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Molecular investigation of *Trichomonas* species in cats in Hungary

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1. Introduction

Veterinarian medicine, particularly parasitology, has a significant role in the guidance and safeguarding of both humans and animals. For instance, against species such as the Trichomonas genus; *Tritrichomonas foetus and Pentatrichomonas hominis,* who are commonly known for causing chronic large bowel disease (all). *Trichomonas* species are flagellated protozoan parasites living on the mucosal surfaces of the reproductive system, oral cavity, and gastrointestinal tract. Although *T. foetus* does not exhibit zoonotic properties, *P. hominis* poses this risk. Since the first description in the late 1800s the relevance of these protozoans in catteries has risen throughout the years. Data relating to the infection rate of these protozoans are significantly lacking in Hungary for both wild and domesticated cats, therefore the aim of the study is to compensate for this.

To investigate the prevalence of these parasites in the feline population oral swabs, rectal swabs, and fresh fecal samples were collected by veterinarians from 102 random domestic cats originating from 18 different locations throughout Hungary. The fecal polymerase chain test (PCR) is considered the most sensitive means of diagnosis for both *T. foetus* and *P. hominis*, therefore was chosen to confirm positive outcomes. The aim of this study is to establish coherent data regarding the infection ratio of wild or domestic cats living in Hungary, through different collection methods and PCR testing. A secondary objective was to assess whether there are associating factors influencing positive PCR results. If the population of *Trichomonas* species continues to rise it will cause an immense number of complications in the feline breeding industry worldwide. The threat extends towards the human population as well since species like *Pentatrichomonas hominis* cause troublesome symptoms for humans too.

2. Literature Review

2.1. Domestic cat (*Felis catus)*

Domestic cats, also known scientifically as *Felis catus*, come from the *Felidae* (cat) family and the *Felis linnaeus* (small cats) genus (77, 83). The common ancestor of cats, big and small, is known as the "true cat", which originated 35 million years ago. 37 species of felines arise from this ancestor, identifiable by the similarities in anatomical features such as reduced number of teeth in the oral cavity. Thanks to modern molecular analysis, Stephan O'brien at the Maryland National institute was able to identify the 36 living wild cats and domestic cats that had appeared in the last 11 million years. This was possible due to the mitochondrial DNA passed down from mothers to daughters. Around 85% of the current cat breeds have surfaced within the past 75 years. Known as top predators, felines have evolved into hypercarnivores (strict meat eaters). Several of their anatomical features such as teeth, tongue and digestive system cater to a high meat consuming diet. Primary prey includes rodents, birds, insects, amphibians and fish. The habitat of felines is extremely diverse as they can be found in deserts, tropical forests, grasslands, and the northern hemisphere. Smaller cats have a more diverse habitat than their larger relatives, however they do not occur naturally on oceanic islands such as Hawaii, Madagascar, or the Galapagos. This is different on continental islands such as Borneo or the Java Chile islands of Chile, they are natural inhabitants (77, 83).

Cats are currently the most common house pet, with an estimated 100 million domestic cats in Europe alone. The majority of the population is made up of non-pedigree forms. While the Ragdoll leads in the pedigree category based on the Finnish Cat Associations survey (47). Cats have significantly surpassed dogs as pets globally. This is primarily due to limitations in wealth, space, and time in the rapidly developing 21st century (7). Some breeds have expressed higher susceptibility than others to infections. An example is the predisposition for Abyssinians, Bengals, Birmans, Ragdolls, and Rexes to feline peritonitis caused by different strains of feline coronaviruses (65). Birmans have even exhibited a specific gene for susceptibility to the virus (44, 65). Genetic diseases are also not an uncommon occurrence in pedigrees. The most prevalent locality being dental and oral diseases followed by respiratory, skin, urinary, digestive, and finally musculoskeletal. Examples of these cases include asthma, urinary tract infection in females especially, tail kinks or the upcoming feline odontoclastic resorptive lesion (FORL). Diseases associated with Ragdolls include reflux nephropathy, behavioral abnormalities, and Hypertrophic

Cardiomyopathy (HCM) mutations (47). Other breeds such as the Devon Rex, are highly liable for skin colonization by *Malessiza* genus fungi (40, 53, 65). It is important to research and understand the variety of diseases that can affect cats in order to prevent deterioration in health and their well-being.

2.2. Feline pathogens

Cats can harbor several pathogens, such as viruses, bacteria, and parasites. Some of them are zoonotic, which means that felines are able to pass them onto humans. Examples of zoonoses include *Rabies*, *Toxoplasma gondii* and the *Hantan* virus. Cats delivering prey such as rats to one's door may put their owner at risk of a fatal disease (77, 83). Felines act as the definitive host for *Toxoplasma gondii*, a global zoonotic parasite that has evolved to infect the most widespread host. Congenital and acquired characteristics of cats has had an important influence in the development of *Toxoplasma gondii*. Selection during breeding would greatly limit the infection possibility of this parasite. Besides the benefits to the health and welfare of cats, selective breeding would also decrease the risk of infection in other hosts such as humans (44, 64, 65). On the other hand, acquiring enteric agents, dermatophytes and ectoparasites depends on the contact type between humans and cats. Cats can act as vectors, reservoirs, sentinels or even amplifying hosts (54). Organisms transmitted by other vectors such as mosquitoes are not directly related to felines (88, 71).

Feline vector borne diseases (FVBD) are rapidly increasing globally making them an important veterinarian matter. Arthropods range from ticks, fleas, phlebotomine sand flies and mosquitoes (26). Living in close quarters and traveling with pets along with climate change have enhanced the spread of diseases such as *anaplasmosis, hepatozoonosis, bartonellosis, babesiosis, leishmaniosis* and *rickettsiosis*. Studies conducted in southern European countries such as Portugal, have come up positive for these parasites in clients owning cats. FVBD are a growing concern regarding human health and the "One health" concept (54, 14). The "One Health" notion aims to stimulate closer collaboration between the human and animal health sectors together with environmental agencies. The goal is to improve public health issues in food safety, zoonosis control and the ecosystem. There are over 200 zoonotic diseases currently threatening the human population (24).

2.3. Feline parasites

A large number of endo and ecto-parasites can infect domestic cats. Their infestation can cause fluctuating clinical signs from mild gastroenteral disorders to anemia. The severity depends on the species and abundance of infection, and usually affects kittens the most (23). 50% of the domestic cat population in Europe carry at least one species of parasite during a given time. Co-infection is highly likely as well (71).

Outdoor access and antiparasitic treatment seem to be the biggest deciding factor during parasitic infections (19, 71). Cats not receiving anthelmintic treatments are more at risk of infection than cats receiving treatment once or twice a year. According to studies done on European cats, age does not always influence the rate of affliction. Global location and climate change has begun influencing the extent of contamination considerably in the past years. Studies show higher rates of positive results in cats living in warmer climates such as Naples, Italy compared to locations like Vienna, Austria. However, lack of awareness and treatment in countries like Hungary, where temperatures are rising has caused results to be alike warmer climate zones (71). Multiple cat households have proven to be the reason for high *Tritrichonomonas foetus* advancement rates, however concerning other parasites this factor is not so significant since they can be eliminated via antiparasitic drugs (28, 71). Most parasites can be found in the feces of healthy cats, making them an easy source for immunocompromised cats or even humans. Cats that suffer from nutritional deficiencies, feline immunodeficiency virus/ leukemia, chronic or systemic diseases are at a higher risk (55).

Parasites can be transmitted in three different ways. Vector-borne arthropods are passed on primarily via blood-feeding include *Leishmania, Trypanosoma*, *Babesia* spp. and the apicomplexan genera (7). A second method is per cutan commonly used by *Ctenocephalides felis, Ixodes ricinus* and *Notoedres cati*. Per oral means vary fecal-oral to ingestion by the host. Transmission per orally is commonly used by protozoa such as *Trichomonas felistomae*. Certain stages of parasites are more likely to be less resistant in the environment and die shortly after being deposited in drinking water or on the surface of the skin after licking. Resistant stages survive in the environment due to their protective wall and once entered the host, persists for long periods. Such parasites include *Giardia duodenalis, Cystoisospora species, Toxoplasma gondii* and *Cryptosporidium felis*. (7). Cats are known as top predators. They consume rodents, birds, insects, amphibians, and fish, who

most likely are carrying a hostile organism (77, 83). *Sarcocysts* species, *T. gondii* and *Hammondia hammondi* are among these species.

In Europe the most important intestinal nematodes of domestic cats are *Toxocara cati* (roundworm), *Ancylostoma tubaeforme* (hookworm) along with *Uncinaria stenocephala* and *Toxascaris leonina*. Parasites such as these are relevant for veterinarian practice, particularly in young animals and kittens, however cats of all ages can be infected. Subclinical infections can occur or in clinical cases, catarrhal enteritis along with vomitus, diarrhea/constipation, delayed development, or migration of worms into other areas of the body. Nematodes such as hookworms even in small quantities can become fatal due to blood and weight loss after severe diarrhea (15). Cardiopulmonary nematodes have recently become a priority in feline veterinary medicine. *Aelurostrongylus abstrusus* and *Troglostrongylus brevior* are widespread parasites in domestic cats throughout Europe. Clinical signs come in varying severity such as dyspnoea, coughing, sneezing or general distress. Life threatening cases can occur in kittens (62). The main source of infection for domestic cats stems from the wild cat population. Migratory birds traveling from Africa infect the European feline population when being preyed upon (87).

Ectoparasites during invasion of a host can cause direct damage, pruritus, allergic reactions, and grave distress. Not only do they cause agonizing symptoms, but they also have a potential vectoring role. Frequent ectoparasites include ear mites (*Otodectes*), fleas and ticks. Cats that frequently go outdoors are more prone to encounter them (22, 23). Fleas play a role in transmission of pathogens such as *Rickettsiosis* or *Bartonella henselae*, which is a zoonotic pathogen responsible for the disease known as cat-scratch (22, 41). Arthropods such as the three main tick species, *Dermacentor reticulatus, Ixodes ricinus* and *Rhipicephalus sanguineus* are becoming more widespread due to human involvement. Traveling with pets and climate change have greatly increased the likelihood of diseases such as tick-borne encephalitis in Europe (17, 22).

Most household pets are well cared for and receive frequent antiparasitic treatment. This is not the same for abandoned or stray animals. Prophylactic initiatives such as ecto- and endoparasiticides should be encouraged along with education and awareness campaigns to prevent the spread and infection of pathogens in cats and other hosts (19, 54). Several types of antiparasitic treatment are available on the market. A common ectoparasite drug for cats is Fipronil, (found under the brand name FRONTLINE or BROADLINE). Fipronil is applied as a spot-on treatment. For internal parasite treatment of cats, drugs are separated

into two categories. Nematode eradicating drugs include Pyrantel, Milbemycin, Fenbendazole, Moxidectin and Emodepside, while cestode eradicating drugs are made up of Praziquantel, Niclosamide and again Fenbendazole (19). Studies have proven that endo and ectoparasites are a growing concern in Europe for domestic cats. Consideration should be given not only for animal health but also for zoonotic prevention. Anthelmintics are strongly advised for the treatment and eradication of both external and internal parasites (23).

2.4. Protozoa

Protozoa are a diverse group of motile unicellular eukaryotic microorganisms (varying shapes and size of 1-300 μm) that have a symbiotic free-living type of entity. Part of the supergroup *Amaobozoa, Excavata*, and SAR (*Stramenopiles, Alvolata, Rhizaria*). The word protozoa are a collective name that does not have a phylogenetic or taxonomic label. There are over 17,000 species of protozoa that are clinically important, especially due to their parasitic characteristics that can infect humans and animals alike. Examples of these include *Trypanosoma, Leishmania, Plasmodium* spp. and *coccidia*. Although like most eukaryotes, protozoa have different cell organelles that make them special. An example is the asexual reproduction capability, which can be done via binary or multiple fission (16).

The classification of different protozoa parasites begins with the phylum. These phylums include *Sarcomastigphora, Apicomplexa, Microspora, Ciliophora* and an unclassified section. *Sarcomastigophora* contain flagella, pseudopodia, or both. Within this phylum are two subphylums; *Mastigophora* (flagella) and *Sarcodina* (pseudopodia). Principle species such as *Giardia* and *Trichomonas* belong to the *Mastigophora* subphylum, while other crucial species like *Babesia* belong to the *Sarcodia* subphylum. *Apicomplexa* genus encompasses *Isospora, Sarcocysts, Cryptosporidium* and *Toxoplasma*. Originally *Microsporidia* was regarded as a protozoon, however now it is believed to be related to fungi and therefore has been assigned another supergroup.

Possessing a complex life cycle, protozoal host species can range from one to several, incorporating phases outside the host in the environment. Generally enclosed in a thin, double-layered cell membrane also known as a unit membrane, protozoans can often form a glycocalyx outer layer as well. Other stages possess zoites, which are thicker, sturdier pellicles, making it possible for extracellular life. The wall of cysts or oocysts facilitates survival externally in the environment. This has enabled protozoa to adapt to diverse habitats from deep sea to polar ice or in affiliation with other ecto- or endoparasites. As heterotrophic organisms, protozoa take up nutrition through their cell membrane via permeation,

endocytosis, and active transport. Endocytosis can be by pinocytosis or phagocytosis. A special region of the cell accommodates these processes such as the flagellar pocket in trypanosomes, the microspores in Apicomplexa, cytostome of ciliates and the peripheral vesicles in Giardia. Anaerobic glycolysis is the main energy producing method for protozoa. The incompletely oxidized products for instance acetate, succinate or pyruvate are excreted. In case of complete aerobic oxidation, the end products of carbohydrates are removed as CO2 and water. (16)

Methods of diagnosis vary from parasite to parasite. The customary sampling method in cats is via fecal collection for gastrointestinal protozoa. *Cytauxzoon felis* a protozoa acquired through tick bites in felines, is diagnosed using blood smear PCR from whole blood (72, 68). Fecal flotation is a general method for protozoa such as *Cystoisospora* species, *Cryptosporidum felis*, *Toxoplasma gondii* and *Giardia* species (18, 68). These parasites relate not only in diagnostic means, but also in the process of obtaining them. Felines encounter them either by ingesting prey or feces that contain the oocystoc or cystic phases. For *Tritrichomonas foetus* and *Pentatrichomonas hominis* this would be ingestion of trophozoites, which are visible under a microscope. *Cryptosporidum felis* can also be proven by fecal ELISA, stained fecal smear and fecal IFA. *Toxoplasma gondii* is diagnosable by antibody titer too. *Giardia* species are like *Tritrichomonas foetus,* therefore direct saline fecal smear, fecal IFA and PCR can be used to detect the flagellates (68, 45). Compared to flagellates such as *Tritrichomonas foetus, Pentatrichomonas hominis* and *Giardia* species, other protozoa namely *Cryptosprodium felis, Cystoisospora species, Toxoplasma gondii* and *Cytauzxoon felis* are treatable with antimicrobial drugs in cats. These antibiotics include Clindamycin, Tylosin, Azythromycin and Sulfadimethoxine. Species such as *Cystoisospora* species react well to antiprotozoal agents such as Ponazuril or *Cytauxzoon felis* to Imiudocarb diprprionate. *Tritrichomonas foetus* and *Pentatrichomonas hominis* are currently only remediable by Ronidazole in cats. Interestingly the *Giardia* species, although like *Tritrichomonas foetus*, responds to anthelmintic agents such as Fenbendazole or antimicrobials like Metronidazole, instead of antiprotozoal (12, 48, 68).

2.5. Trichomonosis

2.5.1. *Tritrichonomonas foetus*

Tritrichomonas foetus is the cause of chronic large bowel disease in cats due to its colonization of the feline alimentary tract (all). Compared to *Tritrichomonas foetus* found in bovines, *T. foetus* in felines does not cause an infection in the genital tract. The parasite can be found worldwide (16), with a prevalence of 17.14% in Italy (67), 15.7% in Germany (50), 31% in the USA (81) and 14-20% in Australia (6). Tritrichonomonas is found to be a naturally occurring parasite in cats (43, 91). A common matter in catteries of pedigree cats, its global importance continues to rise throughout the years, due to the practice of purchasing cats from abroad (6). *Tritrichomonas foetus* was originally described in 1888, by Kunstler, however, it was Mazzanti in 1900, who reportedly cited them in infertile cattle. Between 1924 and 1929, German scientists, Drescher, Riedmüller, and Abelein conducted studies on cattle that aborted fetuses relating to *Trichomonads*, thus giving it its name *Tritrichomonas foetus*. The next reported cases came from America in 1932 (36, 70). It wasn't until 1996, that *Trichomonads* were discovered in Pixie-bob kittens with colitis. In 2001 feline Trichomonas infections were molecularly identified as *Tritrichomonas foetus*.

Various experimental infection studies resulted in diarrhea. Since then, several studies have been carried out to test prevalence and optimal diagnostic methods. Ronidazole was reported to affect *Tritrichomonas foetus* in 2006 and has since been administered as a treatment for the infection. Since 2010 genetic differences have been found between bovine strains and feline strains of *Tritrichomonas foetus*, therefore in 2013 there was a proposition to rename the feline genotype, however, this is still under dispute (33, 39). Sexually transmitted *Tritrichomonas foetus* is a common parasite in cattle. The locality of the infection is in the vagina, cervix, and uterus. Several diseases can develop due to the infection such as pyometra, vaginitis, abortion, and infertility (60). In contrast to the cattle strain of *Tritrichonomonas foetus* the porcine version (*Tritrichomonas suis*) invades the nasal cavity, intestines, and stomach (9). Identification and elimination of *Tritrichomonas foetus* in cattle herds have been a primary goal. Methods used include testing the preputial membranes of bulls. Positive individuals are culled as they cannot be used to breed. These methods cannot be used in feline or porcine *Tritrichomonas* infection since the different genotypes prefer other areas in the body. Investigation of molecular analysis, virulence, cross-transmission studies, geography, and morphology, reveals that there are very few differences between the cattle *Tritrichonomonas* and swine *Tritchonmonas* genotypes (60,

85). Altho cross-transmissibility has been proven experimentally, naturally transpiring *Tritrichomonas foetus* and *Tritrichomonas suis* transmission is rare (63). The 'feline genotype' of *Tritrichomonas foetus*, however, has displayed variability in genetics compared to the 'cattle genotype' of *Tritichomonas foetus*. The ITS-1-5.8 rDNA gene to the ITS-2 differs in one nucleotide polymorphism, which proves there is a possibility of differentiation. Other studies on DNA sequencing have uncovered 10 more genotypic polymorphism differences between the genotypes. This adds up to a total of 1.03% variability, mainly found in the cysteine protease (CP) gene (61). Other studies found these differences in the CP8 and eukaryotic elongation factor 1a (EG-1a) locus (73, 84).

Originally misidentified as *Pentatritrichonomonas hominis* for many years, the protozoan was finally dubbed *Tritrichomonas foetus* (33). Debates are still being conducted to decide whether the *Tritrichomonas* species found in cats should receive the differentiating name "*blagburni*" from the one found in cattle. Genetic differences are present as studies show, however the current minor variability between *Trichomonas foetus* and *Trichomonas "blagburni"* can be due to the evolution/ diversification of the organism. Throughout this study, the trophozoite will be referred to as *Tritrichomonas foetus* and not *Tritrichomonas "blagburni"*. Host specificity is not restricted to a single genotype; therefore, it would cause confusion if it was referred to by another name (8, 18).

Tritrichonomonas foetus is part of the phylum *Parabasalia*, class *Trichomonadida*, Family *Tritrichomadida*, supergroup *Excavata* (unicellular organisms' part of the *Eukaryota* domain) (16, 33). Phylum *Parabasalia*: *Para* meaning next or near and *basis* meaning lat. *Parabasalia* is a group of parasites that can infect the genital system and the digestive system. (16). Class/Order *Trichomonadida*: As microaerophilic/ anaerobic organisms, *Trichomonas* do not have peroxisomes or hydrogenosomes to replace the mitochondria, but instead metabolize pyruvate to produce ATP. End products of their metabolism include acetate and CO_2. They have a double membrane and contain no DNA (16). Family *Trichomonadidae*: *Thrix* meaning hair and *monas* meaning individual being. Unlike in the class *Trichomonadida*, the family *Trichomodidae* contains hydrogenosomes and golgi dictyosomes. The genera are distinguished by the number of anterior flagella; *Tritrichonomonas* (3), *Trichomonas* (4), and *Pentatrichomonas* (5).

Although they can be pleomorphic, *Tritrichomonas* are known for their pyriform-shaped (pear-like) body with a pointed posterior and rounded anterior. They have a ridged axostyle organelle in the shape of a rod that runs through the trophozoite protruding at the

posterior end. Within the organism is a single nucleus. There are 3 anteriorly directed flagellants arising from the kinetosome complex, along with a single posteriorly directed flagellum on the anterior free edge, which journeys along creating an undulating membrane. This vibrating movement is what allows for the diagnosis of *Tritrichomonas foetus*. They are similar in size to *Giardia*, which is around 10-20 x 3-15 μm (8, 16, 33, 70). Unlike the majority of the parabasel parasites, there is no cyst stage in the life cycle of *Tritrichomonas*, only trophozoites, therefore only the trophozoite takes part in the transmission between hosts (34). Transmission is done via ingestion/ fecal-oral route, of the trophozoites. Reproduction is carried out asexually by binary fission (8, 16, 33, 92). A study has found garden slugs such as the Leopard slug (*L. maximus*) and the yellow cellar slug (*L. flavus*) are able to host *Trichonomonad* after ingesting them. This makes slugs a possible source of infection (90). In cattle, *Tritrichonomonas* is sexually transmitted unlike in cats. Research has shown that infection of the reproductive tract of purebred cats with *Tritrichonomonas foetus* did not show signs of colonization after assessment with different diagnostic tools such as the light microscope, immunochemistry, or PCR. This indicates reproductive transmission does not play a role in the infection of purebred cats (13,35).

The natural locality of *Tritrichomonas foetus* in cats is in the superficial mucosa of the outermost epithelium in the large intestines. It has been demonstrated that Tritrichomonas-specific antigens have been taken up by the colon surface epithelium. This means it invades the lymphocytes, neutrophils, and plasma cells of the colonic lamina propria. Some studies have demonstrated it invading the subepithelium too (30, 92). Due to its specific ligand-receptors, *Tritrichomonas foetus* can attach to the monolayers of the intestinal epithelium in vitro (59). Attachment to the alimentary epithelium is possible due to the cysteine protease, which also mediates the cytotoxicity of the parasite. Studies are being conducted for possible treatments that combate this mechanism (86). *Tritrichomonas* is not a hardy parasite, due to its lack of a cystic phase. It cannot withstand drastic environmental changes because it does not have a contractile vacuole to regulate osmolarity (56). A study conducted to test the survivability of *Tritrichomonas foetus* trophozoites in different locations has shown a minimum survival time of 30 min in distilled water and a maximum of 180 min in urine or canned cat food. No live trophozoites were observed in cat litter. This supports the idea that *Tritrichomonas foetus* is mostly transmitted via contaminated food or water, rather than through co-used litter boxes (34, 38, 76).

2.6. Clinical signs

A predominant characteristic of *T. foetus* infection is chronic diarrhea and colitis due to the inflammation of the ileum, colon, and caecum (6, 16, 50, 67). The large bowel diarrhea can abruptly appear and just as promptly disappear. Some studies have documented the duration to range from one day to eight years. During these symptoms, voiding can occur one to eight times a day, with mucus or fresh blood present in the stool. Straining can also transpire during this time. The consistency of the feces is typically semi-solid with a fetid odor. Most cats maintain good general health, appetite, and body weight, during the infection (33, 27). Kittens younger than twelve months are most likely to become infected. They may develop fecal incontinence and have obvious swelling/ inflammation of the anal region due to variation in feces (16, 27, 33, 67). A study on the histological findings of the infection have exhibited varying ranges of neutrophilic and lymphoplasmacytic colitis, hypotrophy in the crypt's epithelium along with increased mitotic activity and goblet cell loss. Microabscesses were present in the crypts and the function of the superficial colonic mucosa was reduced. No eosinophilic inflammation was present indicating it is not an attribute of the infection. Profound penetration of the trichomonads into the lamina propria or further revealed macrophages were present (92).

2.7. Diagnosis

The sampling method for *T. foetus* is by fecal sample collection. Techniques include the fecal loop, collection of freshly voided feces, fecal swabbing, and colon flushing (25, 34, 40, 42, 43). The method of sample collection can influence the results of the PCR test. There are contradicting conclusions regarding which sampling method is the best. One study considers colon flushing provides better results followed by the fecal loop and finally freshly voided feces (33), however, another study found that samples collected using the fecal loop were 2.04 times more likely to produce a positive result than colonic flushing due to the location the protozoa reside in the colon (40). Successful sampling encompasses selecting fresh or diarrhetic feces, withholding antimicrobial agent's days before, and collecting the correct amount of feces (34, 33). Diagnosis of the infection with *T.s foetus* is based on the identification of the protozoa in fecal samples after culturing in media specific for its growth (InPouch TF® culture system from BioMed Diagnostics) or by performing a PCR test on extracted DNA (42, 34, 25, 33). Approaches include direct fecal mounting, fecal culturing, PCR, and histopathology. It is important to know that none of the tests provide 100%

sensitivity for *T.s foetus*, since the shedding of the protozoa is irregular. Negative results do not necessarily mean the cat does not carry them; therefore, retesting is always recommended. Cats that do not show signs typical for *T.foetus* infection, and who have dry or non-diarrhetic ceca scarcely produce positive results (25, 33, 34). The sensitivity of the diagnostic tests varies from method to method, studies done to compare these have observed that PCR testing has the highest sensitivity, followed by the 'In Pouch TF' and microscopy (33,42). Within the medium selection, for culturing *T.s foetus*, a study has detected that commercial pouch systems are more advantageous in producing positive results, compared to modified Diamond mediums (3). Identification is not possible by standard fecal detection such as centrifugation or flotation. *Tritrichomonas foetus* does not survive low temperatures or in the refrigerator (25, 33, 34).

2.7.1. PCR

Requires high-quality DNA to be extracted from fecal samples, which can be strenuous to obtain. This is because the PCR inhibitors removed with the DNA such as bile salts, hemoglobin degraders, complex polysaccharides, and heavy metals, block the process. Factors influencing the composition of the feces, and the number of inhibitors vary based on the animal's diet, species, and disease. Commercial PCR kits for specific fecal specimen identification have increased in the past years. They allow for better DNA extraction from fecal samples without inhibitors, however, they vary in complexity, time, and price (80). Used to detect 10 different species of *Trichomonas* in observed samples. A transcribed spacer region (ITS1 and ITS2) and 5.8s rRNA gene is amplified allowing the diagnosis of naturally infected felines. It is possible to perceive dead or live protozoa with this method (25, 40, 61, 84).

2.7.2. Histopathology

Used when an intestinal biopsy is taken from the lining of the colon. The tissue is examined under a light microscope, however, due to the difficulty in preserving and detecting trichomonads it is not commonly used. Issues with this method include maintaining the fragile protozoa in the biopsy, differentiating individuals, and requiring six layers of tissue at least (92). Procedures that enhance detection in the histological slide include fluorescence in situ hybridization, immunochemistry, and chromatic in situ hybridization. In a study conducting Fluorescence In Situ, Hybridization (FISH) a species-

specific oligonucleotide sequence is removed, and an antisense probe is created to be highly specific in the identification of *T. foetus*. The *T. foetus* oligonucleotide probe, which is labeled as Cy3 antisense (TFR1615) was successful in identifying the protozoon on the mucosa of the colon, using a light microscope. The result was also confirmed to be *T. foetus, its* trophozoites appeared magenta after staining with nuclear DAPI. (31, 92).

2.7.3. InPouch TF

Provides better results for cats that have naturally occurring, symptomless *T. foetus* infection. Within the clear plastic resides antibiotics to suppress unwanted bacterial growth from the feces and a culture medium specific for *T.foetus* culturing (33, 34). Inoculation of the pouch is done via a fecal swab or 0.05g (0.5-1 ml) of feces. The pouch is then incubated upright, at 37°C in the dark. This allows for fast multiplication of the organism, which can be seen under a light microscope in 72 (18-24) hours. Lower temperature incubation will slow down the growth and take longer (12 days) to produce results visible under the microscope (16, 25, 33, 34). Unlike the PCR test, the InPouch requires live *T. foetus* to produce a positive result. This makes the handling of the sample with care essential, to avoid false positives. Sampling and diagnosis within a clinic are suggested rather than externally, to avoid the destruction of the *Tritrichomonas* during transportation. Observation is done through the pouch with a light microscope at 20x or 40x. *Giardia* sp. should not be present in the pouch after 24 hours therefore any trophozoite growth in the medium can be presumed to be *Trichomomonas* (33, 25). Differentiation between which *Trichomonas (T. foetus or P. hominis)* is visible in the pouch, requires PCR testing (16, 33).

2.7.4. Direct fecal wet mount

Used as a direct and fast examination method in clinics to check for the presence of trophozoites in feces. A small amount of saline-diluted feces is smeared onto a glass slide and examined under a light microscope at 20x or 40x. The sample must be fresh and diarrheic because the visualization of the *Trichomonads* depends on their motility. Old or firm feces makes it less possible for the protozoa to be motile, therefore making identification harder. Even though this method is the fastest and less complicated method, it is the least sensitive out of all the detection techniques. It is almost impossible to detect *Trichomonas* in asymptomatic felines (33, 40). Species differentiation is also difficult and can be confused with other similar pathogens such as *Giardia*, due to the identical size,

concave shape, and motility pattern implicative of a “falling leaf”. Special features of *Trichomonas* spp. help differentiate their tear-drop shape and undulating membranes, which produce robust motility. If distinguishing differences is difficult, a *Giardia* species antigen test is available and will not produce a positive in the case of *Trichomonas* species (6, 33).

2.8. Treatment

The only antimicrobial found to affect *T. foetus* infections is ronidazole. It is an off label nitroimidazole that has been shown to improve the consistency and frequency of diarrhea in infected felines (16, 33, 52). Currently, ronidazole is not approved by the US Food and Drug Administration for human or veterinary use. It is strictly banned in food animals because of its danger to human health, however, can be administered by veterinarians in case of confirmed *Tritrichomonas* diagnosis. In Hungary "Ronidazole 50 por” is registered for use on pigeons. It is suggested to package it in a gelatin capsule rather than a flavored fluid due to its unknown stability and repulsive taste (33). The dosage is 30 mg/kg body weight., post orally. This amount needs to be administered every twelve hours for two weeks to interrupt the shedding process of *T. foetus* (6, 16, 33). There has been no evidence to show that increasing the dose or prolonging the duration of administration has higher effectiveness. The pharmacodynamic of ronidazole is to induce free radical formations to inhibit the nucleic acid and protein synthesis of the parasite (16, 33). The pharmacokinetics of this drug is quite good since it absorbs quickly in the intestinal tract and has a long half-time within the circulatory system. The first contact with *Tritrichomonas* in the colon is via the bloodstream (51, 66). Side effects of ronidazole can include neurotoxicity, ataxia, lethargy, seizures, and inappetence (75). To limit the side effects, special colon-targeted capsules are being tested to prevent release prior to arriving in the colon (51). Implications for ronidazole therapy include administration to cats older than twelve weeks of age, avoiding pregnant or nursing queens along with systemically ill cats, using only 100% ronidazole, preventing reinfection by maintaining hygiene or quarantining the animal, monitoring side effects, and preventing toxicity (33).

It is important to make sure *Tritrichomonas* was eradicated from the cat and not just masked after using ronidazole. Clinical signs such as diarrhea can persist or recur within two weeks after drug administration. If this is the case the cat should be retested for *Tritrichomonas* through fecal PCR. Negative results indicate the diarrhea is most likely not from *Tritrichomonas* infection. Repetition of the test helps confirm the results. Since *T.*

foetus infection can have periods where clinical signs are absent, it makes it hard to diagnose these asymptomatic infections. A study suggests inducing soft diarrhea by administering lactulose and then taking a fecal sample to test for *T. foetus* via PCR. If this result proves to be negative as well, it is safe to assume the parasite has been eliminated (28, 33).

Treatment failure can be confirmed if *T.foetus* infection persists, based on testing for the protozoa and not based on the presence or absence of diarrhea. As mentioned in the post-treatment test, diarrhea may persist after ronidazole treatment, however, may not be due to *Tritrichomonas*. Clinical signs of *Tritrichomonas* infection are still not understood, however, it is suggested to search for other causes and to use other empirical treatments such as fenbendazole for general parasite control. Secondary infections can be with *Giardia, Clostridium perfringens* or *Coronavirus* (6, 69). Possible reasons for ronidazole to fail include underdosing or inadequate duration. This can be due to imprecise administration or using less than 100% ronidazole formula (10% powder constitution for avians). This can be resolved by using gelatin capsules with pure ronidazole containing the correct dosage. Another reason for the incompetence of the treatment can be due to the reinfection by another feline within the environment. Other cats can be an asymptomatic carrier of *T. foetus*. Therefore, it is commonly seen in households with multiple cats or catteries. Isolated cats should not be allowed back until the result of the treatment can be confirmed successfully. The last cause of a failed treatment can be caused by a resistant strain of *Tritrichomonas* species to ronidazole (28).

Alternative treatment for *Tritrichomonas* infection is restricted in reliability and efficacy. Changes in diet, antimicrobials, supplements, and probiotics have proven to be unsuccessful in reducing diarrhea. (27, 29) Most diarrhea cases eventually resolve on their own with time. Care needs to be taken when administering any antimicrobial drug as they can subdue the signs of *Tritrichonomonas* infection (27). Although the diarrhea resolves itself on its own, *Tritrichonomonas* infection is still present in the cat. Termination of the symptoms can range from five months to two years with the average being nince months (27). The length of the symptoms suggests that cats are not able to develop an immunity against the parasite. There are currently no studies available on the long-term effect of *Tritrichonomonas* infection (21). The European Advisory Board for Feline diseases suggests that treatment should be applied if the cat has come up positive for *T. foetus* and has diarrhea symptoms. Relapse of symptoms can occur as early as six years and are liable to transmit the infection to other cats in the meantime, therefore need to be treated to prevent this (37).

Metronidazole was considered another possible treatment option; however, it has not proven effective in several studies for *T. foetus* or *P. hominis* (74, 69).

2.9. *Pentatrichomonas hominis*

Pentatrichomonas hominis exists as an obligate protozoon, habitually residing in the large intestines of vertebrates. Having similar characteristics to *Tritrichomona foetus, P. hominis* differs primarily in morphology as it possesses a single recurrent flagellum operating as an undulating membrane and five anterior flagella. An overgrowth of *P. hominis* in the intestines can develop into severe diarrhea (74, 93). Due to its opportunistic and commensal characteristics various cases have deemed its pathogenic significance questionable (5). The zoonotic and pathogenic potential of the protozoa is yet to be ascertained, predominantly due to the limited amount of research relating to the organism (93). *P. hominis* has been observed by several authors to inhabit the gastrointestinal tract of several mammals namely monkeys, humans, dogs, cats, and guinea pigs (5, 46). The clinical manifestations of *P. hominis* in humans are corresponding with those of irritable bowel syndrome. Moreover, isolation of the parasite has occurred in the pleural effusion fluid of a lupus patient and the feces of a man with symptoms of fever, arthralgia, and diarrhea (5, 58). Misidentification of *P. hominis* from *T. foetus* is common in general practice. Proper distinguishing between the two trichomonads is vital for diagnosis and treatment purposes. Species- specific PCR assay is utilized to identify the 18S rRNA gene of *P. hominis* from fecal samples. It is not unusual for cats to be infected with both *P. hominis* and *T. foetus* at the same time making PCR testing even more important (32, 93). If the samples are fresh (not frozen) identification of *P. hominis* is also possible under light microscopy reportedly after staining with methylene blue. Unlike *T. foetus, P. hominis* has not appeared in vaginal samples. Successful treatment of *P. hominis* has been proven to be through the empirical use of metronidazole not ronidazole (93).

3. Aims

The study aims to address the significant lack of data on the infection rate of *T. foetus* and *P. hominis* in both wild and domesticated cats in Hungary. The infection rate of these protozoans is influenced by various factors such as geographical location, household density, breed, and access to the open. The choice of sampling method for *T. foetus* and *P. hominis* PCR testing significantly impacts the accuracy and detection rate of these

infections. Different sampling methods, such as rectal swabs, fecal samples, InPouch TF-Feline tests may yield varying results due to differences in the distribution and concentration of the parasites within the gastrointestinal tract.

4. Materials and methods

4.1 Sample collection

From June of 2021 to September of 2023, 173 samples were harvested from 99 individual cats and five wildcats for *Trichomonas* spp. PCR testing. Repeated testing was conducted on 39 cats. The samples were collected from 18 different locations from Hungary.

Four collection methods were utilized during the study:

- Fecal swabbing (128)
- Voided feces (28)
- Inpouch TF-Feline test from Biomed Diagnostics (12)
- Oral swabbing (5)

During the collection of fresh feces, it was requested the samples be at least the size of a bean, free from litter or other contaminants and collected without the use of alcohol fixation. These freshly voided feces were obtained immediately after evacuation from litter boxes and placed in fecal collection containers. Swabbing was conducted by my supervisor and I or veterinarians on individual domestic cats brought to the clinic. A cotton swab was introduced 3-4 cm into the rectum of the cat and gently rotated several times connecting with the rectal wall (Image 1). Swab samples were then placed in claved tubes. Clave tubes and fecal containers were stored in a freezer (-18°C) until further tests could be conducted.

Similarly, the Inpouch TF-Feline tests were harvested by us upon visits to different catteries. The pouches were inoculated with 0.5g of fecal matter. The Inpouch TF-Feline tests were placed in an incubator (32°C) to propagate possible *T. foetus*. Feasible organisms were observed under a light microscope after 72 hours (Image 2).

Image 1: Fecal swab sample harvesting from a ragdoll breed in a cattery in Kunszentmiklós.

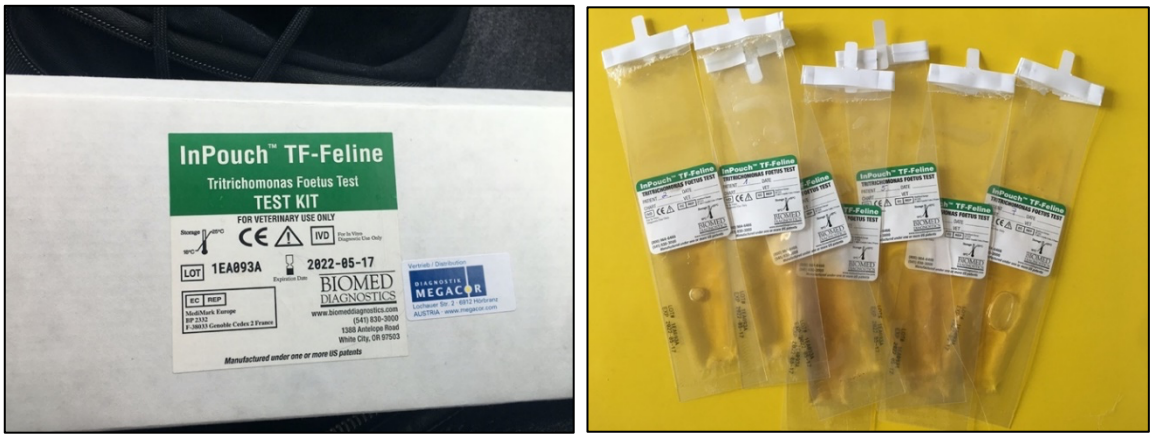

Image 2: InPouch TF-Feline test kit from BIOMED Diagnostics (left) and the pouches (right) prior to sampling of the ragdoll cats in Kunszentmiklós.

4.2 Data gathering

Most of the samples were accompanied with a sample form, gathering information on the cats' location, date of birth, breed, neuter status, sampling method (fecal swab, InPouch TF-Feline, voided feces, oral swab), sample collecting time, symptoms. The submitted form did not specify detailed figures regarding previous treatments. A map of Hungary was created to illustrate the geographic locations the sampled cats originated from using google drawing.

4.3 DNA extraction, PCR, and sequencing

DNA was extracted using the QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions with some modifications. For each fecal swabs and fecal samples, the following steps were performed. Swab or 180-220 mg stool was added to a two ml sterile Sarstedt tube containing 1ml InhibitEx Buffer (Image 3). Thereafter it was vortexed continuously for one minute to get a thoroughly homogenized solution. The suspension was heated for 5 minutes at 70°C, then vortexed for fifteen seconds and centrifuged for one minute using the 14000 rpm (rotation per minute) speed as each time later. 200 μl of the supernatant was pipetted to a 1.5 ml microcentrifuge tube and 20 μl Proteinase K was added, then the solution was thoroughly mixed by pulse-vortexing. Before pipetting 200 μl of Buffer AL the solution was incubated at 56°C for sixty minutes. Then the lysate that already contained the Buffer AL was heated at 70°C for 10 minutes. The DNA was precipitated by adding ethanol (96%) in a volume of 200 μl. The solution was vortexed for fifteen seconds, centrifuged for a short time, and 600 μl from the lysate was carefully applied to the QIAamp spin column, then centrifuged for one minute. The column was placed in a new 2ml collection tube, and the tube containing the filtrate was discarded. All this was followed by the washing process during which 500 μl of Buffer AW1 was used twice, then 500 μl Buffer AW2 was added once to provide the purity of DNA at each step by exchanging the collection tube. After washing with Buffer AW2 the collection tube containing the filtrate was discarded, the spin column was inserted into a new collection tube and centrifuge for one minute to remove residual ethanol. As a last step, the columns were transferred into a new 1.5 ml microcentrifuge tubes and 100 μl Buffer ATE was pipetted directly onto the QIAamp membrane and incubated for 1 minute at room temperature, then centrifuged for one minute to elute DNA. The labeled DNA extracts were stored in -20°C until molecular analyzation by conventional PCR.

For each PCR method, 5 μl of extracted DNA was added to 20 μl of reaction mixture containing 1.0 U HotStar Taq Plus DNA Polymerase (5 U/μl) (Qiagen, Hilden, Germany), 0.5 μl dNTP Mix (10 mM), 0.5 μl of each primer (50 μM), 2.5 μl of 10× CoralLoad PCR buffer (15 mM MgCl2 included) and 15.8 μl distilled water (DW). Further details of the PCRs are summarized in Table 1. In all PCRs sequence-verified positive controls were included. PCR products were electrophoresed in 1.5% agarose gel (100V, 55-60 min), stained with ethidium-bromide and visualized under ultraviolet light.

Purification and sequencing of the selected products were done by Biomi Ltd. (Gödöllő, Hungary). The obtained sequences were manually edited using the BioEdit program, then aligned with GenBank sequences by the nucleotide BLASTN program (https://blast.ncbi.nlm.nih.gov). All sequences retrieved from GenBank were trimmed to the same length prior to phylogenetic analysis. This dataset was resampled 1,000 times to generate bootstrap values. Phylogenetic analysis was conducted with the Maximum Likelihood method (Jukes–Cantor model) by using MEGA 7.0.

Image 3: Steps during DNA extraction. Pipetting InhibitEx (left), thermo block (central), solution centrifuge (right).

Target group	Target gene	Primer name	Primer sequence (5'-3')	Amplicon length (bp)	Thermocycling profile	Ref.
Trichomonadidae screening assay for short fragments	16S rRNA	1055F 16SR1	GGT GGT GCA TGG CCG TCA CCT ACC GTT ACC TTG	~500	95 °C for 5 min; 40× (95 °C for 45 s; 50 °C for 45 s; 72 °C for 1,5 min); 72 °C for 10 min	11
Trichomonadidae seminested PCR assay for long fragments	16S rRNA outer	16SL 16SR1	TAC TTG GTT GAT CCT GCC TCA CCT ACC GTT ACC TTG	~1550	95 °C for 5 min; 45× (95 °C for 45 s; 48 °C for 45 s; 72 °C for 1,5 min); 72 °C for 10 min	10
	16S rRNA nested	16SL 1385R	TAC TTG GTT GAT CCT GCC GAT CCT AAC ATT GTA GC	~1450	95 °C for 5 min; 45× (95 °C for 45 s; 42 °C for 45 s; 72 °C for 1,5 min); 72 °C for 10 min;	
Trichomonadidae	ITS2	TFR1 TFR2	TGC TTC AGT TCA GCG GGT CTT CC CGG TAG GTG AAC CTG CCG TTG G	~330-380	95 °C for 5 min; 40× (95 °C for 30 s; 65 °C for 30 s; 72 °C for 50 s); 72 °C for 5 min	20

Table 1: Primers and details for conventional PCR methods used in this study.

4.4 Data analysis

At first a standard descriptive statistical analysis was used to review the acquired data such as mean and median. Comparison between different factors (sex, age, breed, symptom) was computed into the Fisher Exact Test to determining the p-value.

5. Results

5.1. PCR results, phylogenetic analysis

Based on the results of the PCR tests performed on 137 samples, it can be stated that wild cats did not harbor *Trichomonas* spp., however, 13 (11,1%) domestic cats were positive for *T. foetus* and two (1,7%) for *P. hominis*. In addition, one sample proved to be a different *Tritrichomonas* species (AGCAT16V). Although the latter could not be precisely identified, it was genetically close to *Tritrichomonas casperi* isolated from mouse (*Mus musculus*), since it showed 96,4% (325/337 bp) identity to *T. casperi* (Accession Number: ON927245) based on the examination of the shorter part of 18S rRNA gene.

All six isolates (BT14, BT19, BT30, BT38, BT65, BT71) of *T. foetus*, from which a longer part of the 18S rRNA gene was successfully amplified in this study, had 100% (1462/1462 bp) identical sequences to each other, and to the sequences of *T. foetus* deposited in GenBank from cat (AF466749) or cattle (AY055799), as well as to that of *T. suis* from pig (MK801504). Furthermore, examining the shorter part of the 18S rRNA of the obtained *T. foetus* isolates BT11, BT13-14, BT18, BT20, BT67-70 (Image 4) showed 100% (445/445 bp) identity to *T. foetus* isolated from cat (AF466749) and cattle (AY055799). Based on the examination of ITS gene (Image 5), the sequence of *T. foetus* of samples BT6, BT11, BT13-14, BT18-21, BT30, BT38, BT64-71 showed 100% (333/333 bp and 332/332 bp) identity to the *T. foetus* sequence of a cat from China (OP866181; OP856640 respectively) and a cat from the USA (AF466749). Considering the 18S rRNA gene of the two *P. hominis* positive samples (BT40, AGCAT20V) they showed 100% (445/445 bp) identity to a *P. hominis* isolate from a cat (KC594038) and 99,3% (442/445 bp) identity to *P. hominis* from a dog (AY758392). Regarding the ITS gene, they were 99,7% (294/295) identical to *P. hominis* of a cat from Czechia (KC594038) and of a dog from the USA (AY758392). In addition, they also showed 99,7% (294/295 bp) identity to *P. hominis* of a human sample from Thailand (AF156964).

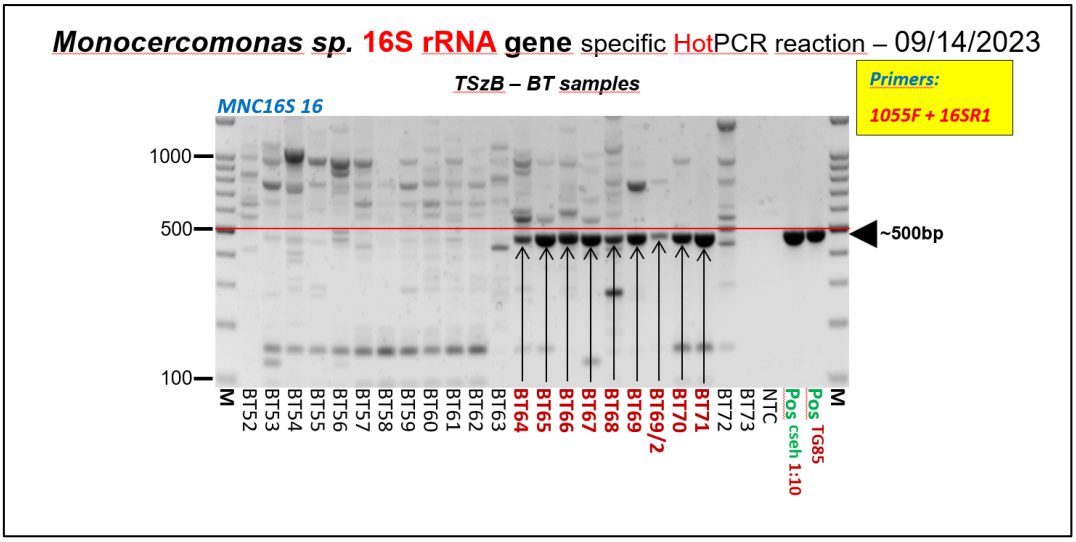

Image 4: Agarose gel electrophoresis of the 18S rRNA PCR products.

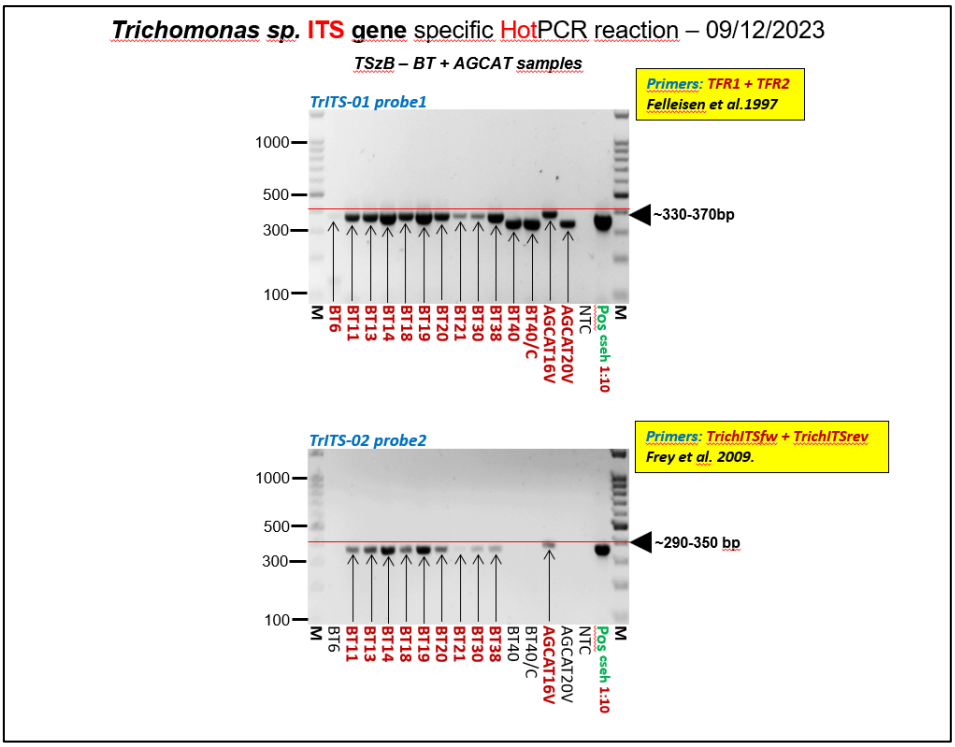

Image 5: Agarose gel electrophoresis of the ITS PCR products.

Based on the phylogenetic analysis conducted, it can be stated that *T. foetus* samples (BT11, BT12, BT13, BT14, BT18, BT20, BT67-70) clustered together and positioned on the 18 S rRNA phylogenetic tree (Figure 1) close to *T. foetus* from another cat (*Felis catus*) and cattle (*Bos taurus*). Examination of the ITS gene of these samples, BT6, BT11, BT13-14, BT18-21, BT30, BT38, BT64-71 found they were in close relation with *T. foetus* from

different cats from China and the USA (Figure 2). Concerning the sequences of the 18S rRNA gene of the two *P. hominis* positive samples (BT40, AGCAT 20V), found they were also clustered together with other *P. hominis* isolates from a cat and dog (Figure 1). In addition, examining the sequence of the ITS gene of the same *P. hominis* samples, they were also found in the same cluster with *P. hominis* of a cat from Czechia, a dog from the USA and of humans in Thailand (Figure 2). Furthermore, one positive sample (AGCAT 16V) interestingly clustered together with different *Tritrichomonas* spp., such as *Tritrichomonas casperi* from a mouse (*Mus musculus*) in both phylogenetic trees (Figure 1,2).

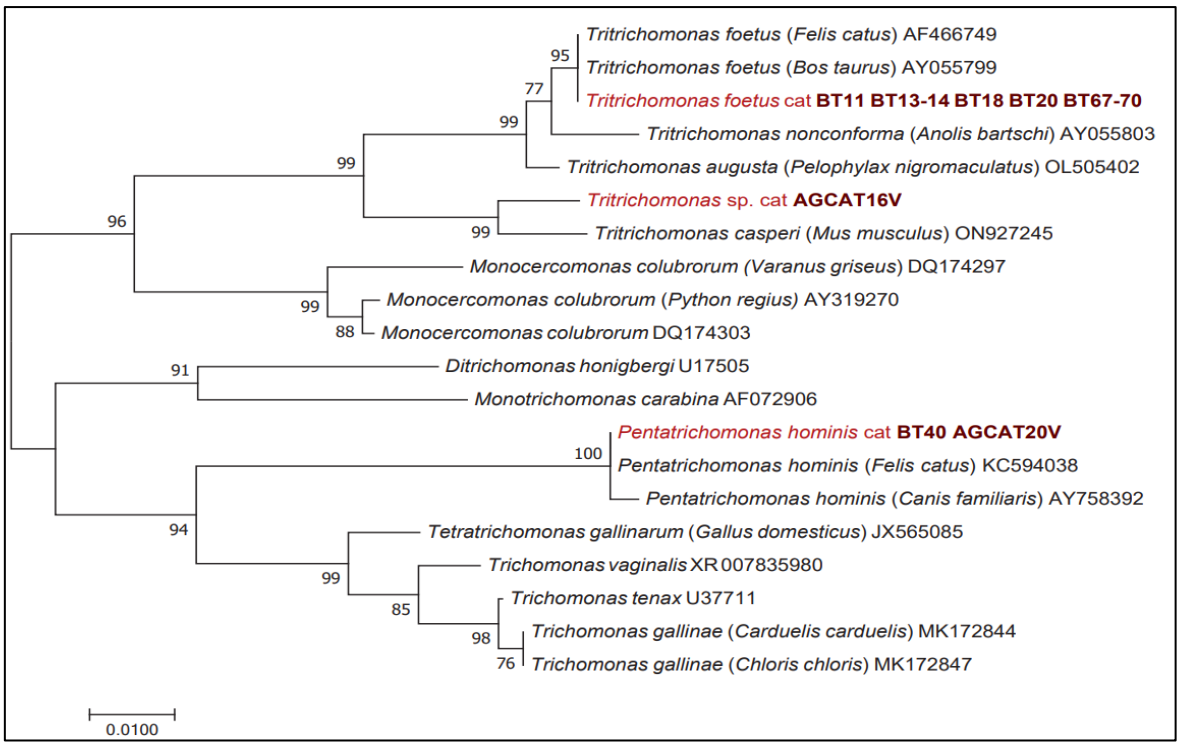

Figure 1: Phylogenetic tree based on the results of 18S gene targeted PCR reaction.

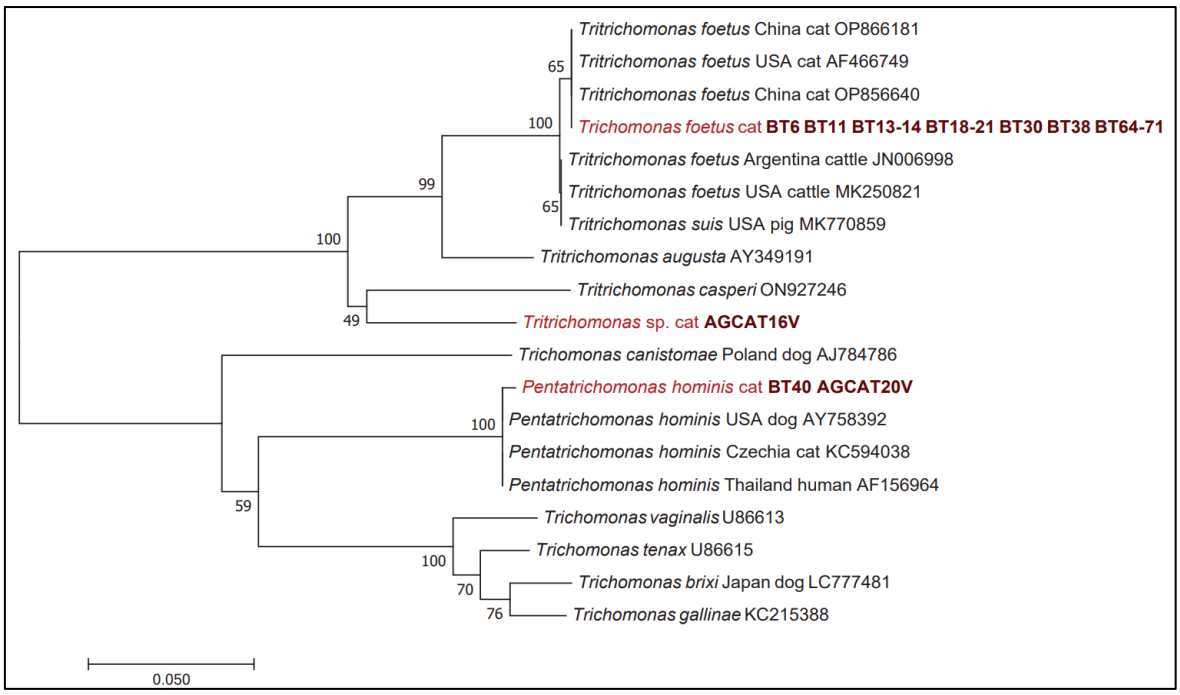

Figure 2: Phylogenetic tree based on the results of ITS gene targeted PCR reaction.

5.2 Geographic locality

The study was carried out using samples from 18 locations throughout Hungary. Samples originated from Kunszentmiklós (30), Budapest (3), Jósvafő (15), Komjáti (6), Sződliget (1), Szinpetri (5), Szendrő (3), Bodrogolaszi (1), Ónod (1), Újszentmargita (1), Múcsony (1), Érd , Székesfehérvár, Ecser, Kaposvár, Keszthely, Kecskemét, Vecsés (1). Among the 104 cats examined, the wildcats which did not harbor any *Trichomonas* spp. and were from the Aggtelek National Park. The domestic cats in this study were from different locations throughout Hungary. Considering the 13 *T. foetus* positive domestic cats and one *P. hominis* positive cat with serious diarrhea, were all from the central part of the country. The other *P. hominis* positive cat was also from the Aggtelek National Park (Komját) (Figure 3).

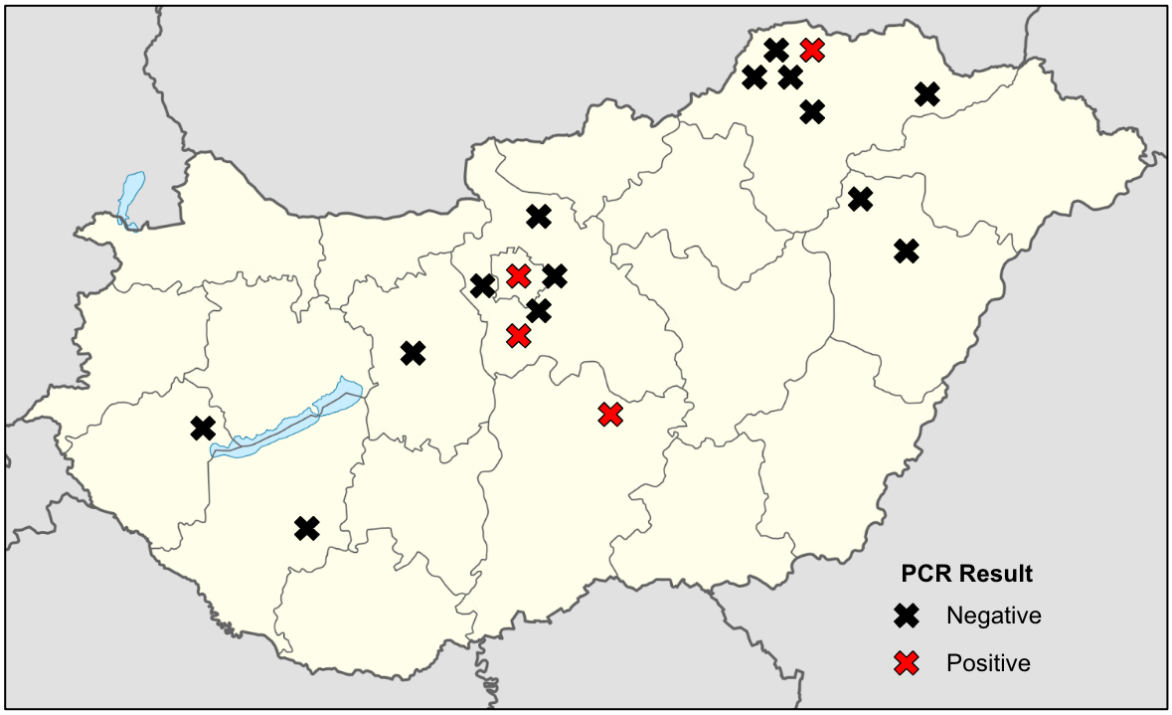

Figure 3: Geographical distribution of acquired samples from which fecal samples were obtained for *Trichomonas* spp. for polymerase chain reaction testing. Negative samples are seen with a black cross, while positive samples with a red cross.

5.3. Signalment

Although breed was reported for all 104 cats, some deficiencies occurred during data collection. Sex was identified in 97 (93%) of them, that being 62 (64%) females and 35 (29%) males. Age was reported for 73 (70%) of the felines participating in the investigation. The mean age of the cats were 6.47 years, and the median age was 6 years (ranging from kittens to 14 years of age). Of the remaining cats, 41 (39%) did not have a known age. During the investigation a total of seven different breeds were sampled:

- Ragdoll (45)
- Devon rex (1)
- Main coon (2)
- European short hair (49)
- Persian- Himalayan (1)
- Felis silvestris (4)
- British shorthair (1)
- Persian (1)

Investigate the correlation between data, we found that among the *T. foetus* positive cats the female and male cats occurred in equal proportions in contrast to the *P. hominis* positive cats, where only females were sampled. In addition, male samples interestingly only carried *T. foetus*, however, these results were not significant. Examining the cats regarding their breed, we found that the most frequently appearing breed was the European shorthair, followed by the Ragdoll. The purebred cats were in larger proportion among positive cats than among negative individuals, however, without significancy. Organizing the cats into two groups, one with cats of two-years-old or younger, the other with more than two-years-old cats. Interestingly, we found that the *T. foetus* positive cats were grouped in older cats comparted to *P. hominis* positive cats (p=0.0952). Out of the 16 infected animals, eight (50%) showed clinical symptoms, such as diarrhea, however, coinfection with the detected protozoa was not found in any of the feline samples. Furthermore, examining the cats regarding gastrointestinal symptoms, we found that the positive cats showed significantly more symptoms than the negative cats (p=0,0016). The important data of positive cats are summarized in Table 2.

#	Site	Sample	Breed	Age	sex	Symptoms	DNA sample	PCR reaction	PCR	Sequence	Microscopic examination
1	Kunszentmiklós	Feces and Swab	**Ragdoll**	4 y	F	No	**BT6 + BT14**	TFR1-TFR2 16SL-SR1/ Monocercomonas/TFR1-TFR2	+	***T. foetus***	
2	Kunszentmiklós	Swab	**Ragdoll**	3 y	F	No	**BT11**	Monocercomonas/TFR1-TFR2	+	***T. foetus***	
3	Kunszentmiklós	Swab	**Ragdoll**	3 y	F	No	**BT 13**	Monocercomonas/TFR1-TFR2	+	***T. foetus***	
4	Kunszentmiklós	Swab	**Ragdoll**	5 y	M	No	**BT18**	TFR1-TFR2/TrichITS	+	***T. foetus***	
5	Kunszentmiklós	Swab	**Ragdoll**	3 y	F	**Diarrhea**	**BT19**	TFR1-TFR	+	***T. foetus***	
6	Kunszentmiklós	Swab	**Ragdoll**	6 y	F	No	**BT20**	Monocercomonas/TFR1-TFR2/TrichITS	+	***T. foetus***	
7	Kunszentmiklós	Swab	**Ragdoll**	3 y	M	No	**BT21**	TFR1-TFR2/TrichITS	+	***T. foetus***	
8	Kunszentmiklós	Inpouch	**Ragdoll**	2 y	F	**Diarrhea**	**B30**	TFR1-TFR2/TrichITS	+	***T. foetus***	InPouch TF-Feline negative
9	Kunszentmiklós	Inpouch	**Ragdoll**	2 y	M	**Diarrhea**	**BT38**	TFR1-TFR2/TrichITS	+	***T. foetus***	InPouch TF-Feline positive
10	Budapest	Swab and Feces	**Persian-Himalayan**	1 y	F	**Strong diarrhea**	**BT 40**	Monocercomonas	+	***P. hominis***	
11	Komját	Swab	**Europ**	Unknown	F	No	**AGCAT16V**	Monocercomonas	+	***Tritrichomonas* sp.**	

			ean short hair							
12	Komját	Swab	**Europ ean short hair**	6 m	F	No	**AGC AT20 V**	Monocercomon as	+	***P. hominis***
13	Kecskemét	Anal swab	**Ragdoll**	2 m	M	**Diarrhea**	**BT64**	TFR1-TFR2	+	***T.foetus***
14	Kecskemét	Anal swab	**Europ ean short hair**	Adult	M	**Diarrhea**	**BT65**	TFR1-TFR2	+	***T.foetus***
15	Kecskemét	Anal swab	**Europ ean short hair**	Adult	M	**Diarrhea**	**BT70**	TFR1-TFR2	+	***T.foetus***
16	Kecskemét	Anal swab	**Europ ean short hair**	Adult	M	**Diarrhea**	**BT71**	TFR1-TFR2	+	***T.foetus***

Table 2: Signalment of the 16 cats, found positive for *Trichomonas* species by polymerase chain reaction (PCR). Abbreviations: m=month, y=year, M=male, F=female.

6. Discussion

There is a limited understanding of the prevalence and distribution of *T. foetus* and *P. hominis* infections in specific geographic regions, including Hungary. This study sampled individual domestic and wild cats at random veterinarian clinics throughout Hungary and the following results where concluded.

6.1 *Tritrichomonas foetus*

Until now, the presence of *T. foetus* in cats has not been proven in Hungary. In this study we found *T. foetus* infection in the domesticated cat population of Hungary with 11,1 % prevalence. The wild cats sampled remarkably harbored none. This may be due to the several factors such as the grouped housing, breed deposition, demographics, or companion animal management. Similar numbers can be found in other countries such as 17.14% in Italy (67), 15.7% in Germany (50) and 14.5% in France (43). Notably the southern part of Europe produced a higher infection rate than countries in northern Europe. The geographical influence of climate on *T. foetus* raises an important question.

Based on the study's findings, it is possible to infer a potential breed predisposition. Among the 104 cats included in the study, 45 were Ragdolls, six were other pure breeds, and 49 were European shorthair (mixed breeds). Notably, only the Ragdoll and European

shorthair breeds yielded positive results. In the UK and Norway, *T. foetu*s has also been reported in other breeds such as Siamese, Bengal and Burmese along with the Ragdoll (42, 57).

No significant association was found between the presence of *T. foetus* and symptoms such as diarrhea at the time of sampling, or with cats whose anamnesis showed enteritic signs. This contradicts previous studies, where symptoms played a key role in the sampling of *T. foetus* positive cats (50, 67). On the other hand, subclinical shedding of *T. foetus* has been reported, therefore isolation from a non-symptomatic/ healthy cat it made possible. Intermittent shedding of *T. foetus* in feline fecal matter is also likely (30). The only noteworthy result is that positive cats showed more symptoms than negative cats. This is supported by the fisher test results (p=0,0016). Sampling was done once in most of the cats during this study. The 39/104 cats who had multiple samples taken also didn't show significate relation between symptoms and presence of the protozoa. Diarrhea in cats infected with *T. foetus* may be multifactorial, therefore further investigation into the pathogenesis is warranted.

Among the 13 *T. foetus* positive 12 (92.3%) were cats above the age of twelve months. Based on the results (Table 2) the mean age of the positive cats were 2.72 years old. Only two (12.5%) were kittens, which suggests that the parasite might be more prevalent in older cats above the age of two. This result is countering other studies who have found kittens below the age of eight months to be more susceptible to the infection of *T. foetus* (43).

The result of the PCR test can be influenced by the method of sample collection, whether its fecal loop, collection of freshly voided feces, fecal swabbing, or colon flushing. There is conflicting evidence regarding the most effective sampling method. One study suggests that colon flushing yields better results, followed by the fecal loop and freshly voided feces (33). However, another study found that samples collected using the fecal loop were 2.04 times more likely to produce a positive result than colonic flushing, due to the location of protozoa in the colon (40). Although in this research we did not have the chance to use fecal loop or colon flushing, there was no association between the sampling methods and positive PCR results, therefore the swab was chosen as the main technique. This limits the studies ability to evaluate whether this was true. On the other hand, there is significate support for the use of InPouch TF-Feline and *T. foetus* PCR due to its culture fluid. During the investigation only two out of ten InPouch TF-feline samples produced a positive PCR

result. PCR on feces from rectal swabs seems to be more sensitive based on the results as out of 16 positive samples 14 (86%) were successful. An issue with this conclusion is, however that most of the samples were swabs (128) and there was not an equal amount of InPouches tested to balance the hypothesis. Regarding additional methods not utilizing a culture medium, the inhibitor in feces raises the question if perhaps that can lead to false negative PCR (43). The initial suspicion was disproven when the use of different tests (swabs and voided feces) resulted in a higher positive PCR rate. This was evident from the two positive results obtained from cats that were repeatedly sampled using these two methods.

6.2 *Pentatrichomonas hominis*

Traditionally, *P. hominis* is a symbiotic protozoon, however, has been reported in several studies as causing diarrhea (25). Many times, it is misidentified as *T. foetus*, therefore the possible potential is probably underestimated (32). In our study two cats were diagnosed with *P. homins* infection. Only one out of the two cats had no symptoms while the other feline endured symptoms of strong diarrhea. This cat was treated with ronidazol, after the time of sampling and their symptoms subside afterwards. This prompts the question of whether ronidazole, is specifically effective against *T. foetus* (48) or if it can also be used for other Trichomonas species. Numerous studies indicate the treatment of *P. hominis* with metronidazole but have been unable to demonstrate its efficacy (32, 74). Further studies are required to prove *P. hominis* is treatable by metronidazole.

Sampling method comparison was not possible in this study since the two samples that appeared positive where via fecal swabs. According to a study there is no difference between the diagnostic techniques of *P. hominis* and *T. foetus* (32). Therefore, the molecular tool such as PCR, is indispensable to confirm the species of Trichomonadidae, especially since their morphology cannot be clearly detected in living, motile specimen.

It was not possible to compared variables such as age, breed, and gender due to the low number of samples producing positive results, however according to the fisher test infection in cats older than two years old is more likely for *T. foetus* than *P. hominis*).

Several studies have reported the zoonotic transmission of *P. hominis*, indicating its ability to infect both humans and animals. For instance, a study investigating the present of *P. hominis* has found two cases in the gastrointestinal tract of humans (58). The prevalence of *P. hominis* in cats (25) suggests that humans living near an infected individual can transmit the organismic and vice versa. According to the sequencing results of our study, it

was found that one of the cats infected with P. *hominis* had a 99.7% similarity to the ones found in humans. To further support the zoonotic potential of *P. hominis*, genetic analysis has been employed in other locations to determine the relatedness of parasite strains found in humans and animals. A study in Thailand utilized molecular techniques to compared *P. hominis* isolates in humans and non-primates, revealed a high degree of genetic similarity (42, 58). The impact of *P. hominis* infections on public health, including their potential role in outbreaks or transmission dynamics within communities along with their zoonotic potential particularly in relation to transmission from animals to humans demands attention in the research field.

6.3 *Tritrichomonas* sp., and its close relation to *Tritrichomonas casperi*

Tritrichomonas casperi is a relatively new species of flagellated protist isolated from the cecum of wild rodents (89). In this research paper a single female cat without any symptoms came up with infestation with a *Tritrichomonas* species and showed 96.42% identity to *Tritrichonomonas casperi*. The flagellate was isolated as a living organism from the rectum via fecal swabbing therefore it had colonized the gastrointestinal tract, leading us to believe it was not a remnant of digestion. Since felines are not the natural host of this species, mice are (89), indicating there might be a similar evolutionary development occurring that we see with *T. foetus*. Further studies are required whether this *Tritrichomonas* sp. is a new species, and regarding *T. casperis*, including its host potential.

7. Conclusion

The present study is the first to report the presence of *T. foetus* and *P. hominis* in cats from Hungary. Based on the results, we can conclude that the prevalence of *T. foetus* and *P. hominis* appears to be highly variable among the cat population examined, with infections being more common in pedigrees from catteries after the age of two. Clear sequences of *T. foetus* and of feline *P. hominis* were provided, along with a *Tritrichomonas* sp. with close relation to *T. casperi* of mice, all by examining 18S rRNA and ITS genes. Limitations of this study include the relatively small number of cats sampled and the narrow variables studied for the investigation. The small number of positive cats overall limits our ability to conduct a multivariable statistical analysis and explore the effect of interactions. Participation rates were also varied as other catteries were less likely to allow testing to be conducted on their cats. To enhance the statistical power for risk factor analysis and explore

factors related to sampling techniques, breed, gender, and age of these felines, it is recommended to conduct a larger study involving more catteries and wild cats across a wider geographical area.

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