THESIS

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Molecular investigation of protozoan parasites that are potential causes of eye lesions in dogs and cats

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1. Introduction:

Protozoa play a major role in the manifestation of a wide variety of diseases in both humans and animals. It has been documented that some protozoa, such as free-living amoeba, act as opportunistic parasites and may causes disease. An example of one such disease is Acanthamoeba Keratitis which is a serious disease of the human eye associated with the use of contact lenses. Although the occurrence of Acanthamoeba Keratitis is rare, reported cases of the disease is described as on the rise. The occurrence of microorganisms like bacteria and viruses is frequently associated to the emergence of eye illnesses in companion animals. Despite being recognised as ocular pathogens in cats and dogs, protozoa like *Leishmania* and *Toxoplasma* may not come to the attention of veterinarians when dealing with ophthalmic patients. This study aimed to determine if *Acanthamoeba* has the potential to cause disease in animals' eyes, in the same way humans may be affected by the amoeba. As some protozoa are thought to becoming more pathogenic, we aimed to find out if other protozoa, such *Leishmania* and *Trichomonas*, were also causing eye lesions, therefore we attempted to detect them also.

2. Literature Review:

2.1 Microorganisms as a source of ocular lesions:

Veterinarians across the globe are regularly presented with small animal patients suffering from painful, swollen and damaged eyes. The way in which cats and dogs acquire such ocular lesions is plentiful, including trauma, allergens, irritants, toxins, mechanical problems and age related deterioration. However, many clinical manifestations of the eye, including conjunctivitis and keratitis, are significantly attributed to infectious agents in the form of microorganisms.

Viruses and bacteria are extensively described in veterinary literature as ophthalmic pathogens enabling veterinarians to effectively make diagnosis and prepare treatment plans when necessary.

→ Viral infections including *Feline Calicivirus Virus (FCV), Canine Distemper (CDV), herpesvirus, influenza viruses* often cause lesions limited to the eye but more frequently accompany a systemic infection.

→ Bacterial infections: a plethora of species can be agents of eye disease including *Staphylococcus* spp., *Corynebacterium* spp., *Bacillus* spp., *Pseudomonas* spp., *Proteus* spp., *Actionbacillus* spp. and *Escherichia coli* [1] [2].

Fungal infections of the eye may also develop when spores of several species come into contact with the ocular surface. Like viruses and bacteria, many species have been recorded as the cause of eye disease in companion animals. Genera of fungi reported to be associated with Canine Fungal Keratitis alone include *Alternaria, Aspergillus, Candida, Cephalosporium, Chrysosporium, Cladosporium, Curvularia, Fusarium, Hormographiella, Penicillium, Phialemonium, Pseudallescheria, and Scedosporium* spp. [3].

The umbrella term 'parasites' elicits thoughts of visible organisms such as worms, mites and flies, all of which can be implicated with a number of eye problems in cats and dogs. However, parasitic organisms in the form of protozoa also have the potential to act as the catalyst of eye infections in our canine and feline companions. The presence of protozoa may not always be anticipated by clinical veterinarians and may frequently be overlooked when cases of conjunctivitis, keratitis, and other similar diseases are presented [4].

2.2 Opportunistic protozoa:

More than 50,000 species of protozoa have been described, the bulk of which are free-living organisms inhabiting almost all environmental niches. Free-living protozoa survive by feeding on bacteria and reproduce asexually, most often via binary fission allowing them to be abundant in numbers. Protozoa however can live on or inside other organisms, making them parasitic. All humans are thought to harbour at least one species of protozoa at any given time, however, most infections are considered commensal.

The term opportunistic protozoa is given to describe those protozoa who are typically free-living organisms but have the capacity to become pathogenic when they come into contact with people and other animals under specific conditions. There are several things that can cause an opportunistic disease to emerge including malnourishment, recurring or simultaneous infections and chemotherapy. However, the effects of opportunistic protozoa are exemplified in individuals who are immunosuppressed or immunocompromised. Considering HIV patients, secondary protozoal infections are major contributing factors to the mortality of sufferers. In immunocompromised patients, protozoa can cause both focal and systemic diseases. *Cryptosporidium parvum* and *Isospora belli* are the most important protozoa causing enteric disease in HIV/AIDS patients, whereas *Toxoplasma* and

Leishmania species are responsible for more generalised systemic infections [5]. Opportunistic protozoa are also shown to be associated with disease in animals. Like humans, the parasites require predisposing factors in order to elicit damaging effects. Enteric disease caused by intestinal protozoa such as *Giardia* and *Cryptosporidium* are common occurrences in young an immunocompromised dogs for example [6].

Acanthamoeba is a genus of free-living protozoa with opportunistic tendencies, which is of interest to the academic research community as it has been identified as a causative agent of sever disease in humans. Acanthamoeba keratitis is one such described disease in medical literature attributed to *Acanthamoeba* and its description as an opportunistic protozoa led us to investigate its potential roll, along with other protozoa, in the manifestation of ocular disease in small animals [7].

2.3 *Acanthamoeba*:

2.3.1 Taxonomy and morphology:

The Greek prefix ‘acantha-’ was added to ‘-amoeba’ to give the name to a genus of single-celled organisms first described by Italian bacteriologist Aldo Castellani in 1930. Acantha-, meaning spike or arrow was chosen to reference the ‘spike like projections’ found on the surface of the organism, these projections later became known as acanthopodia. Since its discovery many studies have been conducted to better describe and understand the organism. Currently Acanthamoeba is classified as outlined below [8].

→ Kingdom: Protozoa
- ↳ Subkingdom: Sarcomastigota
  - ↳ Phylum: Amoeboza
    - ↳ Class: Discosea
      - ↳ Subclass: Longamoebia
        - ↳ Order: Centramoebida
          - ↳ Suborder: Acanthopodina
            - ↳ Family: Acanthamoebidae
              - ↳ Genus: *Acanthamoeba*

Within the *Acanthamoeba* genus DNA analysis of *Acanthamoeba* have successfully identified more than 20 individual species [9]. Below is a list of species that have been identified along with the sample source where the species was first isolated [10].

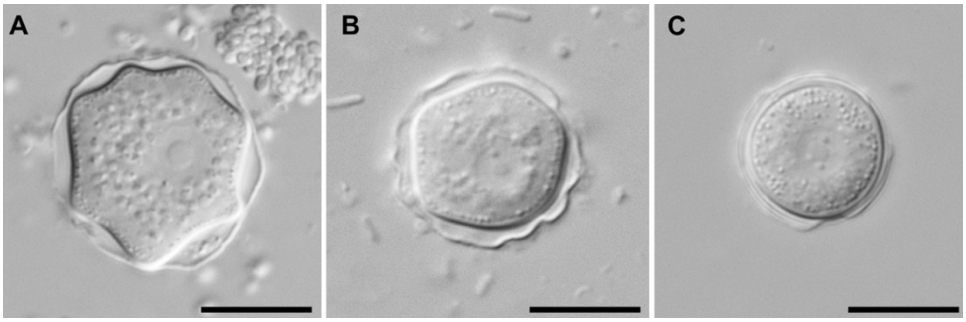
A. astronyxis [water from termite colony]
A. castellanii [yeast culture]
A. commandoni [garden humus]
A. culbertsoni [monkey kidney cell culture]
A. divionensis [soil]
A. echulata [compost]
A. griffin [seawater bottom sample]
A. hatchetti [harbour sediment]
A. healyi [brain tissue]
A. jacobsi [marine sediment]
A. lenticulate [swimming pool]
A. lugdunensis [pool]
A. mauritaniensis [sewer sludge]
A. palestinensis [soil]
A. pearcei [sewage sediments]
A. polyphaga [pond]
A. pusulosa [pool]
A. quina [swimming pool]
A. rhysodes [soil]
A. royreba [human choriocarcinoma cells]
A. stevensoni [shellfish beds]
A. triangularis [human faeces]
A. tubiashi [river water]

Acanthamoeba has two life stages, a trophozoite stage and a cyst stage. The diameter of both trophozoite and cyst ranges between 13-23µm. Differentiation of the various species within the *Acanthamoeba* genera can be difficult to do based on morphology therefore DNA sequencing is necessary to make adequate identifications. However, the species have been assigned into three separate groups (Picture 1.) based on the morphology of the cyst stage, which allows for primary identification to occur [9].

→ Group I: Large cysts.

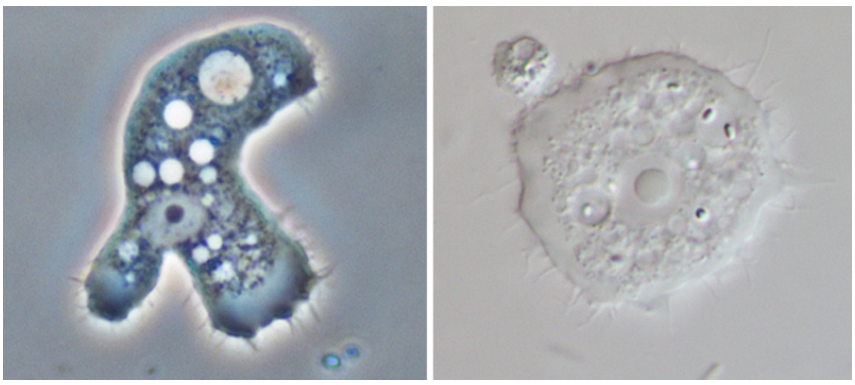
→ Group II: Wrinkled ectocyst and an endocyst which may be stellate, polygonal, triangular or oval.

→ Group III: Thin, smooth ectocyst and round endocyst.



Picture 1: Morphological cyst groups. Group I: A, Group II: B, Group III: C [11].

Solely using morphological characteristics of *Acanthamoeba* should not be relied on when attempting to identify at the species level. The presence of the acanthopodia on the cell surface may be used to identify on a genera level (Picture 2.). *Acanthamoeba* also contains several other organelles that are more often associated with cells of higher organisms. Electron microscope studies of *Acanthamoeba* have identified numerous organelles and structures apart from the acanthopodia. Golgi complexes, smooth endoplasmic reticula, rough endoplasmic reticula, ribosomes, mitochondria to name a few. A digestive vacuole and a large contractile vacuole for controlling the water content inside the cell are also present along with the cell's nucleus [12].



Picture 2: Acanthamoeba trophozoites displaying characteristic acanthopodia [11].

2.3.2 Optimal conditions:

Optimal conditions for *Acanthamoeba* spp. sees the protozoa in its trophozoite form. The optimal conditions include a neutral pH, a temperature of approximately 30°C, an osmotic concentration of 50-80mOsmol and a food supply [13]. *Acanthamoeba* spp. are bacterivores feeding primarily on bacteria but also interact and nourish on other microorganism such as yeast and viruses via phagocytosis [14].

2.3.3 Resistance of *Acanthamoeba*:

When conditions are unfavourable cellular differentiation occurs transforming the trophozoites into cysts. Once in its cyst form, *Acanthamoeba* is extremely resistant to physical and chemical conditions. Numerous studies have shown the protozoa's resistance capabilities which include a pH as low as 2, both gamma and UV irradiation, freezing conditions and moist heat up to 60°C. The duration in which *Acanthamoeba* resistance lasts can be very prolonged as they have been recorded surviving at room temperature for 2 years

and in water with a temperature of 4°C for 24 years [15]. *Acanthamoeba* cysts retrieved from human tissues have the ability to survive after desiccated treatment for more than 20 years [16]. Cysts of *Acanthamoeba* show resistance to several chemical disinfections including chlorine and hydrogen peroxide products. Trophozoites, on the other hand, are considered sensitive to almost all disinfections apart from glutaraldehyde products as some resistance has been recorded. Several strains of *Acanthamoeba* species have been recovered from hospital settings demonstrating that the amoeba poses the ability to evade disinfection used to ensure the highest of sterility [17].

2.3.4 Distribution of *Acanthamoeba*:

The resistance exhibited by *Acanthamoeba* ensures that the protozoa is one of the most abundant protozoa in the environment. Acanthamoeba can be described as an ubiquitous organism as successful isolation of the protozoa has been demonstrated from samples taken from an extensive range of sources. Acanthamoeba is present worldwide with positive sample even been retrieved from samples taken from Antarctica [18].

Water, in numerous environmental states act as a significant habitat for member of the Acanthamoebidae family. The salinity of water does not have a negative impact on the occurrence of *Acanthamoeba* as it has been recorded in salt water, fresh water and brackish waters in both coastal regions and isolated inland natural water systems. Packaged water including bottled mineral water and distilled water have also returned positive results for the presence of *Acanthamoeba*. Chlorinated swimming pools and hot water sources such as Jacuzzis and hot springs among many other water sources have been confirmed to be a habitat for *Acanthamoeba* [13]. *Acanthamoeba* possess the ability to be airborne. Early studies confirmed the presence of *Acanthamoeba* in the nasal cavities of individuals [19]. *Acanthamoeba* are thought to be suspended in the air as an aerosol via dust particles and are regularly isolated from ventilation systems and air conditioning units [20]. Airborne *Acanthamoeba* spp. are of interest as they may play a role in the pathogenicity of diseases occurring from exposure to the amoeba. The thin and narrow nasal vessels, along with the olfactory nerve may serve as an entry point to the bodies central nervous and circulatory systems.

Asides from water and air *Acanthamoeba* can be found in a myriad of solid structures including, plant material, surfaces and domestic utensils and abundantly in soil. The extensive distribution of this protozoa and its resistant nature ensures that all humans and

animals will become exposed to the organism throughout their lifetime. Different *Acanthamoeba* spp. and humans typically interact without any negative effects, however in unhealthy people, the protozoa can behave as an opportunistic parasite and cause a number of diseases.

2.3.5 Pathogenic *Acanthamoeba* strains:

The species of *Acanthamoeba* have allocated into genotype groups based on rRNA sequencing. A minimum of 5% sequence divergence exists between each genotype which currently results in 23 described groups [21]. The genotype clades are designated T1, T2 and so forth all the way up to T23 [22]. Ten of the identified groups have been found to be disease causing agents in humans, with the exception of T1 and T12, which have only been found to cause Granulomatous Amebic Encephalitis (GAE), all genotypes cause keratitis in humans [13, 22]. The T4 and T5 genotypes appear to have the greatest influence on pathogenicity and disease development. The T4 genotype in particular has the greatest pathogenic effect in inciting Acanthamoeba Keratitis in humans as 90% of AK cases are linked to the presence of the T4 genotype [13]. In veterinary scientific literature, the possibility for the T4 strain to induce disease is discussed to a lesser extent. T4 genotypes have nevertheless been found in the corneas of feline patients who are experiencing ocular lesions and were found to be in homology with *Acanthamoeba* strains responsible for causing AK in humans, suggesting there may be a potential zoonotic risk [23].

2.3.6 *Acanthamoeba* as a human pathogen: Opportunistic/Non-opportunistic:

Pathogenically *Acanthamoeba* is an interesting organism as it is a free-living organism that can cause disease in both immunocompromised and immunocompetent individuals. *Granulomatous Amebic Encephalitis* (GAE) is one of the most frightening disease caused by *Acanthamoeba* spp. People who already have underlying illnesses such as cancer, diabetes, renal failure, cirrhosis, Tuberculosis, skin ulcers or HIV infections allow *Acanthamoeba* to cause GAE as an opportunistic parasite [10].

GAE is a disease of the central nervous system (CNS) that can take a subacute or chronic course and although morbidity rates are low it has a mortality rate between 97 and 98%. *Acanthamoeba* spp. in the T4 genotype, which includes the species *A. castellanii* and *A. polyphaga* have been most associated with the occurrence of GAE [24]. Although T4 Acanthamoeba has been associated with the majority of cases, additional species from

different genotype clades have the ability to cause GAE as T2 and T18 have also been recorded inflicting the condition [24, 25]. The olfactory epithelium in the nasal cavity is the primary route of penetration, and infection results via air inhalation or aspiration of water contaminated with the protozoan's invasive forms. Trophozoites travel over the olfactory nerves from the nasal mucosa, the ethmoid bone and the endothelium of the brain's capillaries to the central nervous system [26]. Since GAE's symptoms, which include seizures, headache, stiff neck, body temperature fluctuations, nausea, and vomiting are non-specific, the condition is frequently misdiagnosed as bacterial or viral encephalitis.

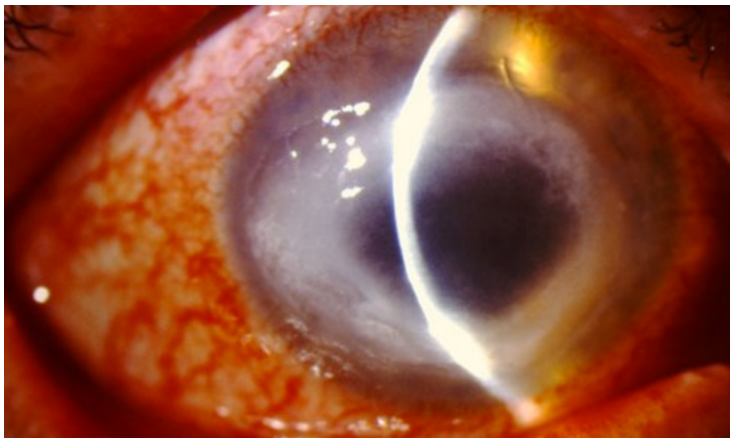
Another disease caused by different *Acanthamoeba* spp. is the *Cutaneous Acanthamebiasis* which is heavily associated the presence of T4 *Acanthamoeba.* A. culbertsoni, a non-T4 genotype member, has also been identified as the illness's cause [27]. Development of firm papulonodules that drain purulent material and then develop into non-healing indurated ulcerations are a common occurrence in HIV patients [28]. Additionally, patients with GEA who are not HIV-positive and receiving immunosuppressive medication for organ transplantation have been shown to experience *Cutaneous Acanthamebiasis* [10]. According to reports, 73% of people die from cutaneous infections without CNS involvement, while 100% of people die from cutaneous infections with CNS signs [29].

2.3.7 Acanthamoeba Keratitis:

Despite the fact that immunosuppressed people are more susceptible to *Acanthamoeba* infections, Acanthamoeba Keratitis (AK), a condition also caused by the protozoa, has been shown to affect immunocompetent people as well. Although immunocompromised individuals are no a prerequisite for the development of AK, certain factors are thought to play a role in the diseases occurrence. AK is most common in contact lens users. Poor hygiene and ocular abrasions are presumed to be certain predisposing factors concerning AK, as *Acanthamoeba* species have been detected from contact lens case, solutions and their accessories [30]. As a rare condition, AK is another protozoal infection that often goes misdiagnosed as a bacterial or viral infection, which can unfortunately result in severe progression ending in eventual blindness.

As the name suggest AK, the presence *Acanthamoeba* species, especially in their trophozoite form cause inflammation to the cornea. The disease's etiological agent has been identified as several species of *Acanthamoeba*, including *A. castellanii, A. polyphaga, A. hatchetti, A. culbertsoni, A. rhysodes, A. griffini, A. quina,* and *A. lugdunensis* [10].

Pathogenesis of Acanthamoeba Keratitis has been described as follows [31]. The attachment of the microbe to the corneal surface is the first stage in the pathogenesis of AK. A number of proteins play a role in the adhesion process, with a mannose-binding protein expressed by the amoeba being the most crucial. *Acanthamoeba* trophozoites continue the process by destroying the epithelial barrier through direct cytolysis, phagocytosis, and the induction of apoptosis. Trophozoites penetrate the underlying collagenous stroma after adhesion and destruction of the corneal epithelium. Numerous amoeba products, including metalloproteinases and serine proteinases, mediate the process of stromal invasion. These proteinases function to provide a strong cytopathic effect that kills host cells and breaks down the stromal matrix and epithelial basement membrane in order to invade deeper layers of the cornea. Typically, stromal involvement appears towards the end of AK. The trophozoites feed on keratocytes and organic matter once they have entered the stroma, which causes keratocyte depletion, the production of a strong inflammatory response, and ultimately stromal necrosis and blindness. Clinically the appearance of a ring-like stromal infiltrate (Picture 3.), presumed to be made up of inflammatory cells like neutrophils, is the most defining clinical hallmark of AK. Scleritis, episcleritis, conjunctival hyperaemia, and corneal inflammation may also develop as a result of infection. Furthermore, the infiltration of ocular nerves by trophozoites might result in neuritis and necrosis and left untreated AK can be extremely detrimental to patients and can result in blindness [32].



Picture 3: A dense ring corneal infiltrate often seen in Acanthamoeba Keratitis sufferers [31].

Acanthamoeba Keratitis is considered a rare disease and the United States CDC suggests that 1-33 cases for every 1 million contact lens users occurs. However, like with animals, humans are prone to several protozoal infections that can cause eye disease.

Giardia, *Leishmania* and *Toxoplasma* spp., among others have been linked to the occurrence to eye disease in humans, like they have been in cats and dogs [33].

2.3.8 *Acanthamoeba* as an animal pathogen: Opportunistic/Non-opportunistic:

As *Acanthamoeba* is an environmentally abundant free-living organism, like in humans, it is reasonable to assume that most animals will come into contact with the protozoa at some point throughout their life. Like in the case of humans, as an opportunistic protozoon, *Acanthamoeba* needs certain circumstances present in animals for disease to manifest. Conditions such as being a stray animal, an animal that is immunocompromised (such as feline immunodeficiency virus and feline leukaemia virus positive cats) or animals which are more likely to fight causing corneal injuries have been suggested [7].

When considering stray animals several studies have been conducted with the aim to detect *Acanthamoeba* species among populations. The results have enabled us to conclude that the incidence of *Acanthamoeba* is higher in stray populations then in domestic populations. An example of one such study is from Turkey and found that among these 184 dogs, 27 of them were found positive for *Acanthamoeba* spp. In the T4 and T5 genotypes and seven of them were noted to present clinical signs (conjunctivitis and keratitis). Interestingly, over 25% of dogs confirmed to be infected with *Acanthamoeba* were showing clinical signs [34] . . Within wild animal populations *Acanthamoeba* has been retrieved from samples taken from various parts of the body. For example, samples taken from the mouth, oesophagus and rectum of squirrels were found to contain *A. polyphaga* and *A. astronyxis* [35]. In relation to *Acanthamoeba* potentially causing eye disease in wild animals, pathogenic strains of *Acanthamoeba* have been recovered from the ocular surface of wild birds also [36]. Even though it is reasonable to say that *Acanthamoeba* are more likely to infect immunocompromised animals, cases of eye disease in healthy cats have also been recorded [37]. In a case, from the United States, examination of the right eye revealed a central superficial corneal ulcer associated with corneal epithelial and subepithelial infiltrates and mild aqueous flare. Structures consistent with amoeboid cysts and trophozoites were detected in the cornea by in vivo confocal microscopy. Suppurative keratitis was identified cytologically. An *Acanthamoeba* of the T4 genotype clade was isolated through culture and identified by a PCR assay of corneal specimens. The author of that study suggests that the natural occurrence of disease caused by *Acanthamoeba* is a significant finding and that *Acanthamoeba* infection should be considered for cats with

superficial corneal disease refractory to appropriate treatments and especially occurring after ocular trauma, including keratotomy [37].

From a human perspective the most common way in which *Acanthamoeba* can cause disease is in the form of keratitis, when considering dogs, investigations have noted a variety of signs, including as anorexia, pyrexia, secretions from the eyes and nose, limb stiffness, and neurological manifestations, suggesting *Acanthamoeba* infections are not just restricted to the eye but can in fact cause disease in other bodily systems in dogs [7]. The symptoms of multisystemic infection of *Acanthamoeba* in the dog have been described as variable, typical cases involve the loss of appetite, fever, discharges from the nose and eyes, and neurological signs such as neck and limb stiffness [38]. Cerebrospinal fluid taken from a dog in Spain, with meningoencephalitis, fever and an apparent difficulty of movement was shown to contain *Acanthamoeba* in a particular case [38]. A Boxer in the United States was presented to a veterinarian with lethargy, fever, and mucopurulent ocular and preputial discharge. On neurologic examination, the gait was characterized by a short stride. After investigation the same dog was found to have *Acanthamoeba* spp. in its lungs, kidneys, and meninges of the brain and spinal cord [39].

The number ocular disease cases due to *Acanthamoeba* in cats and dogs are negligible as naturally occurring infections are not often reported. Studies have shown that the way in which *Acanthamoeba* interacts with corneal epithelium differs between species, for example, humans, pigs and hamsters are such species that share similarities [40]. The similarities demonstrated between these species has allowed for the use of hamsters and pigs in research into the pathogenicity, immunology and possible treatment into human Acanthamoeba Keratitis. *Acanthamoeba* interacts differently with a variety of other species, such as chickens, rabbits, rats, guinea pigs, dogs, and horses, than it does with the corneal epithelium of humans, hamsters, and pigs for example. This differential selectivity to binding corneal tissue may be attributed to species-specific differences in the mannose residues on the surface of the cornea however, the precise mechanisms remain unknown [41]. It has been demonstrated that cats are prone to developing ocular lesions similar to those that AK patients experience, but only after receiving an inoculation of the organism. In that study topically inoculated the eyes of cats with a solution containing *Acanthamoeba* following corneal abrasion, placement of a contaminated contact lens for 7 days, and placement of a contaminated contact lens for 7 days following corneal abrasion [42].

2.4 Other protozoal eye pathogens:

2.4.1 *Leishmania:*

Leishmania is a genus of protozoan parasite in which species are transmitted via the bite of sandflies (*Phlebotomus* spp.). The occurrence of leishmaniosis is usually associated with tropical and subtropical climates but it is encroaching more and more north as global temperatures continue to rise.

Dogs are the primary reservoir of *Leishmania infantum* which results in a multisystemic infection often resulting in death. After been inoculated with *Leishmania* promastigotes are phagocytosed by macrophages and develop further into amastigotes within the call. *Leishmania* is able to avoid the dogs innate immune response through the remodelling of the parasitophorous vacuole, hindering the macrophages signalling pathways to their advantage [43]. The protozoon is transported throughout the body via macrophages enabling a systemic infection to occur. The main clinical signs of *Leishmania infantum* skin peeling, cutaneous lesions (nodular, ulcerative, and pustular), and exfoliative dermatitis. Alopecia, pale mucosa, and erythematous reactions are also common. Kidney involvement in the form of Glomerulonephritis with immune complex deposition and can progress to kidney failure along with epistaxis, haematuria and haemorrhagic diarrhoea due to the coagulation disorders are all common findings of canine leishmaniosis [44]. In dogs the ocular manifestations of leishmaniosis are diverse, and most of the ocular tissues can be affected: blepharitis, periocular alopecia, conjunctivitis, keratoconjunctivitis, keratoconjunctivitis sicca (KCS), corneal ulcers, uveitis, orbital cellulitis and myositis of the extraocular muscles have all been recorded [45]. One study suggests that up to 67% of dogs infected with *Leishmania* will lead to the occurrence of clinical signs in the form of ocular lesions. Furthermore, the same study suggests that in *Leishmania* endemic areas, 33% of all ophthalmic patients are patients infected with Leishmania spp. [46].

Cats are also susceptible to *Leishmania* infections, though to a lesser extent. The leading clinical sign of leishmaniosis in cats is cutaneous presentations follow by ocular disease. Like dogs, all tissues of the eye are vulnerable to infection but uveitis is thought to be the most common eye lesion [47]. *Leishmania* species can be considered as opportunistic parasites when considering cats as they are not deemed to be the natural reservoir of the protozoa. Studies into the occurrence of *Leishmania* in cats have been linked to cats suffering from Feline Immunodeficiency Virus (FIV). Cats infected with FIV have shown to be 2,8 times at a greater risk to developing leishmaniosis [48].

Leishmania spp. have been implicated as causative agents of eye lesions, and going forward, it should be taken into consideration when dealing with patients who have eye diseases given that the existence of these protozoa is becoming more widespread in Europe as a result of changing climates [49].

2.4.2 *Toxoplasma:*

Toxoplasma gondii is an obligate intracellular pathogen which uses felines as its reservoir. It has the ability to infect humans along with all other warm-blooded animals via both vertical and horizontal transmission routes. It is thought that approximately a third of the totally human population is chronically infected with toxoplasma [50]. Toxoplasmic retinochoroiditis is a well describe disease of the human eye. Ocular toxoplasmosis is a progressive and recurring necrotizing retinitis, with vision-threatening complications such as retinal detachment, choroidal neovascularization, and glaucoma, which may occur at any time during the clinical course and is a major cause of visual impairment which accounts for 30-55% of posterior uveitis in developed nations [50].

Like in humans, *Toxoplasma gondii* has the capability of causing ocular diseases in cats and dogs. As cats are the reservoir hosts of the protozoa the manifestation of ocular lesions occurs more often than in dogs. Ocular toxoplasmosis is considered rare in the dog but reports have included episcleritis, scleritis, retinitis, anterior uveitis, ciliary epithelium hyperplasia, optic neuritis and polymyositis [51]. A study in 2009 which identified *Toxoplasma gondii* for the first time as the causative agent of corneal conjunctivitis in a dog, highlighting its rarity [51]. Cats, especially immunocompromised kittens who were infected via the transplacental route are more susceptible to developing toxoplasmosis, making this another opportunistic parasite. Generally, pneumonia is the main clinical sign of disease manifestation followed by the appearance of ocular lesions. Although toxoplasmosis in cats is usually generalised ocular toxoplasmosis has been observed in cats without poly-systemic [52]. Chorioretinitis is the most common ocular manifestation of ocular toxoplasmosis in cats but optic neuritis and anterior uveitis are additional consequences of an infection [53].

Although bacterial and viral infections are more likely to be the cause of eye diseases in cats, the role protozoa should always be considered. One study from 2023, that examined 105 cats with ocular lesions reported that 60 of the cats, or 57,14% of the sample group, tested positive for *Toxoplasma gondii* [54]. Veterinarians should take ocular toxoplasmosis

into account when cats appear with ocular diseases due to the high prevalence rates of toxoplasma infection in the global cat population [55].

2.4.3 *Trichomonas:*

Several species belonging to the *Trichomonas* genus of protozoa have been linked to the occurrence of disease in cats and dogs. *Trichomonas foetus* in cats, and dogs to a lesser extent, is well documented as an inciter of gastrointestinal disease with diarrhoea with or without blood and mucus as a leading symptom. Chronic infections that cause diarrhoea can eventually result in painful, swollen anal areas [56]. Rare incidences of *T. foetus* related illness developing in other body parts are occasionally reported; for instance, pyometra in a cat has been linked to the protozoa's presence in the uterus [57]. *Pentatrichomonas hominis* is described as a commensal parasite which inhabits the large intestine of several mammal species but has opportunistic tendencies as it can play a role in the occurrence of disease either in immunosuppressed animals especially kittens and puppies and as part of a broader coinfection [58].Certain species of trichomonads such as *Trichomonas felistomae* and *Trichomonas canistomae* are well known to be present in the oral cavities of cats and especially dogs. Their presences has been linked to the occurrence of periodontal disease, as has other protozoa *Entomoeba* species [59]. Humans are also known to host *Trichomonas* species, specifically *Trichomonas tenax,* in their oral cavity. However, a study from 2016 found the species to be responsible for a case of canine mandibular sialadenitis, suggesting it may be a shared pathogen between humans and small animals [60]. *Trichomonas* is not known as an important pathogen of the eye, however a case of conjunctivitis caused by an extragenital infection of *T. vaginalis* has been recorded [61]. There is suggestions in the literature there is a widening spectrum of the pathogenicity in humans which led us to question whether it was possible to locate the protozoa in cats' and dogs' eyes [62].

3. Aim of the study:

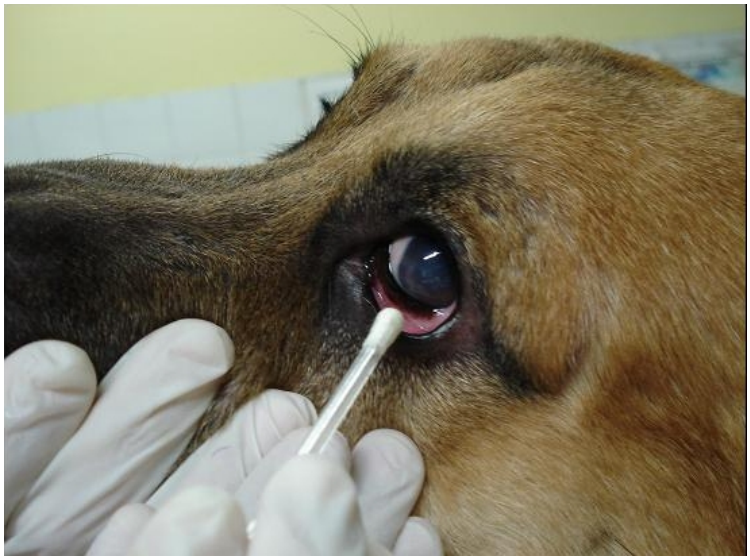
The lack of research conducted on *Acanthamoeba* as a source of animal infection prompted us to investigate if the pathogen was yet another protozoon causing disease, shared between humans and animals. There has been evidence that AK cases are rising in the Netherlands [63]. From a European perspective, we aimed to ascertain whether there was a correlation between the presence of protozoa, notably *Acanthamoeba*, and ocular illnesses in canine and feline patients in light of this development as well as the fact that *Acanthamoeba* is such an environmentally abundant protozoon.

The Study

4. Materials and methods:

4.1 Sampling method and sample pool:

In conjunction with two veterinary ophthalmologists in Hungary, a total of 115 samples from dogs and 45 cat's samples were obtained and used in our investigation. Samples were taken between May and September of 2023 and came from locations distributed throughout Budapest and the northern part of Hungary. All 115 of the canine samples came from domestic, privately owned dogs that presented to veterinarians with clinical ocular signs. The majority (67%) of the feline samples were acquired from feral cats residing in Aggtelek National Park, with only 15 samples coming from privately owned cats. To acquire our conjunctival samples, we gently rubbed sterile cotton swabs along the conjunctiva of our subjects (Picture 4). The cotton swabs were then places and sealed in Sarstedt tubes and stored frozen at -20℃ until processing.

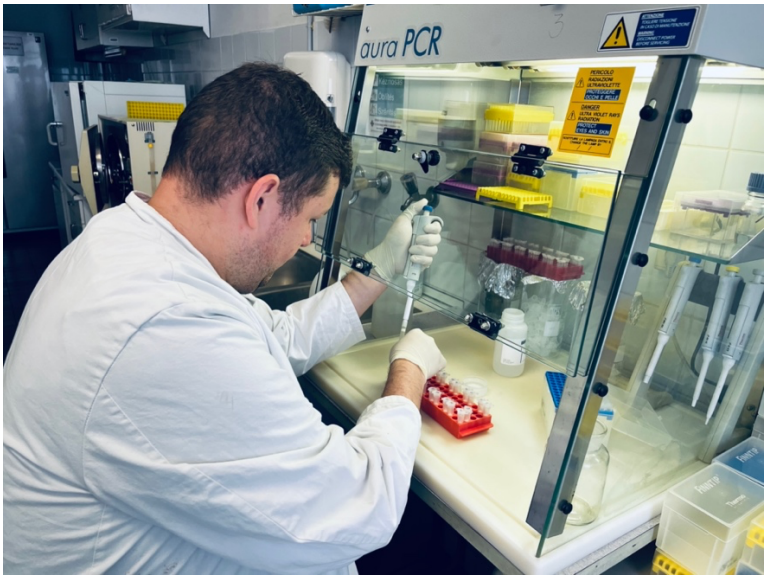


Picture 4: Conjunctival swab sampling [64].

4.2 DNA extraction and PCR methods:

DNA was extracted with the QIAamp DNA Mini Kit (Qiagen, Hilden Germany) according to the manufacturer's blood protocol, with slight modification. In particular, as a first step, the Sarstedt tubes containing the conjunctival cotton swabs were overlaid with 400 µl AL buffer, incubated for 10 min at 56 °C prior to removal of cotton swab from the fluid, followed by adding proteinase-K and continuing the procedure according to the tissue protocol. As

per the protocol 200 µl of ethanol (96-100%) was added then vortexed to assure a homogonous solution for 1 minute. To ensure no solution remained on the lid we placed the tube in a centrifuge briefly. After centrifugation, using a pipet we transferred the solution to a QIAmp Mini spin column, which is essentially a 2ml collection tube. Once ready, the solution was again centrifuged at 6000 x g (8000 rotations per minute) for 1 minute. The flow-through and collection tube were discarded before placing the QIAmp Mini spin column into a new 2ml collection tube and proceed by adding 500 µl of the AW1 buffer (Picture 5). The continuous changing of the collection tube helps to assure no contamination and the purity of the DNA. Centrifugation occurs once again centrifuged at 6000 x g (8000 rotations per minute) again for one minute. This previously described process was done once again but instead with the AW2 buffer. The centrifugation was intensified for the AW2 buffer step, at 20,000 x g (14,000 rotations per minute) and lasts for a longer time, 3 minutes. Finally, the QIAmp Mini spin column were placed into a new 1,5 ml microcentrifuge tubes and 130 µl of elution buffer (AE) was added before once again been centrifuged at 6000 x g (8000 rotations per minute) for 1 minute to elute the DNA.



Picture 5: Pipetting during the process of DNA extraction.

In each group of 23 samples an extraction control (180 µl tissue lysis buffer) was included to monitor cross-contamination and all were labeled (GD1-GD270). All DNA extracts and extraction controls were analyzed with three conventional PCRs. In these PCRs 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× Coral Load PCR buffer (15 mM $MgCl_2$ included), 1 µl extra $MgCl_2$ (25 mM) and 14.8 µl distilled water. Primers and cycling conditions of PCRs are summarized in the 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 ultra-violet light.

Target group	Target gene	Primer name	Primer sequence (5'-3')	Amplicon length (bp)	Thermo cycling profile	Reference
***Acanthamoeba* spp.**	18S rRNA	JDP1 JDP2	GGC CCA GAT CGT TTA CCG TGA A TCT CAC AAG CTG CTA GGG GAG TCA	~480	95 °C for 5 min; 35× (95 °C for 35 s; 56 °C for 45 s; 72 °C for 1 min); 72 °C for 7 min	[65]
***Leishmania* spp.**	kDNA	RV1 RV2	CTT TTC TGG TCC CGC GGG TAG G CCA CCT GGC CTA TTT TAC ACC A	~145	95 °C for 5 min; 40× (95 °C for 30 s; 59 °C for 30 s; 72 °C for 30 s); 72 °C for 10 min	[66]
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	[67]

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

4.3 Sequencing:

The purification and sequencing of the PCR products were performed at Biomi Ltd. (Gödöllő, Hungary). Obtained sequences were compared to GenBank data using the nucleotide BLASTn program (https://blast.ncbi.nlm.nih.gov).

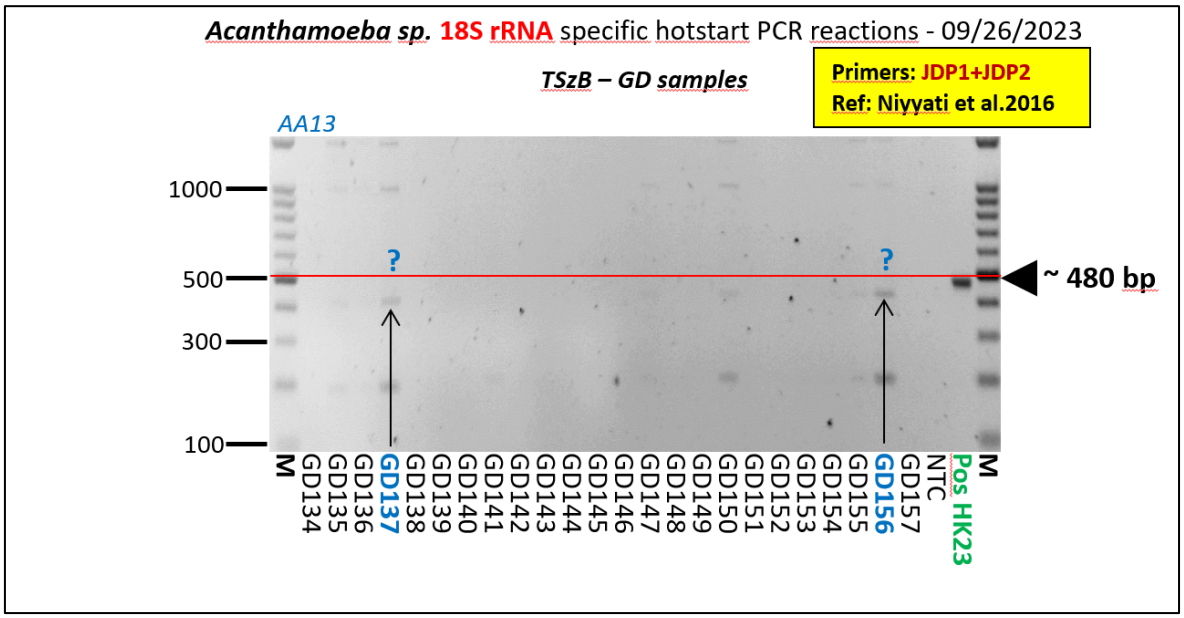
5. Results:

5.1. PCR examination:

All DNA samples extracted from the conjunctival swabs returned negative results from PCR's targeting *Acanthamoeba* spp., *Leishmania* spp. and Trichomonadidae. The results of three samples did however return unexpected appearances of molecular weights.

5.1.1. *Acanthamoeba* PCR results:

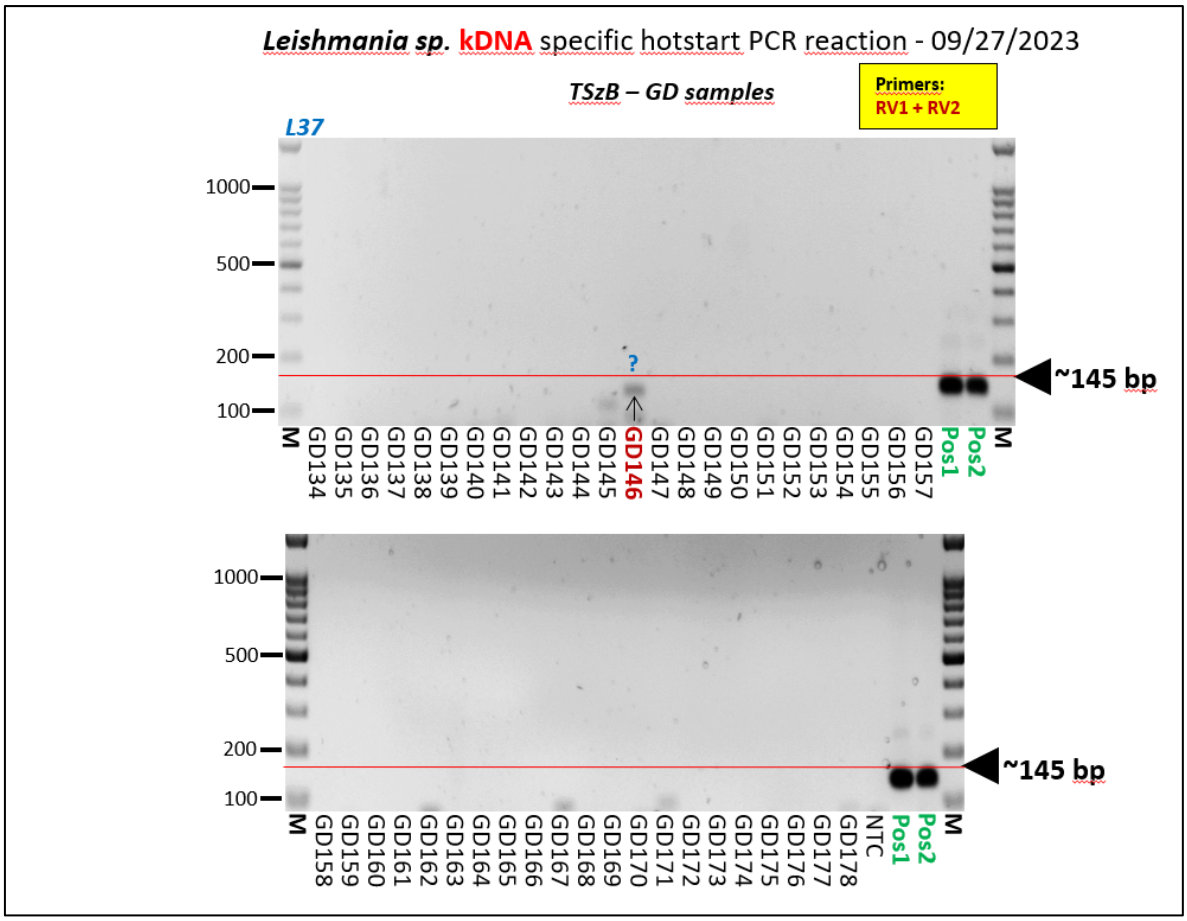
When executing PCR's tests screening for *Acanthamoeba*, on two occasions molecular weights appeared in the agarose gel electrophoresis. Both GD137 (samples taken from the left eye of a canine with bilateral conjunctivitis) and GD156 (samples taken from the right eye of a 10-year-old English Bulldog with bilateral conjunctivitis) had some molecular weights appearing (Picture 6). The subsequent sequencing of both samples was unsuccessful.



Picture 6: Image with results of *Acanthamoeba* PCR (GD134-157).

5.1.2 *Leishmania* PCR results:

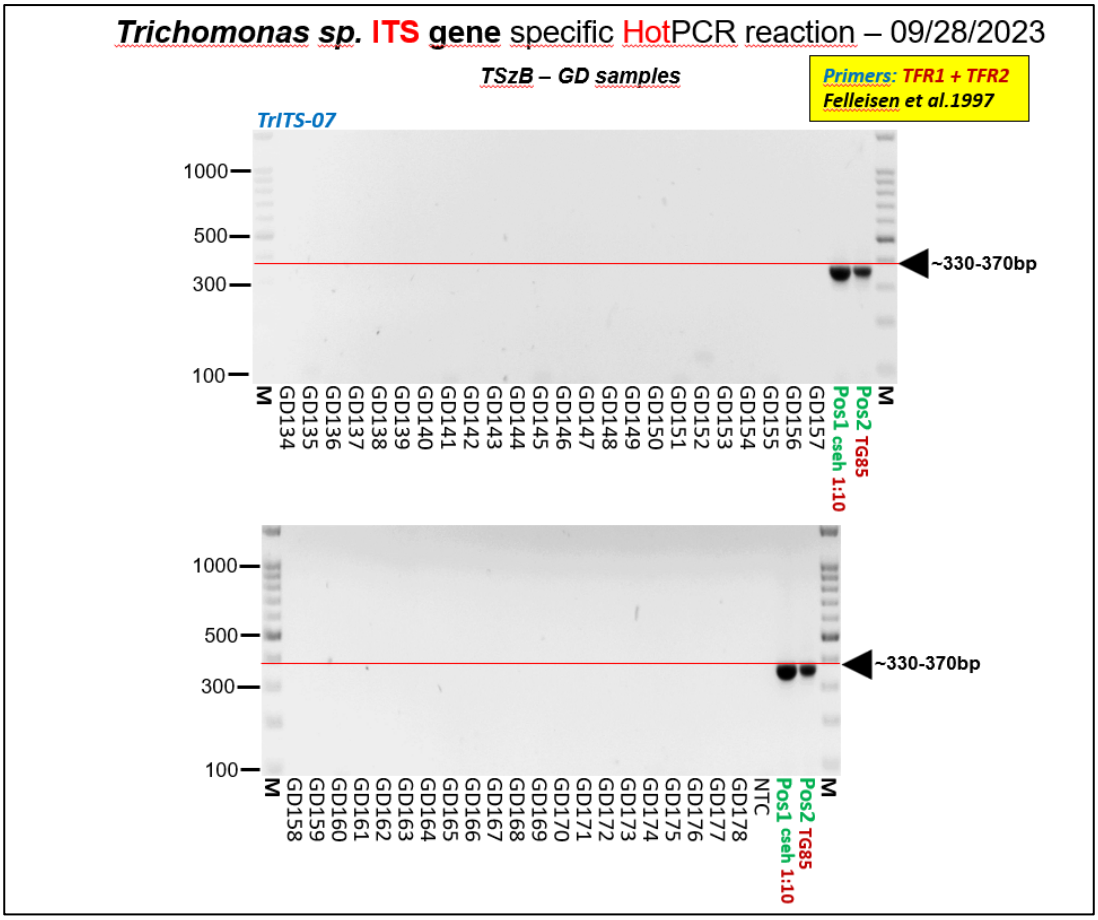
Regarding PCR's targeting the *Leishmania* genus of protozoa one samples also returned molecular weight on the gel plate. Sample GD146 (originating from the right eye of a 7-year-old West Highland White Terrier presenting with bilateral conjunctivitis) caused molecular weight to appear (Picture 7) but again subsequent sequencing was unsuccessful.



Picture 7: Image with results of *Leishmania* PCR (GD134-GD178).

5.1.3 *Trichomonas* PCR results:

All PCR's targeting *Trichomonas* failed to return any results in any capacity. Some of the results are seen in Picture 8.



Picture 8: Image with results of *Trichomonas* PCR (GD134-178).

5.2. Animals examined:

Out of the 160 animals tested, the feral cats were not known to have any ocular lesions at the time of testing and were included to ascertain if any protozoal organisms could be detected in the cat population in the Aggtelek National Park. Of the remaining 130 animals, all but 14 presented with solely conjunctivitis which represents 89.2% of the animals showing clinical signs included in this study. The second most prevalent (7.7%) clinical symptom was keratoconjunctivitis (KCS); 7 animals had bilateral KCS, while 3 had unilateral KCS, with conjunctivitis present in the other eye. The final 4 animals included dogs suffering from either keratitis alone, blepharoconjunctivitis, ectropion or an ocular neoplasia. Only conjunctivitis was present in the 15 cats seen by the ophthalmologist. The locations of sampling and further details are seen in Table 2-4.

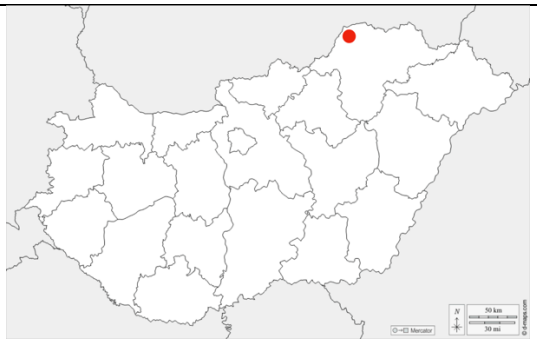
Sample Source	Location in Hungary
30 samples were collected from 30 individual feral cats. No symptoms were present and the age, sex and breed of the cats were incompletely recorded.	

Table 2: Sample source 1.

Sample Source	Location in Hungary
Veterinary ophthalmologist A collect samples only from eyes showing clinical signs. 35 samples were collected from 35 individual dogs and 5 samples taken from 5 individual cats. The animals signalments were recorded and made available.	

Table 3: Sample source 2.

Sample Source	Location in Hungary
Veterinary ophthalmologist B collected samples from several locations limited to Budapest and its immediate environs. In almost all cases the ophthalmologist took samples from both of the patients' eyes. Samples were taken from 13 cats (on 4 occasions only one eye was tested) and 91 dogs (on 7 occasions only one eye was tested). The animals signalments were also recorded and made available to us.	

Table 4: Sample source 3.

The animals' recorded ages ranged from two months to fifteen years. We decided not to include the animals' sex in our study because it was not consistently obtained. In this study,

a wide variety of dog breeds with different sizes, coat types, and eye characteristics were used. Small breed dogs were more likely to be the source of samples as they were more frequently presented to our collaborators. 7 out of the top 10 most common breeds in our study are small breed dogs and include French Bulldogs, Shih Tzu's, West Highland White Terriers, Bichon Bologneses and Dachshunds. All of the cats were listed as house cats or mixes except for 2 Devon Rex's and a Mainecoon.

6. Discussion:

Protozoa are well documented as the causative agents of numerous disease effecting both humans and animals. Studies are suggesting that certain protozoa are becoming more pathogenic. Along with *Acanthamoeba*, meningoencephalitis in humans has also been linked to free-living amoebae including *Balamuthia* and *Sappinia*. *Plasmodium knowlesi*, a zoonotic malarial parasite, has become a major cause of human malaria in Southeast Asia. *Trypanosoma evansi* and *Trypanosoma lewisi*, which normally infect horses and rodents respectively, have been reported to cause human trypanosomiasis in India. *Balantidium coli* is emerging as an important cause of dysentery especially in the immunocompromised population [68]. The geographical distribution of reported cases related to certain protozoal diseases is also altering due to climate change [69].

Since *Acanthamoeba* is a well-known cause of eye disease in humans and is increasingly being reported in Europe, we were encouraged to see if we could isolate it from small animal patients with ophthalmic diseases in Hungary. *Acanthamoeba* is described as an opportunistic parasite which mainly causes disease in immunosuppressed individuals. However, considering the dog from Spain and the Boxer dog from United States. Both cases clearly demonstrate that dogs are susceptible to systemic infections caused by *Acanthamoeba* spp. including *A. castellanii* similar to those seen in cases of GEA in humans. However, neither of the aforementioned examples involved immunosuppressed dogs; both featured young animals, indicating that the *Acanthamoeba* can take advantage of hosts with underdeveloped immune systems also [38, 39]. Samples used in our study included samples taken from both juvenile puppies and geriatric dogs but still no *Acanthamoeba* was detected. Although *Acanthamoeba* is abundant in the environment, and there is an increase in reported cases, AK still remains rare in humans [63]. When discussing AK, *Acanthamoeba* is considered as a non-opportunistic protozoa as the host does not need to be immunosuppressed, instead the leading factor in the appearance of keratitis in humans in the

presence of *Acanthamoeba* species is the use of contact lenses and the unhygienic keeping of them [70].

It is apparent that *Acanthamoeba* has the capacity to be the source of disease in humans in the form of GAE and AK and although the manifestation of diseases in animals as a result of the presence of that protozoon is not as well documented, cases of disease in animals have been described. *Acanthamoeba quina* has been recorded as the probable cause of meningoencephalitis in a Rhesus Macaque and *A. hatchettti* has been recorded causing severe placentitis in a horse [71, 72]. Studies have found that animals have may shed strains of *Acanthamoeba* that have been linked to AK cases in humans, in their faeces, which suggests that disease in humans could potentially be caused by zoonosis. For example, *A. lugdunensis* has been isolated from reptile feaces and the eyes of an AK sufferer [73, 74]. It has been proven that AK like disease can occur in cats, but this has only been proven to be the case when cats were exposed to *A. castellanii* contaminated contact lenses [42]. Naturally occurring ocular lesions due to an *Acanthamoeba* infection in animals is rare and very limited literature sources have it recorded, the case of sclerokeratitis in a cat is just one of very limited examples [37]. Perhaps as the mannose-binding abilities of *Acanthamoeba* differs among species and the unlikely nature of cats and dogs to use contact lenses, the risk of small animals presenting to veterinarians with ocular lesions due to the presence of *Acanthamoeba* is negligible, the result of this study supports this. However, to the best of our knowledge, our study did not utilise any patients who were immunosuppressed and perhaps further studies need to be conducted on immunosuppressed cats and dogs with ocular lesions for further evidence.

In regard to *Leishmania*, the manifestation of ocular lesion due to an infection with *Leishmania* is well known. However, the occurrence of ocular leishmaniosis depends on the presence of the protozoon's vector, the sandfly (*Phlebotomus* spp.). In a recent study, Budapest has been recorded as the northern most point at which two species *Phlebotomus mascittii* and *Phlebotomus neglectus* have been recorded, the discovery of these species in the Budapest area has again been attributed to the continuous climate change altering Hungary's average temperature to more favourable condition of the sandflies [75]. Despite the presence of sandflies in Hungary, the majority of report leishmaniosis cases are imported cases, most often from regions around the Mediterranean basin. 2012 saw for the first time a reported case of an autochthonous case of canine leishmaniosis in Hungary and that dog had uveitis as one of many clinical signs [76]. As leishmaniosis can potentially occur in Hungary and is a known eye pathogen we sought to find additional cases of ocular

leishmaniosis in our study. In addition, it was verified in other studies that *Leishmania* DNA can be amplified from conjunctival swab samples of dogs having ocular lesions due to these protozoa [77]. The negative results suggest that, although the factors are present for the disease to occur, it still remains an uncommon disease in Hungary. Perhaps future surveys in the search for *Leishmania* in the eyes of dogs and cats in Hungary would use a wider field of study by utilising samples obtained from various regions in Hungary, specifically in the south of the country.

The case of conjunctivitis developing in an adult man due to extragenital infection of *Trichomonas* prompted us to include the genus of protozoa in a search for potential ocular protozoan pathogens [61]. To the best of our knowledge there has never been any reported cases of ocular lesions occurring in cats and dogs due to an infection with *Trichomonas*, therefore we were unsurprised to discover we failed to detect any *Trichomonas* in our samples.

7. Conclusion:

Several authors have recently questioned whether *Acanthamoeba* may inflict sickness on animals in a manner comparable to how it can on people. We asked ourselves the same question and wondered should veterinarians be worried about protozoa as eye pathogens in general. Although protozoa have been recorded as ocular pathogens, the incidence of them remains lower than diseases of the eyes caused by bacteria and viruses. In the case of Hungary, we found that protozoa are still not to be considered as a major concern to the eyes of our canine and feline companions. This however could change in the future as protozoa become more pathogenic and climate change drives the emergence of diseases in new and unusual ways.

8. Abstract: *Molecular investigation of protozoan parasites that are potential causes of eye lesions in dogs and cats.*

Bacteria and viruses can be the cause of many ophthalmic lesions of infectious origin, however, protozoan parasites have the potential to also be the source of such lesions. Among the others, opportunistic protozoa, as exemplified by *Acanthamoeba* spp. and *Toxoplasma gondii*, are responsible for the manifestation of several diseases affecting the eyes in both humans and animals. Members of the genus *Acanthamoeba* have been identified as the cause of serious diseases including both Granulomatous Amebic Encephalitis (GAE) and Acanthamoeba Keratitis (AK). Ophthalmic lesions, resulting from acanthamoebosis have been widely investigated from a medical point of view, while such studies are scarce in veterinary scientific literature. The aim of our research was to attempt to detect protozoa, *Acanthamoeba* in particular, from small animal patients who presented to veterinarians with ophthalmic lesions and investigate the correlation between the two. The data used was collected in collaboration with veterinary ophthalmologists in Hungary who collected samples from 115 dogs and from 45 cats on our behalf. Samples were obtained using conjunctival swabs from animals experiencing conjunctivitis and keratitis/blepharitis to a lesser extent. The sample population included a variety of different breeds, a wide age spectrum and came from locations distributed throughout Hungary. Using conventional PCR as the detection method, samples were screened and examined for different protozoa. Although *Acanthamoeba* is a known causative agent of eye disease in humans, the results obtained from this study failed to identify *Acanthamoeba* and other protozoa such as *Leishmania* and *Trichomonas* species as major contributors to the incidence of ophthalmic lesions in small animal medicine.

8. Összefoglaló: *Kutyák és macskák szembetegségeit okozó egysejtű paraziták molekuláris vizsgálata*

A fertőző eredetű szembetegségek hátterében többnyire baktériumok és vírusok állnak, azonban egysejtű paraziták is okozhatnak hasonló elváltozásokat. Többek között opportunista protozoonok, mint például az *Acanthamoeba* fajok és a *Toxoplasma gondii* felelősek számos olyan betegség megnyilvánulásáért, amelyek mind az emberek, mind az állatok szemét érintik. Az *Acanthamoeba* nemzetség tagjait súlyos betegségek kórokozójaként tartják számon, beleértve a granulomatózus amőbás agyvelőgyulladást (GAE) és az *Acanthamoeba* szaruhártyagyulladást (AK). Az *Acanthamoeba* fertőzésből eredő szemgyulladásokat humán egészségügyben ezidáig széles körben vizsgálták, míg az állatorvostudományi szakirodalomban kevés adat található róluk. Kutatásunk során célul tűztük ki, hogy egysejtű parazitákat találjunk, különösképp *Acanthamoeba* fajokat, olyan kutya és macska betegekből, akik szemészeti problémával fordultak állatorvoshoz, majd megvizsgáljuk a kettő közötti összefüggést. A felhasznált adatok összegyűjtése állatorvos szemész szakorvosokkal együttműködésben történt, akik 115 kutyától és 45 macskától gyűjtöttek mintát a számunkra. A vizsgálat során kötőhártya tampon mintavétel történt olyan állatokból, amelyeknél kötőhártya-, szaruhártya-, vagy szemhéj gyulladást tapasztaltak. A mintapopuláció változatos életkorú és fajtájú állatot tartalmazott, melyek Magyarország különböző helyeiről származtak. Kimutatási módszerként hagyományos PCR módszert végeztünk, mely során a mintákat különböző egysejtű parazitákra vizsgáltuk meg. Bár egyes *Acanthamoeba* fajok az emberek szembetegségeinek ismert kórokozói, vizsgálataink során nem tudtuk igazolni az *Acanthamoeba* és más protozoonok jelenlétét, úgymint *Leishmania* és *Trichomonas* fajokat, amelyek fő szerepet játszhatnak különböző szembetegségek előfordulásában a kisállatgyógyászatban.

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Thesis statement for TDK thesis

I, the undersigned dr. Barbara Tuska-Szalay as the supervisor, declare that I have read and approved the thesis "*Molecular investigation of protozoan parasites that are potential causes of eye lesions in dogs and cats*" of the student **Glenn Daly** (Vth year student) and support his participation in the Scientific Student Conference of the University of Veterinary Medicine in 2023. Furthermore, I declare that the uploaded TDK thesis has been successfully checked for plagiarism and that any matches found comply with the University guidelines/rules.

Budapest, 2023. October 17th

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Supervisor

Thesis progress report for veterinary students

Name of student: GLENN DALY

Neptun code of the student: FGNSAI

Name and title of the supervisor: DR. TUSKA-SZALAY BARBARA

Department: PARASITOLOGY AND ZOOLOGY

Thesis title: ~~MOLECULAR INVESTIGATION OF ACANTHAMOEBA KERATITIS AMONG PET ANIMALS~~

NEW TITLE: MOLECULAR INVESTIGATION OF PROTOZOAN PARASITES THAT ARE POTENTIAL CAUSES OF EYE LESIONS IN DOGS AND CATS (09.20 NOV.)

Consultation – 1st semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2023	01.	19.	SAMPLING	
2.	2023	03.	14.	SAMPLING	
3.	2023.	03.	21.	SAMPLING	
4.	2023.	05.	03.	THESIS WRITING	
5.	2023.	05.	05.	THESIS WRITING	

Grade achieved at the end of the first semester: 5

Consultation – 2nd semester

	Timing			Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2023	09.	11.	THESIS WRITING	
2.	2023.	10.	05.	THESIS WRITING	
3.	2023.	10.	09.	THESIS WRITING	
4.	2023.	10.	13.	SUBMISSION, FINALIZING	

5.	2023.	10.	24.	PRESENTATION	dr L

Grade achieved at the end of the second semester: 5

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and found suitable to defence,

dr L –
.....
signature of the supervisor

Signature of the student: .....

Signature of the secretary of the department: .....

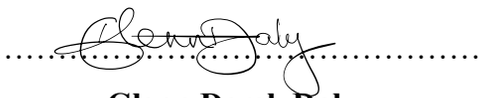
Date of handing the thesis in..... .....

Appendix 5. Declaration regarding TDK research paper-thesis equivalence

DECLARATION

I hereby declare that the thesis entitled "**Molecular investigation of protozoan parasites that are potential causes of eye lesions in dogs and cats**" is identical in terms of content and formal requirements to the TDK research paper submitted in 2023.

Date: Budapest, 2023, November 10



Glenn Derek Daly