attributed to microsporidia, a group of intercellular parasites known to infect a wide range of hosts, including humans.(Desportes et al., 1985)

The designation *Enterocytozoon bieneusi* arises from the Greek *énteron* (intestine), *kútos* (cell), and *zȭion* (animal), along with the surname Bieneus, in honor of the first infected patient.(Moniot et al., 2021)

Subsequent studies highlighted its substantial impact on immunocompromised hosts, particularly those with AIDS or organ transplants, as it can lead to severe and life-threatening diarrhea and wasting syndrome. (Akinbo et al., 2012b; Liguory et al., 2001a; Sadler et al., 2002a; Ten Hove et al., 2009a)

Although zoonotic transmission is plausible due to *E. bieneusi's* ability to infect a wide range of domestic and wild animals, direct evidence supporting animal-to-human transmission remains elusive. Despite documented infections across various animal species, including beavers, calves, cats, chickens, dogs, foxes, goats, llamas, macaques, muskrats, ostriches, otters, pigs, pigeons, rabbits, raccoons, and wild boars, confirming such transmission remains a challenge. These findings highlight the potential reservoir role of mammals and birds in spreading microsporidian spores capable of infecting humans. (Breitenmoser et al., 1999; Chalifoux et al., 1998; Del Aguila, C Izquierdo, F Navajas, R Pieniazek, NJ Miro, G Alonso, AI Da Silva, AJ Fenoy, 1999; Dengjel et al., 2001; Deplazes et al., 1996; Galván-Díaz et al., 2014; Maria Luisa Lobo et al., 2003; Lores et al., 2002; Mansfield et al., 1998; Mathis et al., 1999; Pourrut et al., 2002; Reetz et al., 2002; Rinder et al., 2000; M. Santín et al., 2005; Mónica Santín et al., 2004; Sulaiman et al., 2004)

2.2. Microsporidia

Microsporidia are intracellular eukaryotic spore-forming microorganisms closely related to fungi that demonstrate a wide host range spanning both invertebrates and vertebrates, with more than 1,700 species catalogued, 17 of which pose pathogenic risks to humans. This represents, however, a small fraction of the real diversity as most host lineages have been poorly studied.(Han et al., 2021)

Microsporidia have a fairly cosmopolitan distribution, being described on all continents with the exception of Antartica (P. Keeling, 2009). Microsporidia have been known to infect several commercially important animal species, including bees, silkworms (Ghosh & Weiss, 2009), fish (Sveen et al., 2012), birds and mammals (Breitenmoser et al., 1999; Chalifoux et al., 1998; Del Aguila, C Izquierdo, F Navajas, R Pieniazek, NJ Miro, G Alonso, AI Da Silva, AJ Fenoy, 1999; Dengjel et al., 2001; Deplazes et al., 1996; Galván-Díaz et al., 2014; Maria Luisa Lobo et al., 2003; Lores et al., 2002; Mansfield et al., 1998; Mathis et al., 1999; Pourrut et al., 2002; Reetz et al., 2002; Rinder et al., 2000; M. Santín et al., 2005; Mónica Santín et al., 2004; Sulaiman et al., 2004).

Microsporidia, despite their complex infection mechanism, lack distinguishing characteristics, making them challenging to compare with other eukaryotes. Their taxonomic classification has been unstable, often being grouped with organisms now known to be unrelated.(P. Keeling, 2009)

Historically, the identification of microsporidians as agents of disease dates back to the mid-nineteenth century. Louis Pasteur's work revealed a protozoan pathogen, subsequently named *Nosema Bombycis* as the causative agent responsible for prebrine or pepper disease in silkworms. The hugely significant impact in the silk industry highlighted the significance of this discovery.(Pasteur, 1870) The transition from commercial to medical significance occurred in 1959 with the first documented case of microsporidiosis in humans (Monaghan et al., 2009)

The classification of Microsporidia has been controversial for decades since its discovery. They were first classified as a schizomycete fungi in 1857, as sporozoan protists in 1882 (Balbiani, 1882), and then as a subgroup of the Cnidosporidia within the sporozoan in 1901 (now the Apicomplexa). This was followed for more than 100 years (P. Keeling, 2009). In 1983, a new hypothesis was formulated, inserting Microsporidia in an ancient lineage "Archezoa" (i.e., diverged prior to the origin of mitochondria), as no obvious mitochondria were found (Vossbrinck et al., 1987). As molecular data has evolved, an alternative hypothesis was formulated that microsporidia are related to fungi (P. J. Keeling & Fast, 2002). Recent genomic characterization led to the reclassification of microsporidians, once regarded as protozoa, as fungi (Trew, 2021).

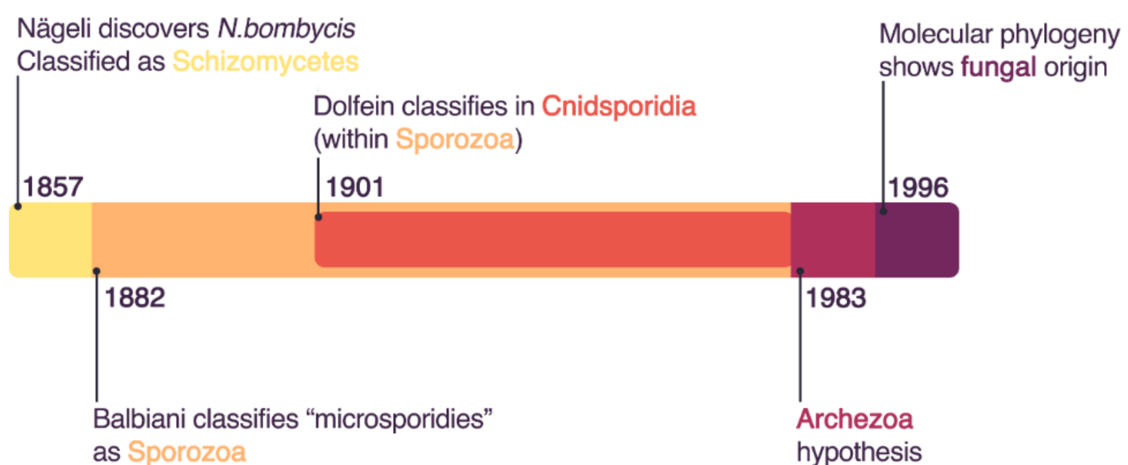


Figure 2 - Timeline of Microsporidia classification Timeline of microsporidia Classification © 2024 by Guilherme Moreira is licensed under CC BY-NC-SA 4.0

Enterocytozoon bieneusi is considered the main cause of human microsporidiosis, accounting for more than 90% of infections worldwide, affecting mostly immunocompromised individuals, e.g. cancer patients, organ transplant recipients and HIV patients.(Sak, Brady, et al., 2011)

2.3. The *Enterocytozoon* spore: infectious unit

In the microsporidian life cycle, the spore represents the infective stage as well as the only stage capable of enduring conditions outside of the hosts' cells. (Vávra & Larsson, 2014a) Microsporidia possess unique features highlighted in Figure 1. Enclosed within the spore is the sporoplasm which contains several organelles: nuclei, ribosomes and endoplasmic reticulum. The nuclei appear in a monokaryon or diplokaryon arrangement (Elizabeth S. Didier & Weiss, 2006). Microsporidia possess unique organelles including mitosomes (thought to be reduced mitochondria), a polar filament or tubule originating in an anterior achoring disk and an atypical Golgi apparatus.(Elizabeth S. Didier & Weiss, 2006) The polar filament is a unique, highly specialized structure which plays a key role in infection, being present in all microsporidia, along with a membranous polaroplast as well as a

posterior vacuole thought to be involved in polar filament extrusion.(Hale-Donze & Didier, 2007; Xu & Weiss, 2005)

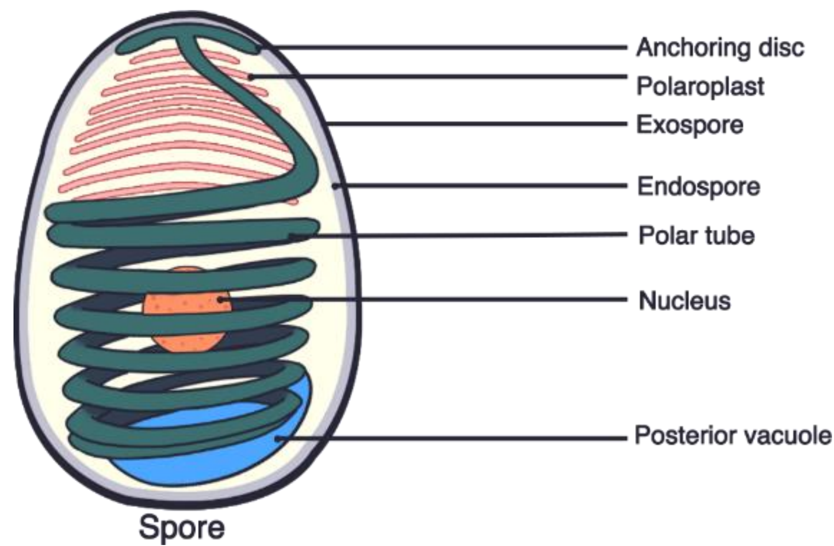


Figure 3 - Generalized Enterocytozoon spore - Enterocytozoon © 2024 by Guilherme Moreira is licensed under CC BY-NC-SA 4.0

2.4. The life cycle of Microsporidia

Spore transmission occurs in a number of ways, including horizontal (Haag et al., 2020; Karthikeyan & Sudhakaran, 2019) transmission through ingestion or other interactions, although some species have been known to transmit vertically(Poley et al., 2017). Horizontal transmission is the most frequent route of transmission and the only route thought to be used by *Enterocytozoon.* Waterborne transmission (fecal-oral) is likely the most frequent route in human infecting species, with microsporidian infections being reported mostly in low income countries with lower water health (Javanmard et al., 2018), with a greater number of spores being found in both treated and raw water.(Galván-Díaz et al., 2014; Javanmard et al., 2018)

Infection occurs as the spore contacts the host's enterocytes. Following ingestion through contaminated food or water, specific conditions (i.e. osmotic pressure, ion concentration, digestive enzymes, redox potential, pH) cause the eversion of the polar tube. (Undeen & Epsky, 1990) The coiled organelle discharges, piercing the host's enterocyte. The posterior vacuole and polaroplast swell, forcing the sporoplasm into the host cell's cytoplasm(P. J. Keeling & Fast, 2002; LOM J, sem data; Mónica Santín & Fayer, 2011b). The next stage is referred to as a meront, with the parasite inside the enterocyte.(Santín-Durán, 2015a) Once inside the cell, nuclei elongate and undergo division (Chalifoux et al., 1998), as the microsporidia undergoes sporogonial division (sporogonic or spore-forming phase).(Franzen, 2005) When enough mature spores are formed, the cell membrane is ruptured infecting new host cells (within the same host) or are released through feces into the environment,

being able to survive long periods outside a host.(Franzen, 2005) Unlike other *microsporidia* (e.g. *Encephalitozoon spp., Nosema spp.*) *E. bieneusi* spores are not packaged in sporophorous vesicles or pansporoblast membranes in host cells (Vávra & Larsson, 2014a). This method of invading cells is one of the most sophisticated mechanisms in biology and ensures that the microsporidia enter the host cell unrecognized and protected from host defence reactions. This life cycle is represented in Figure 3.

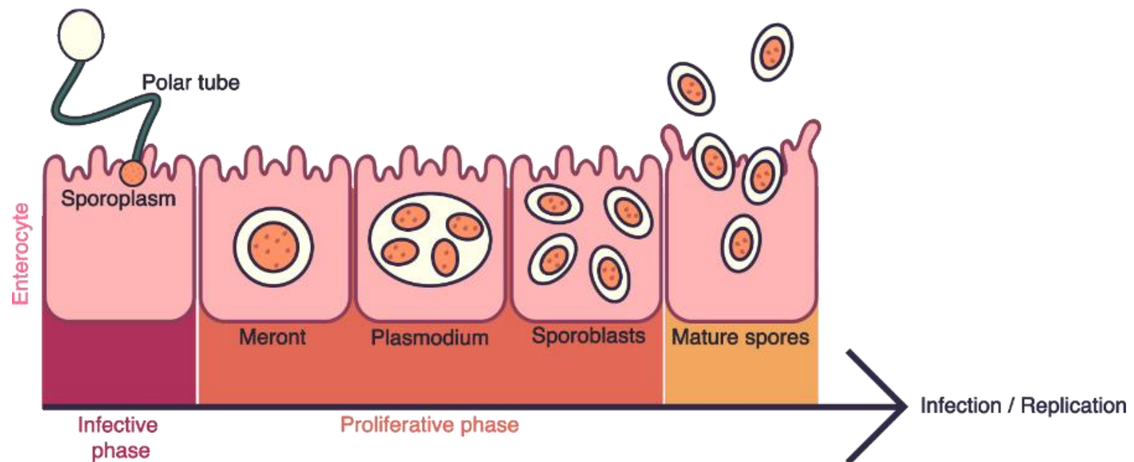


Figure 4 - Generalized Enterocytozoon Life Cycle - Enterocytozoon_lc © 2024 by Guilherme Moreira is licensed under CC BY-NC-SA 4.0

2.5. Survival

The spores of microsporidia can survive for months to years outside the host, depending on exterior conditions(Santin & Fayer, 2015). Environmental factors affecting spore survival include solar radiation, humidity and temperature, and have been studied in multiple microsporidia including *Encephalitozoon* spp., *Enterocytozoon* spp. and other entomopathogenic microsporidia (Fayer, 2004; Kramer, 1976; Solter & Becnel, 2007). UV light exposure can render spores noninfectious within hours, however, protection from sunlight will significantly extend their longevity. Temperature and humidity also play crucial roles, with high temperatures decreasing spore viability and slow dehydration posing a significant threat. Spores within dried feces and cadavers can maintain infectivity for months to years at lower temperatures.(Y. Zhang, 2019) The resilience of the microsporidian spores holds significant implications for human infection in zoo environments.

2.6. Pathogenesis

Typically, *E. bieneusi* human infection is limited to the small intestine (duodenum and jejunum)(Santín-Durán, 2015b) but respiratory tract infections are possible.(Graczyk et al., 2007) The main signs of *E. bieneusi* infection include chronic diarrhea, abdominal pain, vomiting, fever and malabsorbtion accompanied by weight loss (Brasil et al., 2000), although the exact causative mechanism is not yet fully understood.(Santín-Durán, 2015a) *E. bieneusi* is recognized as an

opportunistic agent in HIV/AIDS patients and otherwise immunocompromised individuals such as organ transplant recipients(Akinbo et al., 2012a; Ghoyounchi et al., 2019; Liguory et al., 2001b; Sadler et al., 2002b; Ten Hove et al., 2009b).

Microsporidia do not seem to produce toxins, with the apoptosis of enterocytes during *E. bieneusi* replication being the most likely cause of intestinal disease. The occasional pulmonary complications are characterized by persistent cough, dyspnea and non-purulent sputum in immunocompromised individuals(Y. Zhang, 2019).

In immunocompetent individuals, less severe forms of *E. bieneusi* induced microsporidiosis can occur, usually resolving within a few weeks or months, thus being self-limiting (Cama et al., 2007). Nonetheless, some studies indicate a lack of significant correlation between *E. bieneusi* infection and diarrhea (W. Zhang et al., 2017). This suggests that asymptomatic *E. bieneusi* infection are reasonably common. Certain genotypes (Peru 3-11), appear to exhibit a greater propensity to induce symptoms in humans when compared to others (e.g. Peru 1-2)(Bern et al., 2005) . This highlights the possibility of varying degrees of virulence and pathogenicity among different genotypes. These aspects represent valuable avenues for further investigation within clinical contexts.

2.7. Microsporidian genome structure

Understanding the microsporidian genome and structure is critical for understanding their biochemistry and biology.

The genomes of microsporidia range from 2.5Mb to 25 Mb (Vávra & Larsson, 2014b). The draft genome of *E. bieneusi* is estimated at 3.86 Mb, composed of 6 chromossomes with high gene density (few introns and short intergenic regions). 3804 genes were predicted for *E. bieneusi,* of which 1702 were identified as coding proteins. *E. bieneusi* doesn't exhibit the metabolic pathways present in other species of microsporidia (such as *E. cuniculi*), and its genome is fragmented, complicating comparative analyses. (Akiyoshi et al., 2009)*E. bieneusi* also lacks pathways for glycolysis, pentose phosphate and trehalose metabolism, suggesting a reliance on the host's ATP (Akiyoshi et al., 2009)Moreover, the ribosomes of certain species such as *Nosema bombycis* and *Telohania maenadis*, are characterized by a 70S configuration akin to prokaryotic organisms. (Y. Zhang, 2019)

2.8. Detection and identification of microsporidia

The methods of microsporidia detection have progressed from traditional microscopic diagnosis to serological and molecular based detection. Conventional methodologies include microsporidia stages through electron microscopy (TEM) and/or light microscope. Serological assays comprise enzyme-linked immunosorbent assay (ELISA), chemiluminescent immunoassay (CIA), immunofluorescent

antibody test (IFAT) and Western Blot (WB) analysis. Molecular approaches typically involve polymerase chain reaction (PCR) amplification of a specific DNA region and are prevalent in diagnostic practices.

Given the constraints inherent to microscopic and serological methods, molecular assays have become the main tool for identification and detection of microsporidia. (Bednarska, Małgorzata & Bajer, Anna & Welc-Falęciak, Renata & Czubkowski, Piotr & Teisseyre, Mikolaj & Graczyk, Thaddeus & Jankowska, 2013) These assays offer considerable advantages, namely high specificity and sensitivity.

PCR serves as the foremost nucleic acid-based detection method (Yang & Rothman, 2004), adept at amplifying minute DNA quantities through a process involving thermostable DNA polymerase, nucleotides and oligonucleotide primers. This cyclic amplification process, divided into denaturation, annealing and extension stages, enabling exponential amplification of target DNA. Under perfect conditions PCR reaches 100% specificity and sensitivity (Thellier & Breton, 2008), thereby facilitating the specific detection of microsporidial DNA, inclusive of unknown taxa. Furthermore, coupled with DNA sequencing, PCR enables sub-specific identification and genotypic characterization (Thellier & Breton, 2008) Dependable gene markers are pivotal in epidemiological and systematic inquiries (Schoch et al., 2012). Molecular markers such as ITS (Internal Transcribed Spacer), SSU (Small Subunit) and LSU (Large Subunit) of nuclear ribosomal DNA are employed for *E. bieneusi*. Studies demonstrate that ITS is optimal for species and genotypic identification and characterization, being predominant in epidemiological investigations (Mónica Santín & Fayer, 2009). The ITS region of *E. bieneusi* spans 240-245 bp, distinct in size from other pathogenic microsporidia.

2.9. Molecular epidemiology of *E. bieneusi*

E. bieneusi exhibits wide distribution, having been identified in more than 41 countries. This pathogen demonstrates infectivity across diverse animal taxa, including birds (e.g. Passeriformes, Columbiformes, Falconiformes, Galliformes, Gruiformes, Passeriformes, Psittaformes and Struthioniformes), mammals (e.g. Artiodactyla, Carnivora, Chiroptera, Diprotodontia, Lagomorpha, Perissodactyla, Primates, and Rodentia), Insecta (e.g. Diptera), and Reptilia (e.g. squamata), among others.

Molecular methods coupled with genotype comparisons across diverse hosts and regions have been instrumental in identifying transmission pathways and potential reservoirs of *E. bieneusi* infection (Cama et al., 2007). Genotyping of *E. bieneusi* is an essential tool for epidemiological inquiries. PCR-based sequencing of ribosomal ITS nucleotide sequences stands as the predominant approach for *E. bieneusi* genotyping. To date, 611 distinct genotypes have been catalogued, 594 of which have precise genotype designations. 92 genotypes have been exclusively recorded in human hosts, 423 in animals,

and 56 in both. Moreover, water sources have harbored 63 genotypes, 22 of which were exclusive to aquatic environments. (Y. Zhang, 2019)

2.9.1. *E. bieneusi infection in humans*

E.bineusi demonstartes the capability to infect individuals with varying immune statuses across both developed and developing nations (Mónica Santín & Fayer, 2009, 2011a). 92 *E. bieneusi* genotypes have been documented in human populations across 22 countries, with a wide range of prevalence, from 1.2% to 100% in HIV-positive patients and from 1.4% to 46.7% in HIV-negative individuals(Breitenmoser et al., 1999; Matos et al., 2012; Sak, Kváč, et al., 2011). Comparing these prevalences is, however, challenging due to variations in diagnostic methodologies, sample types and patient demographics.

Due to its significant impact and potential public health threat, *E. bieneusi* has been categorized as a Category B agent by the National Institutes of Health, ranking it among second highest priority organisms/biological agents (Wang et al., 2024) Additionally, *E. bieneusi* has been identified in both HIV-positive and HIV-negative patients from Portugal, with prevalences of 6.3% (59/856) and 29% (20/69)(Ferreira et al., 2001; Maria Luísa Lobo et al., 2012), which highlights the importance of this zoonotic pathogen. This data suggests the presence and circulation of *E. bieneusi* in the Portuguese fauna, as well as its zoonotic potential, evidenced by the infection of individuals with genotypes found in fauna regardless of their immunological condition.

2.9.2. *E. bieneusi* infection in animals

2.9.2.1. **Companion animals**

Pets such as cats, dogs and horses often interact closely with humans and pose a potential risk of transmitting zoonotic *E. bieneusi* genotypes (Mónica Santín et al., 2006). Additionally, feces from contaminated animals pose a risk of environmental contamination, including water sources, thereby endangering public health(Mori et al., 2013).

In total, 32 genotypes have been recorded in dogs, ten of which are present in other species, including humans (i.e., A, D, EbpA, EbpC, O, Peru 5, Peru 8, PigEBITS5, PtEb VI and PtEb VII). Genotype PtEb IX is the most common genotype in dogs and has been found only in cats, dogs and European badgers, pointing towards specificity in relation to these species. In cats, 20 genotypes have been recorded, eight of which have been detected in humans, suggesting that they might be carriers of zoonotic genotypes. In horses, 40 genotypes have been detected, 11 of which have zoonotic potential.(Y. Zhang, 2019)

2.9.2.2. **Livestock animals**

Farm animals, including cattle, pigs and sheep serve as potential reservoirs for *E. bineusi* transmission to humans through direct contact or via water routes (Prasertbun et al., 2017; X. Zhang et al., 2011). Cattle have shown *E. bieneusi* prevalence rates ranging from 2.0% to 37.6% across various regions (Valenčáková & Danišová, 2019; X. Zhang et al., 2011). Pigs exhibit prevalences from 10% to 94% across different regions (Valenčáková & Danišová, 2019). The genotypes most often found in these animals belong to zoonotic group 1, indicating the zoonotic potential of livestock-associated *E. bieneusi.*

2.9.2.3. **Mammalian wildlife and other animals**

Numerous studies suggest that wildlife animals serve as potential reservoirs for *E. bineusi.* It has been documented across more than seven orders of animals, including Artiodactyla, Carnivora, Diprotodontia, Lagomorpha, non-human Primates, Perissodactyla and Rodentia, spanning over 104 species.(Y. Zhang, 2019)

Several investigations have detected *E. bieneusi* in wildlife inhabiting areas near water catchments that supply drinking water, indicating a potential route of transmission through spore-contaminated drinking water (Guo et al., 2014; Sulaiman et al., 2004). Studies assessing *E. bieneusi* in wildlife populations comprising 23 species and in drinking water revealed its presence in both. The prevalent genotype (WL4), found in animals and in water, suggests that animals may contribute to water contamination (Guo et al., 2014). *E. bieneusi* was also found in Rhesus monkeys and water samples from a lake frequented by these primates for bathing, with the same zoonotic genotypes being found in the animals and in the water samples (EbpC, Peru11, Type IV), raising significant public health concerns given the potential for direct contact between monkeys and tourists.

2.9.2.4. **Birds**

Birds harbor multiple species of microsporidia, including *E. bieneusi.* Diverse range of bird populations have been found to be affected by *E. bieneusi* (aviary birds, wild birds, pet store birds, in local markets, public parks and zoos) (Y. Zhang, 2019).

The prevalence of *E. bieneusi* spans multiple orders, including Anseriformes, Columbiformes, Falconiformes, Galliformes, Gruiformes and Passeriformes, with prevalences ranging from 0.89% to 44% (J. Li et al., 2015; Zhao et al., 2016). A total of 24 genotypes have been identified in birds, including 13 previously found in humans and 11 shared between other species.

Transmission of *E. bieneusi* from infected birds to humans can occur through various routes, including air, water or contaminated food (E. S. Didier et al., 2004; Haro et al., 2005; W. Li et al., 2014).

Bird feces pose a risk with potential for inhalation of fecal dust by humans, with particular importance in the case of those handling birds in aviaries or in bird-dense environments such as parks or areas with high pigeon population(Y. Zhang, 2019), in addition to dissemination through water sources.

2.10. Treatment

Current therapy options for microsporidiosis mainly rely on albendazole and fumagillin, which have shown efficacy against microsporidian pathogens(Costa & Weiss, 2000; H. Zhang et al., 2005). Albendazole, a benzimidazole derivative, acts by inhibiting tubulin synthesis, disrupting the microsporidian division(Conteas et al., 1999). It is commonly used for *Encephalitozoon spp.* infections in HIV patients, with minimal side effects (Costa & Weiss, 2000).

Fumagillin, extracted from *Aspergillus fumigatus*, inhibits cellular metalloprotease-methionine aminopeptidase-2 (MetAp2), crucial for protein stability and post-translational modifications (Siddiquee, 2017). This substance blocks microsporidial replication by irreversibly binding to MetAp2's site of action, leading to death (Costa & Weiss, 2000). Despite its efficacy in treating *E. bieneusi* in HIV-positive patients, fumagillin can induce bone marrow toxicity, necessitating close medical monitoring (Elizabeth S. Didier & Weiss, 2006).

Preventive strategies against *E. bieneusi* transmission to humans include hand washing, washing of fresh produce, consumption of boiled or bottled water, and minimizing animal-human contact (Santín-Durán, 2015a). Additionally, surveillance of wildlife and domesticated animals, particularly those living in or around drinking water, is crucial for early detection and prevention of *E. bieneusi* infection.

3. Practical work

My interest and experience in enteric parasites, developed in the clinic, were fortunately complemented by the focus on enteric microorganism microorganisms at MIDlab. This dual exposure allowed me to develop research on *E. bieneusi*, which resulted in a manuscript, which is the basis of this dissertation. The practical work conducted in this study is extensively detailed in **Annex B**. This annex is formatted as a manuscript submitted to the journal *Animals*, ensuring comprehensive documentation and peer-reviewed validation of the methodologies and findings. The document includes an introduction, materials and methods, results, discussion, and the relevant bibliography, effectively substituting the corresponding chapters of this work.

4. Conclusion

This dissertation focused on evaluating the prevalence and genetic diversity of *Enterocytozoon bieneusi* among zoo animals in Portugal, allowing me to further expand my interest and knowledge in enteric parasites that I started to cultivate at CRF-PBG.

Through molecular analysis, our study uncovered significant insights into the presence and potential zoonotic implications of this pathogen in a controlled zoo environment. The genotypes detected in our study varied from those reported in other regions, such as China or Kenya, indicating possible geographical and host-specific differences in *E.bieneusi* distribution. This variability underscores the importance of localized studies to better understand the epidemiology of the pathogen and tailor prevention strategies accordingly.

The overall prevalence of *E. bieneusi* in the sampled zoo animals was relatively low, at 1.57%. However, a notable exception was observed in NHP's, where the prevalence was significantly higher at18.18%. This disparity suggests a susceptibility or exposure risk among NHP's to this pathogen compared to other zoo animals. Diverse genotypes were identified within the primate population, including representatives of group 2, identified through phylogenetic analysis, known for zoonotic potential. This finding underscores the risk of cross-species transmission, including humans, within zoo environments and highlights the necessity for stringent hygiene and monitoring practices in zoos to mitigate the potential spread to humans, particularly zookepers and visitors.

The findings of this study open up several avenues for future research. Building on the insights gained, further investigations can comprehensively assess the epidemiology of *Enterocytozoon bieneusi* in zoo settings, focusing on understanding transmission dynamics and host specificity. Expanding the sample size and including a wider variety of animal species across multiple zoos will provide a more robust dataset, enhancing our understanding of this pathogen's behavior. Additionally, integrating advanced molecular techniques and genomic studies will allow for a deeper exploration of the genetic diversity and evolution of *E. bieneusi*. Furthermore, Studies examining the interactions between zoo animals and humans, particularly zookeepers and visitors, can provide valuable insights into the risk factors and pathways of zoonotic transmission. These efforts will not only enhance the health and well-being of zoo animals but also safeguard public health, contributing to the overall goal of preventing zoonotic outbreaks.

References

Akinbo, F. O., Okaka, C. E., Omoregie, R., Dearen, T., Leon, E. T., & Xiao, L. (2012a). Molecular Epidemiologic Characterization of *Enterocytozoon bieneusi* in HIV-Infected Persons in Benin City, Nigeria. *The American Journal of Tropical Medicine and Hygiene*, *86*(3), 441. https://doi.org/10.4269/AJTMH.2012.11-0548

Akinbo, F. O., Okaka, C. E., Omoregie, R., Dearen, T., Leon, E. T., & Xiao, L. (2012b). Molecular Epidemiologic Characterization of *Enterocytozoon bieneusi* in HIV-Infected Persons in Benin City, Nigeria. *The American Journal of Tropical Medicine and Hygiene*, *86*(3), 441. https://doi.org/10.4269/AJTMH.2012.11-0548

Akiyoshi, D. E., Morrison, H. G., Lei, S., Feng, X., Zhang, Q., Corradi, N., Mayanja, H., Tumwine, J. K., Keeling, P. J., Weiss, L. M., & Tzipori, S. (2009). Genomic Survey of the Non-Cultivatable Opportunistic Human Pathogen, *Enterocytozoon bieneusi*. *PLoS Pathogens*, *5*(1), 1000261. https://doi.org/10.1371/JOURNAL.PPAT.1000261

Balbiani. (1882). Comptes Rendus Hebdomadaires des Seances de L'Academie des Sciences. Em *Journal of the Röntgen Society* (Vol. 8, Número 30). publiés avec le concours du Centre national de la recherche scientifique par MM. les secrétaires perpétuels. https://doi.org/10.1259/jrs.1912.0009

Bednarska, Małgorzata & Bajer, Anna & Welc-Falęciak, Renata & Czubkowski, Piotr & Teisseyre, Mikolaj & Graczyk, Thaddeus & Jankowska, I. (2013). The first case of *Enterocytozoon bieneusi* infection in Poland. *Annals of agricultural and environmental medicine*, 287–288.

Bern, C., Kawai, V., Vargas, D., Rabke-Verani, J., Williamson, J., Chavez-Valdez, R., Xiao, L., Sulaiman, I., Vivar, A., Ticona, E., Ñavincopa, M., Cama, V., Moura, H., Secor, W. E., Visvesvara, G., & Gilman, R. H. (2005). The epidemiology of intestinal microsporidiosis in patients with HIV/AIDS in Lima, Peru. *Journal of Infectious Diseases*, *191*(10), 1658–1664. https://doi.org/10.1086/429674/2/191-10-1658-TBL005.GIF

Brasil, P., De Lima, D. B., De Paiva, D. D., De Castro Lobo, M. S., Sodré, F. C., Da Silva, S. P., Villela, E. V., Da Silva, E. J., Peralta, J. M., Morgado, M., & Moura, H. (2000). Clinical and diagnostic aspects of intestinal microsporidiosis in HIV-infected patients with chronic diarrhea in Rio de Janeiro, Brazil. *Revista do Instituto de Medicina Tropical de Sao Paulo*, *42*(6), 299–304. https://doi.org/10.1590/S0036-46652000000600001

Breitenmoser, A. C., Mathis, A., Bürgi, E., Weber, R., & Deplazes, P. (1999). High prevalence of *Enterocytozoon bieneusi* in swine with four genotypes that differ from those identified in humans. *Parasitology*, *118 (Pt 5*(5), 447–453. https://doi.org/10.1017/S0031182099004229

Cama, V. A., Pearson, J., Cabrera, L., Pacheco, L., Gilman, R., Meyer, S., Ortega, Y., & Xiao, L. (2007). Transmission of *Enterocytozoon bieneusi* between a Child and Guinea Pigs. *Journal of Clinical Microbiology*, *45*(8), 2708. https://doi.org/10.1128/JCM.00725-07

Chalifoux, L. V., Mackey, J., Carville, A., Shvetz, D., Lin, K. C., Lackner, A., & Mansfield, K. G. (1998). Ultrastructural Morphology of *Enterocytozoon bieneusi* in Biliary Epithelium of Rhesus Macaques (Macaca mulatta). *Veterinary Pathology*, *35*(4), 292–296. https://doi.org/10.1177/030098589803500408

Conteas, C., Berlin, O., Ash, L., & Pruthi, J. (1999). Therapy for human gastrointestinal microsporidiosis. *The American journal of tropical medicine and hygiene*, *63*, 121–127. https://doi.org/10.4269/ajtmh.2000.63.121

Costa, S. F., & Weiss, L. M. (2000). Drug treatment of microsporidiosis. *Drug Resistance Updates :*

Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy, *3*(6), 384–399. https://doi.org/10.1054/drup.2000.0174

Del Aguila, C Izquierdo, F Navajas, R Pieniazek, NJ Miro, G Alonso, AI Da Silva, AJ Fenoy, S. (1999). *Enterocytozoon bieneusi* in animals: Rabbits and dogs as new hosts. *JOURNAL OF EUKARYOTIC MICROBIOLOGY*, *46*(5), 8S-9S.

Dengjel, B., Zahler, M., Hermanns, W., Heinritzi, K., Spillmann, T., Thomschke, A., Löscher, T., Gothe, R., & Rinder, H. (2001). Zoonotic potential of *Enterocytozoon bieneusi*. *Journal of Clinical Microbiology*, *39*(12), 4495–4499. https://doi.org/10.1128/JCM.39.12.4495-4499.2001

Deplazes, P., Mathis, A., Müller, C., & Weber, R. (1996). Molecular epidemiology of Encephalitozoon cuniculi and first detection of *Enterocytozoon bieneusi* in faecal samples of pigs. *The Journal of eukaryotic microbiology*, *43*(5). https://doi.org/10.1111/J.1550-7408.1996.TB05018.X

Desportes, I., Charpentier, Y. Le, Galian, A., Bernard, F., Cochand-Priollet, B., Lavergne, A., Ravisse, P., & Modigliani, R. (1985). Occurrence of a new microsporidan: *Enterocytozoon bieneusi* n.g., n. sp., in the enterocytes of a human patient with AIDS. *The Journal of protozoology*, *32*(2), 250–254. https://doi.org/10.1111/J.1550-7408.1985.TB03046.X

Didier, E. S., Stovall, M. E., Green, L. C., Brindley, P. J., Sestak, K., & Didier, P. J. (2004). Epidemiology of microsporidiosis: sources and modes of transmission. *Veterinary parasitology*, *126*(1–2), 145–166. https://doi.org/10.1016/J.VETPAR.2004.09.006

Didier, Elizabeth S., & Weiss, L. M. (2006). Microsporidiosis: current status. *Current Opinion in Infectious Diseases*, *19*(5), 485. https://doi.org/10.1097/01.QCO.0000244055.46382.23

Fayer, R. (2004). Infectivity of microsporidia spores stored in seawater at environmental temperatures. *The Journal of parasitology*, *90*(3), 654–657. https://doi.org/10.1645/GE-3335RN

Ferreira, F. M., Bezerra, L., Santos, M. B., Bernardes, R. M., Avelino, I., & Silva, M. L. (2001). Intestinal microsporidiosis: a current infection in HIV-seropositive patients in Portugal. *Microbes and Infection*, *3*(12), 1015–1019. https://doi.org/10.1016/s1286-4579(01)01465-4

Franzen, C. (2005). How do microsporidia invade cells? *Folia parasitologica*, *52*(1–2), 36–40. https://doi.org/10.14411/FP.2005.005

Galván-Díaz, A. L., Magnet, A., Fenoy, S., Henriques-Gil, N., Haro, M., Gordo, F. P., Miró, G., Águila, C. Del, & Izquierdo, F. (2014). Microsporidia Detection and Genotyping Study of Human Pathogenic *E. bieneusi* in Animals from Spain. *PLoS ONE*, *9*(3). https://doi.org/10.1371/JOURNAL.PONE.0092289

Ghosh, K., & Weiss, L. M. (2009). Molecular diagnostic tests for microsporidia. *Interdisciplinary perspectives on infectious diseases*, *2009*, 1–13. https://doi.org/10.1155/2009/926521

Ghoyounchi, R., Mahami-Oskouei, M., Rezamand, A., Spotin, A., Aminisani, N., Nami, S., Pirestani, M., Berahmat, R., & Madadi, S. (2019). Molecular Phylodiagnosis of *Enterocytozoon bieneusi* and Encephalitozoon intestinalis in Children with Cancer: Microsporidia in Malignancies as an Emerging Opportunistic Infection. *Acta Parasitologica*, *64*(1), 103–111. https://doi.org/10.2478/S11686-018-00012-W

Graczyk, T. K., Sunderland, D., Rule, A. M., Da Silva, A. J., Moura, I. N. S., Tamang, L., Girouard, A. S., Schwab, K. J., & Breysse, P. N. (2007). Urban feral pigeons (Columba livia) as a source for air- and waterborne contamination with *Enterocytozoon bieneusi* spores. *Applied and environmental microbiology*, *73*(13), 4357–4358. https://doi.org/10.1128/AEM.00202-07

Guo, Y., Alderisio, K. A., Yang, W., Cama, V., Feng, Y., & Xiao, L. (2014). Host Specificity and Source of

Enterocytozoon bieneusi Genotypes in a Drinking Source Watershed. *Applied and Environmental Microbiology*, *80*(1), 218–225. https://doi.org/10.1128/AEM.02997-13

Haag, K. L., Pombert, J. F., Sun, Y., De Albuquerque, N. R. M., Batliner, B., Fields, P., Lopes, T. F., & Ebert, D. (2020). Microsporidia with Vertical Transmission Were Likely Shaped by Nonadaptive Processes. *Genome Biology and Evolution*, *12*(1), 3599. https://doi.org/10.1093/GBE/EVZ270

Hale-Donze, H., & Didier, E. S. (2007). Microsporidioses. *Encyclopedia of Life Sciences*. https://doi.org/10.1002/9780470015902.A0001930

Han, B., Pan, G., & Weiss, L. M. (2021). Microsporidiosis in Humans. *Clinical Microbiology Reviews*, *34*(4). https://doi.org/https://doi.org/10.1128/CMR.00010-20

Haro, M., Izquierdo, F., Henriques-Gil, N., Andrés, I., Alonso, F., Fenoy, S., & Del Águila, C. (2005). First Detection and Genotyping of Human-Associated Microsporidia in Pigeons from Urban Parks. *Applied and Environmental Microbiology*, *71*(6), 3153. https://doi.org/10.1128/AEM.71.6.3153-3157.2005

Javanmard, E., Mirjalali, H., Niyyati, M., Jalilzadeh, E., Seyed Tabaei, S. J., Asadzadeh Aghdaei, H., Nazemalhosseini-Mojarad, E., & Zali, M. R. (2018). Molecular and phylogenetic evidences of dispersion of human-infecting microsporidia to vegetable farms via irrigation with treated wastewater: One-year follow up. *International Journal of Hygiene and Environmental Health*, *221*(4), 642–651. https://doi.org/10.1016/J.IJHEH.2018.03.007

Karthikeyan, K., & Sudhakaran, R. (2019). Experimental horizontal transmission of Enterocytozoon hepatopenaei in post-larvae of whiteleg shrimp, Litopenaeus vannamei. *Journal of fish diseases*, *42*(3), 397–404. https://doi.org/10.1111/JFD.12945

Keeling, P. (2009). Five Questions about Microsporidia. *PLOS Pathogens*, *5*(9), e1000489. https://doi.org/10.1371/JOURNAL.PPAT.1000489

Keeling, P. J., & Fast, N. M. (2002). Microsporidia: Biology and evolution of highly reduced intracellular parasites. *Annual Review of Microbiology*, *56*(Volume 56, 2002), 93–116. https://doi.org/10.1146/ANNUREV.MICRO.56.012302.160854/CITE/REFWORKS

Kramer, J. P. (1976). The Extra-Corporeal Ecology of Microsporidia. *Biology of the Microsporidia*, 127–135. https://doi.org/10.1007/978-1-4684-3114-8_5

Li, J., Qi, M., Chang, Y., Wang, R., Li, T., Dong, H., & Zhang, L. (2015). Molecular Characterization of Cryptosporidium spp., Giardia duodenalis, and *Enterocytozoon bieneusi* in Captive Wildlife at Zhengzhou Zoo, China. *Journal of Eukaryotic Microbiology*, *62*(6), 833–839. https://doi.org/10.1111/JEU.12269

Li, W., Diao, R., Yang, J., Xiao, L., Lu, Y., Li, Y., & Song, M. (2014). High diversity of human-pathogenic *Enterocytozoon bieneusi* genotypes in swine in northeast China. *Parasitology research*, *113*(3), 1147–1153. https://doi.org/10.1007/S00436-014-3752-9

Liguory, O., Sarfati, C., Derouin, F., & Molina, J. M. (2001a). Evidence of Different *Enterocytozoon bieneusi* Genotypes in Patients with and without Human Immunodeficiency Virus Infection. *Journal of Clinical Microbiology*, *39*(7), 2672. https://doi.org/10.1128/JCM.39.7.2672-2674.2001

Liguory, O., Sarfati, C., Derouin, F., & Molina, J. M. (2001b). Evidence of Different *Enterocytozoon bieneusi* Genotypes in Patients with and without Human Immunodeficiency Virus Infection. *Journal of Clinical Microbiology*, *39*(7), 2672. https://doi.org/10.1128/JCM.39.7.2672-2674.2001

Lobo, Maria Luisa, Teles, A., Da Cunha, M. B., Henriques, J., Lourenço, A. M., Antunes, F., & Matos, O. (2003). Microsporidia detection in stools from pets and animals from the zoo in Portugal: a

preliminary study. *The Journal of eukaryotic microbiology*, *50 Suppl*(SUPPL.), 581–582. https://doi.org/10.1111/J.1550-7408.2003.TB00638.X

Lobo, Maria Luísa, Xiao, L., Antunes, F., & Matos, O. (2012). Microsporidia as emerging pathogens and the implication for public health: a 10-year study on HIV-positive and -negative patients. *International Journal for Parasitology*, *42*(2), 197–205. https://doi.org/10.1016/j.ijpara.2011.12.002

LOM J, V. J. (sem data). Some results in the study on the microstructure of spores of the fish parasite, Plistophora hyphessobryconis (Microsporidia). *Wiad Parazytol.*

Lores, B., Del Aguila, C., & Arias, C. (2002). *Enterocytozoon bieneusi* (microsporidia) in faecal samples from domestic animals from Galicia, Spain. *Memorias do Instituto Oswaldo Cruz*, *97*(7), 941–945. https://doi.org/10.1590/S0074-02762002000700003

Mansfield, K. G., Carville, A., Hebert, D., Chalifoux, L., Shvetz, D., Lin, K. C., Tzipori, S., & Lackner, A. A. (1998). Localization of persistent *Enterocytozoon bieneusi* infection in normal rhesus macaques (Macaca mulatta) to the hepatobiliary tree. *Journal of clinical microbiology*, *36*(8), 2336–2338. https://doi.org/10.1128/JCM.36.8.2336-2338.1998

Mathis, A., Breitenmoser, A. C., & Deplazes, P. (1999). Detection of new Enterocytozoon genotypes in faecal samples of farm dogs and a cat. *Parasite (Paris, France)*, *6*(2), 189–193. https://doi.org/10.1051/PARASITE/1999062189

Matos, O., Lobo, M. L., & Xiao, L. (2012). Epidemiology of *Enterocytozoon bieneusi* Infection in Humans. *Journal of Parasitology Research*, *2012*, 36–45. https://doi.org/10.1155/2012/981424

Monaghan, S. R., Kent, M. L., Watral, V. G., Kaufman, R. J., Lee, L. E. J., & Bols, N. C. (2009). Animal cell cultures in microsporidial research: their general roles and their specific use for fish microsporidia. *In vitro cellular & developmental biology. Animal*, *45*(0), 135. https://doi.org/10.1007/S11626-008-9172-3

Moniot, M., Poirier, P., & Nourrisson, C. (2021). Etymologia: *Enterocytozoon bieneusi*. *Emerging Infectious Diseases*, *27*(6), 1587. https://doi.org/10.3201/EID2706.ET2706

Mori, H., Mahittikorn, A., Watthanakulpanich, D., Komalamisra, C., & Sukthana, Y. (2013). Zoonotic potential of *Enterocytozoon bieneusi* among children in rural communities in Thailand. *Parasite (Paris, France)*, *20*, 14. https://doi.org/10.1051/parasite/2013014

Pasteur, L. (1870). *Etude sur la maladie des vers a soie*.

Poley, J. D., Sutherland, B. J. G., Fast, M. D., Koop, B. F., & Jones, S. R. M. (2017). Effects of the vertically transmitted microsporidian Facilispora margolisi and the parasiticide emamectin benzoate on salmon lice (Lepeophtheirus salmonis). *BMC genomics*, *18*(1). https://doi.org/10.1186/S12864-017-4040-8

Pourrut, X., Sarfati, C., Liguory, O., Moyou-Somo, R., & Derouin, F. (2002). Search for *Enterocytozoon bieneusi* infection in wild monkeys in Cameroon. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *96*(1), 56–57. https://doi.org/10.1016/S0035-9203(02)90238-5

Prasertbun, R., Mori, H., Pintong, A.-R., Sanyanusin, S., Popruk, S., Komalamisra, C., Changbunjong, T., Buddhirongawatr, R., Sukthana, Y., & Mahittikorn, A. (2017). Zoonotic potential of Enterocytozoon genotypes in humans and pigs in Thailand. *Veterinary Parasitology*, *233*, 73–79. https://doi.org/10.1016/j.vetpar.2016.12.002

Reetz, J., Rinder, H., Thomschke, A., Manke, H., Schwebs, M., & Bruderek, A. (2002). First detection of the microsporidium *Enterocytozoon bieneusi* in non-mammalian hosts (chickens). *International*

Journal for Parasitology, *32*(7), 785–787. https://doi.org/10.1016/S0020-7519(02)00045-0

Rinder, H., Thomschke, A., Dengjel, B., Gothe, R., Löscher, T., & Zahler, M. (2000). Close genotypic relationship between *Enterocytozoon bieneusi* from humans and pigs and first detection in cattle. *The Journal of parasitology*, *86*(1), 185–188. https://doi.org/10.1645/0022-3395(2000)086[0185:cgrbeb]2.0.co;2

Sadler, F., Peake, N., Borrow, R., Rowl, P. L., Wilkins, E. G. L., & Curry, A. (2002a). Genotyping of *Enterocytozoon bieneusi* in AIDS patients from the north west of England. *Journal of Infection*, *44*(1), 39–42. https://doi.org/10.1053/jinf.2001.0934

Sadler, F., Peake, N., Borrow, R., Rowl, P. L., Wilkins, E. G. L., & Curry, A. (2002b). Genotyping of *Enterocytozoon bieneusi* in AIDS patients from the north west of England. *Journal of Infection*, *44*(1), 39–42. https://doi.org/10.1053/jinf.2001.0934

Sak, B., Brady, D., Pelikánová, M., Květoňová, D., Rost, M., Kostka, M., Tolarová, V., Hůzová, Z., & Kváč, M. (2011). Unapparent microsporidial infection among immunocompetent humans in the Czech Republic. *Journal of clinical microbiology*, *49*(3), 1064–1070. https://doi.org/10.1128/JCM.01147-10

Sak, B., Kváč, M., Petrželková, K., Květoňová, D., Pomajbíková, K., Mulama, M., Kiyang, J., & Modrý, D. (2011). Diversity of microsporidia (Fungi: Microsporidia) among captive great apes in European zoos and African sanctuaries: evidence for zoonotic transmission? *Folia parasitologica*, *58*(2), 81–86. https://doi.org/10.14411/FP.2011.008

Santín-Durán, M. (2015a). Biology of Foodborne Parasites. Em *Biology of Foodborne Parasites*. CRC Press. https://doi.org/10.1201/B18317-13

Santín-Durán, M. (2015b). *Enterocytozoon bieneusi*. *Biology of Foodborne Parasites*, 164–189. https://doi.org/10.1201/B18317-13

Santin, M., & Fayer, R. (2015). *Enterocytozoon bieneusi*, Giardia, and Cryptosporidium Infecting White-tailed Deer. *Journal of Eukaryotic Microbiology*, *62*(1), 34–43. https://doi.org/10.1111/JEU.12155

Santín, M., Trout, J. M., & Fayer, R. (2005). *Enterocytozoon bieneusi* genotypes in dairy cattle in the eastern United States. *Parasitology research*, *97*(6), 535–538. https://doi.org/10.1007/S00436-005-1482-8

Santín, Mónica, & Fayer, R. (2009). *Enterocytozoon bieneusi* genotype nomenclature based on the internal transcribed spacer sequence: a consensus. *The Journal of eukaryotic microbiology*, *56*(1), 34–38. https://doi.org/10.1111/J.1550-7408.2008.00380.X

Santín, Mónica, & Fayer, R. (2011a). Microsporidiosis: *Enterocytozoon bieneusi* in domesticated and wild animals. *Research in Veterinary Science*, *90*, 363–371. https://doi.org/10.1016/j.rvsc.2010.07.014

Santín, Mónica, & Fayer, R. (2011b). Microsporidiosis: *Enterocytozoon bieneusi* in domesticated and wild animals. *Research in veterinary science*, *90*(3), 363–371. https://doi.org/10.1016/J.RVSC.2010.07.014

Santín, Mónica, Trout, J. M., & Fayer, R. (2004). Prevalence of *Enterocytozoon bieneusi* in post-weaned dairy calves in the eastern United States. *Parasitology research*, *93*(4), 287–289. https://doi.org/10.1007/S00436-004-1132-6

Santín, Mónica, Trout, J. M., Vecino, J. A. C., Dubey, J. P., & Fayer, R. (2006). Cryptosporidium, Giardia and *Enterocytozoon bieneusi* in cats from Bogota (Colombia) and genotyping of isolates. *Veterinary Parasitology*, *141*(3–4), 334–339. https://doi.org/10.1016/j.vetpar.2006.06.004

Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W., Bolchacova, E., Voigt, K., Crous, P. W., Miller, A. N., Wingfield, M. J., Aime, M. C., An, K. D., Bai, F. Y., Barreto, R. W., Begerow, D., Bergeron, M. J., Blackwell, M., … Schindel, D. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(16), 6241–6246. https://doi.org/10.1073/PNAS.1117018109

Siddiquee, S. (2017). Recent Advancements on the role of biologically active secondary metabolites from aspergillus. *New and Future Developments in Microbial Biotechnology and Bioengineering: Penicillium System Properties and Applications*, 69–94. https://doi.org/10.1016/B978-0-444-63501-3.00004-1

Solter, L. F., & Becnel, J. J. (2007). Entomopathogenic microsporidia. *Field Manual of Techniques in Invertebrate Pathology*, 199–221. https://doi.org/10.1007/978-1-4020-5933-9_9

Sulaiman, I. M., Fayer, R., Yang, C., Santin, M., Matos, O., & Xiao, L. (2004). Molecular characterization of *Enterocytozoon bieneusi* in cattle indicates that only some isolates have zoonotic potential. *Parasitology Research*, *92*, 328–334. https://doi.org/10.1007/s00436-003-1049-5

Sveen, S., Øverland, H., Karlsbakk, E., & Nylund, A. (2012). Paranucleospora theridion (Microsporidia) infection dynamics in farmed Atlantic salmon Salmo salar put to sea in spring and autumn. *Diseases of aquatic organisms*, *101*(1), 43–49. https://doi.org/10.3354/DAO02464

Ten Hove, R. J., Van Lieshout, L., Beadsworth, M. B. J., Perez, M. A., Spee, K., Claas, E. C. J., & Verweij, J. J. (2009a). Characterization of genotypes of *Enterocytozoon bieneusi* in immunosuppressed and immunocompetent patient groups. *The Journal of eukaryotic microbiology*, *56*(4), 388–393. https://doi.org/10.1111/J.1550-7408.2009.00393.X

Ten Hove, R. J., Van Lieshout, L., Beadsworth, M. B. J., Perez, M. A., Spee, K., Claas, E. C. J., & Verweij, J. J. (2009b). Characterization of genotypes of *Enterocytozoon bieneusi* in immunosuppressed and immunocompetent patient groups. *The Journal of eukaryotic microbiology*, *56*(4), 388–393. https://doi.org/10.1111/J.1550-7408.2009.00393.X

Thellier, M., & Breton, J. (2008). *Enterocytozoon bieneusi* in human and animals, focus on laboratory identification and molecular epidemiology. *Parasite (Paris, France)*, *15*(3), 349–358. https://doi.org/10.1051/PARASITE/2008153349

Trew, J. (2021). *The Enterocytozoonidae: the emergence of a microsporidian clade into the aquatic and terrestrial food-chain.*

Undeen, A. H., & Epsky, N. D. (1990). In vitro and in vivo germination of Nosema locustae (Microspora: Nosematidae) spores. *Journal of Invertebrate Pathology*, *56*(3), 371–379. https://doi.org/10.1016/0022-2011(90)90124-O

Valenčáková, A., & Danišová, O. (2019). Molecular characterization of new genotypes *Enterocytozoon bieneusi* in Slovakia. *Acta Tropica*, *191*, 217–220. https://doi.org/10.1016/j.actatropica.2018.12.031

Vávra, J., & Larsson, J. I. R. (2014a). Structure of the Microsporidia. *The Microsporidia and Microsporidiosis*, 7–84. https://doi.org/10.1128/9781555818227.CH2

Vávra, J., & Larsson, J. I. R. (2014b). Structure of Microsporidia. *Microsporidia: Pathogens of Opportunity: First Edition*, 1–70. https://doi.org/10.1002/9781118395264.CH1

Vossbrinck, C. R., Maddox, J. V, Friedman, S., Debrunner-Vossbrinck, B. A., & Woese, C. R. (1987). Ribosomal RNA sequence suggests microsporidia are extremely ancient eukaryotes. *Nature*, *326*(6111), 411–414. https://doi.org/10.1038/326411a0

Wang, Y., Li, X. M., Yang, X., Wang, X. Y., Wei, Y. J., Cai, Y., Geng, H. L., Yang, X. B., Yu, H. L., Cao, H., & Jiang, J. (2024). Global prevalence and risk factors of *Enterocytozoon bieneusi* infection in humans: a systematic review and meta-analysis. *Parasite*, *31*. https://doi.org/10.1051/PARASITE/2024007

Xu, Y., & Weiss, L. M. (2005). The microsporidian polar tube: A highly specialised invasion organelle. *International journal for parasitology*, *35*(9), 941. https://doi.org/10.1016/J.IJPARA.2005.04.003

Yang, S., & Rothman, R. E. (2004). PCR-based diagnostics for infectious diseases: Uses, limitations, and future applications in acute-care settings. *Lancet Infectious Diseases*, *4*(6), 337–348. https://doi.org/10.1016/S1473-3099(04)01044-8

Zhang, H., Huang, H., Cali, A., Takvorian, P., Feng, X., Zhou, G., & Weiss, L. (2005). Investigations into microsporidian methionine aminopeptidase type 2: A therapeutic target for microsporidiosis. *Folia parasitologica*, *52*, 182–192. https://doi.org/10.14411/fp.2005.023

Zhang, W., Ren, G., Zhao, W., Yang, Z., Shen, Y., Sun, Y., Liu, A., & Cao, J. (2017). Genotyping of *Enterocytozoon bieneusi* and subtyping of Blastocystis in cancer patients: Relationship to Diarrhea and assessment of zoonotic transmission. *Frontiers in Microbiology*, *8*(SEP), 267962. https://doi.org/10.3389/FMICB.2017.01835/BIBTEX

Zhang, X., Wang, Z., Su, Y., Liang, X., Sun, X., Peng, S., Lu, H., Jiang, N., Yin, J., Xiang, M., & Chen, Q. (2011). Identification and genotyping of *Enterocytozoon bieneusi* in China. *Journal of Clinical Microbiology*, *49*(5), 2006–2008. https://doi.org/10.1128/JCM.00372-11

Zhang, Y. (2019). *Molecular epidemiological investigations of Enterocytozoon bieneusi*.

Zhao, W., Yu, S., Yang, Z., Zhang, Y., Zhang, L., Wang, R., Zhang, W., Yang, F., & Liu, A. (2016). Genotyping of *Enterocytozoon bieneusi* (Microsporidia) isolated from various birds in China. *Infection, Genetics and Evolution : Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases*, *40*, 151–154. https://doi.org/10.1016/j.meegid.2016.02.037

Annex A - Detection of Enterocytozoon bineusi in non-human primates in Portuguese Zoos

Article

Detection of *Enterocytozoon bieneusi* in non-human primates in Portuguese Zoos

Guilherme Moreira [1], Andreia V.S. Cruz [1], Sérgio Santos-Silva[1], Rafaela São Simão Moreira[1], João R. Mesquita[1,2,3*]

1 Department of Veterinary Clinics, ICBAS – School of Medicine and Biomedical Sciences, Universidade do Porto, Porto, Portugal; gmoreiravet@gmail.com, up201303679@edu.icbas.up.pt, up202110051@edu.icbas.up.pt.
2 Epidemiology Research Unit (EPIUnit), ISPUP – Instituto de Saúde Pública da Universidade do Porto, Porto, Portugal
3 Laboratório para a Investigação Integrativa e Translacional em Saúde Populacional (ITR), ISPUP – Instituto de Saúde Pública da Universidade do Porto, Porto, Portugal
* Professor João R. Mesquita, ORCID ID: 0000-0001-8769-8103, jrmesquita@icbas.up.pt, +351 220 428 000

Simple Summary: *Enterocytozoon bieneusi*, the leading cause of human microsporidiosis, was found in 1.57% of zoo animal samples from Portugal, exclusively in non-human primates, underscoring their potential contribution to the transmission of this microsporidia to humans and other animals.

Abstract: *Enterocytozoon bieneusi*, an intracellular eukaryotic closely related to fungi, is recognized as a significant pathogen affecting humans, particularly those with compromised immune systems. While its transmission routes are still not fully elucidated, fecal-oral transmission remains the primary one. With a wide host range, the zoonotic potential of *E. bieneusi* is a concern, albeit direct evidence of animal-to-human transmission remains scarce. Genotyping based on the internal transcribed spacer (ITS) region facilitates the delineation of genetic diversity, with potentially zoonotic genotypes predominantly associated with Groups 1 and 2. Despite the broad spectrum of susceptible animal hosts, research into microsporidian infection among zoo animals remains limited. This study aimed to evaluate the occurrence of *E. bieneusi* infection across diverse captive animals, focusing on zoo settings in Portugal. Fecal samples were collected from a variety of animals, and molecular detection of *E. bieneusi* was conducted using nested PCR targeting the ITS region. From 127 fecal samples, 1.57% (95% CI: 0.19–5.57) tested positive for *E. bieneusi*, with non-human primates (NHP's) exhibiting an 18.18% (95% CI: 2.28–51.78) occurrence. Phylogenetic analysis revealed clustering within Group 2 genotypes, indicating potential zoonotic implications. The study highlights the need for further research to understand the epidemiology of *E. bieneusi* in zoo environments and its potential transmission pathways to humans.

Keywords: *Enterocytozoon bieneusi*; Microsporidia; Zoo; non-human primates; Portugal

Citation: To be added by editorial staff during production.

Academic Editor: Firstname Lastname

Received: date
Revised: date
Accepted: date
Published: date

Copyright: © 2024 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

37

1. Introduction

Microsporidia are intracellular eukaryotic organism closely related to fungi that demonstrate a wide host range spanning both invertebrates and vertebrates, with 220 genera described and more than 1,700 species catalogued, 17 of which pose pathogenic risks to humans [1]. Of particular prominence in human infections, especially among immunocompromised individuals, is *Enterocytozoon bieneusi* [2]. Initial documentation of *E. bieneusi* as an intestinal pathogen in a HIV-infected patient dates back to 1985 [3], and subsequent studies highlighted its substantial impact on immunocompromised hosts, particularly those with AIDS or organ transplants, as it can lead to severe and life-threatening diarrhea and wasting syndrome [4–7].

Despite considerable research efforts, the precise modes of microsporidian transmission, including *E. bieneusi*, remain incompletely described [8–13]. Nonetheless, infection is acquired via fecal-oral transmission of spores either through direct contact or exposure to contaminated water or food [10]. As infection progresses, spores enter host enterocytes through the discharged polar tube, introducing the sporoplasm. This is followed by the development of meronts, and consequently multinucleated types. After that, these plasmodia undergo sporogony, producing sporoblasts. Fully formed spores emerge, leaving the affected cells and being eventually released through stool [8,14].

Zoonotic transmission is conceivable, given the demonstrated capacity of *E. bieneusi* to infect various domestic and wild animal hosts. However, direct evidence substantiating animal-to-human transmission remains elusive, notwithstanding documented infections across diverse animal species, including beavers, calves, cats, chickens, dogs, foxes, goats, llamas, macaques, muskrats, ostriches, otters, pigs, pigeons, rabbits, raccoons, and wild boars have been shown [15,16]. Furthermore, *E. bieneusi* infection in reptiles and amphibians is not well understood, although there are reports and molecular characterization of the microsporidian in captive snakes [17] and in edible bullfrogs (*Lithobates catesbeiana*) in China [18]. These findings underscore the potential reservoir role of these animals in the dissemination of microsporidian spores capable of infecting humans.

The conventional approach to *E. bieneusi* genotyping is based on the examination of polymorphisms within internal transcribed space (ITS) nucleotide sequences. This region is flanked by ribosomal RNAs, and exhibits notable variety across *E. bieneusi* isolates, facilitating the discernment of intraspecific genetic diversity [8]. Upwards of 600 genotypes of *E. bieneusi* have already been catalogued and stratified into 13 [19] phylogenetic groups. Potentially zoonotic genotypes tend to be associated with Groups 1 and 2, with Group 1

exhibiting the largest representation, comprising over 300 genotypes [10]. Recent investigations have demonstrated the presence of certain genotypes from Groups 1 and 2 across multiple host species, underscoring their broad zoonotic potential. Conversely, genotypes aligned with Groups 3 to 13 display a greater degree of host specificity, and consequently their impact on public health remains to be fully understood [20].

Despite the broad range of susceptible animal hosts, investigation into microsporidian infection among zoo animals remains limited in scope [21]. However, the easy access that both visitors and zookeepers have to zoo animals poses risks related to the transmission of zoonotic pathogens. [21,22]. Therefore, the primary objective of this study is to evaluate the occurrence of *E. bieneusi* infection across a diverse array of captive animals, including birds, reptiles, amphibians, mammals, and arthropods. Additionally, the study aims to genetically characterize the circulating *E. bieneusi* genotypes in these zoo animals.

2. Materials and Methods

2.1. *Sample collection*

This study screened 127 fecal samples from two Zoological Gardens (Maia Zoo and Pedagogical Farm of Canelas), where animals are housed for educational, recreational, and conservation objectives. Both are located in the Porto district of the northern region of mainland Portugal. Feces with a well-formed structure and no other signs of gastrointestinal disease were collected from the soil immediately after excretion by selectively extracting material from the inner core of the fecal matter. From the Maia Zoo, fecal samples (n=76) were collected from 61 species of animals belonging to 39 different families (Supplementary Table 1). From the Pedagogical Farm of Canelas, fecal samples (n=51) were collected from 12 species, from 10 families. Animals from both sites were exposed to regular proximity with human beings, often direct contact. Animals from the Pedagogical farm were subjected to frequent contact with visitors and caretakers. Animals from Maia Zoo had constrained yet recurrent interaction with human visitors, and frequent direct contact with caretaker staff. All samples, collected in September 2023, were immediately kept at -20°C following collection until DNA extraction.

2.2. *Nucleic acid extraction*

Fecal suspensions (10%) were prepared in phosphate-buffered saline pH 7.2. The samples were then homogenized for 5 minutes using the Disruptor Genie (Scientific Industries, Inc., Bohemia, NY, USA) and then centrifuged for 5 minutes at 8000 x *g*.

DNA extraction was carried out using 140 µl of the resultant supernatant and the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, in the automatic extraction machine QIAcube (Qiagen, Hilden, Germany). DNA was eluted in RNase-free water and kept at -20°C until further analysis. Stools positive for *E. bieneusi* were extracted in each batch of 12 samples and used as PCR positive controls.

2.3. *Molecular detection of Enterocytozoon bieneusi*

Detection of *E. bieneusi* was performed using a nested PCR amplifying the internal transcribed spacer (ITS) region as well as the flanking small and large subunits of the ribosomal RNA (rRNA), with the outer primer set EBITS3/EBITS4 (435 bp) and the inner primer set EBITS1/EBITS2.4 (390 bp) (Table 1) [23].

Table 1. Oligonucleotides used for the molecular detection of *Enterocytozoon bieneusi*.

Target	**Locus**	**Primer**	**Sequence (5′-3′)**	**Reference**
Enterocytozoon bieneusi	ITS (and flanking rRNA)	EBITS3	GGTCATAGGGATGAAGAG	[23]
		EBITS4	TTCGAGTTCTTTCGCGCTC	
		EBITS1	GCTCTGAATATCTATGGCT	
		EBITS2.4	ATCGCCGACGGATCCAAGTG	

2.4. *General Procedures*

All PCR reactions were run on T100 thermocycler (Bio-Rad, Hercules, CA, USA). Reaction mixtures were performed using the Speedy Supreme NZYTaq 2x Green Master Mix (NZYTech, Lisbon, Portugal), in accordance with the manufacturer's instructions. The cycling conditions were as follows: initial denaturation at 95°C for 3 minutes, 40 cycles of denaturation at 95°C for 15 seconds, annealing at 57°C for 15 seconds for the first round of PCR or 55°C for 13 seconds for the second round, extension at 72°C for 2 seconds and final extension at 72°C for 10 minutes. The amplified DNA fragments were identified by electrophoresis on 1.5% agarose gels, stained with Xpert Green Safe DNA gel dye (GRiSP®, Porto, Portugal), at 100 V for 30 minutes. UV light irradiation was used to visualize the results.

131 *2.5. Sequencing and phylogenetic analysis*

132 Amplicons with the expected size were purified using GRS PCR & Gel Band Purification Kit (GRiSP®, Porto, Portugal). Following purification, bidirectional sequencing was carried out using the Sanger dideoxy sequencing method and the inner primers for the target gene. The obtained sequences were aligned using BioEdit Sequence Alignment Editor v7.2.3 software package and compared to those found in the NCBI nucleotide database (GenBank, retrieved on February 6th, 2024). MEGA-X version 10.2.6 software [24] was used to calculate the pairwise distances between the sequences obtained in this study. MEGA-X version 10.2.6 software [24] and the interactive Tree of Life (iTOL) platform [25] were used for phylogenetic analysis, including representative sequences from GenBank along with the sequences originated from this work. The Hasegawa Kishino-Yano model was applied, and maximum likelihood (ML) bootstrap values with 1000 replicates were estimated for statistical robustness. This model was determined to be the most effective replacement by Mega X [24]. 133 134 135 136 137 138 139 140 141 142 143 144

145

146 *2.6. Statistical analysis*

147 The occurrence of *E. bieneusi* in animals from the two zoos in Portugal was determined by calculating the proportion of positive samples relative to the total samples analyzed, along with a 95% confidence interval (95% CI). 148 149

150 **3. Results**

151 From the analysis of the 127 fecal samples, 1.57% (2/127; 95% CI: 0.19–5.57) were positive for *E. bieneusi*. The two positive samples derived from two NHPs from Maia Zoo: a *Hylobates lar* (white-handed gibbon) and a *Lemur catta* (ring-tailed lemur). Both were the sole representatives of their species in the zoos. The occurrence in NHPs was 18.18% (2/11; 95% CI: 2.28–51.78), while in all other animal species, the occurrence was 0%. 152 153 154 155

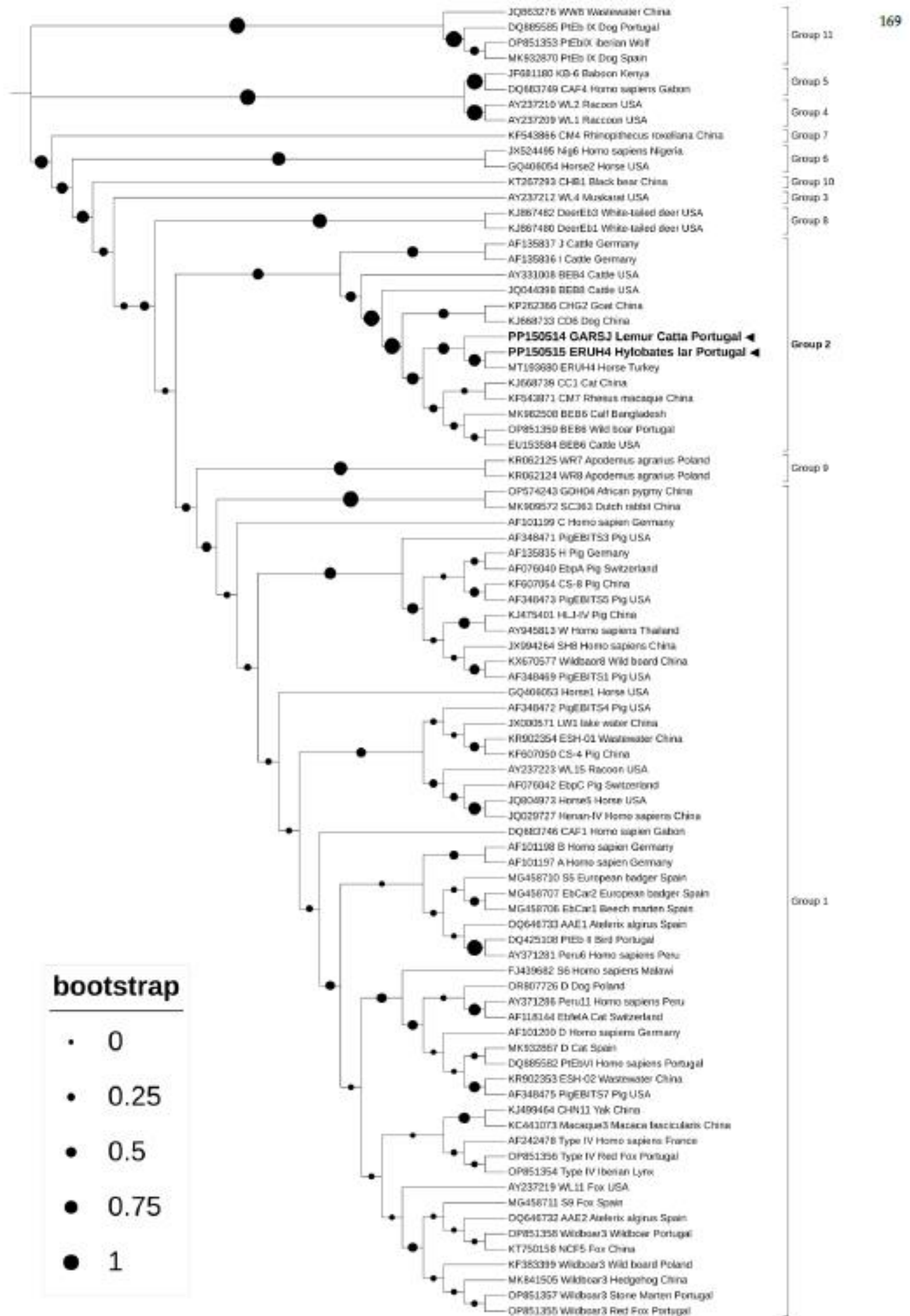
156 The *E. bieneusi* sequences derived from the white-handed gibbon and the ring-tailed lemur were deposited in GenBank under accession numbers PP150515 and PP150514, respectively. Pairwise nucleotide sequence similarity of the two positive samples obtained in the present study was 96.33% 157 158 159

160 BLAST analysis of the obtained sequence from the white-handed gibbon (PP150515) showed highest match (100% identity) with *E. bieneusi* genotype ERUH4 (MT193680), obtained from a horse in Turkey in 2020. The sequence retrieved from the ring-tailed lemur 161 162

(PP150514) showed the highest match (97.02% identity) with *E. bieneusi* genotype BEB6 (MK982508), obtained from a calf in Bangladesh in 2020. 163 164

Phylogenetic analysis of the obtained ITS amplicons showed that the sequences originated in our study grouped with sequences from Group 2 (Figure 1). Genotype ERUH4 and a proposed novel genotype denominated here as "GASRJ" were identified from the white handed gibbon and the ring-tailed lemur, respectively 165 166 167 168

169



170
171

Figure 1. Phylogenetic analysis of *Enterocytozoon bieneusi* sequences obtained in this study (highlighted in bold) and reference genotypes, identified with the respective accession numbers, genotype, host and country of origin. Phylogenetic tree was performed using the maximum likelihood method and the Tamura-Nei model. 172–175

4. Discussion

In this study the occurrence of *E. bieneusi* was assessed in 127 zoo animals from mainland Portugal, also conducting molecular characterization of the detected variants.

In Portugal, *E. bieneusi* has been detected in several animal species, including domestic, wild and zoo animals, in a total of 13 genotypes identified (BEB6, Peru6, PtEb IV, PtEb V, D, PtEb VII, PtEb VIII, PtEb IX, PtEb X, PtEb XI, PtEb XII, Type IV, and Wildboar3) [26–29]. To date, only one study in the country has reported *E. bieneusi* in zoo animals in 2006 [27]. In that study, the presence of *E. bieneusi* was confirmed molecularly in fecal samples from a marmoset (*Callithrix geoffroyi*) and a Kudo (*Tragelaphus strepsiceros*), identified as genotypes PtEb XII and PtEb V, respectively.

In our study, the positive samples derived from NHP's (white-handed gibbon and ring-tailed lemur) and the overall occurrence of *E. bieneusi* was 1.57% from the 127 fecal samples tested, and 18.18% in NHP's. Recent molecular studies conducted in China and Kenya revealed the pathogen's common occurrence and considerable genetic diversity among NHP's [2,21]. The majority of these genotypes belong to genotypic Group 1, some of which have been detected in humans worldwide, raising concerns about the potential role of NHP's in the zoonotic transmission of *E. bieneusi*. A previous study conducted in seven zoos in China tested 496 NHP's fecal samples, using an ITS-based PCR and sequence analyses. From the 36 NHP's species from nine families tested, *E. bieneusi* was detected in 29.8% of the samples, including in the same species as detected here: 24.4% (11/45) in ring-tailed lemur and 62.5% (5/8) in white-handed gibbon [2]. However, the genotypes identified in the present study differed from those found in the study performed in China. Specifically, genotypes Type IV, EbpA, O, CM16, CM10, CM11, and CM18 were found in the ring-tailed lemur, whereas genotypes EbpC, EbpA, BEB4, and CM17 were found in the white-handed gibbon [2]. Other studies have also found evidence of *E. bieneusi* in NHP's. In a study from China, 12.5% of the 369 fecal samples from NHP's tested positive for *E. bieneusi*, specifically from rhesus macaques and northern white-

cheeked gibbons [30]. A study on captive and semi-captive NHP's in Côte d'Ivoire, Sierra Leone, and Peru, in a total of 116 specimens, only detected one positive animal, a sooty mangabey, from Côte d'Ivoire (4.2%; 1/24) [31]. An investigation carried out in six European zoological gardens from France, Germany, and Spain, involving 35 genera of NHP's (n=454) as well as their zookeepers (n=70), detected an occurrence of *E. bieneusi* of 0.9% in NHP's: two gorillas, a saguinus and a saimiri [32]. The occurrences observed in the different mentioned studies, including ours, show occurrences ranging from 0.9% to 29.8%. It is noteworthy that caution should be taken when analyzing the results obtained in this study, given the small sample size of NHP's. Moreover, in the present study, only feces with a well-formed structure and no other signs of gastrointestinal disease were collected which may have reduced the number of detected *E. bieneusi*. Lastly, genomic DNA isolation from feces is known to be difficult, possible inhibitors in feces may affect the results of the study and produce false negatives.

BLAST analysis of the sequence derived from the white-handed gibbon showed 100% identity with *E. bieneusi* sequence genotype ERUH4 from a horse from Turkey (MT193680). The analysis of the entire ITS region allowed the confirmation of the sequence from the white-handed gibbon as genotype ERUH4. This sequence differs in a single SNP (single nucleotide polymorphism) with genotype BEB6. On the other hand, the sequence retrieved from the ring-tailed lemur showed highest identity (97.02%) with sequences of *E. bieneusi* genotype BEB6 from a calf from Bangladesh (MK982508), and a tan sheep from China (MK322762). Further analysis of the ITS region, supported the existence of a new genotype, named "GASRJ". Despite the white-handed gibbon and the ring-tailed lemur belonging to the same zoo, they do not share enclosures. The fact that positive animals do not share the same enclosure, and the sequences exhibit a 96.33% pairwise nucleotide distance, may suggest that the origin of the positive samples is distinct.

The phylogenetic analysis of the ITS amplicons obtained in this study revealed that the sequences clustered with the group containing potentially zoonotic genotypes of *E. bieneusi*, Group 2. Initially considered ruminant-adapted, Group 2 genotypes have since been found in humans and various other animals, including NHP's [20, 10]. Group 1 is the largest group, encompassing genotypes found in both humans and animals. Genotypes within Groups 3 to 13 appear to be more host-adapted and exhibit limited zoonotic potential [20].

The results of the present study enhance our understanding of *E. bieneusi* epidemiology among captive animals in Portugal. The detection of genotypes belonging to the

potential zoonotic Group 2 in NHP's from a zoo underscores their potential role in transmitting this microsporidian to other zoo animals and humans, including zookeepers and visitors. Further studies are required to comprehensively assess the epidemiology of *E. bieneusi* and its impacts, particularly in zoo settings. This is crucial not only due to its potential zoonotic implications but also for the veterinary health of the inhabitants of the zoo, especially considering that the pathological features are poorly understood.

5. Conclusions

In conclusion, animals from 39 different families, including birds, mammals, reptiles, amphibians, and arthropods, from two zoological establishments were tested for *E. bieneusi*, with only NHP's testing positive. The identification of *E. bieneusi* genotypes from Group 2 in NHP's highlights the necessity for additional research to evaluate the zoonotic potential of the identified genotypes. Regarding zoos, it's important to investigate potential transmission routes and implement strategies for disease prevention and control, including appropriate handling and management practices.

Author Contributions: G.M. C data curation, resources, formal analysis, investigation, validation, visualization, writing – original draft, writing – review & editing; A.V.S.C data curation, formal analysis, validation, visualization, writing – original draft, writing – review & editing; S.S.-S. data curation, formal analysis, validation, visualization, writing – original draft, writing – review & editing; R.S.S.M. data curation, resources, investigation, writing – review & editing; J.R.M. conceptualization, funding acquisition, methodology, project administration, resources, supervision, validation, visualization, writing – original draft, writing – review & editing.

Funding: Andreia V. S. Cruz and Sérgio Santos-Silva thank Fundação para a Ciência e a Tecnologia (FCT – Portuguese Foundation for Science and Technology) for the financial support of their PhD works under the Maria de Sousa scholarships 2022.15408.BD and 2021.09461.BD, respectively

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We would like to extend our gratitude to Maia Zoo and Pedagogical Farm of Canelas for their generous permission and collaboration in the sample collection process. 270 271

Conflicts of Interest: The authors declare no conflicts of interest. 272

273

274

275

276

277

278

References

1. Han, B.; Pan, G.; Weiss, L.M. Microsporidiosis in Humans. Clin. Microbiol. Rev. 2021, 34, doi:https://doi.org/10.1128/CMR.00010-20.
2. Karim, M.R.; Dong, H.; Li, T.; Yu, F.; Li, D.; Zhang, L.; Li, J.; Wang, R.; Li, S.; Li, X.; et al. Predomination and New Genotypes of *Enterocytozoon bieneusi* in Captive Nonhuman Primates in Zoos in China: High Genetic Diversity and Zoonotic Significance. PLoS One 2015, 10, e0117991, doi:10.1371/journal.pone.0117991.
3. Desportes, I.; Charpentier, Y. Le; Galian, A.; Bernard, F.; Cochand-Priollet, B.; Lavergne, A.; Ravisse, P.; Modigliani, R. 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.
4. Ten Hove, R.J.; Van Lieshout, L.; Beadsworth, M.B.J.; Perez, M.A.; Spee, K.; Claas, E.C.J.; Verweij, J.J. Characterization of genotypes of *Enterocytozoon bieneusi* in immunosuppressed and immunocompetent patient groups. J. Eukaryot. Microbiol. 2009, 56, 388–393, doi:10.1111/J.1550-7408.2009.00393.X.
5. Liguory, O.; Sarfati, C.; Derouin, F.; Molina, J.M. Evidence of Different *Enterocytozoon bieneusi* Genotypes in Patients with and without Human Immunodeficiency Virus Infection. J. Clin. Microbiol. 2001, 39, 2672, doi:10.1128/JCM.39.7.2672-2674.2001.
6. Sadler, F.; Peake, N.; Borrow, R.; Rowl, P.L.; Wilkins, E.G.L.; Curry, A. Genotyping of *Enterocytozoon bieneusi* in AIDS patients from the north west of England. J. Infect. 2002, 44, 39–42, doi:10.1053/jinf.2001.0934.
7. Akinbo, F.O.; Okaka, C.E.; Omoregie, R.; Dearen, T.; Leon, E.T.; Xiao, L. Molecular Epidemiologic Characterization of *Enterocytozoon bieneusi* in HIV-Infected Persons in Benin City, Nigeria. Am. J. Trop. Med. Hyg. 2012, 86, 441, doi:10.4269/AJTMH.2012.11-0548.
8. Santín, M.; Fayer, R. Microsporidiosis: *Enterocytozoon bieneusi* in domesticated and wild animals. Res. Vet. Sci. 2011, 90, 363–371, doi:10.1016/J.RVSC.2010.07.014.
9. Karim, M.R.; Wang, R.; Dong, H.; Zhang, L.; Li, J.; Zhang, S.; Rume, F.I.; Qi, M.; Jian, F.; Sun, M.; et al. Genetic polymorphism and zoonotic potential of *Enterocytozoon bieneusi* from nonhuman primates in China. Appl. Environ. Microbiol. 2014, 80, 1893–1898, doi:10.1128/AEM.03845-13.
10. Li, W.; Xiao, L. Ecological and public health significance of *Enterocytozoon bieneusi*. One Heal. 2021, 12, doi:10.1016/j.onehlt.2020.100209.
11. Li, W.; Diao, R.; Yang, J.; Xiao, L.; Lu, Y.; Li, Y.; Song, M. High diversity of human-pathogenic *Enterocytozoon bieneusi* genotypes in swine in northeast China. Parasitol. Res. 2014, 113, 1147–1153, doi:10.1007/S00436-014-3752-9.

12. Ghosh, K.; Weiss, L.M. Molecular diagnostic tests for microsporidia. Interdiscip. Perspect. Infect. Dis. 2009, 2009, 1–13, doi:10.1155/2009/926521. 307 308
13. Anane, S.; Attouchi, H. Microsporidiosis: epidemiology, clinical data and therapy. Gastroenterol. Clin. Biol. 2010, 34, 450–464, doi:10.1016/J.GCB.2010.07.003. 309 310
14. Li, W.; Feng, Y.; Xiao, L. *Enterocytozoon bieneusi*. Trends Parasitol. 2022, 38, 95–96, doi:10.1016/j.pt.2021.08.003. 311
15. Santín, M.; Fayer, R. Microsporidiosis: *Enterocytozoon bieneusi* in domesticated and wild animals. Res. Vet. Sci. 2011, 90, 363–371, doi:10.1016/j.rvsc.2010.07.014. 312 313
16. Li, W.; Feng, Y.; Xiao, L. Diagnosis and molecular typing of *Enterocytozoon bieneusi*: the significant role of domestic animals in transmission of human microsporidiosis. Res. Vet. Sci. 2020, 133, 251–261, doi:https://doi.org/10.1016/j.rvsc.2020.09.030. 314 315
17. Karim, M.R.; Yu, F.; Li, J.; Li, J.; Zhang, L.; Wang, R.; Rume, F.I.; Jian, F.; Zhang, S.; Ning, C. First molecular characterization of enteric protozoa and the human pathogenic microsporidian, *Enterocytozoon bieneusi*, in captive snakes in China. Parasitol. Res. 2014, 113, 3041–3048, doi:10.1007/s00436-014-3967-9. 316 317 318
18. Ding H, Zhao A, Wang L, Gao N, Sun Y, Li J, Qi M. Genotypes and zoonotic potential of Enterocytozoon bieneusi in edible bullfrogs (Lithobates catesbeiana) in China. Int J Parasitol Parasites Wildl. 2020 Jan 13;11:103-107. doi: 10.1016/j.ijppaw.2020.01.004. PMID: 32051812; PMCID: PMC7005328. 319 320 321
19. Zhang, T.; Yu, K.; Xu, J.; Cao, W.; Wang, Y.; Wang, J.; Zhou, L.; Chen, J.; Huang, H.; Zhao, W. *Enterocytozoon bieneusi* in Wild Rats and Shrews from Zhejiang Province, China: Occurrence, Genetic Characterization, and Potential for Zoonotic Transmission. Microorganisms 2024, 12. 322 323 324
20. Li, W.; Feng, Y.; Santin, M. Host Specificity of *Enterocytozoon bieneusi* and Public Health Implications. Trends Parasitol. 2019, 35, 436–451, doi:10.1016/j.pt.2019.04.004. 325 326
21. Sak, B.; Kváč, M.; Petrželková, K.; Květoňová, D.; Pomajbíková, K.; Mulama, M.; Kiyang, J.; Modrý, D. Diversity of microsporidia (Fungi: Microsporidia) among captive great apes in European zoos and African sanctuaries: evidence for zoonotic transmission? Folia Parasitol. (Praha). 2011, 58, 81–86, doi:10.14411/FP.2011.008. 327 328 329
22. Lobo, M.L.; Teles, A.; Da Cunha, M.B.; Henriques, J.; Lourenço, A.M.; Antunes, F.; Matos, O. Microsporidia detection in stools from pets and animals from the zoo in Portugal: a preliminary study. J. Eukaryot. Microbiol. 2003, 50 Suppl, 581–582, doi:10.1111/J.1550-7408.2003.TB00638.X. 330 331 332
23. Buckholt, M.A.; Lee, J.H.; Tzipori, S. Prevalence of *Enterocytozoon bieneusi* in Swine: an 18-Month Survey at a Slaughterhouse in Massachusetts. Appl. Environ. Microbiol. 2002, 68, 2595, doi:10.1128/AEM.68.5.2595-2599.2002. 333 334

24. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol. Biol. Evol. 2018, 35, 1547–1549, doi:10.1093/molbev/msy096. 335 336
25. Letunic, I.; Bork, P. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Res. 2019, 47, W256–W259, doi:10.1093/NAR/GKZ239. 337 338
26. Figueiredo, A.M.; Dashti, A.; Santín, M.; Köster, P.C.; Torres, R.T.; Fonseca, C.; Mysterud, A.; Carvalho, J.; Sarmento, P.; Neves, N.; et al. Occurrence and molecular characterization of *Enterocytozoon bieneusi* in wild and domestic animal species in Portugal. Med. Mycol. 2023, 61. 339 340 341
27. Lobo, M.L.; Xiao, L.; Cama, V.; Stevens, T.; Antunes, F.; Matos, O. Genotypes of *Enterocytozoon bieneusi* in Mammals in Portugal. J. Eukaryot. Microbiol. 2006, 53, S61–S64, doi:10.1111/j.1550-7408.2006.00174.x. 342 343
28. Lobo, M.L.; Xiao, L.; Cama, V.; Magalhães, N.; Antunes, F.; Matos, O. Identification of Potentially Human-Pathogenic *Enterocytozoon bieneusi* Genotypes in Various Birds. Appl. Environ. Microbiol. 2006, 72, 7380–7382, doi:10.1128/AEM.01394-06. 344 345
29. Sulaiman, I.M.; Fayer, R.; Yang, C.; Santin, M.; Matos, O.; Xiao, L. Molecular characterization of *Enterocytozoon bieneusi* in cattle indicates that only some isolates have zoonotic potential. Parasitol. Res. 2004, 92, 328–334, doi:10.1007/s00436-003-1049-5. 346 347
30. Zhong, Z.; Li, W.; Deng, L.; Song, Y.; Wu, K.; Tian, Y.; Huang, X.; Hu, Y.; Fu, H.; Geng, Y.; et al. Multilocus Genotyping of *Enterocytozoon bieneusi* Derived from Nonhuman Primates in Southwest China. PLoS One 2017, 12, e0176926, doi:10.1371/journal.pone.0176926. 348 349 350
31. Köster, P.C.; Lapuente, J.; Pizarro, A.; Prieto-Pérez, L.; Pérez-Tanoira, R.; Dashti, A.; Bailo, B.; Muadica, A.S.; González-Barrio, D.; Calero-Bernal, R.; et al. Presence and Genetic Diversity of Enteric Protists in Captive and Semi-Captive Non-Human Primates in Côte d'Ivoire, Sierra Leone, and Peru. Int. J. Parasitol. Parasites Wildl. 2022, 17, 26–34, doi:10.1016/j.ijppaw.2021.12.004. 351 352 353
32. Köster, P.C.; Martínez-Nevado, E.; González, A.; Abelló-Poveda, M.T.; Fernández-Bellon, H.; de la Riva-Fraga, M.; Marquet, B.; Guéry, J.-P.; Knauf-Witzens, T.; Weigold, A.; et al. Intestinal Protists in Captive Non-Human Primates and Their Handlers in Six European Zoological Gardens. Molecular Evidence of Zoonotic Transmission. Front. Vet. Sci. 2021, 8, 819887, doi:10.3389/fvets.2021.819887. 354 355 356 357

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions, or products referred to in the content. 358 359 360

Supplementary Materials:

Table 1. Distribution of animal species used in this study.

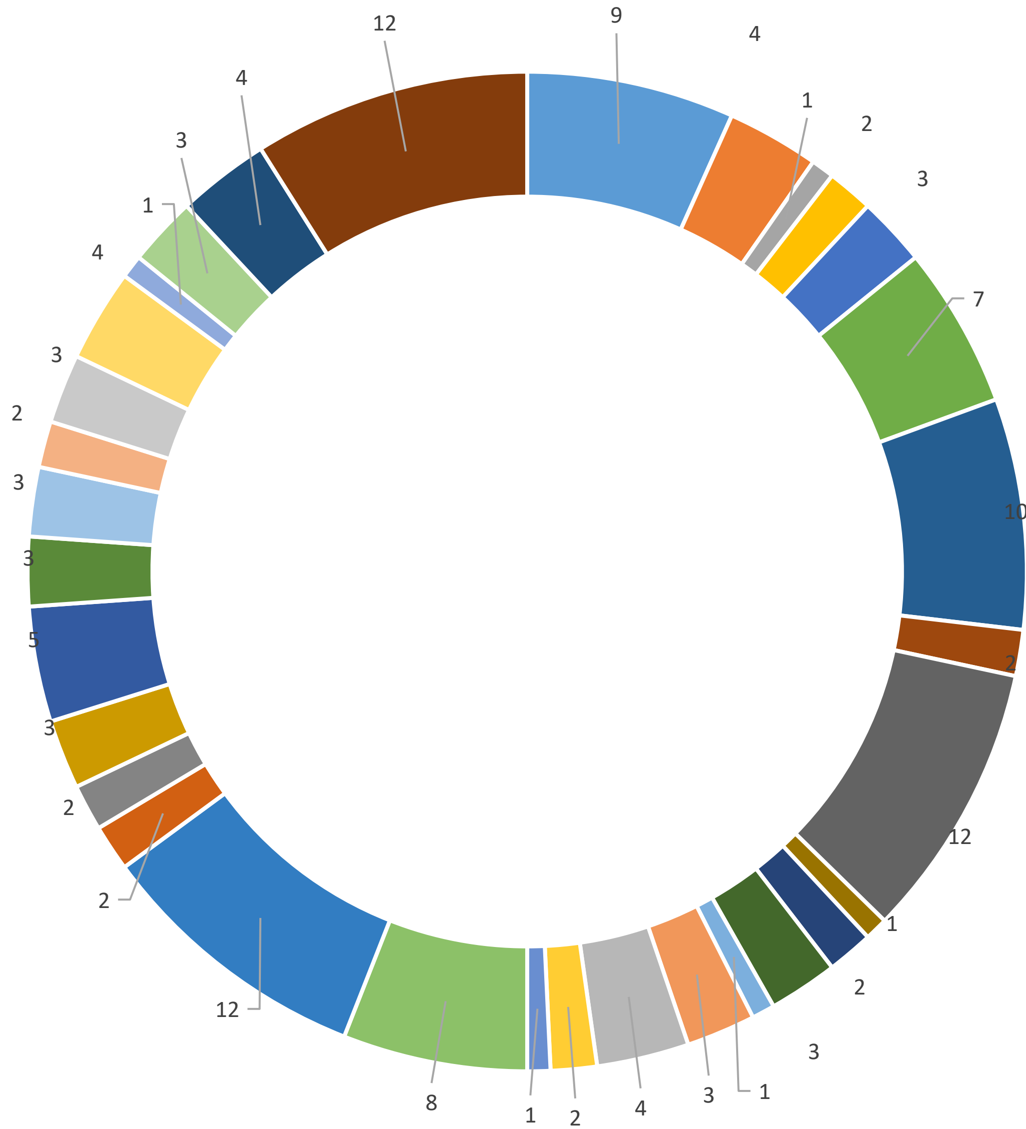
Class	Family	Common name (Scientific name)	Nr samples (n)	Origin
Amphibia	Ambystomatidae	Axolotl (*Ambystoma mexicanum*)	3	Maia Zoo
	Hylidae	Red-eyed tree frog (*Agalychnis callidryas*)	1	Maia Zoo
Aves	Anatidae	Mallard duck (*Anas platyrhyncus*)	2	Pedagogical Farm of Canelas
			1	Maia Zoo
		Greylag goose (*Anser anser*)	1	Pedagogical Farm of Canelas
		Egyptian goose (*Alopochen aegyptiaca*)	1	Maia Zoo
		Mandarin duck (*Aix galericulata*)	1	Maia Zoo
	Casuariidae	Emu (*Dromaius novaehollandiae*)	1	Maia Zoo
	Cracidae	Great curassow (*Crax rubra*)	1	Maia Zoo
		Bare-faced curassow (*Crax fasciolata Spix*)	1	Maia Zoo
	Estrildidae	Gouldian finch (*Chloebia gouldiae*)	1	Maia Zoo
	Gruidae	Grey crowned crane (*Balearica regulorum*)	1	Maia Zoo
		Red-crowned crane (*Grus japonensis*)	1	Maia Zoo
	Phasianidae	Indian peafowl (*Pavo cristatus*)	1	Maia Zoo
		Chicken (*Gallus gallus*)	4	Pedagogical Farm of Canelas
		Wild turkey (*Meleagris gallopavo*)	1	Pedagogical Farm of Canelas
		Golden pheasant (*Chrysolophus pictus*)	1	Pedagogical Farm of Canelas
	Phoenicopteridae	Lesser flamingo (*Phoenicopterus minor*)	1	Maia Zoo
	Psittacidae	Blue-and-yellow macaw (*Ara ararauna*)	1	Maia Zoo
		African grey parrot (*Psittacus erithacus erithacus*)	1	Maia Zoo
		Burrowing parrot (*Cyanoliseus patagonus*)	1	Maia Zoo
		Rainbow lorikeet (*Trichoglossus haematodus*)	1	Maia Zoo
	Psittaculidae	Grey parrot (*Psittacus grandis*)	1	Maia Zoo
	Rheidae	Greater rhea (*Rhea americana*)	1	Maia Zoo
Diplopoda	Spirostreptidae	Giant centipede (*Archispirostreptus gigas*)	1	Pedagogical Farm of Canelas
Mammalia	Bovidae	Domestic goat (*Capra hicus*)	6	Pedagogical Farm of Canelas
		Domestic goat (*Capra hicus*)	1	Maia Zoo
		Domestic sheep (*Ovis aries*)	5	Pedagogical Farm of Canelas
	Callithrichidae	Common marmoset (*Callithrix jacchus*)	3	Maia Zoo

	Camelidae	Alpaca (*Vicugna pacos*)	1	Maia Zoo
	Cebidae	Capuchin monkey (*Cebus capucinus*)	1	Maia Zoo
	Cercopithecidae	Vervet monkey (*Chlorocebus pygerythrus*)	1	Maia Zoo
		Red-tailed monkey (*Cercopithecus ascanius*)	1	Maia Zoo
	Cervidae	Muntjac (*Muntiacus muntjak*)	1	Maia Zoo
	Didelphidae	Virginia opossum (*Didelphis marsupialis*)	1	Maia Zoo
	Equidae	Horse (*Equus caballus*)	24	Pedagogical Farm of Canelas
		Donkey (*Equus asinus*)	1	Pedagogical Farm of Canelas
		Grevy's zebra (*Equus grevyi*)	1	Maia Zoo
	Felidae	Lion (*Panthera leo*)	2	Maia Zoo
		Tiger (*Panthera tigris*)	3	Maia Zoo
		Eurasian lynx (*Lynx lynx*)	1	Maia Zoo
	Herpestidae	Meerkat (*Suricata suricatta*)	1	Maia Zoo
	Hylobatidae	White-handed gibbon (*Hylobates lar*)	2	Maia Zoo
	Hystricidae	African crested porcupine (*Hystrix africaeaustralis*)	1	Maia Zoo
	Lemuridae	Ring-tailed lemur (*Lemur catta*)	1	Maia Zoo
		Common brown lemur (*Eulemur fulvus*)	1	Maia Zoo
		Ruffed lemur (*Varecia variegata*)	1	Maia Zoo
	Leporidae	Domestic rabbit (*Oryctolagus cuniculus domesticus*)	2	Maia Zoo
	Macropodidae	Red-necked wallaby (*Macropus rufogriseus*)	1	Maia Zoo
	Mustelidae	Oriental small-clawed otter (*Aonyx cinereus*)	2	Maia Zoo
	Suidae	Domestic pig (*Sus domesticus*)	3	Pedagogical Farm of Canelas
	Ursidae	Brown bear (*Ursus arctos*)	1	Maia Zoo
Reptilia	Agamidae	Bearded dragon (*Pogona vitticeps*)	2	Maia Zoo
			2	Pedagogical Farm of Canelas
		Saharan uromastyx (*Uromastix geyri*)	1	Maia Zoo
	Anguidae	European legless lizard (*Ophisaurus apodus*)	1	Maia Zoo
	Boidae	Yellow anaconda (*Eunectes notaeus*)	1	Maia Zoo
	Colubridae	California kingsnake (*Lampropeltis getula californiae*)	2	Maia Zoo
		Milk snake (*Lampropeltis triangulum*)	1	Maia Zoo
		Western rat snake (*Pantherophis obsoletus*)	1	Maia Zoo
		Corn snake (*Pantherophis guttatus*)	1	Maia Zoo
	Eublepharidae	Leopard gecko (*Eublepharis macularis*)	1	Pedagogical Farm of Canelas
	Gerrhosauridae	Giant plated lizard (*Zonosaurus maximus*)	1	Maia Zoo
	Iguanidae	Green iguana (*Iguana Iguana*)	2	Maia Zoo
	Pythonidae	Ball python (*Python regius*)	1	Maia Zoo
		Carpet python (*Morelia spilota variegata*)	2	Maia Zoo

		Burmese python (*Python biviattus*)	3	Maia Zoo
		Reticulated python (*Python reticulatus*)	1	Maia Zoo
	Scincidae	Eastern blue-tongued skink (*Tiliqua scincoides*)	1	Maia Zoo
		Eastern water skink (*Egernia striolata*)	1	Maia Zoo
	Teiidae	Nile monitor (*Salvator marinae*)	1	Maia Zoo
	Testunidae	African spurred tortoise (*Geochelone sulcata*)	1	Maia Zoo
Total			127	

Annex B – activities performed at PBG-CRF

	Procedures	Aves	Mammalia	Reptilia	Total
Imaging	X-ray	90	20	5	115
	Endoscopy	2			2
Lab Work	Hematology	52	9	1	62
	Diff Quick stain	30	5	1	36
	Gram stain	18	2	NA	20
	Coprology	250	38	4	292
	Urinalysis	NA	4	NA	4
Administration	Intramuscular	71	36	3	110
	Endovenous	48	34	NA	82
	Subcutaneous	53	36	1	90
	Oral	90	30	2	122
Others	Physical exams	70	22	9	101
	Blood draws	20	10	1	31
	Catheterizations	31	13	NA	44
	Anesthesia	75	43	1	119
	Surgery	5	2	NA	7
	Wound cleanup	23	15	NA	38
	Bandaging	26	14	NA	40
	Euthanasia	43	13	NA	56
	Gavage	67	12	NA	79
	Ophtalmological exa	11	2	NA	13
	Total				1463


Necropsies Performed
9
4
1
2
3
7
10
2
12
1
2
3
1
3
4
2
1
8
12
2
2
3
5
3
3
2
3
4
1
3
4
12
Accipiter nisus
Alca torda
Apodidae
Athene noctua
Bats (various)
Birds (Others)
Buteo buteo
Carduelis carduelis
Ciconia ciconia
Circus pygargus
Columba livia
Corvus corone
Erinaceus europaeus
Falco tinnunculus
Galliformes
Hieraaetus pennatus
Laurus cannus
Laurus michaelis
Mammals (others)
Morus bassanus
Nycticorax nycticorax
Oryctolagus cunniculus
Passeriforme
Perdix perdix
Pica pica
Pyrrhura
Rattus noverigcus
Rhinechis scalaris
Streptotelia decaocto
Trachemys
Uria aalge
Vulpes vulpes

Accipiter nisus	9
Alca torda	4
Apodidae	1
Athene noctua	2
Bats (various)	3
Birds (Others)	7
Buteo búteo	10
Carduelis carduelis	2
Ciconia ciconia	12
Circus pygargus	1
Columba livia	2
Corvus corone	3
Erinaceus europaeus	1
Falco tinnunculus	3
Galliformes	4
Hieraaetus pennatus	2
Laurus cannus	1
Laurus michaelis	8
Mammals (others)	12
Morus bassanus	2
Nycticorax nycticorax	2
Oryctolagus cunniculus	3
Passeriforme	5
Perdix perdix	3
Pica pica	3
Pyrrhura	2
Rattus noverigcus	3
Rhinechis scalaris	4
Streptotelia decaocto	1
Trachemys	3
Uria aalge	4
Vulpes vulpes	12

Coprologies performed

- Accipiter gentilis
- Accipiter nisus
- Agapornis
- Alca torda
- Amazona leucocephala
- Anas platyrrhincus
- Anser anser
- Ara ararauna
- Ara macau
- Athene noctua
- Birda (other)
- Bos taurus
- Bufus bufus
- Buteo buteo
- Carduelis carduelis
- Ciconia ciconia
- Columba livia
- Columba palamus
- Corvus corax
- Corvus corone
- Erinaceus europaeus
- Falco peregrinus
- Falco subbuteo
- Falco tinnuncultus
- Galliformes
- Hiraaetus pennatus
- Laurus cannus
- Laurus fuscus
- Laurus michaelis
- Milvus milvus
- Morus bassanus
- Mustela furo
- Mustela lutrola
- Nycticorax nycticorax
- Oryctolagus cuniculis
- Parabuteo unicinctus
- perdix perdix
- Phasianus colchicus
- Pica pica
- Pluvialis squatarola
- Pyrrhura
- Rattus novergicus
- Streptotelia decaocto
- Strix aluco
- Terrestrial Turtle
- Trachemys scripta
- Turtles (various)
- Tyto alba
- Uria aalge
- Uria aalge
- Vulpes vulpes

Accipiter gentilis	1
Accipiter nisus	9
Agapornis	3
Alca torda	5
Amazona leucocephala	1
Anas platyrrhincus	3
Anser anser	1
Ara ararauna	7
Ara macau	3
Athene noctua	6
Birda (other)	30
Bos taurus	2
Bufus bufus	1
Buteo buteo	7
Carduelis carduelis	4
Ciconia ciconia	5
Columba livia	2
Columba palamus	2
Corvus corax	4
Corvus corone	16
Erinaceus europaeus	30
Falco peregrinus	15
Falco subbuteo	2
Falco tinnucultus	4
Galliformes	25
Hiraaetus pennatus	2
Laurus cannus	1
Laurus fuscus	10
Laurus michaelis	20
Milvus milvus	2
Morus bassanus	1
Mustela furo	3
Mustela lutrola	1
Nycticorax nycticorax	9
Oryctolagus cuniculis	4
Parabuteo unicinctus	2
perdix perdix	8
Phasianus colchicus	2
Pica pica	7
Pluvialis squatarola	2
Pyrrhura	2
Rattus novergicus	2
Streptotelia decaocto	6

Strix aluco	7
Terrestrial Turtle	7
Trachemys scripta	11
Turtles (various)	15
Tyto alba	5
Uria aalge	2
Uria aalge	7
Vulpes vulpes	5

Detection of *Enterocytozoon bieneusiEnterocytozoon bieneusi*

in non-human primates in Portuguese Zoos

Guilherme Ramos Moreira

ICBAS