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Faculty of Tropical AgriSciences

The Occurrence of Apicomplexan Parasites in the Selected Representatives of the Felinae Subfamily at the Zoos in the Czech Republic

MASTER'S THESIS

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DIPLOMA THESIS ASSIGNMENT

Bc. Anita Bírošíková

Wildlife and Livestock Production, Management and Conservation
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Thesis title

The Occurrence of Apicomplexan Parasites in the Selected Representatives of the Felinae Subfamily at the Zoos in the Czech Republic

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Objectives of thesis

The aim of the work will be to determine the occurrence of coccidia of the species Toxoplasma gondii and other zoonotic parasitic pathogens of the genus Cryptosporidium, Giardia, Toxocara and Toxascaris, which will be detected by coprological and molecular analysis (PCR) to evaluate the current state in comparison with previous surveys from the Czech Republic.

Methodology

Faecal samples will be taken from selected members of the Felinae subfamily at zoos and examined by parasitological methods and PCR analyzes in cooperation with the laboratories of the Institute of Parasitology of the Biological Center CAS in České Budějovice. Positive oocyst findings will be recorded in protocols to identify sources and routes of spread of developmental stages of parasitic pathogens.

The proposed extent of the thesis

40

Keywords

Toxoplasma gondii, toxoplasmosis, Cryptosporidium, Giardia, Toxocara, Toxascaris, zoonoses, zoo animals, small felines

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Recommended information sources

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Prague on 22. 04. 2022

Declaration

I hereby declare that I have done this thesis entitled “The Occurrence of Apicomplexan Parasites in the Selected Representatives of the Felinae Subfamily at the Zoos in the Czech Republic” independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA CZU in Prague.

2022, Prague

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Anita Bírošíková

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Abstract

The presented diploma thesis was focused on the occurrence of apicomplexan parasites *Toxoplasma gondii* and *Cryptosporidium* spp. with zoonotic potential in the faeces of selected representatives of the Felinae subfamily at the Czech zoos, with a significant impact on the health of bred animals and public health.

Regular faecal sampling was carried out from June to August 2021 at the Zoo Jihlava and the Zoo Olomouc. A total of 222 samples were taken from eight species of small felines. The samples were examined by flotation centrifugation method, and to diagnose *Cryptosporidium* spp. also by aniline-carbol-methyl violet staining and subsequently microscopically examined. PCR molecular analysis was performed to confirm the parasitic findings of the phylum Apicomplexa and the accompanying parasitic pathogens.

Positive (n=23) and dubious (n=3) findings of *Toxoplasma gondii* oocysts were microscopically detected from samples examined by flotation, and PCR analysis confirmed their occurrence in three bred individuals of Pallas's cat (*Otocolobus manul*) from the Zoo Jihlava.

The staining technique revealed 22 positive and seven dubious findings of *Cryptosporidium* spp. oocysts and according to PCR analysis, the occurrence of *Cryptosporidium felis* (n=10) was subsequently demonstrated from three species of small felines, at both zoos.

Nematode eggs (n=49), the amount of which was quantified by EPG using a modified McMaster's method, were diagnosed by the flotation method. The highest intensity of *Toxocara cati* infection was recorded in Pallas's cat (6,225 EPG) and *Toxascaris leonina* in cheetah (19,725 EPG). The findings of parasitic co-infections of *Giardia intestinalis*, *Toxocara cati* and *Toxascaris leonina* were confirmed by the PCR molecular method. The obtained sequences were 100% identical to the reference sequences stored in the GenBank database. The sources and routes of spreading parasitic pathogens were pointed out and measures were proposed to ensure biosecurity at both Czech zoos.

Key words: *Toxoplasma gondii*, *Cryptosporidium felis*, zoonoses, zoo animals, small felines

Abstrakt

Předložená diplomová práce byla zaměřena na výskyt apikomplexních parazitů *Toxoplasma gondii* a *Cryptosporidium* spp. se zoonotickým potenciálem v trusu vybraných zástupců Felinae v českých zoologických zahradách, s významným vlivem na zdraví chovaných zvířat i zdraví veřejné.

Pravidelný odběr vzorků trusu byl proveden od června do srpna roku 2021 v Zoo Jihlava a Zoo Olomouc. Celkem bylo odebráno 222 vzorků od osmi druhů malých kočkovitých šelem. Vzorky byly vyšetřeny flotačně centrifugační metodou a k diagnostice oocyst *Cryposporidium* spp. i barvící technikou anilin-karbol-metylvioletí a následně mikroskopicky zkoumány. Pro potvrzení parazitárních nálezů rodů kmene Apicomplexa a doprovodných parazitárních patogenů byla provedena PCR molekulární analýza.

Mikroskopicky byly zjištěny ze vzorků zpracovaných flotací pozitivní (n=23) a dubiózní (n=3) nálezy oocyst ***Toxoplasma gondii*** a PCR analýza potvrdila jejich výskyt u tří chovaných jedinců manulů (*Otocolobus manul*) ze Zoo Jihlava.

Barvící technika odhalila 22 pozitivních a sedm dubiózních nálezů oocyst *Cryptosporidium* spp. a dle PCR analýzy byl následně prokázán i výskyt ***Cryptosporidium felis*** (n=10) od tří druhů koček, v obou zoologických zahradách.

Flotační metodou byla diagnostikována vajíčka hlístic (n=49), jejichž množství bylo kvantitativně stanoveno EPG pomocí modifikované McMasterovy metody. Nejvyšší intenzita infekce *Toxocara cati* byla zaznamenána u manula (6 225 EPG) a *Toxascaris leonina* u geparda (19 725 EPG). Nálezy parazitárních coinfekcí druhu *Giardia intestinalis*, *Toxocara cati* a *Toxascaris leonina* byly potvrzeny molekulární metodou PCR. Získané sekvence byly 100% shodné s referenčními sekvencemi uloženými v databázi GenBank. Bylo poukázáno na zdroje a cesty šíření parazitárních patogenů a navržena opatření k zajištění biosecurity v obou českých zoologických zahradách.

Klíčová slova: *Toxoplasma gondii*, *Cryposporidium felis*, zoonózy, zoo zvířata, malé kočkovité šelmy

Contents

List of tables

List of figures

List of the abbreviations used in the thesis

AIDS – acquired immunodeficiency syndrome

APB Research Team – Animal Physiology and Behaviour Research Team

BioLib – Biological Library

BSA – bovine serum albumin

CAS – Czech Academy of Sciences

CDC – Centers for Disease Control and Prevention

CNS – central nervous system

CR – Czech Republic

CZU – Czech University of Life Sciences

DNA – deoxyribonucleic acid

dNTPs – deoxynucleoside triphosphate

e.g. – exempli gratia (for example)

ECDC – European Centre for Disease Prevention and Control

EEA – European Economic Area

EPG – eggs per gram

EU – European Union

FTA – Faculty of Tropical AgriScience

HIV – human immunodeficiency virus

IFAT – indirect fluorescent antibody test

min – minute

NFS standard – National Sanitation Foundation standard

no. – numero (number)

PCR – polymerase chain reaction

RFLP – restriction fragment length polymorphism

sp. – species (singular)

spp. – species (plural)

SSU rRNA – small subunit ribosomal RNA

UV light – ultraviolet light

Zoo – zoological garden

1 Introduction and Literature Review

1.1 Introduction

Intracellular parasite representatives of the phylum Apicomplexa have implications for public health, hence it is necessary to pay attention to their zoonotic potential (Bowman et al. 2002). This thesis is particularly focused on *Toxoplasma gondii* (Nicolle & Manceaux 1908) and *Cryptosporidium felis* (Iseki 1979), which have cats as the definitive host (Frenkel 1970; Iseki 1979; Bowman et al. 2002), however, it can be likewise transmitted to humans, as described by Lucio-Forster et al. (2010), who reported a small number of cases of cryptosporidiosis in humans and by Dubey (2020), who was focused on toxoplasmosis.

Infection with *Cryptosporidium felis*, found and described by Iseki in 1979, can be symptomatic (Bowman et al. 2002) as well as asymptomatic (Fayer et al. 2006). This is also the case with *Toxoplasma gondii* infection. Dubey & Carpenter (1993); Bowman et al. (2002) and Terio et al. (2018) described symptomatic cases and Kenny et al. (2002) asymptomatic. In terms of asymptomatic infection, zookeepers should be very careful when approaching the animals, removing their faeces and cleaning the enclosure, because even at first glance a healthy animal can infect them. This is especially dangerous for female zookeepers, which are in constant contact with animals, putting themselves at risk of infection. If they become *Toxoplasma* infected during pregnancy, the risk of transmission to the fetus is getting higher with each trimester (McAuley 2014; Dubey et al. 2021) and it can even lead to miscarriage or stillbirth (McAuley 2014).

No vaccine, safe to use in humans, is currently available neither for cryptosporidiosis (Zahedi & Ryan 2020) nor toxoplasmosis (Dubey 2020). Accordingly, prevention is the most important aspect to maintain a healthy environment for zookeepers as well as for zoo animals (EAZA 2013; CDC 2018; Dunowska 2019), as some animals are more susceptible to infection (Ketz-Riley et al. 2003) and breeding them can be hence unsuccessful.

For the reasons mentioned above, this issue needs to be addressed and the occurrence of zoonotic pathogens at the Czech zoos should be identified. Previous surveys of

Toxoplasma gondii in zoo animals at the Czech zoos used the direct flotation method (Lukešová & Literák 1998; Kudrnáčová 2014) or indirect fluorescent antibody test (Sedlák & Bártová 2006; Bártová et al. 2018). However, to clearly identify the pathogen, it is needed to perform PCR molecular detection, as mentioned also about *Cryptosporidium* spp. by Fayer et al. (2006), which has not been done in the past.

1.2 Literature Review

1.2.1 Felinae

1.2.1.1 Taxonomy

There are four subfamilies within the Felidae family. Two extinct Proailurinae, which are the ancestors of all Felidae, and Machairodontinae subfamily, both known exclusively thanks to fossils, for example of saber-toothed cats. The Pantherinae and **Felinae** subfamilies contain all currently living species as well as extinct fossil species connected to them (Bellani 2019).

The Pantherinae were believed to differ from the Felinae subfamily by the ability to roar, but not purr. It appeared to be caused by an elastic ligament in the hyoid apparatus, which is located beneath the tongue of the Pantherinae. By contrast, Felinae has a bony hyoid, so they can purr, but not roar. Recent research into the hyoid structure and vocal ability of cats revealed that this distinction is ambiguous because not all the big cats (Pantherinae) are able to roar (Kitchener et al. 2017). The structure of the larynx was discovered to be the important difference in the ability of roaring, but not purring (Hast 1989). Smaller cats have smaller vocal folds within the larynx that allow them to purr solely, but large cats have elasticated vocal folds that allow them to roar (Kitchener et al. 2017), despite the fact that not all can roar (Bellani 2019). Body size differences are not considered a credible factor of taxonomic diversity between them (Kitchener et al. 2017).

In the Felinae subfamily, there were various taxonomic classification issues, especially in the division of the *Felis* genus (Bellani 2019). The current Felinae consists of 12 genera (See Table 1): genus *Acinonyx*, *Caracal*, *Catopuma*, *Felis*, *Herpailurus*, *Leopardus*, *Leptailurus*, *Lynx*, *Otocolobus*, *Pardofelis*, *Prionailurus* and *Puma*.

Table 1 - Classification of current Felinae subfamily

Source: own processing according to Bellani (2019) and BioLib (2022)

Genus	Species
Acinonyx	*Acinonyx jubatus* (Cheetah)
Caracal	*Caracal aurata* (African Golden Cat), *Caracal caracal* (Caracal)
Catopuma	*Catopuma badia* (Bornean Bay Cat), *Catopuma temminckii* (Asiatic Golden Cat)
Felis	*Felis chaus* (Jungle Cat), *Felis margarita* (Sand Cat), *Felis nigripes* (Black-footed Cat), *Felis silvestris* (Wildcat)
Herpailurus	*Herpailurus yagouaroundi* (Jaguarundi)
Leopardus	*Leopardus braccatus* (Pantanal Cat), *Leopardus colocola* (Pampas Cat), *Leopardus emiliae* (Eastern Tigrina), *Leopardus garleppi* (Bolivian Pampas Cat), *Leopardus geoffroyi* (Geoffroy's cat), *Leopardus guigna* (Kodkod), *Leopardus guttulus* (Southern Tigrina), *Leopardus jacobita* (Andean Cat), *Leopardus munoai* (Uruguay Pampas Cat), *Leopardus pajeros* (Patagonian Pampas Cat), *Leopardus pardalis* (Ocelot), *Leopardus tigrinus* (Oncilla), *Leopardus wiedii* (Margay)
Leptailurus	*Leptailurus serval* (Serval)
Lynx	*Lynx canadensis* (Canadian Lynx), *Lynx lynx* (Eurasian Lynx), *Lynx pardinus* (Spanish Lynx), Lynx rufus (Bobcat)
Otocolobus	*Otocolobus manul* (Pallas's Cat)
Pardofelis	*Pardofelis marmorata* (Marbled Cat)
Prionailurus	*Prionailurus bengalensis* (Leopard Cat), *Prionailurus iriomotensis* (Iriomote Cat), *Prionailurus planiceps* (Flat-headed Cat), *Prionailurus rubiginosus* (Rusty-spotted Cat), *Prionailurus viverrinus* (Fishing Cat)
Puma	*Puma concolor* (Puma)

Many of them have a wide distribution. For instance, the European wildcat *Felis silvestris silvestris* (Schreber 1777) is distributed in Europe. In Sub-Saharan Africa, we can find African golden cat (*Caracal aurata* Temminck 1827), African wildcat *Felis silvestris lybica* (Forster 1780), Black-footed cat *Felis nigripes* (Burchell 1824), caracal *Caracal caracal* (Schreber 1776), cheetah *Acinonyx jubatus* (Schreber 1775), and serval *Leptailurus serval* (Schreber 1776). Pallas's cat *Otocolobus manul* (Pallas, 1776) with Eurasian lynx *Lynx lynx* (Linnaeus 1758) is in North Asia. Tropical Asia has a distribution of Asiatic golden cat *Catopuma temminckii* (Vigors & Horsfield 1827), Bornean bay cat

Catopuma badia (Gray 1874), fishing cat *Prionailurus viverrinus* (Bennett 1833), flat-headed cat *Prionailurus planiceps* (Vigors & Horsfield 1827), jungle cat *Felis chaus* (Schreber 1777), leopard cat *Prionailurus bengalensis* (Kerr 1792), marbled cat *Pardofelis marmorata* (Martin 1837), rusty-spotted cat *Prionailurus rubiginosus* (I. Geoffroy Saint-Hilaire 1831) and sand cat *Felis margarita* (Loche 1858). Bobcat *Lynx rufus* (Schreber 1777) with Canadian lynx *Lynx canadensis* (Kerr 1792) are from North America and South America is home to Geoffroy's cat *Leopardus geoffroyi* (d'Orbigny & Gervais 1844), jaguarundi *Herpailurus yagouaroundi* (É. Geoffroy Saint-Hilaire 1803), kodkod *Leopardus guigna* (Molina 1782), margay *Leopardus wiedii* (Schinz 1821), ocelot *Leopardus pardalis* (Linnaeus 1758), oncilla *Leopardus tigrinus* (Schreber 1775), Pampas cat *Leopardus colocolo* (Molina 1782) and puma *Puma concolor* (Linnaeus 1771) as well (O'Brien & Johnson 2007).

1.2.1.2 Distribution in human care in the CR

Small felines were bred in 14 breeding facilities (See Table 2) in the Czech Republic.

The most facilities bred Carpathian lynx, specifically nine including the Zoo Olomouc, from which the samples for this study were taken. The least bred cats, according to this table, would be South African cheetah, Pallas's Cat, Amur leopard cat and Palawan leopard cat, which were all only in three institutions.

The Zoo Jihlava was the institution with the most bred Felinae species. As can be seen in Table 2, the Zoo Jihlava had seven out of eight Felinae species, except for the South African cheetah. As stated in the Table 2, most individuals of South African cheetahs were kept at the Zoo Dvůr Králové. The Zoo Jihlava had the most individuals of European wildcats, the Zoo Ostrava with the Zoo Ústí nad Labem had the most Geoffroy's cats and the Zoo Ostrava kept the most servals. Pallas's Cat with four individuals were the most bred at the Zoo Jihlava, although there was a loss of one male in November 2021, so previously they had five animals in sum.

The Zoo Liberec had the highest number of Carpathian lynxes. Amur leopard cat was the only species with unidentified sexes of kittens and the most individuals were at the Zoo Prague.

At the Zoo Plzeň, four males and four females of Palawan leopard cat could be found, which were highest for both sexes.

Table 2 - Overview of breeding facilities with Felinae at the Czech zoos

Source: own processing according to Species360 (2021) and Species360 (2022)

Common name	Latin name	Institutions	Number of individuals
South African cheetah	*Acinonyx jubatus jubatus*	Zoo Dvur Kralove, a.s.	4,4
		Zoologicka zahrada Olomouc	1,1
		The Prague Zoological Garden	0,1
European wildcat	*Felis silvestris silvestris*	Zoo Decin - Pastyrska Stena	0,1
		Jihoceska zoologicka zahrada Hluboka nad Vltavou	1,0
		Zoologicka Zahrada Jihlava	3,1
		Zoologicka zahrada Olomouc	0,2
Geoffroy's cat	*Leopardus geoffroyi*	Zoologicka Zahrada Jihlava	2,0
		Zoologicka zahrada Olomouc	1,1
		Ostrava Zoological Garden and Botanical Park	2,1
		The Prague Zoological Garden	1,1
		Usti nad Labem Zoo	1,2
Serval	*Leptaiulurus serval*	Zoo Dvur Kralove, a.s.	1,1
		Zoologicka Zahrada Jihlava	1,2
		Zoologicka zahrada Olomouc	1,1
		Ostrava Zoological Garden and Botanical Park	3,1
Pallas's Cat	*Otocolobus manul*	Brno Zoo and Environmental Education Centre, Semi Budgetary Organization	1,2
		Zoopark Chomutov, p.o.*	2,0
		Zoologicka Zahrada Jihlava	2,2
Carpathian lynx	*Lynx lynx carpathicus*	Zoopark Chomutov, p.o.*	1,1
		Zoo Decin - Pastyrska Stena	1,2
		Jihoceska zoologicka zahrada Hluboka nad Vltavou	1,2
		Zoologicka zahrada Hodonin	2,0
		Zoologicka Zahrada Jihlava	1,2
		Zoologicka zahrada Liberec, prispevkova organizace.	3,1
		Zoologicka zahrada Olomouc	1,1
		Ostrava Zoological Garden and Botanical Park	1,1
		Zoologicka Zahrada Tabor	1,0
Amur leopard cat	*Prionailurus bengalensis euptilurus*	Zoologicka Zahrada Jihlava	2,2,1
		Zoologicka zahrada Olomouc	1,1,3
		The Prague Zoological Garden	3,3
Palawan leopard cat	*Prionailurus bengalensis heaneyi*	Zoologicka Zahrada Jihlava	1,1
		Zoologicka a botanicka zahrada Plzen	4,4
		The Prague Zoological Garden	2,1

Legend: The first number indicates the number of males, the second number of females, and the third number of unidentified sex

1.2.2 Intracellular parasites of phylum Apicomplexa

The phylum Apicomplexa (previously known as Sporozoa) contains obligatory intracellular parasites. The phylum is named according to the apical complex, which plays a role in parasite invasion into host cells (Bowman et al. 2002).

As described by Bowman et al. (2002) many representatives of this phylum have cats as final hosts, for instance, the genus *Toxoplasma* and *Cryptosporidium*, on which this thesis is focused, and which are also zoonotic agents with public health implications. Other genera that have cats as final hosts of their life cycles are *Babesia*, *Besnoitia*, *Cytauxzoon*, *Hammondia*, *Isospora* and *Sarcocystis* (Bowman et al. 2002). But they can infect a wide range of species (Cavalier-Smith 1993).

Coccidial parasites such as *Cryptosporidium* and *Isospora* can cause diarrhoea, while infections of the intestines with *Besnoitia*, *Hammondia*, *Sarcocystis* and *Toxoplasma* are commonly without any symptoms. Tick-transmitted blood parasites such as *Babesia* and *Cytauxzoon* can cause anaemia or even death (Bowman et al. 2002).

1.2.2.1 Genus *Cryptosporidium* (Tyzzer 1907)

An apicomplexan protozoan, *Cryptosporidium*, with several species and genotypes, may also infect a wide range of animals and also humans (CDC 2019).

The most common *Cryptosporidium* in cats is *Cryptosporidium felis*, which was first described and named by Iseki (1979). The oocysts of *Cryptosporidium felis* are smaller than *Cryptosporidium parvum* oocysts (Sargent et al. 1998; Zajac & Conboy 2012). *Cryptosporidium felis* oocyst is 3.5-5 µm in diameter (with a mean of 4.3 µm), while the oocyst of *Cryptosporidium parvum* is bigger with a mean of 5 µm (Bowman et al. 2002).

Its life cycle is direct (in a single host). Felines get infected by eating oocysts. *Cryptosporidium* oocysts are infectious immediately after being passed through the faeces (Zajac & Conboy 2012). There are four sporozoites in each sporulated oocyst. Released sporozoites from the oocyst invade the intestinal epithelial cells or also respiratory tract (CDC 2019).

In the epithelial cells, phagocytosis is caused by the sporozoites, but in contrast with coccidias, the sporozoites stay on the surface with the cell membrane around them (See Figure 1). The parasite develops an apical organelle, where it proceeds schizogony and creates eight daughter merozoites (See Figure 1), that infects other cells (Bowman et al. 2002).

After the asexual cycle, the sexual cycle begins, which develops microgamonts (male) and macrogamonts (female). The macrogamonts are fertilized by the microgamonts and develop oocyst, which sporulates within the host (CDC 2019). The sporulated oocysts include four sporozoites that are infectious (Bowman et al. 2002).

Two types of oocysts are created, thick-walled, which are excreted out of the host in faeces and thin-walled, which participate in the internal autoinfective cycle (CDC 2019). Oocysts leave the host in the defecation (Zajac & Conboy 2012) and are infectious, which allows direct faecal-oral transmission (CDC 2019).

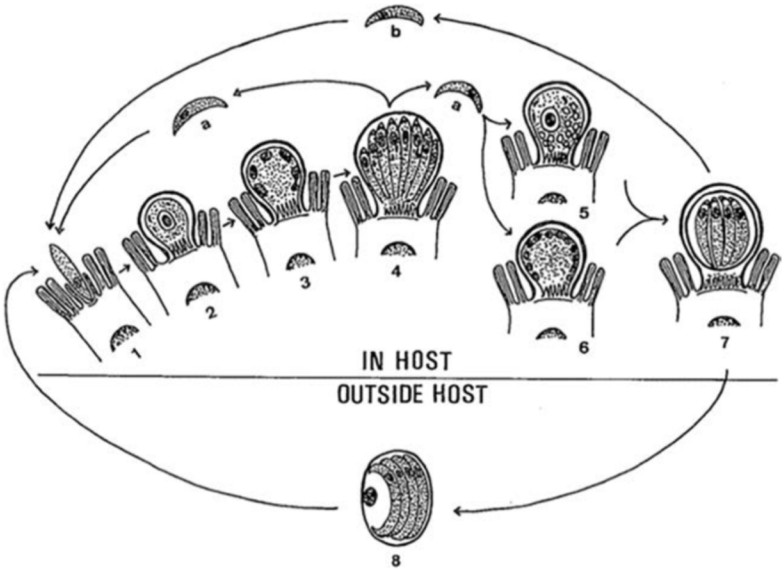

Figure 1 - Life cycle of *Cryptosporidium felis*

Source: Iseki (1979)

1. Intestinal epithelial cell penetrated by zoite; 2. Trophozoite in full development; 3. Meront with 6 N; 4. Mature meront with 8 merozoites; 5. Young microgametocyte; 6. Young macrogametocyte; 7. Mature oocyst with 4 sporozoites; 8. Oocyst in faeces; a. Merozoites; b. Sporozoites have been released from mature oocyst.

1.2.2.2 Cryptosporidiosis in cats

Cryptosporidium infected cats can be either asymptomatic or symptomatic. Many studies including Fayer et al. (2006) found out that asymptomatic cats were shedding *Cryptosporidium* oocysts, hence the infection was present without any clinical signs including diarrhoea. In their study, six cats had *Giardia duodenalis* infection coexisted with *Cryptosporidium felis* infection, during the patency. On one or more days, all 18 tested cats were determined infected with *Cryptosporidium felis* and *Giardia duodenalis* infection was discovered in six cats.

After immunofluorescence microscopy, they performed a molecular detection and the sequence data showed 100% similarity with *Cryptosporidium felis* (GenBank accession number, AF112575) and 100% similarity with *Giardia duodenalis* genotype F (GenBank accession number, AF199444). In terms of identifying infections, PCR was, according to Fayer et al. (2006), considerably more sensitive than immunofluorescence microscopy. Other authors, such as Hajdušek et al. (2004) and Kváč et al. (2017) also identified, by molecular method, sequence, which matched with *Cryptosporidium felis* from cats (*Felis catus*) from the Czech Republic. Kváč et al. (2017) revealed the presence of *Cryptosporidium felis*, sharing the identity with GenBank accession no. AF112575 from the faecal samples of pet and stray cats.

Rambozzi et al. (2007) collected 200 faecal samples from domestic cats in Italy and with the use of the flotation method analysed the samples, from which 49 were detected as *Cryptosporidium* positive. It was found that kittens are particularly likely to be infected, probably due to their undeveloped immune system and a lack of acquired immunity from past exposure to the parasite. Rambozzi et al. (2007) also demonstrated that infected cats with other intestinal parasites were three times more likely to test positive for *Cryptosporidium* spp.

In some cases, infected cats can have repeated outbreaks of diarrhoea. Because *Cryptosporidium* is reproducing within the intestinal epithelial cells, the infection is connected with loss of water (Bowman et al. 2002).

1.2.2.3 Genus *Toxoplasma* (Nicolle & Manceaux 1909)

A protozoan parasite *Toxoplasma gondii* can infect animals and humans around the globe due to its wide distribution (Dubey 2020). Despite the fact that *T. gondii* has a worldwide distribution and probably the broadest host range, the genus *Toxoplasma* contains just one species *Toxoplasma gondii* (Dubey 2008).

In the tissues of a North African hamster-like rodent called the "gundi" *Ctenodactylus gundi* (Rothmann 1776), Nicolle & Manceaux (1908) first discovered *Toxoplasma gondii*. It got a name by Nicolle & Manceaux (1909) based on its morphology and the host. *Toxo* means arc or bow, and *plasma* means life from the Greek. The parasite should have been called *Toxoplasma gundi* instead of *T. gondii*, as Nicolle & Manceaux (1909) had misidentified the host as *Ctenodactylus gondi* (Dubey 2020). However, its full life cycle was discovered in 1970, when Frenkel (1970) found the sexual stage in the cat's small intestine and *T. gondii* oocysts were reported to be excreted only by felids.

Toxoplasma gondii's only known definitive hosts are members of the Felidae family as previously mentioned according to Frenkel (1970). Cats are shedding unsporulated oocysts in their faeces usually from one to three weeks. The oocysts then become infectious after one to five days after the sporulation in the environment. Intermediate hosts (birds and rodents included) get infected after consuming oocyst-contaminated soil, water or plant material (See Figure 2). Shortly after the ingestion, oocysts turn into tachyzoites, which develop into tissue cyst bradyzoites in muscular tissue. Cats, its final host, can get infected directly by ingestion of sporulated oocysts or by eating an intermediate host with tissue cysts (CDC 2020).

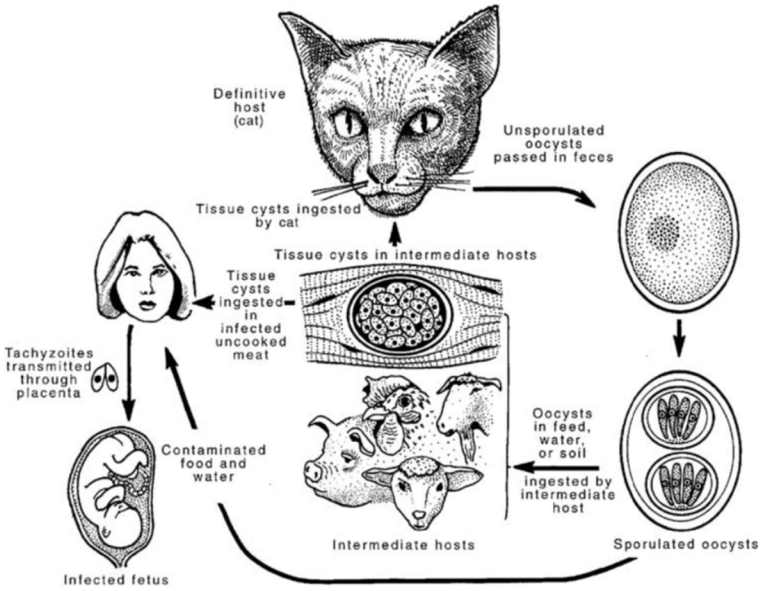

Figure 2 - Life cycle of *Toxoplasma gondii*

Source: Dubey (2020)

1.2.2.4 Toxoplasmosis in cats

Most *Toxoplasma gondii* infected cats do not show any symptoms of toxoplasmosis. However, Pallas's cats in captivity are highly susceptible to infection with *Toxoplasma gondii* with a high neonatal mortality rate (Kenny et al. 2002). The cause of this high susceptibility is uncertain, although Brown et al. (2005) proposed, based on their findings, that Pallas's cats live in an environment, which is biologically unique and an exposure to *Toxoplasma gondii* in their natural habitat is low, therefore, the infection most likely occurs in captivity.

Pallas's cat is endemic to Central Asia and the severe winters and significant seasonal variations in the Pallas's cat's native habitat have resulted in several biologic adaptations. Of all the felines, it has the thickest fur and the longest hairs (Bellani 2019). Their reproduction is season-dependent, oestrous cyclicity is usually only seen in females during the winter months and each year from late spring to early summer, they have a single litter (Brown et al. 2002). In contrast to pregnant domestic cats and other cat species, maternal immune responses do not appear to be protective in seropositive (for anti-*T. gondii* immunoglobulins) Pallas's cats during pregnancy (Kenny et al. 2002).

Toxoplasmosis has been shown to cause 60-65% death rates in Pallas's cat's kittens in managed care around the time of weaning when exposure is highest, but

immunity is still developing (Girling et al. 2020). Necrotizing encephalitis, pneumonia, and hepatitis are all common lesions in newborns (Terio et al. 2018). Girling et al. (2020) demonstrated a statistically significant reduction in first-year mortality when using a clindamycin treatment for kittens. Meanwhile, survival rates improved statistically significantly over time as well. After two years, a reduction in the first-year death rate resulted in a 5.88% mortality rate in the animals treated with clindamycin (Girling et al. 2020). Immune weaknesses, according to Ketz-Riley et al. (2003), may also play a role in susceptibility. In the Oklahoma City Zoo, toxoplasmosis with parallel herpesvirus infection killed five newborn Pallas's cats (Ketz-Riley et al. 2003).

Felines infected with toxoplasmosis can have a fever between +40-41,7°C. Shortness of breath, polypnea and jaundice as well as indicators of abdominal pain are frequently present. Many organs can have signs of macroscopic or microscopic lesions, this is especially present in the lungs. From abdominal organs, the liver is the most impacted (Bowman et al. 2002) Beside these signs, ocular lesions and congenital toxoplasmosis can also occur (Dubey & Carpenter 1993; Bowman et al. 2002).

1.2.2.5 Previous surveys of *Toxoplasma gondii* in zoo animals (Felinae) in the Czech Republic

Lukešová & Literák (1998) examined, using a flotation method, faecal samples of Felidae in six Czech zoos, including two zoos, from which this thesis also examined samples. Some of the animal species previously tested from 1995 to 1996 were identical to those tested in this thesis. From the Zoo Jihlava, it was a Geoffroy's cat (*Leopardus geoffroyi*). Five collections were made at the Zoo Jihlava at intervals of every three months, but after a positive finding of *Toxoplasma* oocysts, samples from Geoffroy's cat were tested for 32 days straight. The collections were into plastic sample containers, samples were then at a temperature of +20°C to allow oocysts to sporulate and after that stored at +4°C.

The diet at the Zoo Jihlava consisted of beef, laboratory mice from their own breeding sources (freshly killed) and live chicks in the age of 5 to 6 days. The water for cats was enriched with minerals by adding soil (Lukešová & Literák 1998).

The oocysts of *T. gondii* were detected in four samples from Geoffroy's cat (See Table 3). As the potential source of the spread of the infection was indicated the water

with added soil, as already mentioned before, due to soil storing, which posed a risk, because feral domestic cats could easily approach it (Lukešová & Literák 1998).

Kudrnáčová (2014) collected 700 faecal samples into plastic sample containers from five feline species from three zoos, including the Zoo Jihlava and the Zoo Olomouc. The sampling was three times a week from July to October 2013 and weekly from November 2013.

The samples were then kept in a refrigerator from +4 to 8°C. Similarly to the present study, the samples from Amur leopard cat (*Prionailurus bengalensis euptilurus*) and European wildcat (*Felis silvestris silvestris*) were collected at the Zoo Olomouc. The sampling at the Zoo Jihlava was from the Amur leopard cat (*Prionailurus bengalensis euptilurus*), European wildcat (*Felis silvestris silvestris*) and Geoffroy's cat (*Leopardus geoffroyi*). Amur leopard cat at the Zoo Olomouc was kept in a mixed enclosure of two females and one male, but later the male killed the rest. European wildcat from the Zoo Olomouc was in a pair (male with female). For the Zoo Jihlava, the Amur leopard cat and European wildcat were kept as a single female, similarly to a female of Geoffroy's cat, which left in September (Kudrnáčová 2014).

At the Olomouc Zoo, the feeding of Amur leopard cat and European wildcat consisted of chicks, hamsters or mice, chicken and beef. At the Jihlava Zoo, the diet was with freshwater fish (such as perch or crucian carp), chickens, rabbits, rats and mice, and guinea pigs. Hamsters, quails, and beef flesh are also served as food. The Zoo Jihlava had their own breeding sources of rats, mice, and guinea pigs. The beef was from abattoirs or private owners. Factory farming provided rabbits and fish and chickens were killed at the zoo when needed (Kudrnáčová 2014).

The examination of samples performed by Kudrnáčová (2014) was done using a flotation method with sucrose solution of a specific density of 1.15 and by followed microscopy. For samples from the Zoo Jihlava, one sample from the Amur leopard cat was indicated as a positive. From the Zoo Olomouc, the oocysts of *Toxoplasma gondii* were positively detected in four samples from Amur leopard cat and one sample from European wildcat (See Table 3). One sample collected from Amur leopard cat was dubious. All samples from Geoffroy's cat were negative. Summer had the highest incidence of *T. gondii* oocysts. Pork and rabbit meat appeared to be cause of the infection in the opinion of Kudrnáčová (2014).

Table 3 - Evaluation of findings of *Toxoplasma gondii* using direct flotation methods in the selected Felinae at the Czech zoos

Source: own processing according to Lukešová & Literák (1998) and Kudrnáčová (2014)

Zoo	Species	Positive	Dubious	Total samples (n)	Author
Olomouc	***Felis silvestris silvestris***	**1 (2.63%)**	0	38	(Kudrnáčová 2014)
Jihlava	***Leopardus geoffroyi***	**4 (10.26%)**	0	39	(Lukešová & Literák 1998)
Jihlava	***Prionailurus bengalensis euptilurus***	**1 (1.75%)**	0	57	(Kudrnáčová 2014)
Olomouc	***Prionailurus bengalensis euptilurus***	**4 (10.53%)**	**1 (2.63%)**	38	(Kudrnáčová 2014)

With the use of different detection methods, Sedlák & Bártová (2006) used an indirect fluorescent antibody test (IFAT) to examine the sera of 286 cats from the Czech Republic for antibodies to *Toxoplasma gondii*. The authors detected in eight cats (2.8%) *T.gondii* IgM antibodies and in 126 cats (44.1%) IgG antibodies. Bártová et al. (2018) mentioned that fatal toxoplasmosis has been found in zoo animals, including eight Pallas's cats, in the Czech Republic. According to the study of Bártová et al. (2018), it was found that 13 (81%) serum samples from cheetahs, one sample from Pallas's cat and one sample from serval were seropositive. The samples were collected at the Czech zoos from 1998 to 2016. By PCR, Bártová et al. (2018) found DNA of *Toxoplasma gondii* in the tissue of a stray cat from the Czech zoo, however full genotyping was not achieved.

1.2.2.6 Transmission

Parasites of the genus ***Cryptosporidium*** can be transmitted in both direct and indirect routes. Direct routes involve human to human and animal to human transmission, meanwhile indirect are via contaminated water and food (Cacciò & Tasini 2017; Shrivastava et al. 2017), see Figure 3. All domestic animals, cattle, wildlife, and humans are potential reservoirs for *Cryptosporidium* spp., which can be transferred to new hosts via the faecal-oral pathway (Pumipuntu & Piratae 2018).

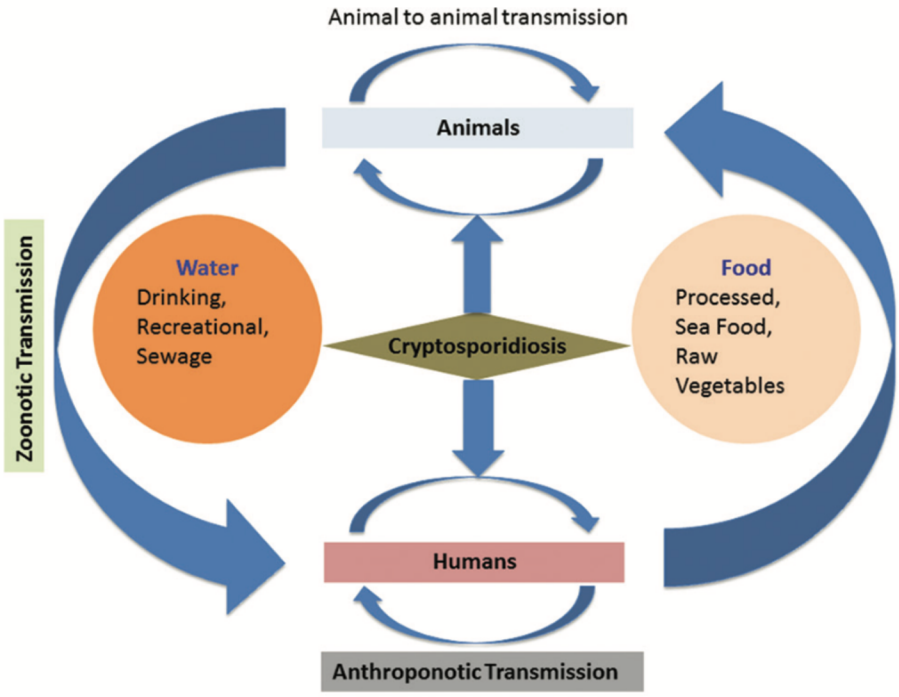

Figure 3 - Transmission cycle of *Cryptosporidium* spp.

Source: Shrivastava et al. (2017)

Except for cases of congenital toxoplasmosis, which is transferred from mother to child, blood transfusion or organ transplantation, **toxoplasmosis** is not transmitted from person to person (CDC 2018).

Humans can get infected by *Toxoplasma* through a variety of routes, for instance, consumption of undercooked meat with tissue cysts, consumption of contaminated food or water with cat faeces, by faeces-contaminated soil or when cleaning a litter box of their pet cat. Other ways, as previously mentioned, include transfusion of blood or transplantation of organs and transplacental transmission from the mother to the fetus (CDC 2020).

According to CDC (2018), we can divide these routes of transmission into categories of foodborne (tissue form in food), zoonotic (oocyst form in contaminated soil, water, litter box), congenital (mother to child) and rare instances (blood or organs from *Toxoplasma*-infected donor).

1.2.2.7 Zoonotic potential

Both cryptosporidiosis and toxoplasmosis are zoonoses possessing a risk of transmission to humans. The problem is that infected cats are often asymptomatic (absent of clinical signs) as mentioned by Fayer et al. (2006) about cryptosporidiosis or by Kenny et al. (2002) about toxoplasmosis.

As mentioned in the previous chapter (1.2.2.6 Transmission), congenital toxoplasmosis can be transmitted from mother to child. Congenital toxoplasmosis causes a significant amount of morbidity in people. The majority of these ***Toxoplasma***-infected infants will most likely acquire clinical toxoplasmosis symptoms (Dubey et al. 2021). The worldwide rate of prenatal transmission is 29% (Dunn et al. 1999).

When a woman gets infected during the first trimester, the risk of congenital infection is lowest (10-15%) and during the third trimester, the risk is highest. The trimester of pregnancy, when the mother got infected, also determines the severity of symptoms (Dubey et al. 2021). According to McAuley (2014) the transmission rate for untreated women is around 25% in the first trimester, 54% in the second trimester and 65% in the third.

Congenital toxoplasmosis includes a wide range of clinical symptoms, although, in around 75% of infected newborns, it is asymptomatic (McAuley 2014). CDC (2020) also noted that patients with congenital infection are frequently asymptomatic until they develop lesions in their eyes in their second or third decade of life. Spontaneous abortion, prematurity, or stillbirth are possible outcomes of congenital toxoplasmosis, further especially CNS involvement. Other typical symptoms include the development of chorioretinitis, intracranial calcifications, and hydrocephalus (McAuley 2014).

From the latest available data from ECDC (2021), there were 208 confirmed cases of congenital toxoplasmosis from 22 countries in the EU/EAA in 2018 (See Figure 4). Because of active and compulsory screening of pregnant women, France had the highest rate of 73% of all cases. Other countries with a high rate included Slovenia, Estonia, and

Poland. There were no cases in 13 countries. Only Austria, Belgium, France, Slovakia and Slovenia are five countries in the EU/EAA that require pregnant women to be screened, but on the other hand, Austria or Belgium do not send their data to the ECDC at all, so the situation is unknown. In total, the notification rate was reported to be 5.8 per 100,000 living newborns (ECDC 2021).

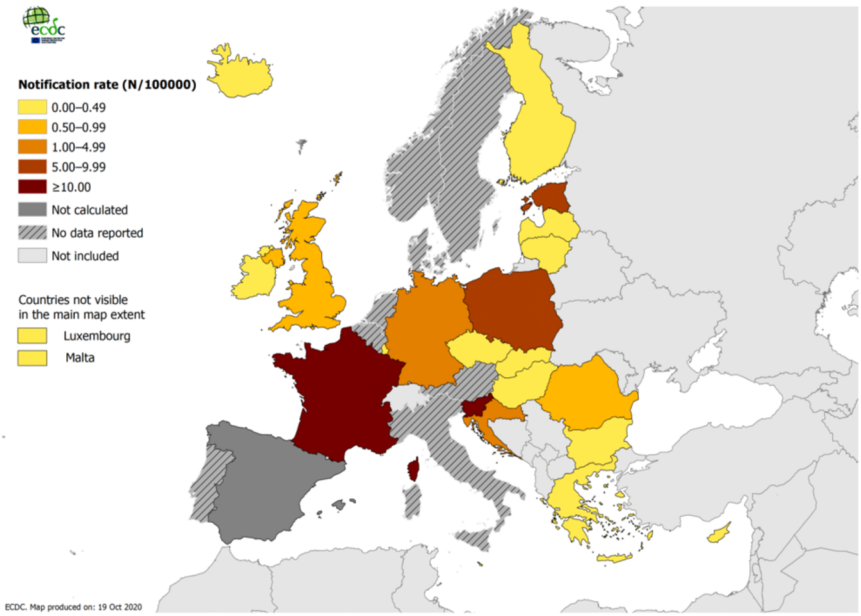

Figure 4 - Map showing the distribution of confirmed cases of congenital toxoplasmosis per 100,000 live births in the EU/EAA in 2018

Source: ECDC (2021)

In the Czech Republic, voluntary serological screening is used and only some regions and gynaecological clinics had offered them. Another disadvantage is that statutory health insurance does not cover it. From available previous data from 2014 to 2018, there was one confirmed case of congenital toxoplasmosis in 2014, another one in 2015, none in 2016, two in 2017 and no confirmed case in 2018 (ECDC 2021).

In recent years, public health concerns that cats and dogs may host ***Cryptosporidium*** species with zoonotic potential (Lucio-Forster et al. 2010). Humans can also be infected by *Cryptosporidium felis* (CDC 2019), although it results in only a small number of cases in humans (Lucio-Forster et al. 2010). *Cryptosporidium* spp. have the capability to cause gastrointestinal illness, have a widespread distribution in the environment and have the potential for waterborne and foodborne outbreaks. (Lucio-Forster et al. 2010).

Cryptosporidiosis is characterized by diarrhoea, nausea, vomiting, stomach pain, and overall malaise. In individuals with immunodeficiencies, infections of other organs such as the pancreas, liver, bile ducts, and respiratory tract can arise, especially in those with acquired immunodeficiency syndrome (AIDS). In patients with undeveloped or compromised immune systems, such as the very young and old, people infected with the human immunodeficiency virus (HIV), those on immunosuppressive medication, and those who are malnourished, a chronic form of the illness can cause dehydration and electrolyte imbalance. Cryptosporidiosis can be fatal in such situations (Tzipori & Widmer 2008).

From the latest available data from ECDC (2021), cryptosporidiosis was confirmed in 14,252 cases from 20 EU/EAA countries in 2018 (See Figure 5). The notification rate was reported to be 4.4 confirmed cases per 100,000 people, which was higher than in the previous year 2017. As in the previous years, the United Kingdom and Germany reported the majority of confirmed cases, although Netherlands, Ireland and Belgium had the highest notification rates (as can be seen in the Figure 5), suggesting a higher reporting and laboratory testing.

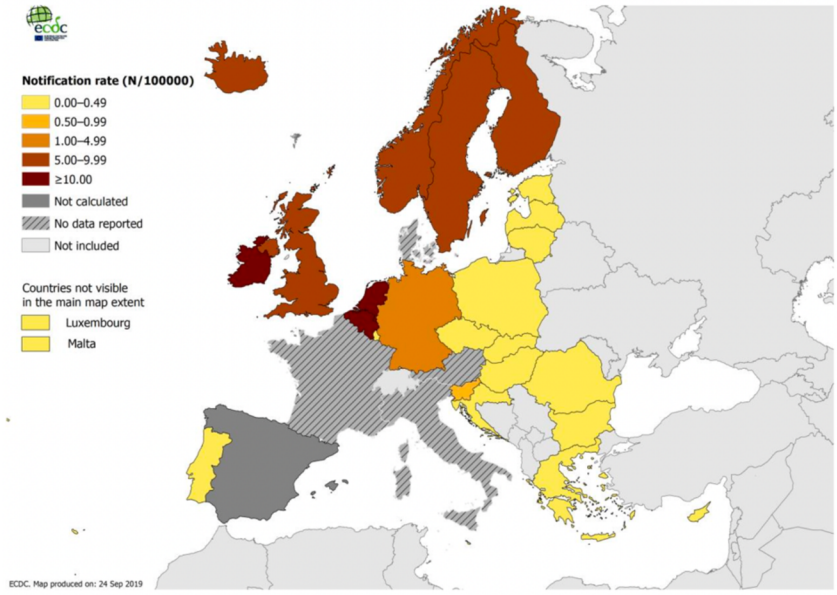

Figure 5 - Map showing the distribution of confirmed cases of cryptosporidiosis per 100,000 population by country in the EU/EAA in 2018

Source: ECDC 2021

For the Czech Republic, available data from ECDC (2021) showed that there was one case of cryptosporidiosis in 2014, two cases in 2015 as well as two cases in 2016, five cases in 2017 and lastly six confirmed cases in 2018, implying that the number of cases is increasing at an exponential rate.

1.2.2.8 Laboratory diagnosis

Cryptosporidium can be detected using both classic detection methods, such as microscopy, antibody, and enzyme-based techniques, but also molecular methods like DNA from oocysts in faecal samples (Gerace et al. 2019; Holubová et al. 2019; Ježková et al. 2021).

In the Figure 6 from Gerace et al. (2019), we can see *Cryptosporidium* oocysts (A) stained with a modified Ziehl–Neelsen stain for microscopic identification. The oocysts are stained bright red against a background of a blue-green colour consisting of faecal debris and yeasts. Staining can also be done with a method by Miláček & Vítovec (1985), used also by many other authors including Kváč et al. (2018), Holubová et al. (2019) and Ježková et al. (2021) on samples from rats, Psittaciformes birds and nutria. *Cryptosporidium* oocyst in faecal samples can be detected using an immunochromatographic technique (B) (Ekici et al. 2021). In order to identify *Cryptosporidium* antigen in faeces, an ELISA technique was developed (C), as mentioned by Gerace et al. (2019) and Beyhan & Yılmaz (2020). Arrows are pointing at the *Cryptosporidium* oocysts, which are not stained with Lugol's iodine solution (D) and resemble yeasts but are colourless and finally *Cryptosporidium* detection via PCR (E) (Hajdušek et al. 2004; Kváč et al. 2017). According to Fayer et al. (2006) and Morris et al. (2019) it is impossible to clearly identify neither the species of *Cryptosporidium* oocysts nor their involvement in illness without molecular methods.

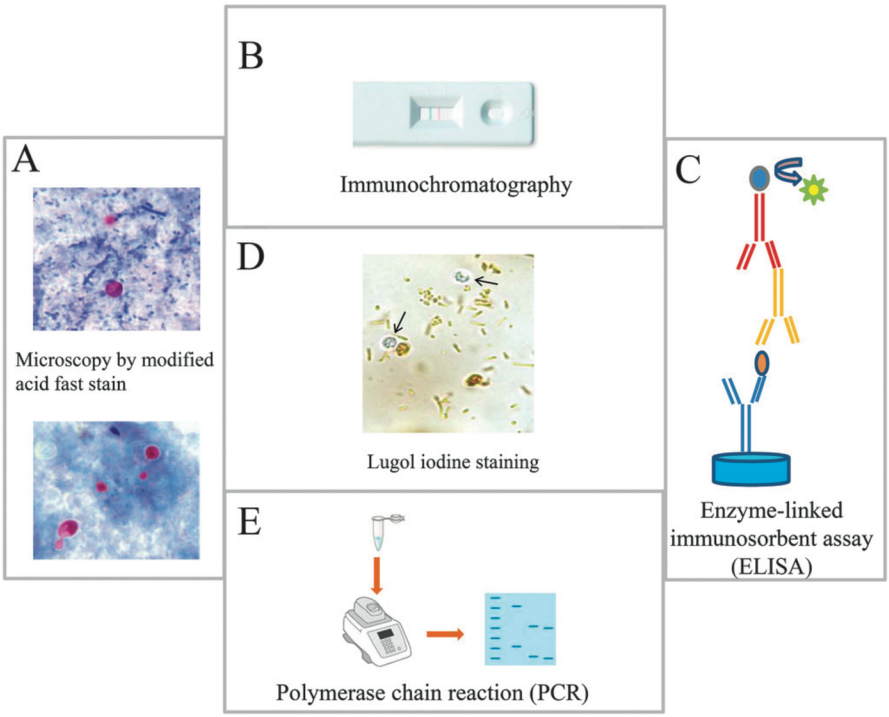

Figure 6 - The most common methods for detecting *Cryptosporidium* spp. in faecal samples

Source: Gerace et al. (2019)

Serologic tests, amplification of certain nucleic acid sequences (PCR), histologic evidence of the parasite or its antigens (immunoperoxidase stain), or isolation of the organism can all be used to diagnose *Toxoplasma gondii* infection (Montoya 2002; Fernández-Escobar et al. 2020). Furthermore, antigenemia and antigen in serum or body fluids, a toxoplasmin skin test and antigen-specific lymphocyte transformation are some of the less common procedures for diagnostics (Montoya 2002). For instance, Sedlák & Bártova (2006) used an indirect fluorescent antibody test as described in the previous chapter (1.2.2.5).

1.2.2.9 Treatment and prevention

As mentioned by Zahedi & Ryan (2020) a vaccine for cryptosporidiosis is currently not commercially available, despite the risk of zoonotic transmission. Many medications have been studied in animals for cryptosporidiosis, including nitazoxanide (Alinia®, Tampa, Florida), which is licensed for use in humans but has little effectiveness in animals (Schnyder et al. 2009; Zahedi & Ryan 2020).

The oocyst of *Cryptosporidium* spp. can stay infectious and resistant to a variety of environmental factors, as well as several common disinfectants, such as chlorination, which is commonly used in water treatment (Pumipuntu & Piratae 2018). However, systems that use UV light or ozone as alternative disinfection can be considered used. Reliable methods include boiling water to kill or inactive *Cryptosporidium* or using filters with labels such as reverse osmosis, absolute pore size of 1 micron or smaller and also tested and certified by NSF Standard 53 for cyst removal/reduction (CDC 2015; Steinbach et al. 2021).

Toltrazuril (Baycox®, Bayer, Canada) is an antiprotozoal/anticoccidial drug that can be used to treat coccidiosis in dogs, cats, calves, lambs, rabbits, mice, camelids, and birds. It has been used to treat toxoplasmosis in cats during the oocyst shedding stage (Plumb 2018). There is currently no vaccine, which would be safe to use in humans, however, spiramycin (Liofilchem, Italy) has been used in pregnant women to prevent transmission from mother to fetus because it is non-toxic and does not cross the placenta (Dubey 2020). Toxoplasmosis is however usually recovered even without treatment in healthy persons, if needed non-pregnant women can be treated with a combination of medications, including pyrimethamine and sulfadiazine, as well as folinic acid (CDC 2018).

According to ECDC (2021), the public should be educated on how to reduce their risk of contracting cryptosporidiosis, which includes appropriate hand hygiene and safe handling of raw or minimally processed fruits and vegetables, including washing, peeling, and boiling if required (ECDC 2021; Lyashchuk et al. 2021). Families with young children should be aware when visiting public pools, zoos and farms, where the kids can even pet the animal. The increased risk of gastrointestinal diseases and particularly cryptosporidiosis is also for participants in mass sporting events, such as open-water swimming (Cristiane Pinto et al. 2020; ECDC 2021), hence they should be its aware.

Similar prevention is needed also for toxoplasmosis, (CDC 2018) during cooking food until it reaches a safe temperature, for instance at least +74°C for cooking poultry, freezing the meat for several days (before cooking), peeling or washing vegetables and fruits, not drinking unpasteurized goat milk, not eating raw meat (for example oysters), washing hands, changing the cat litter box daily and wear gloves when gardening.

On a daily basis, zookeepers have to remove animal faeces (EAZA 2013; Jensen et al. 2021), which could possibly attract pests and also contain a wide range of parasites, posing a danger of infection (Müller-Klein et al. 2019) or reinfection (EAZA 2013). Cleaning prevents infection from spreading and hence maintains a healthy environment for both animals and employees. Diseases may spread throughout enclosures if biosecurity measures to prevent infectious agent transfer are not implemented, resulting in clinical illness, reproductive failure, and even death (Jensen et al. 2021). Controlling the flow of feed, water, and waste might be crucial in managing the disease spread (Animal Health Australia 2014).

According to EAZA (2013), it is crucial that the tools used to clean animal cages are thoroughly cleaned after each usage. Infectious agents can be mechanically distributed on inert items such as automobiles, clothes, and equipment (Animal Health Australia 2014). Furthermore, different separate tools should be used for each animal house to keep infections and parasites from spreading (EAZA 2013; Remes 2021). Hygiene, including cleaning, disinfecting, pest control, but also personal hygiene, helps to keep diseases under control (Remes 2021). Personal hygiene and safety should be emphasized to animal keepers because they are in close contact with the animals. Before eating, smoking, or entering another animal (after animal handling), zookeepers should always wash their hands. Since zookeepers frequently walk from one animal enclosure to the next and can potentially spread diseases across the zoo, it is also recommended to use a disinfectant footbath while entering and exiting an animal area with a high risk of illness (EAZA 2013; Dunowska 2019).

The veterinarian should be in charge of devising a preventative strategy and conducting frequent testing on the substrate, dung, or live animals. New animals, those that have come from another institution, but also animals with an infectious disease should be quarantined for a period of time (EAZA 2013; Al Badi & Ahmad 2019). Disease spread is more likely to occur when zoo animals move between facilities and have direct or indirect contact with domestic animals or wildlife. Sick zoo animals should be isolated from other zoo animals until a diagnosis is verified or the danger of infectious disease was treated (Animal Health Australia 2014). Parasites and viruses should also be checked in the faeces and blood of the animals under quarantine. Quarantining an animal before releasing it into the collection will prevent infection outbreaks (Al Badi & Ahmad

2019). Barrier nursing, cleaning and sanitation of equipment, and adequate movement control inside the zoo are all established biosecurity measures for managing sick animals (Animal Health Australia 2014). Handwashing before and after feeding, cleaning and a disinfectant footbath at the entrance to disinfect the shoes of keepers and veterinarians, reduce the risk of pathogen transmission from quarantine animals (EAZA 2013).

According to Schares et al. (2016), climate conditions were shown to be linked with seasonality in the percentage of domestic cats shedding *T. gondii*. Schares et al. (2016) also described that during the summer and fall, cats shedding *Toxoplasma gondii* oocysts were discovered the most, therefore, sanitation procedures should also be adapted accordingly.

Animals are vulnerable to stress because it weakens them physically and psychologically. The animals will be more prone to illness as a result of this, therefore a zoo must ensure that animals' anxiety and stress levels are kept to a minimum (EAZA 2013).

1.2.3 Coinfections in the Czech Republic

Kudrnáčová (2014) also found in *Toxoplasma* positive sample, from the female Amur leopard cat bred at the Zoo Jihlava, other parasites, e.g. *Cystoisospora* spp. and *Toxocara cati*. In other samples of this particular individual, Kudrnáčová (2014) did not detect *Toxoplasma gondii* oocysts, but she found *Giardia* spp., *Cystoisospora* spp. and *Toxocara cati*, so there was a chance of coinfection. There were no findings of *Toxoplasma gondii* in the samples of a female Geoffroy's cat from *Jihlava*, but in some samples from this animal, *Giardia* spp., *Cystoisospora* spp., *Toxacara cati* and *Toxascaris leonina* were found. In one sample on a female Geoffroy's cat, there were occurrences of *Toxocara cati* and *Toxascaris leonina* at the same time. For species European wildcat from the Zoo Olomouc, where mixed samples were taken from a couple, one sample was identified as *Toxoplasma* positive as well as *Cystoisospora* positive. Other samples from this couple were *Toxoplasma* negative, but findings of *Giardia* spp., *Toxocara cati*, *Toxascaris leonina* and *Cystoisospora* spp. were made. In the samples from the male Amur leopard cat, which killed other females that were previously in the enclosure with him (as mentioned in Chapter 1.2.2.5 Previous surveys of *Toxoplasma gondii* in zoo animals (Felinae) in the CR), no other parasites were found in four *Toxoplasma gondii* positive and one dubious sample, however, in other samples, Kudrnáčová (2014) made findings of *Giardia* spp., *Toxascaris leonina*, *Toxocara cati* and *Cryptosporidium* spp. This indicates that the male could probably have ongoing polyinfections of *Toxoplasma gondii* with other found parasites including *Cryptosporidium* spp. All samples were examined using the flotation method (Kudrnáčová 2014).

2 Aims of the Thesis

The aims of the diploma thesis were to find the occurrence of apicomplexan parasites, specifically *Cryptosporidium* spp. and *Toxoplasma gondii* in the faeces of the selected representatives of the Felinae subfamily by coprological and molecular methods at the Czech zoos and to evaluate the current situation in comparison with previous surveys from the Czech Republic. Other parasitic accompanying pathogens, on which this thesis was not focused, of the genus *Cystoisospora*, *Giardia*, *Capillaria*, *Toxocara* and *Toxascaris* were also identified and alternatively verified by PCR methods. In view of the findings, the sources and routes of spread were investigated.

Based on the objectives of the thesis, hypotheses were established:

H_1 – *Toxoplasma* infection was the most prevalent in Pallas's cat *Otocolobus manul* (Pallas 1776) due to their high susceptibility.

H_2 – Oocysts of *Toxoplasma gondii* and *Cryptosporidium* spp. and other accompanying parasitic pathogens were detected in the faecal samples of small felids kept at the Czech zoos.

H_3 – Findings of oocysts of *Toxoplasma gondii* and *Cryptosporidium* spp. and other accompanying parasitic pathogens were verified by PCR analyses.

3 Material and Methods

3.1 Theoretical part

Available sources from scientific and specialised literature were used to process the theoretical part of the diploma thesis, which were searched through scientific databases such as Web of Science, Science Direct, PubMed, Google Scholar and Scopus.

The database of the Library of the Czech Academy of Sciences was used to search for scientific monographs. In addition, available information from websites such as CDC, ECDC and also BioLib for biological taxonomy was used. The University Information System of the Czech University of Life Sciences in Prague was also used to obtain a diploma thesis from Kudrnáčová (2014), which dealt with *T. gondii* in small felines.

Relevant sources were focused on the genus *Toxoplasma* and *Cryptosporidium* from the phylum Apicomplexa concerning infectious diseases with zoonotic potential in small cats.

Keywords such as: *Toxoplasma gondii*, *Cryptosporidium*, toxoplasmosis, cryptosporidiosis, small felines, zoo animals and various combinations were used to search the mentioned databases.

The used sources were cited according to the citation rules set by the Dean of FTA CZU Prague in 2017 according to the journal Conservation Biology.

3.2 Practical part

The aim of the practical part was to obtain faecal samples from two Czech zoos and process these samples using direct coprological and molecular methods to determine the developmental stages of *Toxoplasma gondii* and *Cryptosporidium* spp. or other agents of parasitic polyinfections.

A database in Microsoft Excel software was created from the obtained data, which enabled their sorting and creation of tables. Using simple statistical calculations, it was possible to compare the detection methods used, such as the number of positive samples in each species of felines to the total number of samples taken.

3.2.1 Tested animals

From the Zoo Jihlava, the samples were taken from 11 animals (see Table 4) for detection of oocysts of Toxoplasma gondii and Cryptosporidium spp. and other parasitic pathogens in their faeces. There were five species, specifically European wildcat (*Felis silvestris silvestris*), serval (*Leptailurus serval*), Pallas's cat (*Otocolobus manul*), Amur leopard cat (*Prionailurus bengalensis euptilurus*) and Palawan leopard cat (*Prionailurus bengalensis heaneyi*). Five males and two females of small felines in total. One male of European wildcat was kept separately. A male with a female of servals, which inhabited the same place in the Cat house. Three males of Pallas's cat (see Figure 7), which were kept individually in the back facilities, one separated female of Pallas's cat and another separated female, which was in an exposition. A male Amur leopard cat was likewise kept separately. And a pair of Palawan leopard cats was kept together in the Cat house. The youngest individual (Pallas's cat) was born in April 2020 and the oldest one (Palawan leopard cat) in August 2014. One male of Pallas's cat was put to death in November 2021 due to long-term complications. The placement of tested animals from the Zoo Jihlava can also be seen in Appendix 1.

Figure 7 - Pallas's cat at the Zoo Jihlava

Source: Bírošíková (2021)

Table 4 - Tested animals from the Zoo Jihlava

Source: own processing according to Species360 (2021; 2022)

Common name	Latin name	Origin facility	Sex	Birth/Death date		Number of individuals (n)
European wildcat	*Felis silvestris silvestris*	Zoo Jihlava	M	*1st May 2020		1
Serval	*Leptailurus serval*	Zoo Jihlava	F	*25th May 2017		2
		Aachener Tierpark AG	M	*14th May 2017		
Pallas's cat	*Otocolobus manul*	Parco Faunistico La Torbiera	M	*1st April 2020		5
		Zoopark Chomutov	F	*6th March 2016		
		Zoo Brno	F	*8th April 2016		
		Novosibirsk Zoo	M	*7th May 2018	†18th November 2021	
		Nordens Ark Zoo	M	*8th April 2020		
Amur leopard cat	*Prionailurus bengalensis euptilurus*	Zoo Olomouc	M	*23rd April 2019		1
Palawan leopard cat	*Prionailurus bengalensis heaneyi*	Tierpark Berlin-Friedrichsfelde GmbH	F	*2nd August 2014		2
			M	*20th February 2018		

From the Zoo Olomouc, the samples were taken from 12 animals. There were six species, cheetah (*Acinonyx jubatus*), European wildcat (*Felis silvestris silvestris*), Geoffroy's cat (*Leopardus geoffroyi*), serval (*Leptailurus serval*), Carpathian lynx (*Lynx lynx carpathicus*) and Amur leopard cat (*Prionailurus bengalensis euptilurus*), see Table 5. Five males and seven females. One pair of cheetahs was kept together on a grassy enclosure, but during the testing period, a female cheetah passed away on 13th August 2021. Another female of cheetah was kept alone sideways from the exhibits. The oldest European wildcat inhabited a separate accommodation. A pair of Geoffroy's cats were kept together, but servals were separated except of the mating. Carpathian lynx were together mostly in the outdoor exhibition. A male and a female of Amur leopard cat were housed separately in an interconnectable facility and on 3rd June 2021, during the testing period, the female had three kittens. Beyond these latest additions, the youngest animal

(Geoffroy's cat) was born in October 2018 and the oldest (European wildcat) in 2005. The placement of tested animals from the Zoo Olomouc can also be seen in Appendix 2.

Table 5 - Tested animals from the Zoo Olomouc

Source: own processing according to Species360 (2021; 2022)

Common name	Latin name	Origin facility	Sex	Birth/Death date		Number of individuals (n)
Cheetah	*Acinonyx jubatus*	-	F	*10th June 2007	†13th August 2021	3
		-	F	*27th May 2009		
		Ree Park Safari, Ebeltoft	M	*11th June 2007		
European wildcat	*Felis silvestris silvestris*	ZOO PARK Vyškov	F	*2005		1
Geoffroy's cat	*Leopardus geoffroyi*	Ústí nad Labem	M	*14th May 2017		2
		Zoo Aschersleben	F	*6th October 2018		
Serval	*Leptailurus serval*	Animal Source, Fraňková	M	*2008		2
		Animal Source, Fraňková	F	*12th August 2013		
Carpathian lynx	*Lynx lynx carpathicus*	Zoo Liberec	F	*24th May 2013		2
		Ohrada Zoo	M	*15th May 2015		
Amur leopard cat	*Prionailurus bengalensis euptilurus*	Zoo Jihlava	M	*20th June 2017		2(+3)
			F	*29th June 2017		

3.2.2 Faecal sample collection, storing and transport

Sampling took place at regular intervals three times a week in both zoos for six weeks (June to August 2021). A total of 222 faeces samples were taken, with 102 samples from five species of small felines from the Zoo Jihlava (see Table 4 in the previous chapter Tested Animals) and 122 samples from six species of small felines from the Olomouc Zoo (see Table 5).

Faecal samples were collected by the zookeepers in plastic bags during routine cleaning, marked with the date of collection, sex and species of the animal. Subsequently, the samples were stored at the zoo at a temperature of +4°C until transport and transported in a cooling box to the laboratory of the APB team at FTA CZU in Prague. They were later delivered to the Institute of Parasitology, Biology Centre CAS in České Budějovice and stored at +4 ° C until further laboratory analyses.

3.2.3 Coproscopical analyses

Faecal samples were processed under laboratory conditions first using the flotation and McMaster's methods, followed by the microscopic diagnosis of parasitic objects using an Eclipse E200 light microscope (Nikon Corporation, Tokyo, Japan). Furthermore, the faeces were smeared on the glass slide, followed by the use of the staining technique according to Miláček & Vítovec (1985). Positive findings of parasitic objects, in samples from previous coprological examinations, were later subjected to PCR molecular analysis.

3.2.3.1 Flotation centrifugation method

Faecal samples were analyzed for the presence of *Toxoplasma gondii* oocysts or other parasitic developmental stages, such as *Giardia* cysts or helminth eggs, and flotation centrifugation was used, followed by microscopic examination as described by Zajac & Conboy (2012). 222 faecal samples were examined in the laboratory of the APB Research Team at the Faculty of Tropical AgriScience, CZU in Prague.

The faecal samples were crushed in a porcelain mortar with a pestle along with distilled water, filtered through gauze or sieve into test tubes (numbered with a marker in advance). Each sample was filtered in two test tubes, the other tube was for control. The samples were then centrifuged at 2,000 rpm for 2 minutes. The supernatant was discarded

and the sediment remaining was mixed with Sheather's sugar solution with a specific gravity of 1.25 g/ml and thoroughly mixed. The samples were centrifuged again at 2,000 rpm for 2 minutes (see the result in Figure 8). The intact surface film was transferred to microscope slides using a wire loop and covered with a coverslip. These slides were viewed under a microscope Nikon Eclipse E200 (Nikon Corporation, Tokyo, Japan) with a magnification eyepiece of 10× and an objective of 10× for examination at first and then at 40× for detailed morphological identification of parasitic stages.

Figure 8 - The sample centrifuged a second time

Source: Bírošíková (2021)

3.2.3.2 The Egg-counting procedure (McMaster's method)

A modified McMaster's egg counting method for the quantitative determination of the number of eggs per one gram of faeces (EPG) was used according to the manufacturer's instructions (Chalex Corporation, USA) and Williamson (2013), see Appendix 3, for the previously positively detected samples with roundworms (Nematoda) during the flotation (n=49 samples). The 26:4 ml ratio of prepared flotation solution (saturated solution of sodium chloride) to faeces was respected and the contents were pipetted into a two-chambered McMaster's counting chamber (Chalex Corporation, USA), see Figure 9.

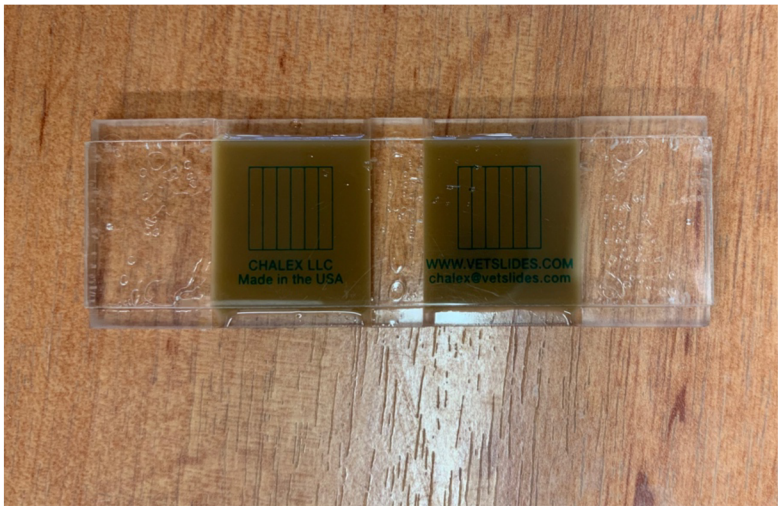

Figure 9 - Filled two-chambered McMaster's counting chamber

Source: Bírošíková (2021)

I visualized the chambers under a light microscope (Olympus BX51, Tokyo, Japan) with a magnification eyepiece of 10× and an objective of 10× in the Institute of Parasitology, Biology Centre CAS, České Budějovice.

The EPG was calculated by the total number of eggs in both chambers multiplied by 25. However, some samples were too dry or of insufficient weight, so for these samples, the estimation of EPG was made. In total, 25 samples were calculated by McMaster's method and the remaining 24 samples were estimated with the use of specific coefficients, which were set specifically for each parasite species.

In the previous flotation method, I recorded the total number of eggs observed during the microscopic examination. Based on these records, I was able to calculate the coefficients using the relationship between the total number of eggs observed by the flotation method and the calculated EPG by McMaster's method. I obtained the ratio of EPG per egg observed by flotation. I then used these coefficients for the remaining 24 samples for which the McMaster method could not be performed properly to obtain at least an EPG estimate. The calculation was performed by multiplying the total number of eggs observed during flotation and the calculated coefficient for each parasite species.

Estimation = number of eggs (from the flotation) * calculated coefficient (from the McMaster's method)

Coefficients: *Toxocara cati* = 1:14.84, *Toxascaris leonina* = 1:17.82, *Capillaria* sp.= 1:42.33

3.2.4 Miláček-Vítovec (1985) staining method

Thin smears from all faecal samples (n=222) were made on slides, fixed with methyl alcohol, and passed through a flame. Subsequently, the samples were stained according to the Miláček-Vítovec staining method (Miláček & Vítovec 1985), see Appendix 4, for the detection of *Cryptosporidium* spp. oocysts. Finally, they were rinsed and dried on filter paper, and with a drop of immersion oil, the stainings on glass slides (see Figure 10) were placed on the workbench of an Olympus BX51 microscope (Tokyo, Japan) with a magnification eyepiece of 10× and an objective of 100 × in the Institute of Parasitology, Biology Centre CAS, České Budějovice.

Figure 10 - Stained faecal smear

Source: Bírošíková (2021)

3.2.5 DNA isolation

Isolation of DNA from faecal samples was done with Exgene™ Stool DNA mini kit (GeneAll Biotechnology Co. Ltd, Seoul, Korea). I followed the manufacturer's instructions. In total 40 samples were isolated in the Institute of Parasitology, Biology Centre CAS, České Budějovice. The detailed procedure is shown in Table 6.

Table 6 - DNA isolation instructions

Step	Instruction
1.	Put approximately 200 mg of stool sample into the Safe-Lock Tube, add glass and zirconia beads and add 1-1.3 ml of buffer FL, homogenize by using vortex and break with a FastPrep ® 24 Instrument (MP Biomedicals, CA, USA) for 1 minute at 5.5 m/s.
2.	Incubate for 5 min. at laboratory temperature, centrifuge for 5 min./ 14,000 rpm.
3.	Transfer the supernatant to an EzPass filter (white column).
4.	Centrifuge 1 min./ 14,000 rpm, discard the pass-through out of the collection tube
5.	Add 100 µl of buffer EB to the EzPass filter, incubate for 1 minute at laboratory temperature, centrifuge 1 minute/ 14,000 rpm.
6.	Discard the white column and pipette 500 µl of buffer PB to the collection tube, mix by pipetting.
7.	Transfer the mixture of the collection tube to a mini spin column (green column).
8.	Centrifuge 1 min./14,000 rpm, discard the pass-through out of the collection tube.
9.	Add 500 µl of buffer NW into the centre of the column.
10.	Centrifuge 1 min./ 14,000 rpm, discard the pass-through out of the collection tube.
11.	Centrifuge again for 1 min./ 14,000 rpm, remove residual buffer, transfer the mini spin column to a new microtube.
12.	Pipette 200 µl of buffer EB to the column, incubate for 1 minute, centrifuge 1 min./ 14,000 rpm.

The obtained DNA was kept frozen at -20 °C until PCR analyses.

3.2.6 Genotypization

In order to confirm the occurrence of parasitic objects and developmental stages of parasites of *Toxoplasma gondii*, *Cryptosporidium* spp., *Giardia* spp. and last but not least *Toxocara cati* and *Toxascaris leonina* in faeces of felines, PCR analysis was performed in the laboratory of the Institute of Parasitology, Biology Centre CAS, České Budějovice. A total of 40 samples were analyzed using species-specific primers for the detection of *Cryptosporidium* spp. oocysts, seven samples for the detection of *Toxoplasma gondii* oocysts, four samples for the detection of *Giardia* spp. cysts and 12 samples for the detection of *Toxocara cati* and *Toxascaris leonina* helminth eggs.

3.2.6.1 Toxoplasma gondii

The obtained DNA from isolation was used to amplify short regions of DNA (SAG1) for PCR in the laboratory of the Institute of Parasitology, Biology Centre CAS in České Budějovice according to Grigg et al. (2001) from the Department of Microbiology and Immunology at Stanford University.

External primers (ext) were used for PCR amplification, followed by nested amplification using internal primers (int) using a template of 1 μl of the primary product. PCR mixtures consisted of 5 mL of PCR buffer (15 mM $MgCl_2$ in 10 × Perkin-Elmer PCR buffer), 0.1 mM dNTP mix, 1.5 U of Taq polymerase and 10 pmol each primer in a reaction volume of 50 μl. SAG1 primers used were F_{ext}, GTT CTA ACC ACG CAC CCT GAG, F_{int}, CAA TGT GCA CCT GTA GGA AGC, R_{ext}, GTG GTT CTC CGT CGG TGT GAG, and R_{int}, TTA TCT GGG CAG GTG ACA AC. PCR water was used as a negative control.

3.2.6.2 *Cryptosporidium* spp.

The obtained DNA from isolation was used to amplify the small ribosomal subunit rRNA gene (SSU) of *Cryptosporidium* spp. using a nested PCR method according to the methodology from Holubová et al. (2019) from the Institute of Parasitology, Biology Centre CAS in České Budějovice.

The primers 5′TTC TAG AGC TAA TAC ATG CG3′ and 5′CCC ATT TCC TTC GAA ACA GGA3′ were used for primary amplification and the primers 5′GGA AGG GTT GTA TTT ATT AGA TAA AG3′ and 5′AAG GAG TAA GGA ACA ACC TCC A3′ were used to perform secondary amplification. The primary PCR mixtures were made up of 2μl of template DNA, 2.5 U of TaqDNA Polymerase (DreamTaq Green DNA Polymerase, Thermofisher Scientific, Waltham, MA, USA), 0.5 × PCR buffer, 6mM $MgCl_2$, 200 μM each dNTPs, 100 mM each primer and 2 μl BSA (10mg ml^{-1}; New England Biolabs, Beverly, MA, USA) in a reaction volume of 50 μl.

The secondary PCR mixtures were similar to the primary PCR mixtures, except for the fact that the template was 2 μl of the primary PCR product, the concentration of $MgCl_2$ was 3 mM and BSA was not used. For positive controls, I used isolated DNA of *Cryptosporidium parvum* and PCR water was used as a negative control.

3.2.6.3 Giardia spp.

The obtained DNA was used to amplify the triosephosphate isomerase gene (TPI) of *Giardia* spp. using a nested PCR method according to the procedure of Sulaiman et al. (2003) from The United States Department of Health and Human Services and Mynářová et al. (2016) from the Faculty of Science, the University of South Bohemia in České Budějovice.

The primers 5′-AAA TIA TGC CTG CTC GTC G-3′ and 5′-CAA ACC TTI TCC GCA AAC C-3′ were used to perform primary amplification and the primers 5′-CCCTTCATCGGIGGTAACTT-3′ and 5′-GTGGCCACCACICCCGTGCC-3′ for the secondary amplification. The primary PCR mixtures consisted of 0.25–2.0 µl of template DNA, 200 µM each dNTPs, 1 × PCR buffer (Perkin Elmer, Wellesley, MA), 3 mM $MgCl_2$, 5 U of Taq polymerase (GIBCO BRL, Frederick, MD) and 200 nM each primer in a reaction volume of 100 µl.

The secondary PCR was performed under the same circumstances as the primary PCR with 2.5 µl of the primary PCR reaction. PCR water was used as a negative control and Giardia intestinalis DNA as a positive control.

3.2.6.4 *Ascaris* sp.

A primer for the Ascaridae family was used to sequentially distinguish between *Toxocara cati* and *Toxascaris leonina*, which anneals to a species-specific identical location.

Using the primer set AsITS1 reported by Carlsgart et al. (2009), the ITS (Internal Transcribed Spacer) region of nematodes in the Ascaridae family was amplified using nested PCR techniques. The primers were following: 5′-CTT GAA CCG GGT AAA AGT CG-3′ and 5′- ATG TGT CTG CAA TTC GCA CT-3′. The primary PCR mixtures were made up of 0.2 mM each dNTP (Ampliqon, Denmark), 0.4 mM each primer pair, 2.0 µl 10 × Taq-buffer (Ampliqon, Denmark), 3.5 mM $MgCl_2$ and 1 U TEMPase Hot Start DNA polymerase (Ampliqon, Denmark).

The multiplex PCR was performed under the same circumstances as the primary PCR. The ITS1 primer pair was utilized in conjunction with 0.1 mM As-Co1 primers at a concentration of 0.3 mM. Negative controls contained PCR water, while positive controls included eggs or single adult worms (ITS1) DNA.

3.2.7 Electrophoresis in agar gel

Agarose gel electrophoresis was used to analyse the PCR products. 1 % agarose gel was used in the laboratory of the Institute of Parasitology, Biology Centre CAS in České Budějovice, according to the methodology in Table 7.

Table 7 - Agarose gel electrophoresis instructions

Step	Instruction
1.	Mix TAE buffer with agarose.
2.	Dissolve the TAE buffer with agarose in an Erlenmeyer flask in the microwave.
3.	Cool under running water to the temperature that can be kept in a hand.
4.	Pippete 1 µl EtBr into the liquid gel and mix gently.
5.	Pour the gel into a prepared mold with a holder and a comb and allow to solidify.
6.	The solidification of the gel is about 5-10 min.
7.	After solidification, remove the comb and insert the gel into an electrophoretic bath with TAE buffer. Insert in a way that the starts are to the left of the negative electrode (the negative electrode is black, the positive red).
8.	Pipette DNA Ladder into the first well and the PCR secondary reaction products into the other wells. Last one is a negative control.
9.	Switch on the power supply and set the voltage between 65 and 70 V for approximately one hour.
10.	Using a UV transilluminator, visualize the electrophoresis result and document using a camera connected to a computer.

3.2.8 Gel isolation

According to the manufacturer's instructions (see Table 8), gel electrophoresis products were isolated using a commercial GenElute (Sigma-Aldrich, St. Louis, MO, USA) kit.

Table 8 - Gel isolation instructions

Step	Instruction
1.	Using a clean scalpel, cut out a DNA fragment from the gel and place it in a 1.5 ml microtube.
2.	In the microtube with the gel fragment, add 500 µl Gel Solubilization Solution (300 µl per 100 mg gel).
3.	Incubate at 50 °C for 10 minutes, stirring every 2-3 minutes to ensure complete dissolution (yellow colour develops).
4.	Pipette 500 µl of Column Preparation Solution into the assembled Binding Column G and centrifuge for 1 minute at 16,000 g.
5.	At 65 °C, incubate the PCR water to elute.
6.	Into the sample, pipette 150 µl of isopropanol and mix.
7.	Pipette the entire sample volume onto Binding Column G and centrifuge at 16,000 g for 1 minute.
8.	Pour the waste out of the collection tube.
9.	Pipette 700 µl Wash Solution G and centrifuge at 16,000 g for 1 minute.
10.	Pour the waste out of the collection tube and centrifuge for 1 minute at 16,000 g.
11.	In a centrifuge, rotate the tube by 180° and centrifuge for 3 minutes at 16,000 g.
12.	Elute by adding 30 µl PCR of water to the centre of the column in a new 1.5 ml tube.
13.	After 1 minute of incubation, centrifuge for 1 minute at 16,000 g.
14.	Using a NanoDrop spectrophotometer, determine the DNA concentration in the obtained eluate according to the manufacturer's instructions.
15.	Prepare DNA for commercial companies' sequencing requirements.

3.2.9 Sequencing and phylogenetic analyses

In a commercial company (SEQme, Ltd., Dobříš, Czech Republic), secondary PCR products were sequenced using secondary primers. The Chromas Pro software, version 2.1.10 (Technelysium Pty Ltd., Tewantin, Australia) was used to analyse the nucleotide sequences. After that, the sequences were compared using the BioEdit program to those stored in GenBank.

4 Results

4.1 Coprological findings of flotation centrifugation method

Based on flotation centrifugation techniques, faecal samples (n=222) were microscopically evaluated for the presence of *Toxoplasma gondii* oocysts and other parasitic pathogens that may have caused polyinfections. In addition to the already mentioned genus *Toxoplasma*, there were other parasitic genera *Cystoisospora*, *Giardia*, *Toxocara*, *Toxascaris* and *Capillaria*.

4.1.1 Occurrence of *Toxoplasma gondii* in captive cats

Table 9 shows that most *Toxoplasma* positive samples were detected in *Otocolobus manul* (Pallas's cat), which also confirmed the established hypothesis H_1, which assumed the predominant *Toxoplasma* infection in this particularly susceptible species for the presence of *Toxoplasma gondii* oocysts.

Hypothesis H_2 was also confirmed, discussing the presence of parasitic *Toxoplasma gondii* in the faeces of small felines bred in the Czech zoos. In addition to positive findings, dubious findings were also recorded in Pallas's cat and European wildcat (*Felis silvetris silvestris*), which were bred at the Zoo Jihlava, as shown in the Table 9.

Dubious findings were examined by the molecular PCR method to confirm or refute the presence of *Toxoplasma gondii* in the examined faecal samples. *Toxoplasma gondii* oocysts were not detected in other species of small felines.

Table 9 – *Toxoplasma gondii* findings in different feline species (June-August 2021)

Source: Bírošíková (2022)

Zoo	Species	Positive	Dubious	Negative	Total samples (n)
Olomouc	*Acinonyx jubatus*	0	0	30	30
Jihlava	***Felis silvestris silvestris***	0	1 (5.56%)	17	18
Olomouc		0	0	9	9
Olomouc	*Leopardus geoffroyi*	0	0	18	18
Jihlava	*Leptailurus serval*	0	0	18	18
Olomouc		0	0	25	25
Olomouc	*Lynx lynx carpathicus*	0	0	6	6
Jihlava	***Otocolobus manul***	23 (76.67%)	2 (6.67%)	5	30
Jihlava	*Prionailurus bengalensis euptilurus*	0	0	18	18
Olomouc		0	0	32	32
Jihlava	*Prionailurus bengalensis heaneyi*	0	0	18	18
Total		23 (10.36%)	3 (1.35%)	196 (88.29%)	**222**

The Table 10 provided an overview of the breeding of small cats from two zoos with results where the Zoo Jihlava was dealing with a problem of the spread of *T.gondii* oocysts in Pallas's cats, compared to other species of small felines, as already described in the literature review. Of the total number of faeces tested, positive samples of Pallas's cat represented 10.36%.

All positively or dubiously detected faecal samples for the presence of *Toxoplasma gondii* were taken at the Zoo Jihlava, as shown in Table 10, but each zoo bred unique species, so it was not possible to make an objective comparison.

Table 10 – Comparison of *Toxoplasma gondii* findings in two zoos (June-August 2021)

Source: Bírošíková (2022)

Zoo	Positive	Dubious	Negative	Total samples (n)
Jihlava	23	3	76	102
Olomouc	0	0	120	120
Total	23 (10.36%)	3 (1.35%)	196 (88.29%)	**222**

4.1.2 Occurrence of *Cystoisospora rivolta* in captive cats

The flotation method revealed the occurrence of *Cystoisospora rivolta* oocysts (see Table 11) in the faeces of bred zoo cats, in species Pallas's cat and Amur leopard cat. Dubious findings were found in both species as well as one dubious finding in cheetah. Positive samples predominated in the Amur leopard cat (12.50%) of all samples tested in this species. These findings also confirmed H_2.

Table 11 – *Cystoisospora rivolta* findings in different feline species (June-August 2021)

Source: Bírošíková (2022)

Zoo	Species	Positive	Dubious	Total samples (n)
Olomouc	***Acinonyx jubatus***	0	1 (3.33%)	30
Jihlava	*Felis silvestris silvestris*	0	0	18
Olomouc		0	0	9
Olomouc	*Leopardus geoffroyi*	0	0	18
Jihlava	*Leptailurus serval*	0	0	18
Olomouc		0	0	25
Olomouc	*Lynx lynx carpathicus*	0	0	6
Jihlava	***Otocolobus manul***	3 (10.00%)	3 (10.00%)	30
Jihlava	***Prionailurus bengalensis euptilurus***	0	0	18
Olomouc		4 (12.50%)	2 (6.25%)	32
Jihlava	*Prionailurus bengalensis heaneyi*	0	0	18
Total		7 (3.15%)	6 (2.70%)	**222**

4.1.3 Occurrence of *Giardia* spp. in captive cats

Other flotation findings in the faeces of small felines included cysts of *Giardia* spp., visible only when using an eyepiece of 10× and an objective of 40×. Of the total number of tested samples (n=222), they were detected in ten samples (4.50%) from small felines of the species: Pallas's cat, European wildcat and cheetah, as shown in Table 12. The occurrence was recorded in both monitored zoos, which contributed to the confirmation of H_2.

Table 12 - *Giardia* spp. positive findings in different feline species (June-August 2021)

Source: Bírošíková (2022)

Zoo	Species	Positive	Total (n)
Olomouc	***Acinonyx jubatus***	1 (3.33%)	30
Jihlava	***Felis silvestris silvestris***	1 (5.56%)	18
Olomouc		0	9
Olomouc	*Leopardus geoffroyi*	0	18
Jihlava	*Leptailurus serval*	0	18
Olomouc		0	25
Olomouc	*Lynx lynx carpathicus*	0	6
Jihlava	***Otocolobus manul***	8 (26.67%)	30
Jihlava	*Prionailurus bengalensis euptilurus*	0	18
Olomouc		0	32
Jihlava	*Prionailurus bengalensis heaneyi*	0	18
Total		10 (4.50%)	**222**

4.1.4 Occurrence of Nematoda in captive cats

Nematode eggs (phylum Nematoda) were detected in the tested samples, which contributed to the confirmation of H_2.

Of the total number of faecal samples (n=222), the most findings of roundworm eggs *Toxocara cati*, with zoonotic potential, were generally recorded in 33 samples (representing 14.86% of all tested samples) as can be seen in Table 13. Of these 33 samples, most *T.cati* eggs were detected in Pallas's cat and serval species. Only one finding was recorded in species: cheetah, Carpathian lynx and Amur leopard cat.

Another species of roundworm detected was *Toxascaris leonina*, which occured mainly in lynx and cheetahs bred at the Zoo Olomouc.

The eggs of the genus *Capillaria* occurred three times in only one male Pallas's cat from the Zoo Jihlava (see Table 13).

Table 13 - Nematoda positive findings in different feline species (June-August 2021)

Source: Bírošíková (2022)

Zoo	Species	*Toxocara cati*	*Toxascaris leonina*	*Capillaria* sp.	Total samples (n)
Olomouc	***Acinonyx jubatus***	1 (3.33%)	14 (46.67%)	0	30
Jihlava	***Felis silvestris silvestris***	0	0	0	18
Olomouc		0	1 (11.11%)	0	9
Olomouc	***Leopardus geoffroyi***	3 (16.67%)	0	0	18
Jihlava	***Leptailurus serval***	1 (5.56%)	0	0	18
Olomouc		5 (20.00%)	1 (4.00%)	0	25
Olomouc	***Lynx lynx carpathicus***	1 (16.67%)	3 (50.00%)	0	6
Jihlava	***Otocolobus manul***	21 (70.00%)	0	3 (10.00%)	30
Jihlava	***Prionailurus bengalensis euptilurus***	0	0	0	18
Olomouc		1 (3.13%)	0	0	32
Jihlava	*Prionailurus bengalensis heaneyi*	0	0	0	18
Total		33 (14.86%)	19 (8.56%)	3 (1.35%)	**222**

4.2 Evaluation of McMaster's method

For faecal samples that were positively detected by the flotation for nematodes, McMaster's counting technique was performed, which is designed to prove the number of eggs in one gram of faeces (EPG) to determine the intensity of parasitic infection in the faeces of small cats kept in the Czech Republic.

4.2.1 The intensity of parasitic infection at the Zoo Jihlava

At the Zoo Jihlava, faecal samples from Pallas's cats showed positive findings of nematode eggs. In one sample of the bred serval pair (see Table 14), the presence of feline roundworm *Toxocara cati* eggs was detected by flotation and the number of eggs in the faeces per gram was found to be 4,200 EPG using the McMaster's method, which is the second largest infection in the tested samples. The highest number of *Toxocara cati* roundworm eggs was found in the male Pallas's cat (6,225 EPG), while the lowest value (25 EPG) was found in the other male Pallas's cat.

As mentioned in the previous chapter (4.1.4 Occurrence of Nematoda in captive cats), the finding of three positive samples for the presence of capillaries (*Capillaria* sp.) in one male Pallas's cat was proved, when the highest EPG was 775.

The average value of positive findings of *Toxocara cati* eggs in faeces was 1,144.32 EPG and in samples with *Capillaria* sp. it was 516.67 EPG. A larger parasitic infection of *Toxocara cati* was found at the Zoo Jihlava in Pallas's cats and *Toxascaris leonina* did not occur in the samples from the Zoo Jihlava.

Table 14 - EPG of captive cats from the Zoo Jihlava

Source: Bírošíková (2022)

Species	ID	*Toxocara cati* EPG	*Capillaria* sp. EPG	Calculated or Estimated
Leptailurus serval	LSC1	4,200	N	C
Otocolobus manul	OMM1	75	N	C
Otocolobus manul	OMM1	25	N	C
Otocolobus manul	OMM1	75	N	C
Otocolobus manul	OMM1	300	N	E
Otocolobus manul	OMM1	75	N	E
Otocolobus manul	OMM1	300	N	C
Otocolobus manul	OMM1	75	N	E
Otocolobus manul	OMM1	1,025	N	C
Otocolobus manul	OMM1	550	N	E
Otocolobus manul	OMM1	100	N	E
Otocolobus manul	OMM1	200	N	C
Otocolobus manul	OMM1	200	N	E
Otocolobus manul	OMM2	1,175	775	C
Otocolobus manul	OMM2	325	400	C
Otocolobus manul	OMM2	1,025	375	C
Otocolobus manul	OMM3	3,200	N	C
Otocolobus manul	OMM3	1,125	N	E
Otocolobus manul	OMM3	6,225	N	C
Otocolobus manul	OMF1	1,500	N	E
Otocolobus manul	OMF1	1,750	N	C
Otocolobus manul	OMF1	1,650	N	C
Mean (positive samples)		1,144.32	516.67	

Legend: N – negative; C – calculated by McMaster; E – estimated with coefficient

4.2.2 The intensity of parasitic infection at the Zoo Olomouc

The Zoo Olomouc had the highest incidence of *Toxascaris leonina* with the largest value of 19,725 EPG in the female cheetah (see Table 15), other high EPG values also belonged to this particular individual (as can be read from the ID in Table 15).

Samples from a pair of cheetah, European cat and Carpathian lynx had the lowest value (50 EPG) of *Toxascaris leonina*. In addition, the occurrence of the *Toxocara cati* eggs was recorded in the serval, with the highest value (3,225 EPG). The lowest value, specifically 25 EPG *Toxocara cati*, had a sample from Geoffroy's cat.

The mean values of the positive samples were 220.83 EPG for *Toxocara cati* and 2,109.26 EPG for *Toxascaris leonina*. It follows that at the Zoo Olomouc there was a greater infection with the roundworm *Toxascaris leonina* than at the Zoo Jihlava, where this infection did not occur at all.

Table 15 - EPG of captive cats from the Zoo Olomouc

Source: Bírošíková (2022)

Species	ID	*Toxocara cati* EPG	*Toxascaris leonina* EPG	Calculated or Estimated
Acinonyx jubatus	AJC1	N	1,875	E
Acinonyx jubatus	AJC1	125	50	E
Acinonyx jubatus	AJC1	N	3,950	C
Acinonyx jubatus	AJC1	N	850	E
Acinonyx jubatus	AJC1	N	125	C
Acinonyx jubatus	AJF1	N	125	E
Acinonyx jubatus	AJF1	N	19,725	C
Acinonyx jubatus	AJF1	N	2,125	E
Acinonyx jubatus	AJF1	N	425	E
Acinonyx jubatus	AJF1	N	6,800	C
Acinonyx jubatus	AJF1	N	8,900	C
Acinonyx jubatus	AJF1	N	8,725	C
Acinonyx jubatus	AJF1	N	2,000	C
Acinonyx jubatus	AJF1	N	850	E
Felis silvestris silvestris	FSSF1	N	50	E
Leopardus geoffroyi	LGC1	50	N	E
Leopardus geoffroyi	LGC1	25	N	E
Leopardus geoffroyi	LGC1	125	N	E
Leptailurus serval	LSF1	50	50	E
Leptailurus serval	LSM1	150	N	C
Leptailurus serval	LSM1	475	N	E
Leptailurus serval	LSM1	700	N	C
Leptailurus serval	LSM1	3,225	N	E
Lynx lynx carpathicus	LLCC1	75	200	E
Lynx lynx carpathicus	LLCC1	N	50	E
Lynx lynx carpathicus	LLCC1	N	75	C
Prionailurus bengalensis euptilurus	PBEM1	300	N	C
Mean (positive samples)		220.83	2,109.26	

Legend: N – negative; C – calculated by McMaster; E – estimated with coefficient

4.3 Staining method findings

Based on the staining technique according to Miláček & Vítovec (1985), faecal samples (n=222) were examined and subsequently microscopically evaluated for the presence of *Cryptosporidium* spp. oocysts.

4.3.1 Occurrence of *Cryptosporidium* spp. in captive cats

The staining technique according to Miláček & Vítovec (1985) revealed a total of 22 positive and seven dubious samples out of a total of 222 faecal samples of small cats at the Czech zoos (see Table 16).

The most positive samples (see Figure 11) with this method were detected in the species of Pallas's cats (43.33%) out of 30 samples. Positive or dubious findings were also recorded in all other species except the Carpathian lynx, as shown in Table 16. The results confirmed the established hypothesis H_2.

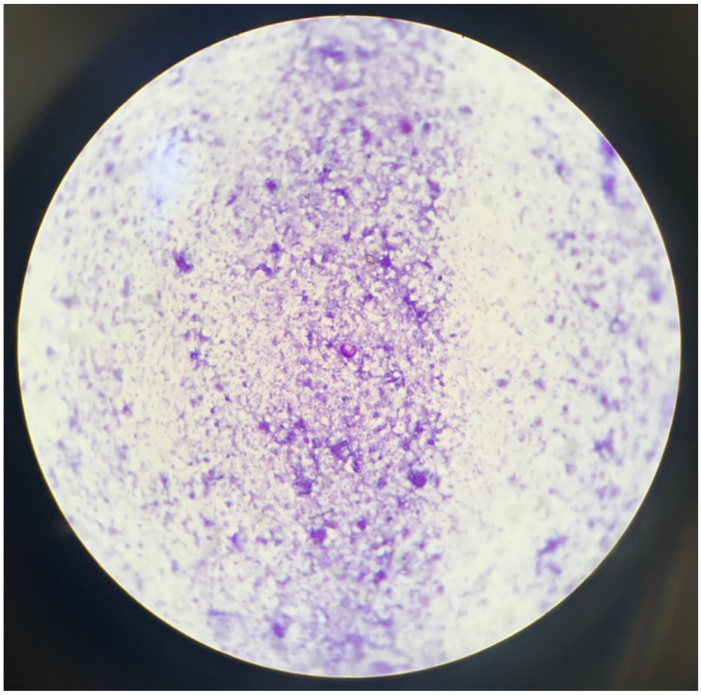

Figure 11 - Stained oocyst of *Cryptosporidium felis* from Pallas's cat

Source: Bírošíková (2021)

Table 16 - *Cryptosporidium* spp. findings in different feline species (June-August 2021)

Source: Bírošíková (2022)

Zoo	Species	Positive	Dubious	Negative	Total samples (n)
Olomouc	***Acinonyx jubatus***	2 (6.67%)	2 (6.67%)	26	30
Jihlava	***Felis silvestris silvestris***	2 (11.11%)	0	16	18
Olomouc		1 (11.11%)	0	8	9
Olomouc	***Leopardus geoffroyi***	2 (11.11%)	0	16	18
Jihlava	***Leptailurus serval***	0	0	18	18
Olomouc		0	1 (4.00%)	24	25
Olomouc	*Lynx lynx carpathicus*	0	0	6	6
Jihlava	***Otocolobus manul***	13 (43.33%)	1 (3.33%)	16	30
Jihlava	***Prionailurus bengalensis euptilurus***	1 (5.56%)	0	17	18
Olomouc		1 (3.13%)	2 (6.25%)	29	32
Jihlava	***Prionailurus bengalensis heaneyi***	0	1 (5.26%)	17	18
Total		22 (9.91%)	7 (3.15%)	193 (86.94%)	**222**

Regarding the representation of positive samples in individual zoos, it can be observed in Table 17 that 15.69% of the tested samples from the Zoo Jihlava were positive, while at the Zoo Olomouc it was 5% of the tested samples in this zoo. Thus, according to this staining method, oocysts of *Cryptosporidium* spp. were detected at both tested zoos.

Table 17 - Comparison of *Cryptosporidium* spp. findings in two zoos (June-August 2021)

Source: Bírošíková (2022)

Zoo	Positive	Dubious	Negative	Total samples (n)
Jihlava	16 (15.69%)	2 (1.96%)	84 (82.35%)	102
Olomouc	6 (5.00%)	5 (4.17%)	109 (90.83%)	120
Total	22 (9.91%)	7 (3.15%)	193 (86.94%)	**222**

4.4 PCR confirmation

The PCR method was used to confirm positive or dubious findings based on flotation and staining techniques. Seven faecal samples of small cats from the Czech zoos were tested by molecular analysis to confirm the presence of *Toxoplasma gondii* oocysts.

A total of 40 faecal samples were tested for the presence of *Cryptosporidium* spp. and 12 faecal samples were isolated and detected by PCR for *Toxocara cati* and *Toxascaris leonina*. Last but not least, four faecal samples were used to confirm the detection of *Giardia* spp. cysts (phylum Mastigophora).

4.4.1 Toxoplasma gondii

Table 18 showed that out of seven isolated samples, three faecal samples were positive for *Toxoplasma gondii* in three individuals of the Pallas's cat species from the Zoo Jihlava. According to the ID, there were two males and one female of this species, which confirmed hypotheses H_1 and H_3. All obtained sequences were 100% identical to the sequence stored in the GenBank database (reference sequence no. KT881323).

Table 18 - PCR results for positive findings of *Toxoplasma gondii* by flotation

Source: Bírošíková (2022)

Species	ID	Zoo	Flotation	Genotypization
Felis silvestris silvestris	FSSM1	J	D	N
Otocolobus manul	OMM1	J	P	N
Otocolobus manul	OMM1	J	P	*Toxoplasma gondii*-KT881323
Otocolobus manul	OMM2	J	P	*Toxoplasma gondii*-KT881323
Otocolobus manul	OMM3	J	D	Unreadable sequence
Otocolobus manul	OMF1	J	P	*Toxoplasma gondii*-KT881323
Otocolobus manul	OMF2	J	P	N

Legend: J – Zoo Jihlava; P – positive; D – dubious; N – negative

4.4.2 *Cryptosporidium* spp.

Out of a total of 23 tested faecal samples from the Zoo Jihlava using the molecular method, eight positive samples (see Table 19) were confirmed for the presence of *Cryptosporidium* spp. and H_3 was confirmed.

These were three individuals of the Pallas's cat species, according to ID two males and one female. *Cryptosporidium felis* specific DNA was detected and all sequences were 100% identical to the KF287127 reference sequence stored in the GenBank database.

Table 19 - PCR results for *Cryptosporidium* spp. in feline faecal samples from the Zoo Jihlava

Source: Bírošíková (2022)

Species	ID	Staining	Genotypization
Felis silvestris silvestris	FSSM1	N	N
Felis silvestris silvestris	FSSM1	P	N
Felis silvestris silvestris	FSSM1	P	N
Felis silvestris silvestris	FSSM1	N	N
Leptailurus serval	LSC1	N	N
Otocolobus manul	OMF1	P	*Cryptosporidium felis* - KF287127
Otocolobus manul	OMF1	P	N
Otocolobus manul	OMF1	P	*Cryptosporidium felis* - KF287127
Otocolobus manul	OMF2	N	N
Otocolobus manul	OMM1	P	N
Otocolobus manul	OMM1	P	N
Otocolobus manul	OMM1	P	N
Otocolobus manul	OMM1	P	N
Otocolobus manul	OMM1	P	N
Otocolobus manul	OMM1	P	*Cryptosporidium felis* - KF287127
Otocolobus manul	OMM1	P	*Cryptosporidium felis* - KF287127
Otocolobus manul	OMM1	N	*Cryptosporidium felis* - KF287127
Otocolobus manul	OMM2	N	N
Otocolobus manul	OMM3	P	*Cryptosporidium felis* - KF287127
Otocolobus manul	OMM3	P	*Cryptosporidium felis* - KF287127
Otocolobus manul	OMM3	P	*Cryptosporidium felis* - KF287127
Prionailurus bengalensis euptilurus	PBEM2	P	N
Prionailurus bengalensis heaneyi	PBHC1	D	N

Legend: P – positive; D – dubious; N – negative

In the case of Olomouc Zoo (see Table 20), the presence of specific DNA of *Cryptosporidium felis* was also molecularly confirmed with 100% identity to the sequence KF287127 stored in the GenBank database and the hypothesis H_3 was confirmed. These were samples from a pair of cheetahs and a male serval.

The samples were intended for the detection of another parasitic pathogen, therefore it could happen that according to the staining technique the sample from the male serval was not positive, but the presence of *Cryptosporidium felis* was detected by the molecular PCR method.

Table 20 - PCR results for *Cryptosporidium* spp. in feline faecal samples from the Zoo Olomouc

Source: Bírošíková (2022)

Species	ID	Staining	Genotypization
Acinonyx jubatus	AJC1	P	*Cryptosporidium felis* - KF287127
Acinonyx jubatus	AJC1	N	N
Acinonyx jubatus	AJC1	D	N
Acinonyx jubatus	AJF1	P	N
Acinonyx jubatus	AJM1	D	N
Felis silvestris silvestris	FSSF1	P	N
Leopardus geoffroyi	LGC1	N	N
Leopardus geoffroyi	LGC1	P	N
Leopardus geoffroyi	LGC1	P	N
Lynx lynx carpathicus	LLCC1	N	N
Leptailurus serval	LSF1	N	N
Leptailurus serval	LSF1	D	N
Leptailurus serval	LSM1	N	*Cryptosporidium felis* - KF287127
Prionailurus bengalensis euptilurus	PBEFY1	D	N
Prionailurus bengalensis euptilurus	PBEM1	P	N
Prionailurus bengalensis euptilurus	PBEM1	D	N
Prionailurus bengalensis euptilurus	PBEM1	N	N

Legend: P – positive; D – dubious; N – negative

4.4.2.1 Staining method vs PCR (*Cryptosporidium* spp.)

The following Table 21 shows the positive detection of individual methods, which were the aniline-carbol-methyl violet staining method (Miláček & Vítovec 1985) and the PCR molecular method. According to the staining method, a total of 22 samples of feline faeces, out of a total of 40 samples, were marked as positive and six samples as dubious.

PCR analysis confirmed only ten positive samples of *Cryptosporidium felis* (n=40). As mentioned, samples tested for the presence of *Cryptosporidium* spp. oocysts were not all isolated to detect this particular species of parasite, so some negative samples in the staining technique were PCR positive or vice versa. Possible causes will be discussed in the Discussion chapter.

Table 21 - Evaluation of staining method vs PCR for *Cryptosporidium* spp. at two zoos (June-August 2021)

Source: Bírošíková (2022)

Zoo	Species	P (Staining)	D (Staining)	N (Staining)	P (PCR)	N (PCR)	Total (n)
O	***Acinonyx jubatus***	2 (40.00%)	2 (40.00%)	1	1 (20.00%)	4	5
J	***Felis silvestris silvestris***	2 (50.00%)	0	2	0	4	4
O		1 (100%)	0	0	0	1	1
O	***Leopardus geoffroyi***	2 (66.67%)	0	1	0	3	3
O	***Leptailurus serval***	0	1 (25.00%)	3	1 (25.00%)	3	4
O	*Lynx lynx carpathicus*	0	0	1	0	1	1
J	***Otocolobus manul***	13 (81.25%)	0	3	8 (50.00%)	8	16
J	***Prionailurus bengalensis euptilurus***	1 (100%)	0	0	0	1	1
O		1 (25.00%)	2 (50.00%)	1	0	4	4
J	***Prionailurus bengalensis heaneyi***	0	1 (100.00%)	0	0	1	1
Total		22 (55.00%)	6 (15.00%)	12 (30.00%)	10 (25.00%)	30 (75.00%)	**40**

Legend: J – Zoo Jihlava; O – Zoo Olomouc; P – positive; D – dubious; N – negative

4.4.3 *Giardia* spp.

Of the four isolated faecal samples of small felines from the Zoo Jihlava (see Table 22), all were 100% identical to the reference sequence of *Giardia intestinalis* LC437479, which also helped to confirm hypothesis H_3. It was one male of the species European wildcat, two male Pallas's cats and one female Pallas's cat according to the assigned ID.

Table 22 - PCR results for positive findings of *Giardia* spp. by flotation

Source: Bírošíková (2022)

Species	ID	Zoo	Genotypization
Felis silvestris silvestris	FSSM1	J	*G. Intestinalis* - LC437479
Otocolobus manul	OMM1	J	*G. Intestinalis* - LC437479
Otocolobus manul	OMM3	J	*G. Intestinalis* - LC437479
Otocolobus manul	OMF1	J	*G. Intestinalis* - LC437479

Legend: J – Zoo Jihlava

4.4.4 Nematoda

Of the 12 isolated faecal samples for the detection of *Toxocara cati* or *Toxascaris leonina* (see Table 23), three samples were positive with *Toxascaris leonina* specific DNA and 100% matched the reference sample of accession no. MK315217 from the GenBank database. Seven positive samples with *Toxocara cati* specific DNA were 100% identical to reference sample MK309924. *Toxascaris leonina* was molecularly confirmed in a pair of cheetahs, a female cheetah bred individually and a pair of Carpathian lynx. *Toxocara cati* was confirmed in a pair of Geoffroy's cats, a pair of servals, a male of serval, three individual males of Pallas's cat and one female Pallas's cat. Detection of nematodes was confirmed in faecal samples at both zoos and H_3 was confirmed for both species of roundworms.

Table 23 - PCR results for positive findings of Nematoda by flotation

Source: Bírošíková (2022)

Species	ID	Zoo	Genotypization
Acinonyx jubatus	AJC1	O	*Toxascaris leonina* – 100% identity with MK315217
Acinonyx jubatus	AJF1	O	*Toxascaris leonina* – 100% identity with MK315217
Leopardus geoffroyi	LGC1	O	*Toxocara cati* – 100% identical with MK309924
Lynx lynx carpathicus	LLCC1	O	*Toxascaris leoni*na – 100% identity with MK315217
Leptailurus serval	LSC1	J	*Toxocara cati* – 100% identical with MK309924
Leptailurus serval	LSF1	O	Unreadable sequence
Leptailurus serval	LSM1	O	*Toxocara cati* – 100% identical with MK309924
Otocolobus manul	OMM1	J	*Toxocara cati* – 100% identical with MK309924
Otocolobus manul	OMM2	J	*Toxocara cati* – 100% identical with MK309924
Otocolobus manul	OMM3	J	*Toxocara cati* – 100% identical with MK309924
Otocolobus manul	OMM3	J	Unreadable sequence
Otocolobus manul	OMF1	J	*Toxocara cati* – 100% identical with MK309924

Legend: J – Zoo Jihlava; O – Zoo Olomouc

4.5 Sources and routes of spread of parasites

Possible routes of pathogens, such as *Toxoplasma gondii* and *Cryptosporidium* spp., spread were investigated at the Zoo Jihlava and the Zoo Olomouc, from which faecal samples of felines were regularly sampled. Prevention, as already mentioned in the literature review, is a key condition for maintaining a healthy environment for both kept animals and for management that may come into close contact with animals. The following subchapters have described the approaches of individual zoos to the issue and point out the risks of sources and routes of transmission of parasitic pathogens. Attention was paid to diets, environmental risks, breeders' behaviour, cleanliness, disinfection, isolation and possible treatments.

4.5.1 Risk factors at the Zoo Jihlava

Unique species of small felines such as Pallas's cat, but also Amur leopard cat, European wildcat, Palawan leopard cat and servals are bred at the Zoo Jihlava. Breeding risks were evaluated according to my own observations and personal interviews with Mgr. Richard Viduna.

Feed: The animals were fed on rabbits, mice, chickens, occasionally quails and some of them even fish. The meat was from local sources from Kostelec town, chickens from the hatchery (veterinary treated) and Sokol Falco company, mice, rats and quails from a private breeder. The rabbits were from a private breeder as well. Small cats were not fed with too big pieces of meat such as beef or pork. Fish and mice were mostly frozen before.

Feed ration contained fish once a week (alternates freshwater and marine). Furthermore, one of the options of beef, rabbit and chicken was chosen. The pork was not given much because of its fatness. Fasting was once or twice a week. They received supplementation with vitamins B and C. Females after birth received greater vitamin supplementation, as needed. The kittens were provided with a supplemental feed from the first month. For instance, Pallas's cat was without fasting and was fed mostly mice and quails that were previously frozen due to parasites. Amur leopard cat was given a quarter chicken and two to three rats.

Water source: Water at the zoo was from potable inspected sources. Some animals also had water ditches with non-potable water, but that was very occasional in the enclosure with the servals. Nowadays, it was not present anymore.

Cleaning: The cleaning was done every day. The substrate was changed two to three times a year. Manure was exported to compost in a container in front of the staff entrance.

Disinfection: Detergent of brand Jar, natural soap or disinfection was used if a problem occurred. Disinfection mats were not used at all.

Environmental risks: Servals were kept on concrete with an outdoor nature exposure. Palawan leopard cats were kept in the Cat house with wood chips, bark and other natural material with also outdoor exposure. Amur leopard cat was in a cage with sand and wood chips. Especially Pallas's cat had as a plain cage as possible, without any substrate, they were in a cage with sand used as a toilet. Only one female of Pallas's cat was in the exposition on natural material. European wildcat male was individually kept on wood and sand.

The sand was taken from outside, where it was induced. Wood chips were stored under the roof, where wild cats could access them. However, the bark and wood chips were stored for a limited time. They were usually used straight from the wood processing industry.

Occurrence of wild animals: Stray cats could be found at the zoo and they could access some of the stored materials, which were used as a substrate or for a cat toilet.

Vaccination: Cats were once a year revaccinated. The Zoo Jihlava did not vaccinate against rabies. For cat diseases, kittens were vaccinated twice a year and revaccinated once.

Deworming: Deworming was done once a year or in case of any visible problem. Cats with outside enclosures were dewormed once every six months. Once a year the faecal examination was also done. Antihelmintic Caniverm® (Bioveta, Ivanovice na Hané) was provided to the Palawan leopard cat on 13th August 2021 and to the European wildcat on 29th September 2021 two millilitres for three days. Anthelmintic Panacur® (Intervet, the Neatherlands) was given to servals on 13th August 2021.

Other treatment: Antiprotozoics Entizol® (Polpharma, Poland) was provided to European wildcat on 21st July 2021 and to Pallas's cat also on 21st July 2021, in both cases half a tablet for five days. These antiprotozoics are also effective against *Giardia* spp. Antibacterial Sumetrolim® (EGIS Pharmaceuticals PLC, Hungary) was given to Pallas's cat for 5 days from 11th June 2021, but the individual was not eating these tablets, so further treatment was done on 14th June 2021 one and a half millilitre for three days by active substance toltrazuril (Baycox®, Bayer, Canada).

Historical problems: Historical problems were *Giardia* and *Chlamydia* occurrence, which were the two most often occurring. Youngs of Pallas's cats died of toxoplasmosis, although *Toxoplasma* infection had never been confirmed. When *Toxoplasma* infection was asymptotic, no treatment was given. Otherwise, they received antibiotics.

Currently bred individuals of Pallas's cat were all asymptomatic. Besides this, only one-time problems occurred. They brought a Pallas's cat infected with *Giardia* from Italy. The animal was quarantined for some time, but not in 100% isolation. The female of Pallas's cat who was exposed in the exposition had confirmed herpes infection, so she was given antibiotics.

They were not succeeding to cure the *Toxocara* and *Cystoisospora* infections. Animals were re-treated three times, but infections were still present. Medical products were put in the feed (e.g. into mice), but there was a chance that the animals did not eat them and throw them away from their food. It was hard to provide the medication by hand as the Zoo Jihlava did not want to cause stress to animals.

After treatment, a check-up was done, however, if the treatment had not been effective and the animal did not show clinical signs, treatment was interrupted. It was not possible

to treat and re-treat animals over and over again. Therefore, they tried to keep them in a good state of health.

Import of new animals: Imported animals for replenishment were in quarantine. As a standard, they were investigated at the place of dispatch.

Isolation of new animals: Some animals were brought to each other immediately, because of a lack of space. Imported Pallas's cat and Amur leopard cat from Russia had a mandatory quarantine there, because they were coming to the zoo from outside Europe. However as mentioned before, sometimes the isolation was not 100%, so the *Giardia* infection occurred. Animals coming from Europe were examined and sometimes put straight to other kept animals.

Breeders: One breeder took care of multiple animals, for instance, Palawan leopard cats and servals had one breeder. Three breeders were usually switching.

Risky objects: The breeders neither changed their rubber shoes, nor used disinfection mats. Usually one cleaning equipment per area was used. A different shovel was used for servals and Palawan leopard cats, so they had their own. However, for individuals kept in back facilities, Pallas's cats, Amur leopard cat and European wildcat, only one common cleaning equipment was used.

To sum it up, food risks were rather unlikely, the only problem could occur from meat provided by private breeders. Environmental risks posed a greater risk as stray cats had access to some stored substrates and other materials. Breeders also posed a risk of pathogen transmission as they did not use disinfectant mats or change their shoes when approaching different animal. The use of the same tools could also significantly increase the risk of transmission. And last but not least, 100% insolation could be more effective, although it could not always be possible due to the limited capacity of zoos.

4.5.2 Risk factors at the Zoo Olomouc

At the Zoo Olomouc are bred cheetahs, European wildcat, Geoffroy's cat, servals, Carpathian lynx and Amur leopard cat, which were the subjects of testing in this thesis. Breeding risks were evaluated according to own observations and personal interviews with Ing. Jitka Vokurková.

Feed: Cats were regularly fed in the afternoon. In terms of meat sources, the Zoo Olomouc provided the animals meat for human consumption from Holešov town, quality beef for cheetahs from supermarket Makro, and rabbits were from a factory farm (eviscerated and without hair). They also got fish once a year, which were caught as less desirable during fish harvest, and were frozen for later. Rodents such as mice or guinea pigs were from breeders, or some animals were gained by zoo staff. One day old chicks were obtained directly from the hatchery. The meat provided to cheetahs was the best, certified for human consumption at the same time. For example, the lynx could tolerate fatter meat.

Water source: The water source of drinking water was from a regularly inspected well. This water was provided to animals in bowls. Transmission of pathogens was unlikely.

Cleaning: Enclosures were cleaned of faeces every day. There was irregular sweeping as well. Wet cleaning was done twice a season. The inner enclosures of the cheetahs were sprayed with water. Wet cleaning was not possible in winter due to ice formation. The bedding was completely replaced once a year in the spring.

Disinfection: Disinfection was performed approximately two to three times a year. Disinfection mats were only used in enclosures with primates, not with cats.

Environmental risks: A possible environmental threat was outdoor enclosures with grass, which could not be disinfected. For example, lynxes were actually just outside, they did not even have water established. The sand was mostly freshly brought from the

construction site but was used minimally. The sand was also not stored anywhere, so there is no danger of using it as a toilet for feral cats.

Occurrence of wild animals: There were wild foxes and cats at the zoo, but they did not have a chance to get into enclosures to kept animals.

Vaccination: Vaccination was done in the spring.

Deworming: The animals were dewormed regularly twice a year. It was done in the spring before vaccination and in the autumn for a check-up. It could also be done during the year in case of any problem. The animals were also dewormed before and after transport, including kittens. Anthelmintic Panacur® (Intervet, the Neatherlands) was given to the cheetahs from 16th to 18th July 2021, to Amur leopard cat on 15th July, 16th August and 24th September 2021 and to servals on 16the August. Anthelmintic Cestal® (Ceva Animal Health, Bratislava) was given to Carpathian lynx on 14th June 2021. Verm-X® granules (Paddocks Farm Partnership Ltd, UK) were also provided to cheetahs to control intestinal parasites.

Historical problems: There were no historical problems. Nematodes were sometimes present, and cheetahs had more likely visible nematodes, therefore they were getting granules Verm-X® (Paddocks Farm Partnership Ltd, UK), so monthly deworming was not necessary.

Import of new animals: If new individuals were imported into the zoo, a parasite control statement was required, and control was also done during the importing of the individuals.

Isolation of new animals: Isolation varied from animal to animal, especially according to stress management. In general, a new animal was next to an original one without direct contact, but it could hear the other animal, which could be comforting.

Breeders: One breeder took care of multiple animals. The breeders were switching, it was different on weekdays and weekends.

Risky objects: The breeders did not change their shoes, which could be the possible transfer of pathogens. However, each cage had its own equipment. Each shovel was their own, they only used a common bucket to export manure.

In conclusion, the risk of persistent infection with, for example, nematodes was mainly on grassland enclosures with cheetahs and lynx, where it was impossible to disinfect surfaces.

Furthermore, the zookeepers might pose a risk of transmission because one breeder was in charge of more animals but did not change shoes and a disinfection mat was not present. Food, water and substrates should not pose a risk. However, caution should be exercised with rodents obtained from private breeders.

5 Discussion

By my results of the **flotation method**, oocysts of ***Toxoplasma gondii*** occurred the most frequently in the faeces of Pallas's cat *Otocolobus manul* (Pallas 1776), which was expected with H_1 due to the high susceptibility of this species to *Toxoplasma* infection, reported by Kenny et al. (2002), Brown et al. (2005) and Girling et al. (2020), who particularly found a death rate of 60-65% in Pallas's cat's kittens in captivity.

According to my own personal communication with Mgr. Richard Viduna, kittens of Pallas's cats died of toxoplasmosis at the Zoo Jihlava, however, the infection had never been confirmed. In this thesis, PCR analyses confirmed the presence of *Toxoplasma gondii* (Genbank accession no. KT881323) in the faeces of two males and one female of Pallas's cats bred at the Zoo Jihlava (confirming H_3), indicating that the infection could be present. A dubious finding of *Toxoplasma gondii* was also found at this zoo by flotation in the faeces of European wildcat, however, the PCR result was negative. The rest of the tested small cats in this thesis, including Geoffroy's cat and Amur leopard cat, had all negative results by the flotation method, which contradicts the findings of Lukešová & Literák (1998) and Kudrnáčová (2014). Lukešová & Literák (1998) detected four *Toxoplasma* positive faecal samples in Geoffroy's cat from the Zoo Jihlava. Kudrnáčová (2014) found one positive sample from a European wildcat from the Zoo Olomouc, one in an Amur leopard cat and four positive with one dubious findings in an Amur leopard cat from the Zoo Jihlava, Nevertheless PCR analyses were not done at that time, so their findings were not confirmed.

Another author, who performed PCR analyses on serum samples from the Czech zoos was Bártová et al. (2018), who detected *Toxoplasma gondii* DNA in a stray cat from the Czech zoo, even though they did not achieve full genotyping. In their study, they performed an indirect fluorescent antibody test (IFAT) and found samples from cheetah, Pallas's cat and serval seropositive.

With the use of the **staining method** according to Miláček & Vítovec (1985), it was found that positive or dubious findings of ***Cryptosporidium* spp.** were present in all species of Felinae subfamily in both monitored the Czech zoos, except of Carpathian lynx, confirming H_2. Subsequent PCR detection confirmed *Cryptosporidium felis* (Genbank Accession no. KF287127) in two males and one female of Pallas's cat from the

Zoo Jihlava and in cheetah and serval samples from the Zoo Olomouc, confirming hypotheses H_3 of this theses as well. Other authors, Hajdušek et al. (2004) and Kváč et al. (2017) identified *Cryptosporidium felis* from samples from the Czech cats (*Felis catus*).

Kudrnáčová (2014) recorded findings of *Cryptosporidium* oocyst by the flotation method. In my case, I did not rely on the flotation method, because of the occurrence of yeasts in the faeces, which were hard to tell apart. Gerace et al. (2019) also mentioned their similarity and suggested Lugol iodine staining. Therefore, the staining method by Miláček & Vítovec (1985) was used to determine the occurrence of *Cryptosporidium* spp. followed by PCR, as recommended by Fayer et al. (2006) to clearly identify the species.

Some samples were intended for the detection of **another parasitic pathogen** but were nevertheless tested for the presence of *Cryptosporidium* spp. by PCR, therefore, some of the samples marked as negative by the staining technique were positive by PCR. This was a case with one faecal sample from Pallas's cat's male, which, however, turned out to be positive in other samples and in one sample from serval's male, which was not recorded as positive at all by the staining method. This could be due to the large amount of hair in the faeces, which in general caused difficult smears for the staining method, impracticable McMaster's method and a difficult sample amount for DNA isolation.

In the case of other pathogens found, on which this work was not focused, the flotation method revealed positive and dubious findings of ***Cystoisospora rivolta*** in the faecal samples of three species, however, we were not able to obtain primer to confirm it. Further *Giardia* spp. was found in three species based on the flotation method and confirmed by PCR in two species (European wildcat and Pallas's cat). The occurrence of Nematoda was also noted in this thesis, specifically ***Toxocara cati*, *Toxascaris leonina*** and ***Capillaria* sp.** PCR molecular method revealed *Toxocara cati* in faecal samples of Geoffroy's cat, serval and Pallas's cat and *Toxascaris leonina* in cheetah and Carpathian lynx. *Capillaria* sp. was found only in one individual of Pallas's cat and was not molecularly confirmed. These findings led to suspicions of co-infections in some individuals as detected in the thesis of Kudrnáčová (2014).

In the faecal samples from Felinae subfamily examined by Kudrnáčová (2014) with the flotation method, the Amur leopard cat from the Zoo Olomouc could have **polyinfections** of ***Toxoplasma gondii*** with ***Giardia* spp.**, ***Toxascaris leonina*, *Toxocara***

cati and ***Cryptosporidium* spp**. According to the findings of this thesis, the Amur leopard cat from the Zoo Olomouc could have postinfection of *Toxocara cati* with *Cryptosporidium* spp., but PCR analyses did not confirm it. Confirmed coinfections by PCR were *Toxoplasma gondii* with *Cryptosporidium felis*, *Giardia intestinalis* and *Toxocara cati* in one male of Pallas's cat. *Toxoplasma gondii* infection with *Toxocara cati* in another male of Pallas's cat. Pair of cheetahs had confirmed *Cryptosporidium felis* with *Toxascaris leonina*, so polyinfection could be or could not be present, because it was a mixed sample. The female of Pallas's cat had confirmed *Toxoplasma gondii* infection with *Cryptosporidium felis*, *Giardia intestinalis* and *Toxocara cati*. This particular female of Pallas's cat had also confirmed herpes infection (personal interview, Mr. Viduna). A similar finding was made by Ketz-Riley et al. (2003), who found out toxoplasmosis with parallel herpesvirus infection in Pallas's cats caused kitten's death.

Plumb (2018) mentioned **toltrazuril** Baycox® (Bayer, Canada) to treat toxoplasmosis in cats, which was also used at the Zoo Jihlava for Pallas's cats (personal interview, Mr. Viduna), however, they did not want to treat an animal indefinitely without results and expose the animal to stress from approaching, so the treatment was done with a check-up and then interrupted. EAZA (2013) also recommended to keep stress levels to a minimum.

As the potential source of the spread of infection in the past, Lukešová & Literák (1998) described water enriched with soil at the Zoo Jihlava, because feral cats had the access to the stored soil. Nowadays, water with soil was not used anymore, however, for the Zoo Jihlava, the occurrence of stray cats was still a problem (personal interview, Mr. Viduna). According to Kudrnáčová (2014), the highest risk came from pork and rabbit meat. This should not be the problem today. Kudrnáčová (2014) and Schares et al. (2016) marked summer as the period of the greatest oocyst shedding of *Toxoplasma gondii*. The sampling for this work was carried out in the summer, which could explain the high *Toxoplasma gondii* prevalence of Pallas's cats. This seasonality should also be taken into account when ensuring biosecurity. Clean separate tools for each animal house (EAZA 2013, Remes 2021) or even disinfectant footbath (Dunowska 2019) could improve the current situation.

6 Conclusions

Based on direct coprological and molecular methods, the occurrence of parasites of the species *Toxoplasma gondii* and *Cryptosporidium felis*, on which this work was focused, was confirmed.

The flotation method revealed 10.36% positive and 1.35% dubious findings in faecal samples of small felines for the presence of *Toxoplasma gondii*. The presence of *Toxoplasma gondii* was confirmed by PCR molecular analysis in three samples (three individuals) of Pallas's cat. Specific DNA of *Toxoplasma gondii* showed 100% identity with Genbank accession no. KT881323.

By using the staining technique, 9.91% of feline faecal samples were marked positive for *Cryptosporidium* spp. presence and 3.15% as dubious. Subsequent PCR analysis showed *Cryptosporidium felis* specific DNA 100% identical to Genbank accession no. KF287127, specifically for ten faecal samples (four individuals and one co-collected pair).

There were also other findings of species of parasites that this thesis was not directly focused on. These species were *Toxocara cati*, *Toxascaris leonina* and *Giardia intestinalis*, which were also subsequently confirmed by PCR analysis. The reference sequences stored in the GenBank database showed 100% identity with the sequences obtained in this thesis. *Cystoisospora rivolta* and *Capillaria* sp. were also found in faecal samples, unfortunately, specific primers for PCR analysis and confirmation of their occurrence were not obtained, on which further research could focus in the future.

The intensity of parasitic nematode infection was evaluated using the McMaster's method, which revealed the highest value of 6,225 EPG *Toxocara cati* in a male Pallas's cat from the Zoo Jihlava, 775 EPG *Capillaria* sp. in another male of Pallas's cat from the same Zoo Jihlava and 19,725 EPG in a female cheetah bred at the Zoo Olomouc. The results indicated that the *Toxocara cati* infection was greater at the Zoo Jihlava. *Toxascaris leonina* infection was recorded only at the Zoo Olomouc.

Both flotation and staining methods should always be confirmed by molecular method (PCR) to confirm the relevant pathogenic species that is causing changes in the health of the animal being kept.

All three established hypotheses H_1, H_2 and H_3 were confirmed, which means that apicomplexan parasites with zoonotic potential have occurred in the Czech zoos, therefore it is necessary to ensure proper biosecurity in the environment, biosecurity of water resources and feed and to pay attention to preventive measures and sanitation not only at zoos but also in the domestic environment of feline breeding in human care.

At both zoos, food and water posed a minimal risk of transmission of parasitic pathogens. For the Zoo Jihlava, environmental risks represented the greatest risk, because feral cats had access to stored materials subsequently used for bred animals. Therefore, stored material should be better stored. Common tools were used for some small cats at the Zoo Jihlava. In contrast, the Zoo Olomouc used different tools for each animal, however, the biggest risk for them was grassy enclosures without the possibility of disinfection. None of the zoos used disinfectant mats and zookeepers did not change their shoes when approaching different individual animals, which could be a recommendation for future biosecurity maintenance.

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Appendices

List of the Appendices:

Appendix 1: Placement of tested animals at the Zoo Jihlava

Source: Bírošíková (2021)

Zoo	Species	Number and sex	Place
Jihlava	*Felis silvestris silvestris*	1♂	separated, back facilities
	Leptailurus serval	1♂ + 1♀	together, Cat house
	Otocolobus manul	1♂	separated, back facilities
		1♂	separated, back facilities
		1♂	separated, back facilities
		1♀	separated, back facilities
		1♀	separated, exposition
	Prionailurus bengalensis euptilurus	1♂	separated, back facilities
	Prionailurus bengalensis heaneyi	1♂ + 1♀	together, Cat house

Appendix 2: Placement of tested animals at the Zoo Olomouc

Source: Bírošíková (2021)

Zoo	Species	Number and sex	Place
Olomouc	*Acinonyx jubatus*	1♂ + 1♀	together, outdoor grassy enclosure
		1♀	separated, back facilities
	Felis silvestris silvestris	1♀	separated, small glass pavilion
	Leopardus geoffroyi	1♂ + 1♀	together, small glass pavilion
	Leptailurus serval	1♂	separated, small glass pavilion
	Leptailurus serval	1♀	separated, small glass pavilion
	Lynx lynx carpathicus	1♂ + 1♀	together, outdoor exhibition
	Prionailurus bengalensis euptilurus	1♂	separated, small glass pavilion
		1♀+3 kittens	together, small glass pavilion

Appendix 3: Modified McMaster's Method

Source: Williamson (2013); Chalex Corporation

Materials: fecal floatation solution, graduated beaker, tongue depressors, weigh scale, cheesecloth, pipettes, McMaster's counting slide*, paper towels, compounded microscope and fecal samples.

Procedure:

1. Weigh out 2g (for sheep/goats) or 4g (for cattle and horses) of feces in small beaker (Nalgene 50 ml beakers work well).
2. Add 28 ml (2g) or 26 ml (4g) of sodium nitrate flotation solution to feces, and mix well.
   - For sheep and goats -- unless feces are very soft, add only a few ml. of solution at first and let feces soak for 10-15 min to soften them, then break up with tongue depressor and add remainder of solution
3. Strain through 1 or 2 layers of cheesecloth (or tea strainer), mix well.
4. Immediately pipette a sample of the suspension and fill both sides of counting chamber. Work quickly, stirring with pipette as you draw up fluid. If it takes more than a few seconds to load the first chamber, then mix fecal solution again and refill pipette before loading the second chamber. Let stand for several minutes to allow eggs to float to top. If visible air bubbles are present, remove the fluid and refill.
   - Steps 3 and 4 should be done at the same time without letting sample sit between steps - eggs are in flotation fluid and will immediately start floating. You want to add a homogeneous sample of fecal float solution to chamber.
   - Once chambers are filled, step 3 can be started for the next sample
   - Once filled, the chambers can set for about 60 min before counting without causing problems - longer than this and drying/crystal formation may begin
5. Count all eggs inside of grid areas (greater than 1/2 of egg inside grid) using low power (10x) objective. Focus on the top layer, which contains the very small air bubbles (small black circles). Count only trichostrongyle eggs (oval shaped, ~ 80-90 microns long) in both chambers. (Notations are made as to the presence of other types of eggs and oocysts).
6. Total egg count (both chambers) x 50 (2 gm) or 25 (4gm) = EPG (eggs per gram).

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Appendix 4: Miláček-Vítovec staining

Source: Miláček & Vítovec (1985)

Staining process: 1. Dry smears or scrapings fix in methyl alcohol for 5 min at room temperature.
2. Stain with a solution of anilin-carbol-methyl violet for 30 min and rinse in tap water.
3. Differentiate in 1—2 % sulphuric acid for 30 sec up to 2 min, till the sample is pale blue-violet and then again rinse in tap water.
4. Counterstain with tartrazine for 30 sec—1 min, briefly rinse in tap water and leave to dry.
5. Smears or scrapings used for the examination can be dry or covered with a thin layer of paraffin oil or mounted in Canada balsam.
Result of staining: Cryptosporidia stain blue to blue-violet on a yellow to yellow-green background.

Appendix 5: DNA isolation

Source: Bírošíková (2021)

Appendix 6: PCR Thermal Cycler

Source: Bírošíková (2021)

Appendix 7: Example of a cage in the back facilities at the Zoo Jihlava

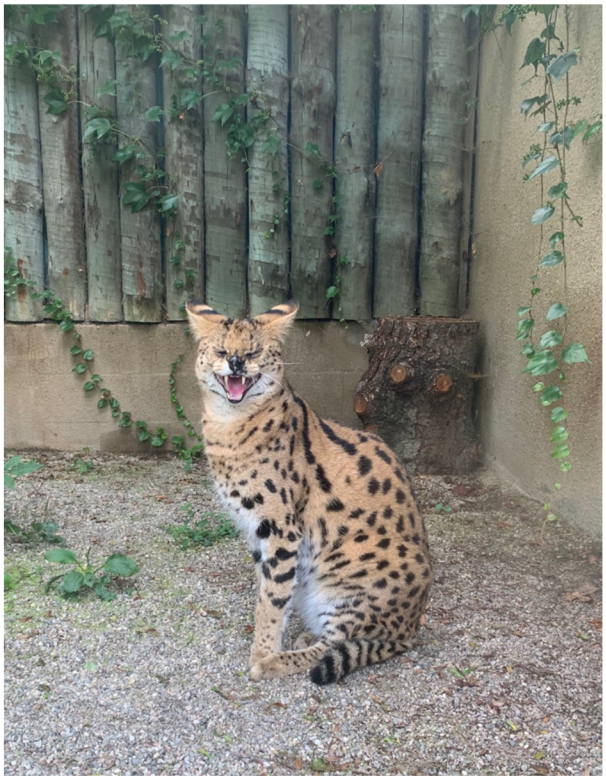
Source: Bírošíková (2021)

Appendix 8: Serval from the Zoo Jihlava

Source: Bírošíková (2021)

Appendix 9: Serval from the Zoo Olomouc

Source: Bírošíková (2021)



Appendix 10: ♀ Amur leopard cat from the Zoo Olomouc

Source: Bírošíková (2021)

Appendix 11: ♀ Amur leopard cat with kittens from the Zoo Olomouc

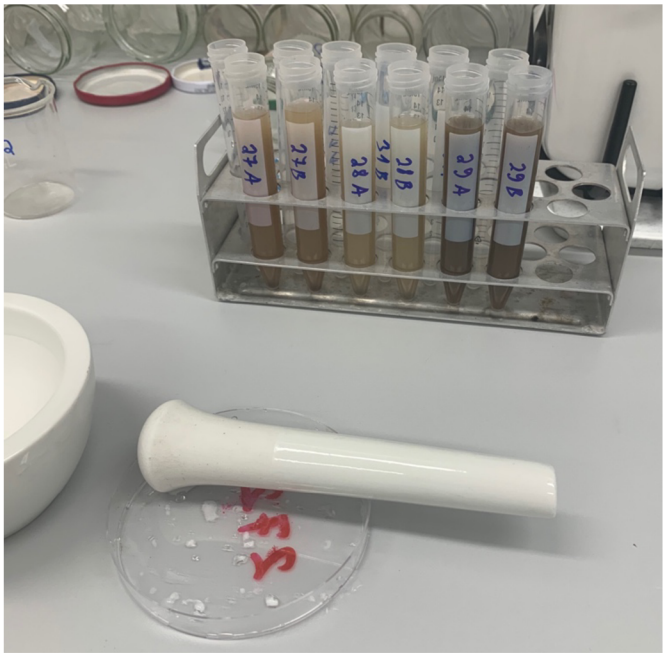
Source: Bírošíková (2021)

Appendix 12: Cheetah from the Zoo Olomouc

Source: Bírošíková (2021)

Appendix 13: Processing of faecal samples

Source: Bírošíková (2021)

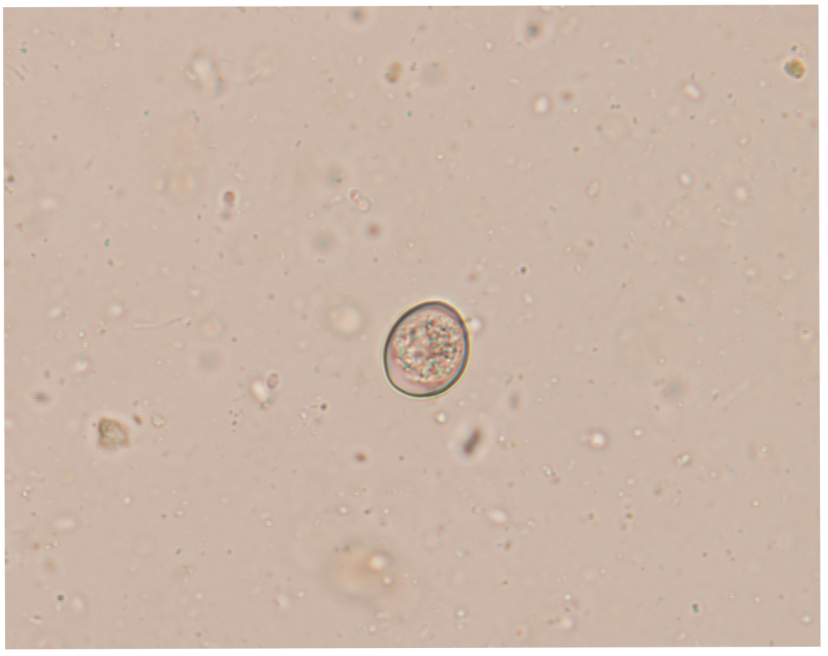


Appendix 14: Microscopy of stained faecal smears

Source: Bírošíková (2021)

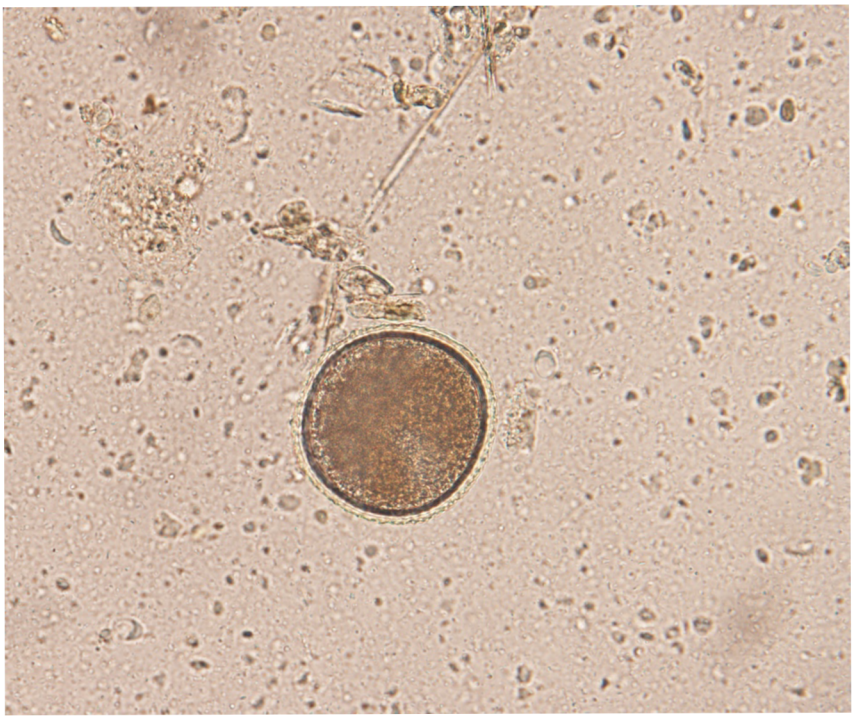
Appendix 15: *Toxoplasma gondii* from Pallas's cat (400×)

Source: Bírošíková & Kváč (2021)



Appendix 16: *Toxocara cati* from Pallas's cat (400×)

Source: Bírošíková & Kváč (2021)



Appendix 17: *Toxascaris leonina* from cheetah (400×)

Source: Bírošíková & Kváč (2021)