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DOTTORATO IN SCIENZE VETERINARIE XXXVI CICLO

Tesi di Dottorato

"Hookworm infections and other geohelminthic infections in dogs: epidemiological and molecular studies"

Candidato

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List of abbreviations

AC *Ancylostoma caninum*

AL Larvae of *A. caninum*

CAPC Companion Animal Parasite Council

CI Confidence interval

CPG Cysts per gram of feces

E.G. exempli gratia

EPG Eggs per gram of feces

ESCCAP European Scientific Counsel Companion Animal Parasites

HK Hookworms

I.D. id est

LPG Larvae per gram of feces

LM *Larva migrans*

NEG Negative

OPG Oocysts per gram of feces

P.I. Post infection

PCR Polimerase chain reaction

POS Positive

UE Eggs of *U. stenocephala*

UL Larvae of *U. stenocephala*

US *Uncinaria stenocephala*

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Abstract

Hookworms (*Ancylostoma caninum* and *Uncinaria stenocephala*) and ascarids (*Toxocara canis* and *Toxoascaris leonina*) are among the most common geohelminths of dogs in Europe. Their importance arises from their possibility to affect both dogs and humans' health. They are common causes of diarrhea, anemia and growth retardation especially in puppies. Moreover, they cause several important syndromes such as *coutaneous larva migrans* (*A. caninum*) and *visceral larva migrans* (*T. canis*) in human hosts. The perpetuation of their biological cycle is supported by their great fertility, the high environmental contamination that allows a high rate of infection for other animals and humans, and their different routes of infection. Prevalence rates of hookworms and ascarids are extremely variable in different parts of the world according to climatic regions and dogs' population. Despite the use of broad-spectrum deworming treatments, hookworms and roundworms persist to affect canine health. In order to deepen the knowledge on hookworm and ascarid infections in dogs, the present thesis entitled "**Hookworm infections and other geohelminthic infections in dogs: epidemiological and molecular studies**" had three specific objectives: i) to update the epidemiological scenario of ascarid infections in dogs in southern Italy; ii) to advance epidemiological data about hookworms' infection in dogs in southern Italy and to assess through molecular investigations the prevalence of hookworm species (*A. caninum* and *U. stenocephala*) in the same area; iii) to evaluate the diagnostic accuracy of different molecular protocols for the detection of *A. caninum* and *U. stenocephala* in dogs using various matrices of samples. The PhD thesis consists of two parts, according to the European standard requirements. The first part - entitled "Literature Review" - summarizes information from the scientific literature about aetiology, biology, epidemiology, clinical signs, diagnostic concerns, control approaches and zoonotic risk of hookworm and ascarid infections in dogs. The second part entitled "Own research" presents the general and specific objectives of the thesis followed by three original studies that address the epidemiology and the diagnosis of hookworms and ascarid infections in dogs.

The literature review in **Chapter 1** provides an overview of both hookworm and ascarid infections in dogs. Data regarding aetiology, life cycle and other biological aspects, pathogenesis, clinical signs, diagnosis, control and zoonotic risk are discussed in detail. This chapter underlined the lack of new and detailed studies on the prevalence of hookworms and ascarids in dogs especially in southern Italy.

Chapter 2 reports the findings of a 5-year retrospective analysis on ascarid infections in dogs in southern Italy. A total of 8,149 dogs, referred to our labs for copromicroscopic analysis using the FLOTAC technique, was considered. A sub-sample of 500 fecal samples were analysed also with the Mini-FLOTAC technique. Of the overall dog samples analysed, 9.2 % (95 % CI = 8.6–9.8) resulted positive for *T. canis* while 0.5 % (95 % CI = 0.4–0.7) resulted positive for *T. leonina*. Co-infections with *T. canis* and *T. leonina* were found in 0.1 % of dogs (95 % CI = 0.0–0.1). The results obtained by the FLOTAC and Mini-FLOTAC examinations showed a nearly perfect k agreement (k = 0.99, P < 0.001) between these two techniques. Chi-square test showed positivity to *T. canis* and *T. leonina* significantly (P < 0.001) associated with dogs housed outdoor (i.e., that lived in garden or in kennel). Moreover, the positivity for *T. canis* was significantly associated (P <0.001) also with age (i.e., puppies), as shown by the logistic regression.

Chapter 3 updates the epidemiological scenario of hookworm infections in dogs in southern Italy. Moreover, in this study the first identification of hookworm species in dogs through molecular studies was conducted. A retrospective analysis was performed over 10 years (2011-2021), including a total of 7008 owned dogs and 5642 stray dogs referred to our laboratory for copromicroscopic examinations. Moreover, 72 fecal samples, from dogs naturally infected by hookworms, were used to discriminate between *A. caninum* and *U. stenocephala* using two PCR protocols. Prior to molecular analyses, a subsample of 40/72 positive fecal samples were used for morphometric investigations on hookworm eggs. The results of the ten-year retrospective analysis (2011-2021) showed an overall prevalence of hookworm infection of 9.16%, specifically 5.1% in owned dogs and 14.2% in stray dogs. Logistic regression showed a significant association between positivity to hookworms and the variable “puppies” both in stray (13.84%; OR=2.4) and owned (7.07%; OR=2.2) dogs. The results of molecular analyses showed that positivity was confirmed only in 21/72 samples, specifically, 6 samples using protocol A and 19 with protocol B. Sequencing revealed 15 samples positive to *U. stenocephala* and 6 to *A. caninum*.

Chapter 4 reports the outcomes of the comparison of different molecular protocols using various matrices of samples for the differentiation of *U. stenocephala* and *A. caninum* in hookworm infected dogs. To this end, the DNA extraction was performed on the following matrices of samples: (i) larvae of *U. stenocephala* obtained from experimentally infected dogs with *U. stenocephala* with different larvae counts per microliter (µl); (ii) pure *U. stenocephala* eggs suspension in distilled water with different egg counts per µl; (iii) spiked dog fecal samples with different *U. stenocephala* eggs per gram (EPG) of feces; (iv) feces from dogs naturally infected with hookworm eggs; (v) fecal

suspension with hookworm eggs recovered from the FLOTAC apparatus. All the samples were tested with four different PCR protocols targeting specific regions for the detection of *A. caninum* and *U. stenocephala* as follows: Protocol A (ITS1, 5.8S, ITS2) and Protocol B (18S) for the detection of both species, Protocol C (ITS1) for the detection of *A. caninum* and Protocol D (ITS1) for the detection of *U. stenocephala*. The best results were obtained with DNA extracted from *U. stenocephala* larvae matrix obtained from experimentally infected dogs, showing a detection limit of 3.5 larvae/ml for the protocols A, B and D. A moderate correlation was found between the FLOTAC technique and PCR protocols B and D with respect to fecal samples from dogs naturally infected with hookworms. Indeed, PCR protocols B (18S) and D (ITS1) gave the best results for feces and fecal suspension from naturally infected dogs. However, all the PCR protocols used showed lower sensitivity than FLOTAC technique.

Chapter 5 is an overall discussion aimed at promoting "good diagnostic practices" of intestinal helminth infections in dogs in order to foster "good clinical practice" aimed at safeguarding the health and well-being of dogs through the use of broad-spectrum anthelmintic products that are effective, safe and easy to administer according to the guidelines of the European Scientific Counsel Companion Animal Parasites (ESCCAP, 2021).

Introduction

The relationship between humans and dogs is something that has spanned the millennia of world history, and just as man has settled occupying the different continents of the planet in the same way the synanthropic fauna has distributed itself in every part of the world bringing with it its own pathogens (Morey et al., 1994). Over time, the relationship between humans and dog has evolved by increasingly taking on an affective denotation, and therefore relational habits have also changed: in fact, most dogs live inside homes sharing more common space with their owners. Just as the relationship between humans and dog has evolved over time from an affective point of view, the most different pathogens such as bacteria, viruses and parasites have evolved by broadening their spectrum of host specificity and acquiring specific characteristics that have granted to their respective diseases to absorb the concept of "zoonoses". According to the definition given by the World Health Organization (WHO), *zoonotic diseases* are diseases and infections naturally transmitted between people and vertebrate animals. It is estimated that, globally, about one billion cases of illness and millions of deaths occur every year from zoonoses. Some 60% of emerging infectious diseases that are reported globally are zoonoses. Over 30 new human pathogens have been detected in the last three decades, 75% of which have originated in animals (Jones et al., 2008).

Geohelminths, also known as soil-transmitted helminths (STHs), are a group of intestinal parasites that can affect both animals and humans' health. Roundworms, hookworms and whipworms are the most important relevant canine geohelminths which have importance both for their wide geographic distribution and for clinical aspects (Traversa et al., 2014, Genchi and Rinaldi, 2016). The biological cycle of these parasites strongly requires the environment in which the elimination of parasitic elements such as eggs through canine feces takes place and, after their emission, they accomplish under certain conditions such important stages of their development becoming infectious (e.g, L3 stage larvae or larvated eggs) (Traversa et al., 2012). Several studies (e.g. Rinaldi et al., 2006; Genchi et al., 2007; Zanzani et al., 2014_a, 2014_b; Traversa et al., 2014; Simonato et al., 2019; Bojar et al., 2018) have shown an important presence of these infectious parasitic elements especially in those places (e.g. parks, public gardens, beaches) where both owned and stray dogs, who can either become infected by direct contact with these parasitic elements or represent themselves a source of infection, persist at the same time with human beings for whom a zoonotic risk is widely demonstrated in the scientific literature worldwide, especially in rural and poorest communities of the world (Puhalenti et al., 2023). Roundworms and hookworms include different species distributed worldwide with a strong variability in prevalence rates. Commonly, *Toxocara canis* and *Ancylostoma caninum* are the most representative species of roundworms and hookworms, respectively. Moreover, other important species may be present in particular areas, e.g., *Toxascaris leonina* in Europe and USA, *Uncinaria*

stenocephala in colder areas of temperate and subarctic regions, *Ancylostoma braziliense* in the southern hemisphere and *Ancylostoma ceylanicum* in the Asia Pacific region (Traversa et al., 2012,2014; Traub et al., 2021). Additionally, the whipworm *Trichuris vulpis* is another common geohelminth that affects the large intestine of dogs (Traversa et al., 2011), but it will not be discussed in this PhD thesis and therefore reference is made to other texts or papers already published. There are few and not very recent studies on epidemiological scenario of these parasitic infection in Italy, especially in southern Italy.

Like mentioned above, roundworms and hookworms affect both dogs and humans' health. Patent intestinal infections with roundworms and hookworms strongly occurred in dogs of all ages, especially in puppies and of all categories especially in stray dogs that represent an important source of infection for all the possible hosts (Traversa et al., 2012). Moreover, wild animals such as foxes and grey wolves that strongly populate sub-urban and sometimes urban areas must be considered as possible source of infection and dissemination of these parasitic infections (Brochier et al. 2007; Karamon et al., 2018; Perrucci et al., 2023). Generally, common clinical signs such as diarrhea, vomiting, hypothermia, lethargy, anemia (only in hookworms' infection) are correlated to the age, the burden of the parasites and the general condition (e.g., body condition score, status of hydration) of the animal (Traversa et al., 2012).

Infections with roundworms (especially *T. canis*) and hookworms (especially *A. caninum*) in humans are highly reported (Deplazes et al., 2011; Traversa et al., 2012) . The infection by *T. canis* in humans occurs generally through the ingestion of embryonated eggs from the soil or from the direct contact hand-to-mouth with the embryonated eggs present on the fur of the dogs (Aydenizoz et al., 2008; Keegan et al., 2010; Maurelli et al., 2019), causing *visceral, neural, ocular larva migrans* and covert toxocarosis. Conversely, human infection by hookworms occurs more frequently by the penetration of the larvae (L3) present in contaminated soil through the skin, causing *cutaneous larva migrans* (Del Giudice et al., 2009).

Accurate and effective diagnosis is the first step for proper control of these parasitic infections. Generally, the diagnosis of these nematodes is made through the detection of eggs in dogs feces by flotation techniques (Cringoli et al., 2010, 2017). Moreover, the differentiation of all the several hookworm species through morphological examination of the eggs is not possible for the similarities between all of them. Hence, molecular tests such as PCR and sequencing are necessary to pursue this objective (Traub et al., 2021). Anyway, several studies reported variable outcomes on the sensitivity of different PCR protocols described for the detection of hookworm species (Oliveira-Arbex et al., 2017; Massetti et al., 2020; Strkolcova et al., 2022).

There are several drugs on the market with a broad spectrum of action for the treatment of these parasitic infections and from this arises the convince that intestinal worms of the dog do not need a high level of attention neither by the veterinary class nor by the scientific community, rather often forgetting their important implications for both animal and human health (Traversa et al., 2014)

However, several studies show that drugs alone are not sufficient for the control of these parasitic infections, but precise prophylactic actions must be pursued, aimed at reducing the environmental parasite load and thus the chances of infection for both animals and humans.

Due to the relevant impact of hookworm and ascarid infections on dog's and human's health worldwide, this PhD thesis aimed at providing new insights into epidemiology and diagnosis of these parasites in dogs in southern Italy.

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Chapter 1

Literature review on ascarid and hookworm infection in dogs: aetiology, biology, epidemiology, clinical signs, diagnosis and control.

1.1 Aetiology and Morphology

1.1.1 Hookworms' aetiology and morphology

Ancylostoma caninum

Domain: Eukaryota

Kingdom: Animalia

Subkingdom: Eumetazoa

Branch: Bilateria

Superphylum: Aschelmintae

Phylum: Nematoda

Class: Secernentea

Subclass: Rabditia

Order: Strongylida

Family: Ancylostomatidae

Genus: *Ancylostoma*

Species: *Ancylostoma caninum*

Uncinaria stenocephala

Domain: Eukaryota

Kingdom: Animalia

Subkingdom: Eumetazoa

Branch: Bilateria

Superphylum: Aschelmintae

Phylum: Nematoda

Class: Secernentea

Subclass: Rabditia

Order: Strongylida

Family: Ancylostomatidae

Genus: *Uncinaria*

Species: *Uncinaria stenocephala*

Ancylostoma caninum is a bursate nematode that has a characteristic hook shape of the anterior part, hence the name "hookworm," in which there is a large buccal capsule with three pairs of teeth placed on the margin and a pair of teeth placed ventrolaterally (Fig. 1.1). It is equipped with a funnel-shaped buccal capsule on the ventral outer margin of which two plates are inserted, each of which is provided with 3 curved, pointed denticles. Other denticles are found on the bottom of the buccal capsule. Adult specimens vary in size, depending on sex, between 1 and 2 cm: the male, on average 1 cm long, has a trilobed caudal bursa, equipped with spicula with a very reduced dorsal lobe. The female, 1.5 to 2 cm long, has vulval orifice that opens toward the lower third of the body (Chabaud et al., 1974; Companion Animal Parasite Council-CAPC, 2023).

Eggs (Fig. 1.2) are ellipsoidal in shape with dissimilar poles, "barrel" side walls and measure 56-67 x 34-47 μm. They contain 2 to 8 blastomeres whose number rapidly increases during passage through the gut and environment (Epe et al., 2009).

Uncinaria stenocephala can reach 1 cm in length, and a morphological difference between the two sexes has been described as well: females measure 7-12 mm, males 5-8.5 mm. The male also has a well-developed copulatory bursa in the caudal end with a short dorsal lobe and two large lateral lobes and thin spicula. The genus *Uncinaria* has typical morphological features of the family Ancylostomatidae but also some peculiar connotations. The parasite has the anterior end, characterized by a typical hook shape, a funnel-shaped buccal capsule with a pair of sharp chitinous plates on the edge and a pair of denticles at the base (Fig. 1.1).

Eggs measure 65-80 x 40-50 μm, are oval, lateral margins are parallel, shell thin and smooth (Fig. 1.3) (CAPC, 2023).

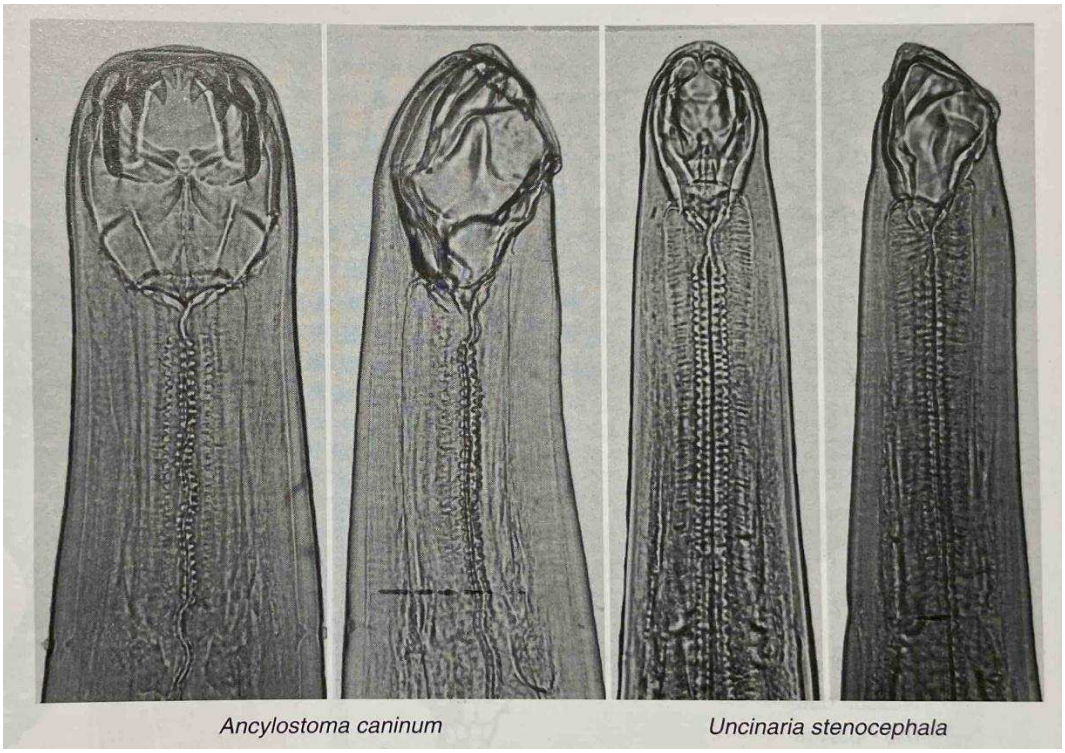

Figure 1.1: Dorsoventral and lateral shapes of the buccal regions of *A. caninum* and *U. stenocephala* (Bowman et al., 2019).

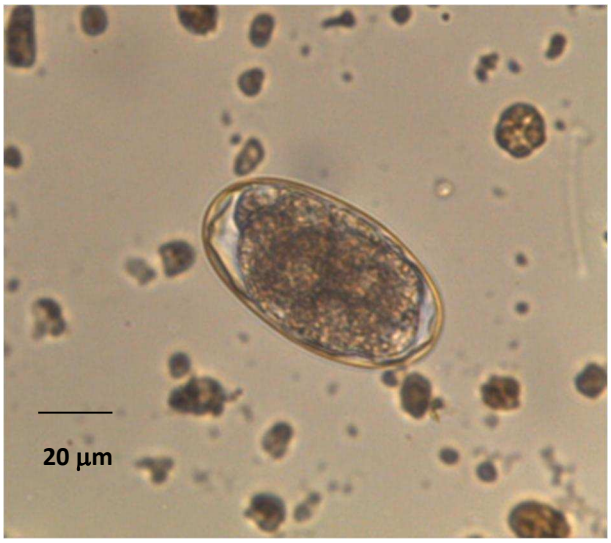

Figure 1.2: Egg of *A. caninum* (40X) (original photo).

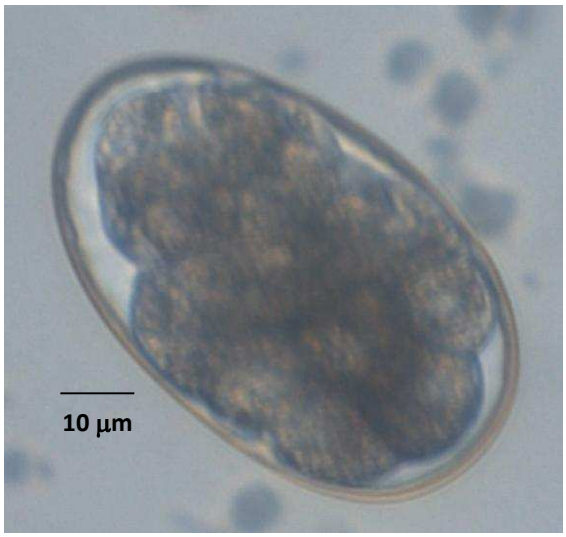

Figure 1.3: Egg of *U. stenocephala* (100X) (original photo).

1.1.2 Ascarids' aetiology and morphology

Toxocara canis

Domain: Eukaryota

Kingdom: Animalia

Subkingdom: Eumetazoa

Branch: Bilateria

Superphylum: Aschelmintae

Phylum: Nematoda

Class: Chromadorea

Order: Ascaridida

Family: Toxocaridae

Genus: *Toxocara*

Species: *Toxocara canis*

Toxascaris leonina

Domain: Eukaryota
Kingdom: Animalia
Subkingdom: Eumetazoa
Branch: Bilateria
Superphylum: Aschelmintae
Phylum: Nematoda
Class: Chromadorea
Order: Ascaridida
Family: Toxocaridae
Genus: *Toxascaris*
Species: *Toxascaris leonina*

Toxocara canis is a nematode with long three lips in the proximity of the buccal capsule, long and narrow cervical alae and oesophagus with ventricle (Fig. 1.4). Adult specimens vary in size, depending on sex, between 10 and 18 cm: the male, on average 10-12 cm long, has 2 spicula without bursa while the female is generally 12-18 cm long. Eggs measure 80-94 x 65-83 μm. They are thick-walled, spherical, greyish in colour with pitted surface (golf ball-like) and with cotent dark, unsegmented and grainy (Brunaska et al., 1995; Companion Animal Parasite Council -CAPC, 2022) (Fig. 1.5).

Toxascaris leonina has long and small cervical alae and oesophagus without ventricle (Fig. 1.4). Adult males measure 4-7 cm while adult females 5-12 cm long. The eggs are 76-95 μm in size, globular to elliptic, surrounded by a thick, lightish, smooth shell (CAPC, 2022) (Fig. 1.6).

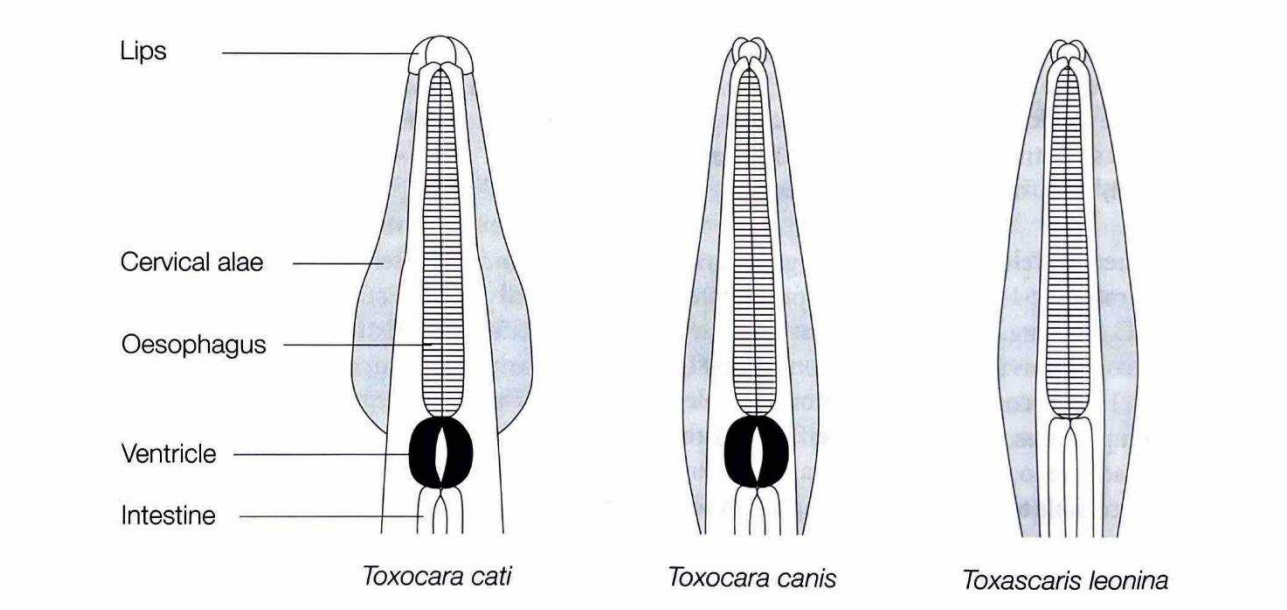

Figure 1.4: Anterior ends of *Toxocara* and *Toxascaris* (Deplazes et al., 2016).

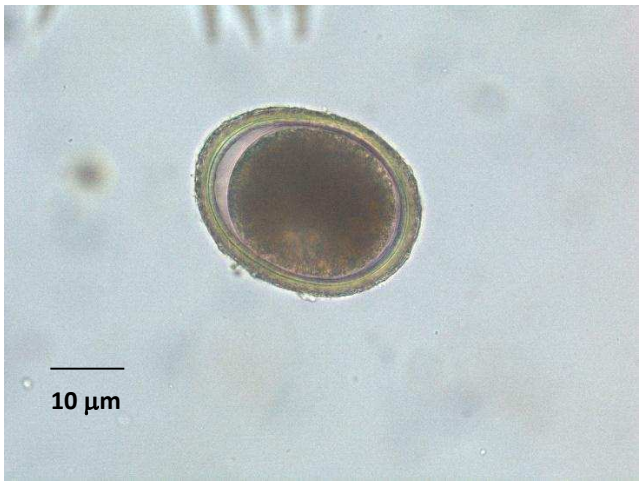

Figure 1.5: Egg of *T. canis* (40X) (original photo).

Figure 1.6: Egg of *T. leonina* (40X) (original photo).

1.2 Life cycle

1.2.1 Hookworms' life cycle

Ancylostoma caninum is a monoxenous parasite, i.e., it has a direct biological cycle (Fig. 1.7). Females of *A. caninum* are remarkably prolific and release thousands of eggs per day, which are eliminated in the outdoor environment with the dog's feces. Under certain conditions (high humidity and temperature between 16 and 22 °C), the eggs mature in about 1-2 days with the formation of an L1 larva, a noninfectious, rhabditiform stage. The L1 undergoes two molts that lead it to become larva L3, filariform, infecting stage, in about 1 week. Host infection at this stage can occur either orally or percutaneously (Epe et al., 2009). Once penetrated, the L3 larva, depending on the route of infection, can either directly reach the small intestine where it moults into L4 and L5 and reaches sexual maturity in order to perpetuate the cycle (oral route) or, otherwise, it gains the blood route by penetrating the subcutaneous venous bed and reaches the lung level where, via the orotracheal route, it reaches the esophagus and then the intestine (percutaneous route). In both cases, part of the penetrated larvae performs somatic migration: starting from the subcutis, intestine and lungs, in fact, they disseminate into muscle tissue and adipose tissue. This migration creates the possibility of other routes of infection for the host such as the transmammary route, endogenous self-infection or through ingestion of a paratenic host (e.g., rodents). In adult dogs, both male and female, once L3s reach the lungs, they reach, via the systemic circulation, various tissues and organs, where they arrest development while remaining alive and viable for years (hypobiosis) (Traversa et al., 2012). Generally, the prepatent time is between 15 and 26 days and is influenced by both the route of

infection and the age of the subject: in fact, if the infection is carried out percutaneously, the prepatent time in puppies of dogs turns out to be 14-17 days while in older dogs it is 26 days (Epe et al., 2009).

The biological cycle of *Uncinaria stenocephala* is very similar to the one of *A. caninum* with a prepatent period of about 15 days (Traversa et al., 2012). The parasitized animal excretes eggs through feces, which, under ideal temperature conditions, mature and in 1-2 days there will be the formation of a first-stage larva (L1) or also referred to as a rhabditiform larva. Following the hatching of the eggs within 5-10 days, under suitable conditions (temperature 7.5 -27 C°), the L1 larvae will develop evolving into third stage (L3) or filariform infective larvae. This larval stage can survive 3-4 weeks under favorable environmental conditions (high humidity) and infesting a definitive host exclusively through the oral route. These, once swallowed, reach the stomach and, releasing the cuticle, remain there for about two days. They then establish themselves in the distal part of the small intestine, evolving into the full-grown adult form and reaching sexual maturity. Lung migration is not present in this biological cycle, but instead somatic migration is found following oral infection. The latter occurs when a portion of the ingested larvae penetrates the mucosa of the most proximal portions of the gastrointestinal tract and migrates to various tissues such as muscle tissue and adipose tissue. This migration gives to the parasite the possibility of infestation to definitive hosts through the ingestion of paratenic hosts, such as rodents (Epe et al., 2019; Traversa et al, 2012). The possibility of infection through the percutaneous route is unlikely to exist. Transmission through the transmammary route and intrauterine infection has not been demonstrated (Traversa et al., 2012).

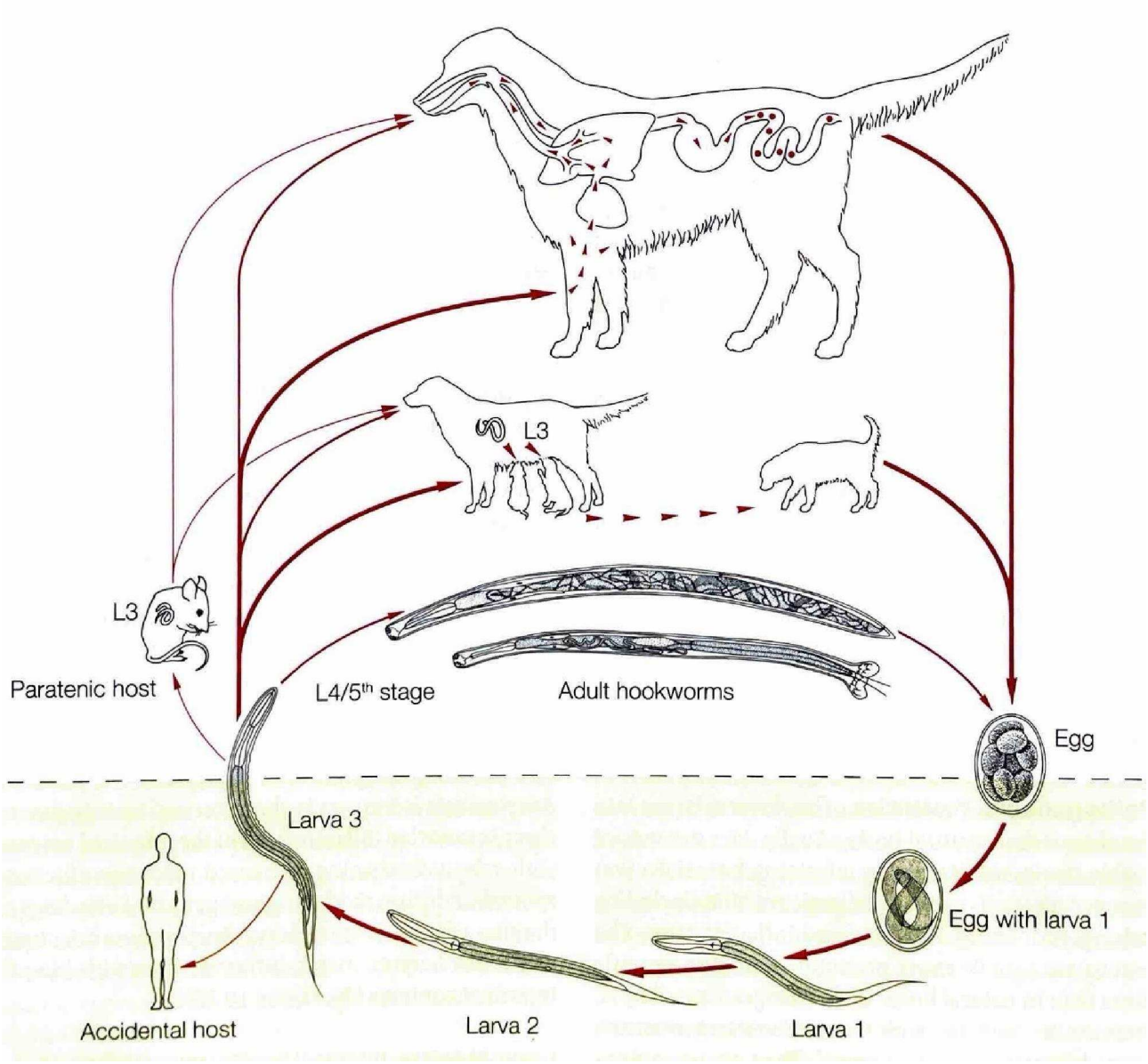

Figure 1.7: Life cycle of *A. caninum* (Deplazes et al., 2016)

1.2.2 Ascarids' life cycle

Toxocara canis is a monoxenous parasite as well (Fig. 1.8). Adults (males and females) copulate in the small intestine of the host producing several thousand of eggs which are eliminated in the environment through dogs' feces. At suitable conditions of temperature (25 - 27°C), humidity (85 - 95%) and oxygenation, L1 stage larvae start to moult and in two weeks they change their shapes in L2 larval stage and then in infective L3 larval stage (Brunaska et al., 1995). The host becomes infected by the ingestion of the eggs with L3 stage larvae inside (Traversa et al., 2012) or. Upon reaching the intestine, the eggs hatch and the larvae penetrate the intestinal wall, reach the bloodstream and

through the mesenteric veins they reach the liver, leaving deep lesions. The cycle continues with migration to the heart and lungs, where they cause rupture of the pulmonary alveoli. In the ascent of the bronchial tree, they mutate to the L4 larval stage and return to the intestine by passage to the oral cavity and subsequent swallowing after 10 days post infection. After a final moult they attain sexual maturity (Schnieder et al., 2011). Prepatency lasts 30-29 days in young dogs slightly longer (40-56 days) in dogs over 1 year. Generally, in young individuals the cycle is complete as described above while in older dogs L3 larvae after their ingestion often migrate in various tissues and organs (somatic migration) including liver, lung, brain, heart, episkeletal musculature and wall of the intestinal tract, in a state of hypobiosis. In males they may remain for several months and then die. Conversely, in females this migration gives the possibility of several option for vertical transmission such as: (i) prenatal (transplacental) transmission; (ii) lactogenic (transmammary) transmission. Transplacental transmission is possible in the last third of pregnancy approximately at 42 days after conception when hypobiotc larvae in pregnant bitches are hormonally activated. They invade the bloodstream, pass through the placenta to the fetus and infect especially liver. After birth, the cycle is completed immediately by migration of the larvae to the intestine on the tracheal migration route. Prepatency is in this case 21-25 days (Traversa et al., 2012). Conversely, transmammary transmission occurs during lactation period when migrating *T. canis* larvae reach the mammary glands through the bloodstream. Prepatency after lactogenic transmission lasts 27-35 days. Moreover, another route of infection for dogs is the ingestion of paratenic hosts such as rodents, birds in which hypobiotic larvae can infect dogs through their ingestion after predation (Strube et al., 2013; Overgaauw e al., 2020). *T. leonina* generally inhabit the small intestine of dogs. The biological cycle is very similar to the one of *T. canis* but without any migration. Prepatency lasts 7-10 weeks (Traversa et al., 2012).

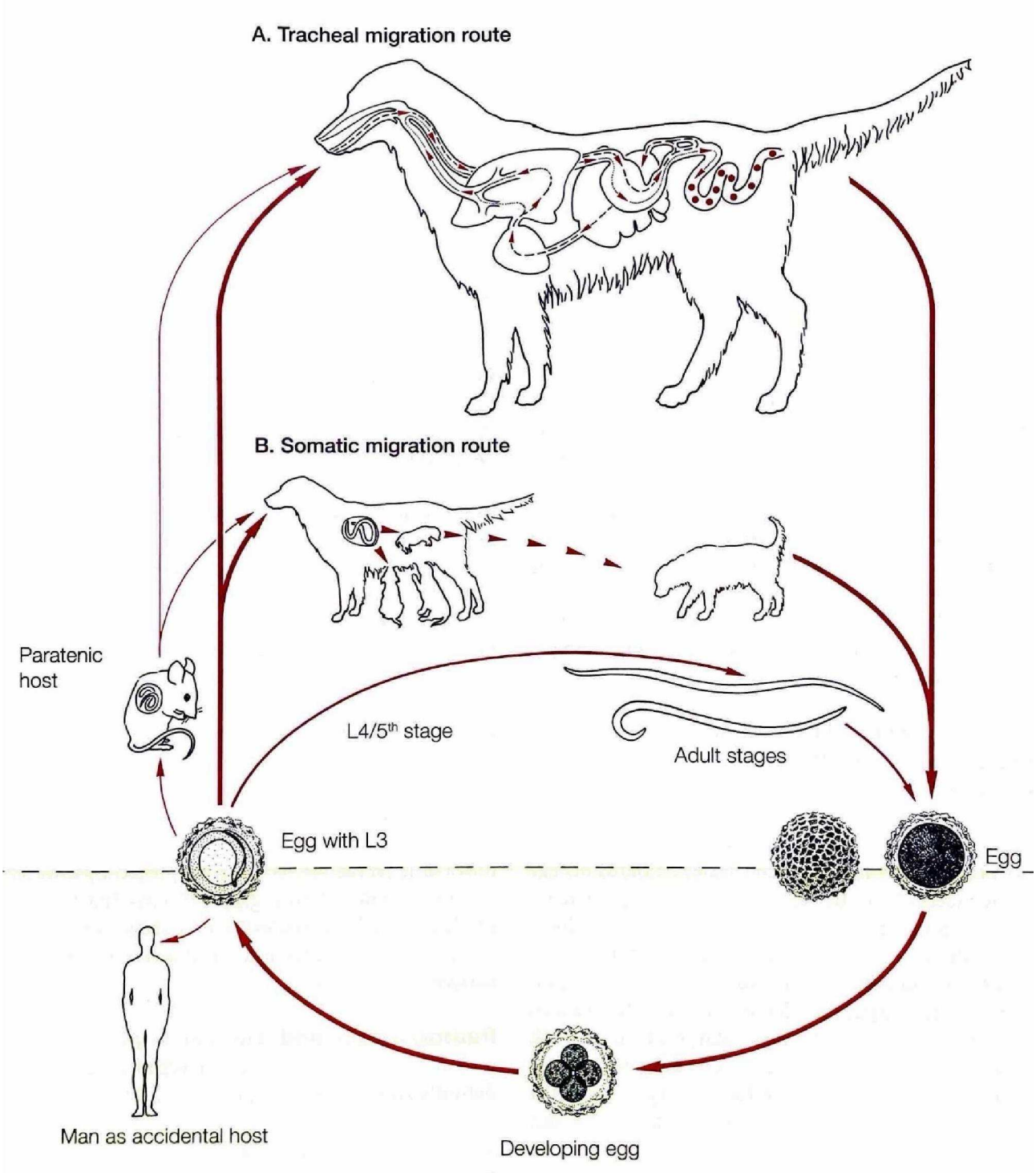

Figure 1.8: Life cycle of *T. canis* (Deplazes et al., 2016)

1.3 Epidemiology

1.3.1 Hookworms' epidemiology

Ancylostoma caninum is mainly distributed in countries with warm climates although, being a nematode with cosmopolitan distribution, it is not impossible to find it in countries further north such as southern Canada or Denmark, with obviously harsher climates (Traub et al., 2021). In Kenya, a study was carried out on the molecular identification of the main species of hookworms causing zoonoses, and out of 1621 stool samples exclusively from dogs from different climatic regions of Kenya such as Narok and Meru (regions with higher rainfall), Isiolo and Turkana (regions with drier climate), 490 were positive for hookworms, with higher prevalence in regions with higher rainfall than those with drier climate. Of these 490 positive samples, 70 were subjected to molecular investigations such as PCR-RFLP or DNA sequencing and, 59 of these, tested positive for *A. caninum* (Mulinge et al., 2019). Additionally, a similar study was performed on 66 stool samples from dogs in Morogoro city in Tanzania that were positive for hookworms and subjected to molecular investigations such as PCR-RFLP. All samples were identified as *A. caninum* (Merino-Tejedor et al., 2019). In a systematic review and meta-analysis conducted in Asia in 2018, the weighted prevalence of *A. caninum* in dogs was found to be 23% with confidence interval ranging from 7% to 53% (Zibaei et al., 2020). In Morocco, a study was conducted in a population of 291 dogs belonging to different age groups: puppies (<6 months) (24.3%, n = 70), young adults (6-12 months) (10.7%, n = 31), adults (1-6 years) (56.9%, n = 164), and elderly (>6 years) (7.9%, n = 23). In this study, the overall prevalence of intestinal nematodes was 58% (95% CI 52.1-63.8) and the prevalence of *Ancylostoma*/*Uncinaria* spp. species was 31.9% (95% CI 26.6-37.6) (Idrissi et al., 2022). In Europe, prevalence rates of *Ancylostoma* infections in dogs range from 1.2% to 34% (Bajer et al., 2011; Wright et al., 2016; Lledó et al., 2015; Ilić et al., 2021; Drake et al., 2022). Also in Europe, in wild carnivores the prevalence of hookworms in general ranges from 10 to 12% in red foxes (Dybing et al., 2013; Lledó et al., 2015;) and from 30 to 90% in wolves (Bindke et al., 2017; Al-Sabi et al., 2018;). In Spain, in a study performed on 400 red foxes (*Vulpes vulpes*), 73.25% tested positive for gastrointestinal nematodes of which *A. caninum* was the most prevalent (12.5%) (Lledò et al., 2015). In addition, a recent study of intestinal parasites in dogs in Western European cities revealed a prevalence of hookworms of 3.2% (Drake et al., 2022). In Italy, hookworm infections have been reported in many studies, with high prevalence rates in stray (67.7%) and owned dogs (18.9%) in the southern area (Rinaldi et al., 2012; Brianti et al., 2018), followed by prevalence rates between 0-9.3% in stray dogs and 0.4-3.6% in owned dogs in the northern area (Zanzani et al., 2010, 2014; Simonato et al., 2015; Traversa et al., 2017; La Torre et al., 2018; Simonato et al., 2020).

Uncinaria stenocephala, also known as the "northern hookworm," is considered widespread in temperate and North America, Asia, and Europe (Traub et al., 2021). Several studies especially in Central Europe highly reported its presence (Demkowska- Kutrzepa et al., 2018;Štrkolcová et al., 2022). Also in Central Europe, *U. stenocephala* turns out to be the most prevalent species not only in dogs but also in foxes. In fact, in both rural and urban fox populations, *U. stenocephala* prevalences are ranges from 60% to 90% percent (Deplazes et al., 2022). In Italy, *U. stenocephala* is considered a rather rare parasite. A study was conducted in 2013, in which fecal samples from 239 owner dogs from central Italy were analyzed to assess the prevalence of the main intestinal and pulmonary parasites. Thirty-one percent of the dogs tested positive for at least one parasite species of the intestinal and pulmonary tracts, and specifically, the prevalence of *U. stenocephala* was 1.25 percent (Riggio et al., 2013).

The definitive hosts of *A. caninum* and *U. stenocephala* are mainly carnivores, specifically the dog, jackal, wolf, and fox, and generally the prevalence is higher in stray animals or animals living in groups, such as dogs in kennels or often hunting dogs (Traversa et al., 2012). No sex or breed predisposition has been demonstrated while age appears to be an important factor both for the possible pathway of trans-mammary infection (that is not considered for *U. stenocephala* infection), which is only contemplated during the lactation period, and for the higher pathogenicity expressed by *A. caninum* in young animals under 1 year of age. Indeed, in older animals there is an important degree of immunity acquired over time, especially in dogs from endemic areas. Other factors such as malnutrition may increase host susceptibility (Traversa et al., 2012). In addition to definitive hosts, *A. caninum* and *U. stenocephala* also possesses paratenic or occasional hosts that have an importance in the epidemiology of this parasitic infection, behaving as a possible source of an additional route of infection: rodents in particular possess this role. Humans themselves turn out to be occasional (blind bottomed) hosts as well. The main risk factors for hookworms' infection are described in Table 1.1. Moreover, the exogenous stages of the parasite are able to survive in shaded, warm, moist, well-drained and oxygenated soils that are rich in humus and plants that can protect the larvae even during summer periods. The survival temperature is between 15 and 37 °C with an optimum between 25 and 30 °C for *A. caninum* and of 15-20 °C for *U. stenocephala*. Dog shelters in kennels can also be an excellent breeding ground for the development and survival of the larval stage of hookworms, especially if poor hygienic conditions, high humidity, and the presence of porosity or lesions in the floor that can promote the infiltration of larvae insist. In the optimum of the required conditions, hookworms' larvae can survive in the environment for 2 to 3 weeks, differently they are inactivated in about 1 day (Traversa et al., 2012).

Table 1.1 : Risk factor of intestinal worms (ESCCAP, 2021).

Worm species	Dog type			Health	Environment		Nutrition			Location and travel
	Pup	Lactating	Stray	Fleas or lice	In kennels	Outdoors	Rodents/ amphibians / reptiles	Molluscs	Raw meat/ viscera	
INTESTINAL WORMS										
Ascarids										
Toxocara canis	■	■	■		■	■	■		■	
Toxascaris leonina			■		■	■	■			
Hookworms										
Ancylostoma caninum	■	■	■		■	■				More in southern Europe
Uncinaria stenocephala			■		■	■				In colder climate (northern Europe)
Threadworms (*Strongyloides*)										
Strongyloides stercoralis	■	■			■	■				
Whipworm										
Trichuris vulpis			■		■	■				
Tapeworms										
***Taenia* spp.**			■			■	■		■	
***Mesocestoides* spp.**			■			■	■			
Dipylidium caninum			■	■	■					
Echinococcus granulosus*			■						■	Central, southern and eastern Europe
Echinococcus multilocularis			■			■	■			Central, eastern and northern Europe

1.3.2 Ascarids' epidemiology

T. canis is distributed worldwide in dogs and wild carnivores such as foxes, racoon dogs and other canids (Otranto et al., 2019). A recent meta-analysis carried out by Rostami et al. (2020$_b$) showed an overall prevalence of *T. canis* infection in dogs of 11.1% (95% CI, 10.6-11.7%). The estimated prevalence in the different WHO-regions ranged from 6.4% to 19.2%: Eastern Mediterranean (19.2%, 13.7-25.5%), Africa (18.5%, 13.7-23.9%), South-East Asia (11.9%, 6.8-18.2%), North America (11.1%, 10.6-11.7%), South America (10.9%, 7.6-14.6%), Europe (10.8%, 8.9-12.9%) and Western Pacific (6.4%, 3.3-10.2%) (Fig. 1.9) Moreover, in the same study young (<1 year of age), stray, rural and male dogs had a significantly ($P<0.001$) higher prevalence of infection than older, pet, urban or female dogs (see Tab. 1.1). According to Traversa et al. (2012), kenneled and coprophagic dogs had

significantly higher prevalences. According to another study carried out by Rostami et al. (2020$_a$), *T. leonina* infection global prevalence in dogs is 2.9%. Similar prevalences were confirmed also in Europe (Overgauw et al., 2020). In Italy, a national survey on endoparasites of dogs showed a prevalence of 9.0% of *T. canis* and 1.0% of *T. leonina*, respectively (Brianti et al., 2018). Moreover, a study conducted in the city of Naples (southern Italy) on canine fecal contamination revealed a prevalence of 0.7% for *T. canis* and 1.4% for *T. leonina* whereas high prevalence values for *T. canis* (14.8%) were found in kennel dogs of the Campania region of southern Italy (Rinaldi et al., 2006; 2015). As above mentioned, foxes and other canids represent source of infection for both animals and humans in urban and suburban areas (Wolfe et al., 2001; Craig et al., 2005; Mackestendt et al., 2015). The persistence of the transmission cycle of both *T. canis* and *T. leonina* is ensured by multiple epidemiological factors such as the high prevalence in final host as reported above, the great fertility of this species that contaminate the environment (daily egg production of a *T. canis* female is estimated at 25,000-85,000), the several options of transmission routes (ingestion of larvated eggs, transplacental route, lactogenic route) (Traversa et al., 2012).

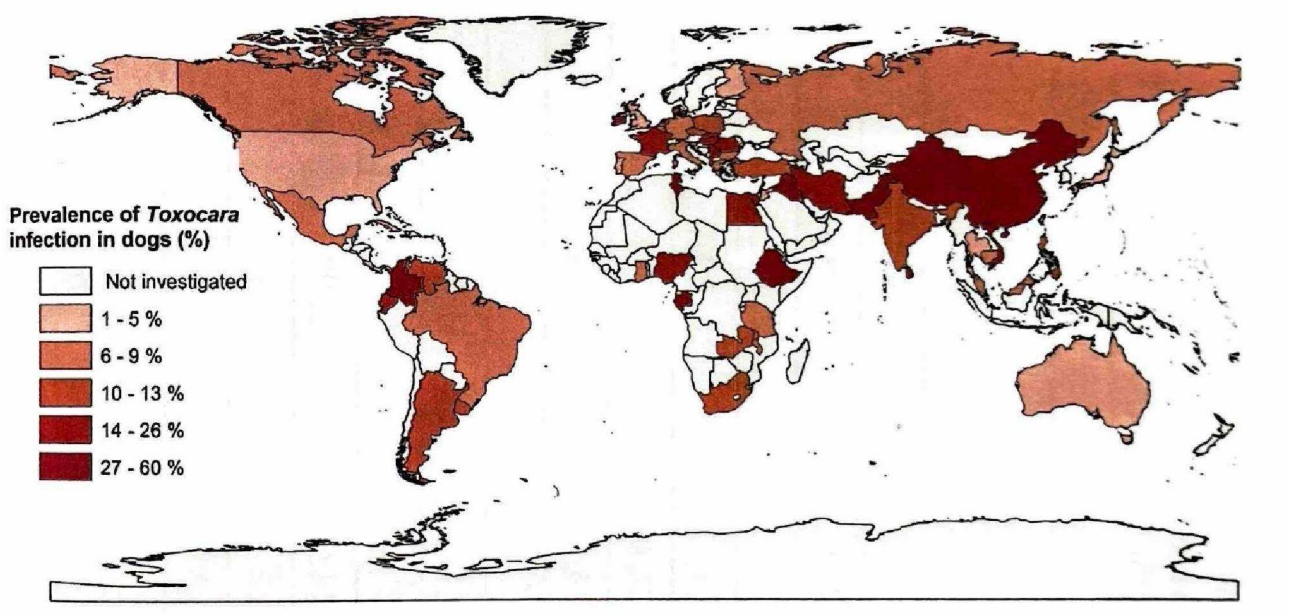

Figure 1.9: Prevalence of *Toxocara* infection in dogs in different countries using geographic information system (GIS) (Rostami et al., 2020$_b$).

1.4 Pathogenesis and clinical signs

1.4.1 Hookworms' pathogenesis and clinical signs

During the stages of its biological cycle, *A. caninum* and *U. stenocephala* exhibit different pathogenetic mechanisms that vary depending on the route of infection. In the percutaneous route of infection, penetration of L3 larvae into the skin of definitive hosts does not cause obvious lesions, which, on the contrary, become clear and explicit in superinfections or reinfections, causing a dermatologic symptom picture of an allergic nature. During migration, the larvae cause an inflammatory state at the pulmonary level and small hemorrhages at the tracheal level: this condition becomes particularly evident only in massive infestations (Traversa et al., 2012). All species are hematophagous and having arrived at the intestinal level, both larval stages and adults adhere to the mucosa of the latter. Around day 8 post infection they develop within the buccal capsule 3 pairs of teeth placed externally on the margin and a pair placed ventro-laterally that will allow them to injure the mucosa and vascular endothelium, thus having free access to the circulatory stream in order to feed (Traversa et al., 2012). According to Bowman et al. (2010), on average, an adult of *A. caninum* is capable of ingesting about 50-200 µl per day of blood while *U. stenocephala* about 0.3 µl/day per adult. Larvae localized in the intestine, at different stages, take on the ability to produce secretions from their esophageal and intestinal glands. These secretions are released into the larva's intestinal tract or partially excreted into the host intestine and consist of molecules that inhibit host blood clotting, such as anticoagulant peptide 5 (AcAP5) (Harrison et al., 2001), which dissolves formed platelet aggregates due to its fibrinolytic properties. Thus, *A. caninum* leads to the establishment of anemia that in acute infestations turns out to be normocytic-normochromic while in chronic ones microcytic-hypochromic due to sideropenia and will be more or less severe depending on the parasite load, age of the subject (especially puppies) and general conditions of the animal (Traversa et al., 2012) In addition to the anemia, both *A. caninum* and *U. stenocephala* are also responsible for a severe inflammatory state in the intestinal mucosa. Typically, this nematode changes the attachment site at which it feeds every 4-6 hours, leaving small lesions that heal within 24 hours (Fig. 1.10).

In dogs, symptomatology in cases of hookworms' infection is related to the parasite load, age, and general condition of infested individuals. Generally, the main clinical sign is anemia (especially for *A. caninum* infection), particularly marked in puppies with massive infestation, where this can become lethal due to the immaturity of the bone marrow, which is not able to supply the blood loss caused by the parasite. Puppies will present anemic explorable mucous membranes, hypothermia, lethargy, anorexia or sometimes allotriophagia, and diarrhea characterized by pultaceous feces,

sometimes hemorrhagic with unchanged frequency of defecation. The anemia and gastroenteric symptoms, in severe cases, may in turn lead to a condition of metabolic acidosis, hypoprotinemia, heart failure, hypovolemic shock, and death. Initially, drowsiness, weakness, anorexia, and, very often, intermittent epistaxis appears, mostly in mild form. This symptom can be considered almost pathognomonic (Flahaut's sign) and reveals the reduced blood clotting ability caused by the parasitic enzymes. Asymptomatic or subclinical infestations are not uncommon in adult animals, while the most severe clinical forms are observed in pups following infestation by the galactogenic route (CAPC, 2023). In chronic infestations, on the other hand, cachexia and coat shedding are added as clinical signs. Although rare, following massive infestations, clinical signs afferent to the respiratory system such as cough, dyspnea and cyanosis are possible, due to both the migration damage of hookworms' larvae and the establishment of the anemic state that does not allow proper hematosis at the pulmonary level. Still very rarely, lesions caused by penetration of L3 larvae into the skin of the abdomen, sternum, and interdigital spaces are observed in percutaneous reinfections or superinfections especially by *U. stenocephala*. These lesions consist of papules, erythematous plaques associated with itching and possible hyperkeratosis of the plantar pads (Chu et al., 2013).

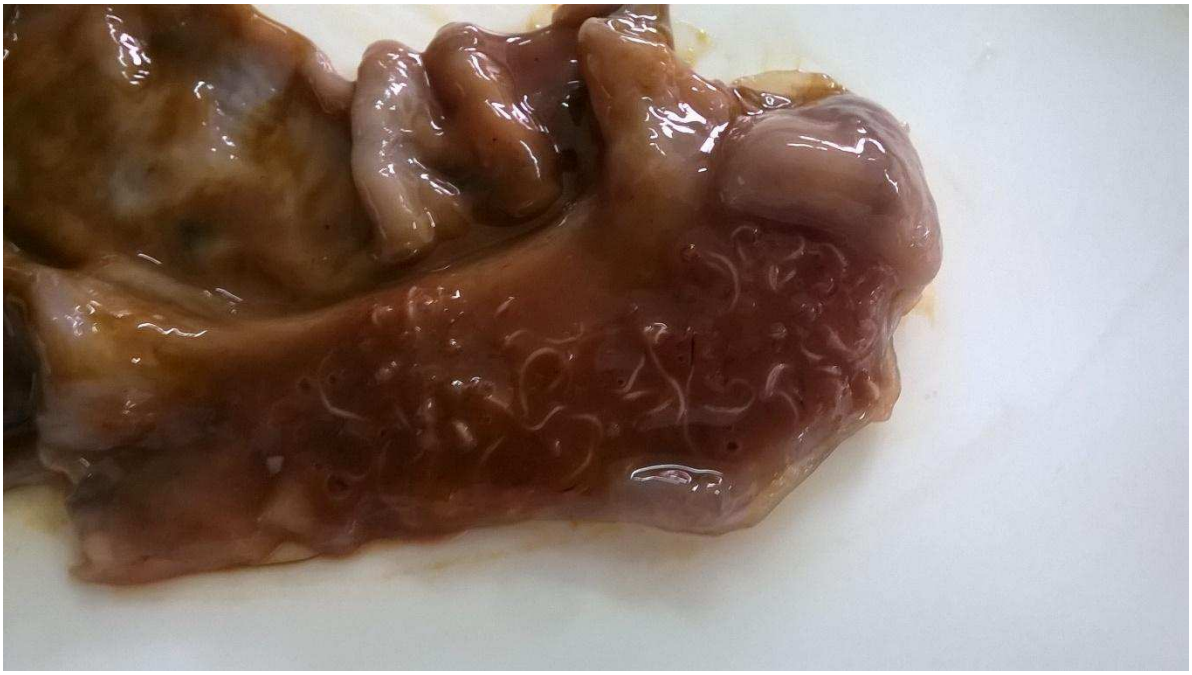

Figure 1.10: Adult hookworms in the intestinal mucosa during necropsy (original photo).

1.4.2 Ascarids' pathogenesis and clinical signs

Toxocara canis infections can cause different types of disease in definitive hosts. Light/moderate intestinal infection in puppies and older dogs that are generally asymptomatic or occasional vomiting, diarrhea, mucous feces and retarded development in young animals (Despommier et al., 2003). Moreover, infection of older dogs by somatic larvae are generally asymptomatic as well. Additionally, heavy infections in puppies can occur with tissue demage caused by migrating lavae that produce different granulomas in several organs such as liver, lungs, kidney or retinitis in the eyes and intestinal infection (Fig. 1.11) with atrophy of villi, gastroenteritis, diarrhea, vomiting, intermittent fever, anorexia, neurological disorders. Occasionally perforation of the intestine can occur. This infection could be possibly fata, from the 2nd and 3rd week of life (Traversa et al., 2012). Conversely, *T.leonina* is of low pathogenicity; but heavy infections may cause enteritis.

Figure 1.11: *T. canis* in a small intestine content of a puppy (original photo).

1.5 Diagnosis

1.5.1 Hookworms' diagnosis

Clinical signs and anamnestic data need confirmation on copromicroscopic examination using flotation-based techniques, which is the gold standard for the detection of hookworms' eggs. There are several flotation techniques in the parasitological diagnostic landscape such as simple flotation, McMaster (Becker at al., 2016), FLOTAC (Cringoli et al., 2010) and Mini-FLOTAC (Cringoli et al., 2017). The most sensitive techniques for the diagnosis of hookworms are FLOTAC and Mini-FLOTAC techniques using a supersaturated solution of sodium chloride with specific gravity 1200 as a floating solution for samples mainly fresh or preserved in 5% formalin (Cringoli et al., 2010, 2017). However, it is not possible with copromicroscopic examination (morphological features of hookworm eggs) alone to detect hookworm species, which can only be discriminated through molecular techniques such as Polymerase Chain Reactions (PCR) and subsequent DNA sequencing (Traub et al., 2021). Conversely, several studies confirmed the possibility to differentiate hookworms' larvae (L3) after coprocolture and according their morphological and morphometric features (Hill et al., 1985, Strkolcova et al., 2022).

It is also possible to use tests for fecal antigens of *A. caninum*, to be combined with the coprological examination, which increases the diagnostic sensitivity and allows the detection of infection even during the prepatent period that are potentially symptomatic but cannot be diagnosed on fecal microscopic examination (Sweet et al., 2021).

1.5.2 Ascarids' diagnosis

Ascarid infections n dogs are diagnosed by copromicroscopical detection of the eggs. The eggs of *T. canis* and *T. leonina* can be easily differentiated. Moreover, molecular analyses could be useful for diagnosis especially for discriminate *T.canis* from *T.cati* (Mikaeli et al., 2017). Anyway, copromicroscopical examination is the gold standard for ascarid diagnosis (Oge et al., 2019). Additionally, a study conducted by Kazemi et al. (2021) showed a greater sensitivity of loop-mediated isothermal amplification (LAMP). Serological tests are useful especially in humans (Skulinova et al., 2022).

1.6 Control

1.6.1 Hookworms' control

The most widely used antiparasitic drugs used to control of hookworms in dogs are broad-spectrum anthelmintics belonging to different classes such as the tetrahydropyrimidines, benzimidazoles, avermectins, and milbemicins. All these molecules generally rather than being used as monotherapy are, in order to amplify the spectrum of action toward other parasites, used in combination with other molecules (Tab.1.2,1.3). According to the guidelines of the European Scientific Counsel Companion Animal Parasites (ESCCAP, 2021), puppies should be treated starting from the second week of age, every two weeks until two weeks after weaning, and then monthly until the sixth month of age, with effective molecules, following the weight and minimum age of administration guidelines for each molecule. Dogs older than 6 months that have access, even sporadically, to the outdoor environment should be treated with broad-spectrum antiparasitics at least 4 times a year, after diagnosis. In case of reported infection and targeted treatment, it is advisable to treat all cohabiting dogs as well. A study conducted in the US in 2019 (Jimenez Castro et al., 2019) reported increasing resistance of *A. caninum* to the most common drug therapies.

Environmental control is of paramount importance, having the aim of eliminating the eggs and larvae of the parasite, significantly reducing the risk of infection for both animals and humans. Spray products or washed with sodium hypochlorite solutions with a concentration greater than 25 percent; iodine solutions (50-60 ppm) at 15-30 °C ; boiling water at a temperature greater than 80 °C; 70% ethanol solutions; chlorhexanol-based disinfectants are useful against hookworms. Moreover, a vaccine had been developed and used in the USA and after a short time withdrawn from the market due to economic problems (1975). This vaccine was based on the establishment of protective immunity stimulated by larvae irradiated with x-rays or gamma rays. Further immunology studies have been conducted on recombinant secretory proteins of L3 larvae or hemoglobin protease of adult parasites as antigens and have been noted to be capable of developing partial immunity (Epe et al., 2009).

1.6.2 Ascarids' control

Pyrantel derivates, benzimidazoles, macrocyclic lactones and emodepside are available for the treatment of adult *Toxocara* and *Toxascaris* in dogs (Tab. 1.2-1.3). The drugs are effective and well tolerated. Anthelmintic resistance was not observed so far. According to ESCCAP guidelines (ESCCAP, 2021), control measures are similar to these recommended for hookworms' control.

Table 1.2: Antiparasitic mono-products registered for use in dogs for the treatment of hookworms and roundworms (Deplazes et al., 2016).

Active substance (generic name) and chemical group[1]	Dose: mg/kg b.w. days of application (d)	Application	Spectrum of drug efficacy[2]	
			Hookworms (*Ancylostoma*, *Uncinaria*)	Roundworms (*Toxocara*, T*oxascaris*)
Fenbendazole (BZ)	50 x 3d	Per os	+	+
Flubendazole (BZ)	22 x 2-3d	Per os	+	+
Mebendazole (BZ)	20-22 x 3-5 d	Per os	+	+
Selamectin (ML)	6	Spot-on	+	+
Nitroscanate (VO)	50-100	Per os	+	+
Pyrantel-base (PY)	20	Per os	+/-	+

Legend: [1] Chemical group: BZ= benzimidazoles; ML = macrocyclic lactones; PY= pyrimidines; VO= various other active substances. [2] Efficacy; + = good to very high ; +/- = partially effective or only in increased dose or with repeated application; - = insufficient effect.

Table 1.3: Antiparasitic combination products registered for use in dogs for the treatment of hookworms and roundworms (Deplazes et al., 2016).

Active substance (generic name)	Dose: mg/kg b.w. days of application (d)	Application	Spectrum of drug efficacy[1]	
			Hookworms (*Ancylostoma*, *Uncinaria*)	Roundworms (*Toxocara*, T*oxascaris*)
Fenbendazole + Praziquantel+ Pyrantel embonate	15+5+5	Per os	+	+
Fenbendazole + Praziquantel	50+ 5 x 2-3 d	Per os	+	+
Praziquantel+ Pyrantel embonate	20+5	Per os	+/-	+
Pyrantel base + Epsiprantel	5+ 5.5	Per os	+	+
Pyrantel+ Oxantel+ Praziquantel	5+20+5	Per os	+	+
Mylbemicin oxime + Praziquantel	0.5+5	Per os	+/-	+
Mylbemicin oxime + Lufenuron	20.5+10	Per os	+/-	+
Mylbemicin oxime + Lufenuron + Praziquantel	0.5+10+5	Per os	+/-	+
Moxidectin + Imidacloprid	2.5+10	Spot on	+	+
Eprinomectin + Praziquantel + Fipronil + S- Methoprene	0.5+10+10+12	Spot on	+	+
Emodepside + Praziquantel	3+12	Spot on	+	+
Emodepside + Praziquantel	1+5	Per os	+	+
Emodepside + Toltrazuril	0.45+9	Per os	+	+

Legend: [1]Efficacy; + = good to very high ; +/- = partially effective or only in increased dose or with repeated application; - = insufficient effect.

1.7 Zoonotic risk

1.7.1 Hookworms' zoonotic risk

Infection with *A. caninum* is a zoonotic infection in which humans are occasional blind-bottom hosts. L3 stage larvae penetrate through the skin of buttocks, feet, and limbs through the action of secreted proteases. Having passed the stratum corneum (outermost layer of the epidermis), larvae lose its natural cuticle and begins a migration but, while in the natural host it is able to penetrate the dermis (deepest part of the skin) and be transported via the lymphatic vessels and venous system to the lungs, in humans it is obliged to confine itself to the epidermis (the superficial part of the skin), presumably due to lack of enzymes such as collagenases, which allow it to pass the basement membrane. The larvae's points of penetration are made evident by the development of red, tortuous, serpiginous skin pathways, characterized by local inflammatory and itchy reaction, given that the parasites are able to produce hydrolytic enzymes that allow them to advance through the thickness of the host's skin by a few centimeters per day to a length of about 20 centimeters, with a marked advancing front, whereas, the caudal portions appear desquamated or hyperpigmented. The skin manifestations are caused by anaphylactic reactions to the parasite and its metabolites and being very itchy, due to the mechanical action from continuous scratching, can lead to the development of secondary bacterial pyoderma. Resolution of the symptoms occurs in a few weeks to a few months and is due to the death of the parasite (Bowman et al., 2010; Traversa et al., 2012). This set of symptoms is known as *Cutaneous Larva Migrans Syndrome* (Fig.1.12), which, in the past, was prevalent mainly in tropical countries but is also spreading to Europe with a steadily increasing incidence in recent years. According to a 2019 systematic review, a total of 55 cases have been reported in Europe from 1994 to 2018: 15 from Spain, 13 from France, 9 from the United Kingdom, 8 from Italy, 7 from Germany, 2 from Serbia, and 1 of dubious origin between Spain and Portugal (Del Giudice et al., 2019). *Cutaneous Larva Migrans Syndrome* outbreaks have also been reported in the city of Naples attributable to gardening practices with material contaminated with dog feces infested with *A. caninum* (Galanti et al., 2002). In addition to the classic aspect above described, other clinical forms were recorded such us a bullous form of *larva migrans* was observed, distinguished by vesicles with serous content. In addition to cutaneous forms, enteric forms can also occur in which eosinophilic enteritis occurs with acute abdominal pain, apparently without cause, accompanied or not by eosinophilia. Conversely, the zoonotic power of *U. stenocephala* has been established, although there is not a large case history of infestations by this parasite in the literature and the relationship between parasite and clinical symptomatology is unclear. However, in Europe, and more specifically in England, France and Italy, cases of autochthonous *cutaneous larva migrans* syndrome strongly attributable to *U. stenocephala*

have been reported, but as no real diagnosis of certainty has been possible, the correlation between this syndrome and the nematode remains unclear.

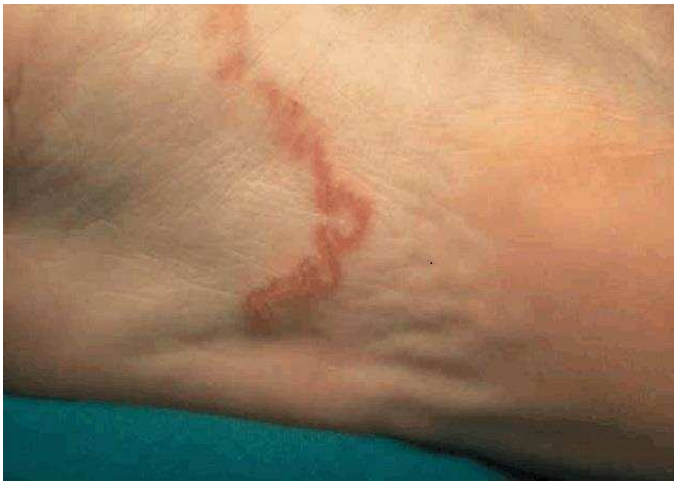

Figure 1.12: Cutaneous *larva migrans.* Courtesy: Prof. C. Tomasini, University of Pavia

1.7.2 Ascarids' zoonotic risk

Toxocara species have been recognized to transmit diseases to humans by zoonotic means. Several studies conducted worldwide have confirmed this, as well as the fact that ascarids species that affect dogs' health can infect humans as well (Despommier et al., 2003; Lee et al., 2010). Humans can be infected by ascarids through the ingestion of *Toxocara* eggs containing infective L3 stage larvae (geophagia, contaminated food, hand-mouth contact) (Macpherson et al., 2013; Traversa et al., 2014). Moreover, coats of dogs can be source of infection as well when possibly contaminated with *T. canis* eggs (Overgauuw et al., 2009; Maurelli et al., 2019). In humans the L3 stage larvae migrate on the somatic route, invading several organs and tissues (liver, lungs, muscles) and causing important lesions. A number of infections are asymptomatic, and the degree of damage and symptoms depend on the tissue or tissues invaded, the number of migratory larvae, the age of the host, and the immune system's reaction (Despommier et al., 2003; Dogan et al., 2007). When symptoms are present, there are two major syndromes that can occurr: "ocular *larva migrans*" (OLM), which is caused by damage to the optic nerve, and "visceral *larva migrans*" (VLM), which affects important organs like the brain, liver, and lungs. Other minor syndromes like covert, neural, and atopyc toxocarosis have also been reported (Despommier et al., 2003; Traversa et al., 2012). Children, especially toddlers, mostly suffer from VLM with important clinical including eosinophilia, splenomegaly, hepatomegaly, fever, asthma, and pneumonia. This high occurrence in children can be explained by their frequent exposure

to areas that can be infected with *Toxocara* eggs such as gardens, playgrounds, sandboxes. Moreover, another explanation could be the low hygiene standards especially in rural areas (Overgauuw et al., 1997; Traversa et al., 2012). OLM, which can cause endophthalmitis, retinal granulomas, and macula detachment, is characterized by reduced vision to complete loss of sight and typically manifests without indications of VLM in children aged 5 to 10 years as well as in adults . Additional symptoms include squint, glaucoma, and complaints of "seeing lights." More significantly, OLM can mistakenly be confused with retinoblastoma and several needless eye enucleations are also reported as a result of retinoblastoma misdiagnosis (Lopez-Velez et al.1995). Moreover, covert toxoacariosis can cause several and aspecific symptoms in children such as behavioral problems, seizures, altered sleep patterns, cough, asthma, headaches, and gastrointestinal discomfort while in adults, it can cause weakness, rash, itching, abdominal pain, and difficulty breathing (Traversa et al., 2012).

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Objective of the thesis

To achieve the overall objective of the PhD thesis entitled "Hookworm infections and other geohelminthic infections in dogs: epidemiological and molecular studies", the following specific objectives were pursued:

- To update the epidemiological scenario of ascarid infections in dogs in Southern Italy.

- To advance epidemiological data about hookworms' infection in dogs in Southern Italy and to assess through molecular investigations the prevalence of hookworm species (*Ancylostoma caninum* and *Uncinaria stenocephala*) in the same area.

- To evaluate the diagnostic accuracy of different molecular protocols for the detection of *A. caninum* and *U. stenocephala* in dogs using various matrices of samples.

Chapter 2

A five-year retrospective study on ascarid infections in dogs in southern Italy

Maurelli MP, Pepe P, **Illiano S**, Nocerino M, Ciuca L, Saralli G, Cringoli G, Rinaldi L. A five-year retrospective study on ascarid infections in dogs in southern Italy. Schweiz Arch Tierheilkd. 2022:164(1),79-88.

2.1 Abstract

A 5-year retrospective analysis of ascarid infections (*Toxocara canis* and *Toxascaris leonina*) in dogs from southern Italy was performed to update the epidemiological scenario of these parasites and to identify the risk factors which may favour these infections in animals in this study area. A total of 8,149 dogs, referred to our labs for copromicroscopic analysis using the FLOTAC technique, was considered. A sub-sample of 500 fecal samples were analysed also with the Mini-FLOTAC technique. Of the overall dog samples analysed, 9.2 % (95 % CI = 8.6–9.8) resulted positive for *T. canis* while 0.5 % (95 % CI = 0.4–0.7) resulted positive for *T. leonina*. Co-infections with *T. canis* and *T. leonina* were found in 0.1 % of dogs (95 % CI = 0.0–0.1). The results obtained by the FLOTAC and Mini-FLOTAC examinations showed a nearly perfect k agreement ($k = 0.99$, $P < 0.001$) between these two techniques. Chi-square test showed positivity to *T. canis* and *T. leonina* significantly ($P < 0.001$) associated with dogs housed outdoor (i.e., that lived in garden or in kennel). Moreover, the positivity for *T. canis* was significantly associated ($P < 0.001$) also with age (i.e., puppies), as shown by the logistic regression. The decreasing overall prevalence both for *T. canis* and *T. leonina* during the years of monitoring, showed that, as suggested by the European Scientific Counsel Companion Animal Parasites, the regular diagnosis could contribute to an efficient control of these parasites.

2.2 Introduction

Ascarids (also known as «roundworms») are large (10–15 cm in length) nematodes of medical and veterinary significance commonly found in the intestine of vertebrate hosts (Otranto et al., 2015; 2019). Of these, two species may affect the small intestine of dogs, namely *Toxocara canis* and *Toxascaris leonina*, the most important and widespread dogs' parasites worldwide (Traversa et al., 2012). Besides their impact on the health and welfare of dogs, *T. canis* is also of zoonotic importance, causing human toxocariasis, an inner systemic illness syndrome complex which can be highly pathogenic (Despommier, 2023). Humans become infected following the accidental ingestion of embryonated *Toxocara* eggs from contaminated soil, unwashed hands and food (Deplazes et al., 2011; Mikaeili et al., 2017). The ascarid life cycle is direct but can include paratenic hosts as a source of infection for definitive hosts (Overgaauw e al., 2020). Briefly, thick-shelled eggs are released in the environment by definitive hosts and L3s develop within 2–6 weeks under suitable environmental conditions (i.e., 28–33 °C) (Otranto et al., 2019). After ingestion of larvated eggs, the L3s invade the intestinal wall and, for *T. canis* and to a lesser extent also *T. leonina*, migrate to the liver and lungs («hepato-pulmonary migration»), reach the trachea and are swallowed and develop to adult males and females within 21–29 days in the small intestine (Otranto et al., 2015). In addition, *T. canis* may be transmitted also through transplacental and transmammary routes due to the arrest of some larvae in somatic tissues (Schnieder et al., 2011), which are then reactivate in the bitch during the last trimester of pregnancy when they are transmitted to the litter in utero. *Toxocara canis* can cause serious disease in puppies with entailing respiratory signs, general failure to thrive and intestinal disorders (Deplazes et al., 2016), whereas *Toxascaris* infection in adult dogs is usually well-tolerated (Macpherson et al., 2013; Eslahi et al., 2020), but may cause pica, digestive disturbances and reduced growth in juveniles (Traversa et al., 2012). From an epidemiological perspective, animal hosts parasitized by adult worms in their gut, can shed parasite eggs, hence being considered as a source for dissemination of the infection (Eslahi et al., 2020). A thorough understanding of the epidemiology and risk factors associated with infection is required for defining effective strategies to control the infection in dogs also preventing the risk of human infection (McNamara et al., 2018; Lejeune et al., 2020; Overgaauw et al., 2020) as recommended by the European Scientific Counsel Companion Animal Parasites (ESCCAP). According to ESCCAP guidelines, a regular coprological examination, at least one-two times a year for dogs that live indoor and four times a year for dogs that live outside or have contact with other animals or frequent places at risk (e.g., parks, sandpits, playgrounds, etc.), is a good alternative to standard deworming advice (ESCCAP, 2021). Moreover, a lifelong control strategy is suggested, because not only puppies are exposed to parasites, but the risk continues

throughout the whole lifetime (ESCCAP, 2021). For this purpose, an accurate diagnosis is crucial for an appropriate treatment. Routine diagnosis of patent infections with ascarids is mostly carried out by egg identification in feces using copromicroscopic techniques (Deplazes et al., 2011). Furthermore, to differentiate the eggs of *T. canis* and *T. cati* that are not clearly distinguishable by microscopy (Uga et al., 2000). A PCR targeting the second internal transcribed spacer (ITS-2) of ribosomal DNA (rDNA) was developed for species identification (Jacobs et al., 1997; Fahrion et al., 2011). Epidemiological surveys on endoparasites prevalence in dogs have been conducted in many countries, the reported infection rates, however, depend on the country, the age of the animals, the lifestyle of the animals (e.g., stray, kennelled or owned dogs), and the fecal examination method used (Overgaauw et al., 2020). Recently, Rostami et al. (2020_a , 2020_b) assessed the global prevalence of *T. canis* (Rostami et al, 2020_b) and *T. leonina* (Rostami et al., 2020_a) infections in dogs, resulting in an overall prevalence of 11,1% and of 2,9%, respectively. Similar prevalence values were reported in Europe with values of 10,8% and 2,0% for *T. canis* and *T. leonina*, respectively (Rostami et al., 2020_a ; $2020_{b)}$ The same scenario was revealed during a nationwide survey in Italy on endoparasites of dogs showing a prevalence of 9,0% of *T. canis* and 1,0% of *T. leonina*, respectively (Brianti et al., 2018). A study conducted in the city of Naples (southern Italy) on canine fecal contamination revealed a prevalence of 0,7% for *T. canis* and 1,4% for *T. leonina* whereas high prevalence values for *T. canis* (14,8%) were found in kennel dogs of the Campania region of southern Italy (Rinaldi et al., 2006;2015). A 5-year retrospective analysis of ascarid infections (*T. canis* and *T. leonina*) in dogs from southern Italy was performed to update the epidemiological scenario of these parasites and to identify the risk factors which may favour these infections in animals in this study area.

2.3 Materials and methods

2.3.1 Study Design

A retrospective study was conducted reviewing the data from 5-year of routine diagnostic activity (2015–2020) performed at the Laboratories of Parasitology and Parasitic Diseases at the Department of Veterinary Medicine and Animal Production (PAR-UNINA), at the Centre of Monitoring of Parasitosis (CREMOPAR) and at the Veterinary Hospital of Naples «Frullone» (VHFrullone),

University of Naples Federico II, Italy. A total of 8,149 dogs from southern Italy, referred to our labs for routine copromicroscopic analysis, was considered. The most part of the dogs was apparently healthy (90%), while in few cases showed abnormalities in fecal consistency or diarrhoea (10%).

2.3.2 Copromicroscopic analysis

Each canine fecal sample was screened for intestinal parasites (helminths and protozoa) using the FLOTAC dual technique with sodium chloride (specific gravity, s.g. = 1.20) and zinc sulphate (s.g. = 1,20) as flotation solutions and a detection limit of 2 eggs/oocysts/cysts/ larvae per gram (EPG/OPG/CPG/LPG) of feces (Cringoli et al., 2010). Furthermore, at random, a total of 500 fecal samples, positive and negative for ascarid eggs, were tested also with the Mini-FLOTAC technique (Cringoli et al., 2017) using sodium chloride and a detection limit of 5 EPG to compare the performance of the two flotation-based techniques on the detection of these parasites.

2.3.3 Statistical analysis

2.3.3.1 Risk factors analysis and evaluation of prevalence per year

Dogs were classified into five age groups: puppies (less than 1 year); young (1–3 years); adult (4–6 years); old (7–10 years); and very old (> 10 years). Furthermore, the dogs belonged to 98 breeds were classified into three groups (small, medium and large) based on their breed size. The prevalence and the 95% confidence intervals (95%CI) were calculated using the free online software «Sample Size Calculator» (Creative Research Systems, CA, USA). The positivity for *Toxocara* and *Toxascaris* eggs were analysed in association with the variables (housing, sex, age and dog breed size) using the Chi-square test and Logistic Regression analysis. Moreover, the association of the factor «year of monitoring» with positivity was also evaluated. The association was considered significant at $P < 0.05$. All statistical analyses were performed using the SPSS® software (version 22,0, IBM Corporation, Armonk, USA).

2.3.3.2 EPG evaluation

The arithmetic mean EPG, min and max values were calculated for each parasite. Differences between the EPG obtained for each parasite and the housing (indoor/ outdoor) were analysed using

one-way ANOVA and Kruskal–Wallis test, for both *T. canis* and *T. leonina*. Differences were considered significant at $P < 0,05$. All statistical analysis were performed using SPSS® software (version 22,0, IBM Corporation, Armonk, USA).

2.3.3.3 K-agreement

The Cohen's κ value was calculated to evaluate the agreement between the FLOTAC and the Mini-FLOTAC techniques in detecting ascarid eggs. The κ measure was interpreted as follows: 0, no agreement; 0.01–0.20, poor agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement; and 0.81–1.0, nearly perfect agreement (Thrusfield et al., 2007). All statistical analyses were performed using the SPSS® software (version 22,0, IBM Corporation, Armonk, USA) and the significance level was set at $P < 0.05$.

2.4. Results

Results Of the 8,149 dog samples analysed, 9.2% (95%CI= 8.6–9.8) resulted positive for *T. canis* while 0.5% (95%CI= 0.4–0.7) resulted positive for *T. leonina*. Co-infections with *T. canis* and *T. leonina* were found in 0.1% of dogs (95%CI= 0.0–0.1). Furthermore, co-infections with other helminths and protozoa were found (data not shown). In addition, different morphotypes of *T. canis*. eggs were found (freshly shed, embryonated, larvated) (Figure 2.1). The samples analysed per year and positivity was reported in Table 2.1. A statistically significant decreasing ($P< 0.001$) overall prevalence both for *T. canis* and *T. leonina* was registered during the years of monitoring.

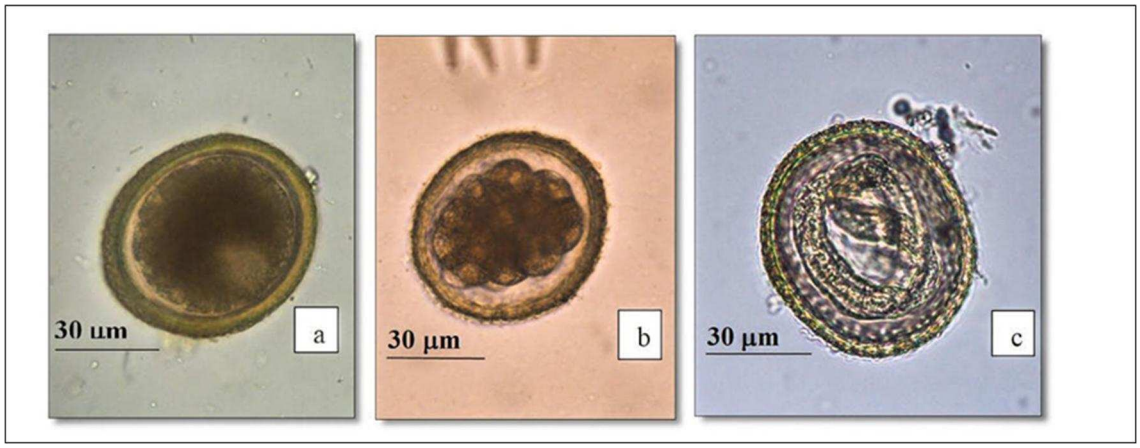

Figure 2.1 Different morphotypes founded of *T. canis* eggs: a) classical form of the egg; b) embryonated egg; c) larvated egg. (40X, magnification).

Table 2.1: Number of analysed dogs, positive dogs and 95 % confidence of interval (95 % CI) per year.

Year	Positive samples/total samples		Prevalence (%) (95 % CI)	
	T. canis	***T. leonina***	***T. canis***	***T. leonina***
2015	116/957	10/957	12.1 (10.2–14.4)	1.0 (0.5–2.0)
2016	138/1170	9/1170	11.8 (10.0–13.8)	0.8 (0.4–1.5)
2017	142/1267	13/1267	11.2 (9.6–13.1)	1.0 (0.6–1.8)
2018	154/1467	6/1467	10.5 (9.0–12.2)	0.4 (0.2–0.9)
2019	112/1583	3/1583	7.1 (5.9–8.5)	0.2 (0.1–0.6)
2020	85/1705	3/1705	5.0 (4.0–6.2)	0.2 (0.1–0.6)

2.4.1 Analysis of risk factors

A total of 4,618 dogs analysed were male. while 3,531 were female. Age of dogs ranged from 1 month to 23 years (median =16 months). Statistical analyses showed positivity to *T. canis* and *T. leonina* significantly ($P < 0.001$) associated with dogs housed outdoor (i.e., that lived in garden or in kennel). Moreover, the positivity for *T. canis* was significantly associated ($P < 0.001$) with age (i.e., puppies) and breed size (i.e., medium). Detailed results according to the different variables considered (housing, sex, age and dog breed size) are reported in Table 2.2. The logistic regression identified a strong association between positivity to *T. canis* and the variables: «outdoor», «puppies», and «dog breed size medium and large». In contrast, the variable «old» was associated with a low *T. canis* prevalence. The Odds Ratios and related P values are reported in Table 2.3 Regarding *T. leonina*, the logistic regression indicates only a strong association with outdoor housing (Odds Ratio = 6.60; P = 0.000).

Table 2.2 Number of dogs analysed, positive dogs, prevalence and 95% confidence of interval (95%CI) for each variable considered in statistical analyses for *T. canis* and *T. leonina* infection. Moreover, the *P value* for each variable is reported.

		T. canis		***T. leonina***	
	No. analysed	**Positive**	**% (95%CI)**	**Positive**	**% (95%CI)**
Life habit					
Indoor	6,972	311	4.5 (4.0-5.0)	21	0.3 (0.2-0.5)
Outdoor	1,177	436	37.0 (34.3-39.9)	23	2.0 (1.3-3.0)
P value			*P* < 0.001		*P* < 0.001
Sex					
Male	4,618	429	9.3 (8.5-10.2)	26	0.6 (0.4-0.8)
Female	3,531	318	9.0 (8.1-10.0)	18	0.5 (0.3-0.8)
P value			*P*= 0.511		*P*= 0.972
Age					
Puppies(<12 months)	2,846	458	16.1 (14.8-17.5)	20	0.7 (0.4-1.1)
Young (1-3 years)	1,569	172	11.0 (9.5-12.6)	16	1.0 (0.6-1.7)
Adult (4-6 years)	2,676	65	2.4 (1.9-3.1)	6	0.2 (0.1-0.5)
Old (7-10 years)	683	38	5.6 (4.0-7.6)	2	0.3 (0.1-1.2)
Very old	375	14	3.7 (2.1-6.3)	0	0 (0.0-1.3)
P value			*P* < 0.001		*P*= 0.786
Dog breed size					
Small	1,713	42	2.5 (1.8-3.3)	4	0.2 (0.1-0.6)
Medium	4,593	618	13.5 (12.5-14-5)	34	0.7 (0.5-1.1)
Large	1,845	87	4.7 (3.8-5.8)	6	0.3 (0.1-0.7)
P value			*P* < 0.001		*P*= 0.394

Table 2.3 Results of the logistic regression analysis.

Variable	Standard Error	Odds ratio	*P value*
Outdoor	0.099	9.27	0.000
Puppies	0.153	1.39	0.031
Old	0.228	0.56	0.007
Dog breed size medium	0.160	2.17	0.000
Dog breed size large	0.192	1.67	0.007

2.4.2 EPG evaluation

Regarding EPG, the higher means were obtained for *T. canis* and *T. leonina* in outdoor dogs. All the results obtained are reported in Table 2.4 (min, max and mean EPG) for each parasite and each housing (i.e., outdoor and indoor). The ANOVA test showed that the *T. canis* mean EPG of outdoor dogs was statistically different from the mean EPG revealed in indoor dogs (P 0.05) for *T. leonina* between medians of EPG in outdoor and indoor dogs. The overall samples were classified in three EPG classes: 2–100, 101–500 and >500. Higher number of fecal samples analysed showed EPG from 2 to 100 for both *T. canis* (73.6; 95%CI = 70.3–76.7) and *T. leonina* (80.0; 95%CI= 64.3–89.7), as reported in Figure 2.2. The overall mean EPG decreased both for *T. canis* and *T. leonina* during the years of monitoring as shown in Figure 2.3.

Table 2.4 Min-max EPG value and mean for *T. canis* and *T. leonina* in two different life habit groups.

	***T. canis* EPG**		***T. leonina* EPG**	
Life habit	**mean**	**min-max**	**mean**	**min-max**
Outdoor	1,118.5	2-34,600	1,364.8	4-22,640
Indoor	48.0	4-1,174	24.4	10-50.0
Overall	494.1	2-34,600	664.1	4-22,640

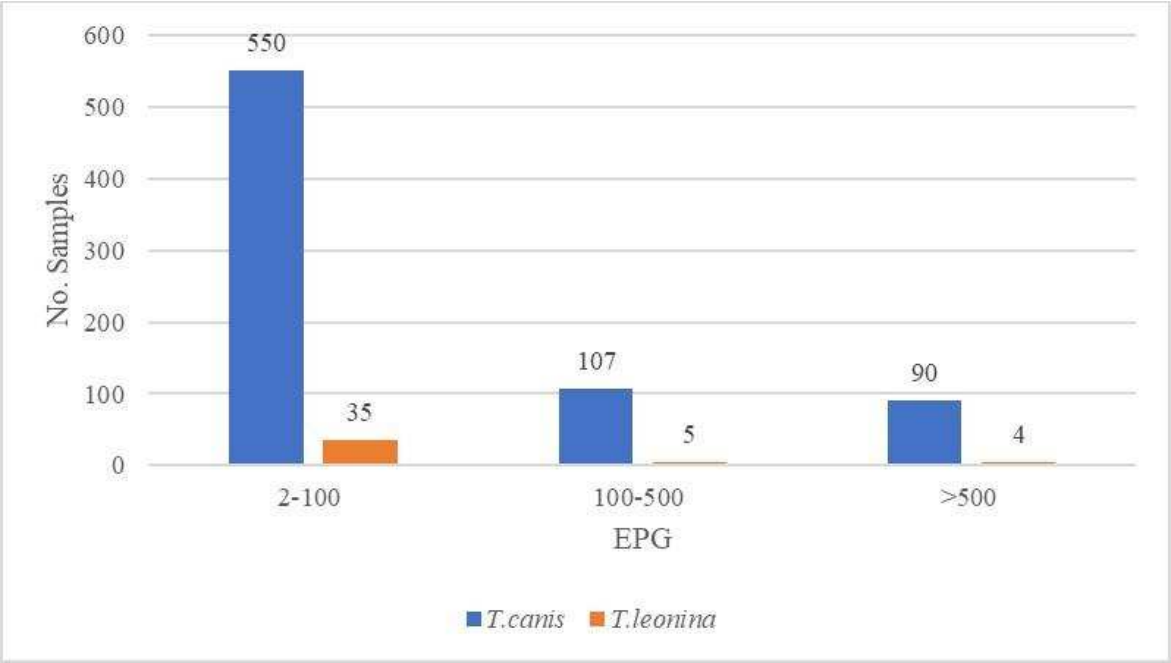

Figure 2.2 Number of positive samples for each EPG class (2-100, 100-500 and >500) for *T. canis* and *T. leonina.*

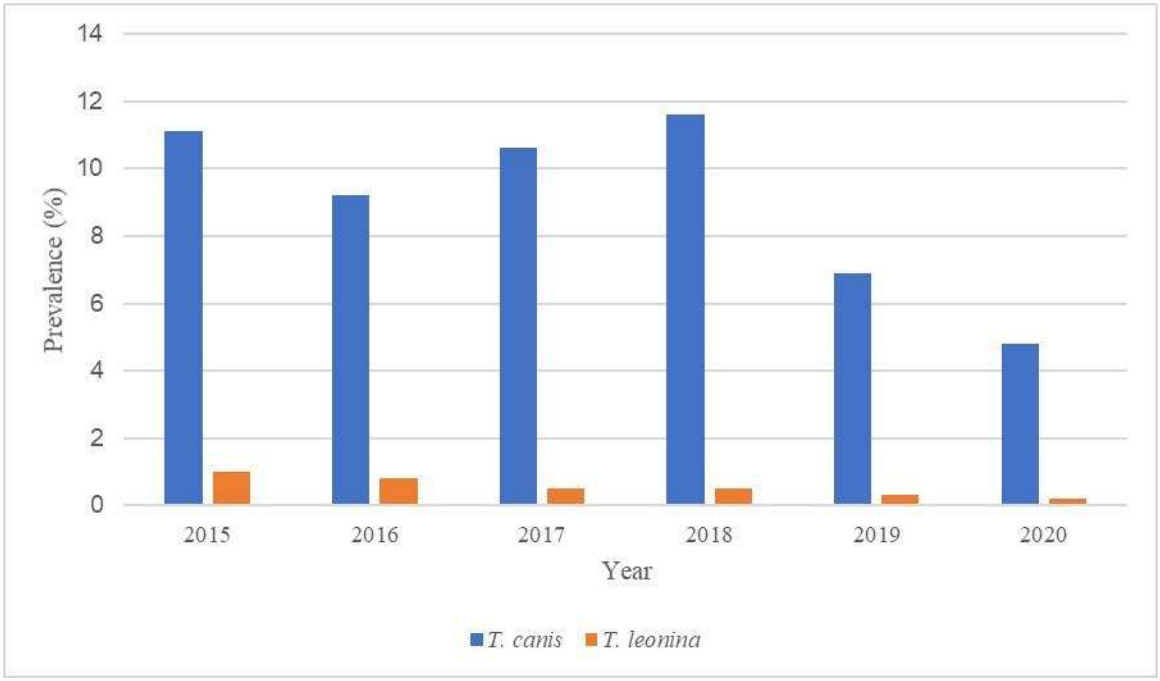

Figure 2.3 Prevalence of *T. canis* and *T. leonina* for each year of monitoring.

2.4.3 Comparison of results obtained with FLOTAC and Mini-FLOTAC

Of the 500 fecal samples analysed by FLOTAC and Mini-FLOTAC, 146 resulted positive for *T. canis* and 8 for *T. leonina* with FLOTAC, while 144 were positive for *T. canis* and 8 for *T. leonina* with Mini-FLOTAC. Therefore, a nearly perfect k agreement ($k = 0.99$, $P < 0.001$) was found. The mean EPG by FLOTAC and Mini-FLOTAC for *T. canis* was 122.0 (min-max EPG: 2–6,800) and 120.0 (min-max EPG: 5–6,725) respectively, with no statistically significant difference ($P > 0.05$). For *T. leonina* the mean EPG was 202.0 (min-max EPG: 10–4,528) and 195.0 (min-max: 10–4,500) respectively, with no statistically significant difference ($P > 0.05$).

2.5 Discussion and conclusions

The results of this 5-year retrospective study show prevalence values of *T. canis* and *T. leonina* in dogs in southern Italy very similar to the national prevalence reported by Brianti et al. (2018) (i.e., 9.0% for *T. canis* and 1.0% for *T. leonina*). In agreement with other studies, *T. canis* resulted most prevalent in puppies, due to higher number of sources (e.g., transplacental and transmammary transmission) and of risk factors (e.g., puppies stay close with other dogs; immune system of young dogs is not completely developed) of infections in puppies than in other age categories (Overgaauw et al., 2013; Baneth et al., 2016; Otranto et al., 2019;Nagamori et al., 2020). This is of great importance considering that many authors have shown that the puppies could represent a potential risk of infection not only for other dogs, but also for humans. Indeed, in some studies on dogs' fur a higher prevalence of subjects with eggs of *T. canis* was shown, representing another route of transmission through contact with coat for humans, as reported in a recent systematic review on this topic (Maurelli et al., 2019). As for the logistic regression analysis, the age class «puppies» was associated with the positivity to *T. canis*, while the age class «old» was associated with a low positivity to the parasite. For both *T. canis* and *T. leonina* a higher prevalence was found in dogs that lived in garden or in kennel than those with an indoor lifestyle. Living outdoors or having access to a garden seems to be a risk factor for ascarid infections in dogs, as also shown for cats (Zanzani et al., 2014; Nijsse et al., 2016). It could be due to the higher probability of outdoor dogs to become infected by capturing paratenic hosts (e.g., rodents and birds), especially those without supervision, or by ingestion of infectious roundworm eggs from the environment. It is well recognized, in fact, that public parks, playgrounds, sandpits etc. can be an important source of infection for both dogs and

humans In Italy, environmental contamination with *T. canis* eggs was evaluated in different cities, with a prevalence of 33.6% in the Marche region, 7.0% in Milan, 3.6% in Messina and Teramo, 2.5% in Bari, 1.9% in Rome, 0.7% in Naples and in Padua and 0.5% in Alghero, as reviewed by Traversa et al. (2014). Regarding EPG, higher means of roundworms were found in outdoor dogs than indoor for both parasites, contributing to the spread of infection and environmental contamination, also on vegetables for human consumption (Reperant et al., 2009; Guggisberg et al., 2020). Some studies investigating lettuce purchased in farmer markets and supermarkets have been conducted to assess the environmental contamination with the fecal matter of canids, cats and other hosts. The results obtained confirmed that stray dog, cats and wild animals can spread in the environment resistant parasite stages (e.g., *Toxocara* spp. eggs) that can contaminate food, water and soil, representing a risk for humans (Guggisberg et al., 2020). The environmentally spreading of *Toxocara* eggs by dogs can represent a problem also for wild carnivores, representing a connection between domestic and wildlife cycle, complicating the epidemiological scenario of this parasite (Deplazes et al., 2011). However, in our retrospective study a small reduction during the last two years for both the parasites was found. This decreasing may be due to a monitoring and control plan (at least one-two times a year for dogs that live indoor and four times a year for dogs that live outside, as suggested by ESCCAP), named «FLOTAC and PETS», started in 2014 by the PAR-UNINA-CREMOPAR, that involved an increasing number of veterinary clinics, veterinary practitioners and the VH-Frullone. This project implemented the awareness of veterinarians concerning the parasite control practices of dogs and cats and the importance of using an accurate copromicroscopic diagnosis before treatment (Maurelli et al., 2014_a) Indeed, the use of specific, sensitive, precise and accurate quantitative copromicroscopic techniques, as the FLOTAC and Mini-FLOTAC, can be very useful for a reliable diagnosis and an effective treatment (Cringoli et al., 2010, 2017; ESCCAP, 2021). These techniques have been successfully used in different studies on roundworms detection in pets (Maurelli et al., 2014_b) as well as in this study. A nearly perfect k agreement was found between the FLOTAC and Mini-FLOTAC techniques and no statistically significant difference was found between the mean EPG obtained. Therefore, an easy-to-use Mini-FLOTAC technique can be useful for a rapid, reliable and point-of-care diagnosis of roundworms in pets, also in labs or ambulatories with basic equipment, because at difference of FLOTAC, Mini-FLOTAC doesn't require centrifuge or other specific instruments, but only the microscope (Cringoli et al., 2017). However, this study shows some limitations. Indeed, we didn't perform a pre-programmed sampling considering the ESCCAP classification: dogs that live indoor (group A), dogs that live outdoor (group B), dogs that live outdoor and eat prey animals (group C), dogs that goes outdoor to hunt without supervisors (group D), because we used findings of our diagnostic routine, analyzing them by a retrospective analysis. Moreover, we

didn’t evaluate the difference of prevalence or intensity of egg shedding, between dogs considered healthy and those with abnormal fecal consistency / diarrhea. Finally, we didn’t perform either an accurate morphological or molecular differentiation between *T. canis* and *T. cati* eggs, because usually during our diagnostic routine we don’t use further analyses above the copromiscopic techniques (Jacobs et al., 1997; Fahrion et al., 2011). Since the environmental control of roundworms is difficult, due to different ways of transmission for dogs, the ESCCAP in Europe and the Companion Animal Parasite Council in USA recommend a regular diagnosis and treatment (CAPC, 2012; ESCCAP, 2021). Moreover, we have seen that following these suggestions, a decreasing of *Toxocara* infections was registered in our region during last years. For these reasons, a standardization and harmonization of diagnostic tools and treatment protocols could be very important.

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Chapter 3

Epidemiological and molecular updates on hookworm species in dogs from southern Italy

Illiano S, Ciuca L, Maurelli MP, Pepe P, Caruso V, Bosco A, Pennacchio S, Amato R, Pompameo M, Rinaldi L. Epidemiological and molecular updates on hookworm species in dogs from southern Italy. BMC Vet Res. 2023: 19(1),204.

3.1 Abstract

The zoonotic hookworms *Ancylostoma caninum* and *Uncinaria stenocephala* are widespread soil-transmitted helminths in dogs in Europe. Given the veterinary and public health importance of hookworms in dogs and the recent changes in the molecular epidemiology of some species, there is a need to continuously monitor the epidemiological and molecular prevalence of these parasites also at the "local" level. The present study aimed to update the epidemiological scenario of hookworm infections in both owned and stray dogs in southern Italy and to discriminate between different hookworm species (*A. caninum* and *U. stenocephala)* through molecular analyses. For this purpose, a retrospective analysis was performed over 10 years (2011-2021), including a total of 7008 owned dogs and 5642 stray dogs referred to our laboratory for copromicroscopic examinations. Moreover, 72 fecal samples, from dogs naturally infected by hookworms, were used to discriminate between *A. caninum* and *U. stenocephala* using two PCR protocols. Prior to molecular analyses, a subsample of 40/72 positive fecal samples were used for morphometric investigations on hookworm eggs.

The results of the ten-year retrospective analysis (2011-2021) showed an overall prevalence of hookworm infection of 9.16%, specifically 5.1% in owned dogs and 14.2% in stray dogs. Logistic regression showed a significant association between positivity to hookworms and the variable "puppies" both in stray (13.84%; OR=2.4) and owned (7.07%; OR=2.2) dogs. The results of molecular analyses showed that positivity was confirmed only in 21/72 samples, specifically, 6 samples using protocol A and 19 with protocol B. Sequencing revealed 15 samples positive to *U. stenocephala* and 6 to *A. caninum*.

The findings of this study showed a high prevalence of hookworm infections in dogs in southern Italy and update the epidemiological scenario of the last decade. Moreover, the results of the study revealed the first identification of hookworm species in dogs in Italy by molecular studies, highlighting that *U. stenocephala* is more prevalent than *A. caninum.*

3.2. Introduction

Among the intestinal parasites that infect dogs, the hookworms *Ancylostoma caninum* and *Uncinaria stenocephala* play an important role in the health and welfare of canine populations worldwide, as well as in public health, due to their zoonotic potential (Mircean et al., 2017; Kostopoulou et al., 2017; Raza et al., 2018). Both pathogens might cause *larva migrans* syndrome or "ground itch" in humans (Bowman et al., 2010; Del Giudice et al., 2019). Moreover, the possibility of causing eosinophilic enteritis in human hosts with diarrhea, abdominal pain and weight loss has also been described (Traub et al., 2021).

The main source of infection in dogs is the soil contaminated with eggs excreted in dog feces, where larvae hatch and develop to the infective stage L3 at suitable temperatures and humidity rates. Infection occurs mainly by percutaneous penetration of L3 or their ingestion *per os* (Epe et al., 2009). In addition, hookworms are known to cause anaemia and hypoproteinemia in dogs, especially in puppies (Bajer et al., 2011; Traversa et al., 2012).

Hookworms are common parasites in dogs and wild carnivores throughout the world, with prevalence values varying by climatic regions and dog population. In Europe, prevalence rates of hookworm infections in dogs range from 1.2% to 34% (Bajer et al., 2011; Lledò et al., 2015; Wright et al, 2016; Ilic et al., 2021; Drake et al., 2022). In particular, in Italy, hookworm infections have been reported in many studies, with high prevalence rates in stray (67.7%) and owned dogs (18.9%) in the southern area (Rinaldi et al., 2012; Brianti et al., 2018), followed by prevalence rates between 0-9.3% in stray dogs and 0.4-3.6% in owned dogs in the northern area (Simonato et al., 2015; Traversa et al., 2017; La Torre et al., 2018; Simonato et al., 2020) . However, there are few studies to support the identification of hookworm species in dogs around the world. For example, in Central Europe, according to a recent study, *U. stenocephala* infection seems to be more prevalent than *A. caninum* infection in dogs (Strkolcova et al., 2022). On the other hand, in Africa (Mulinge et al., 2019; Merino-Tejedor et al., 2019; Ngcamphalala et al., 2019), Asia (Ng-Nguyen et al., 2015; George et al., 2016; De Silva et al., 2022) and Brasil (Oliveira-Arbex et al., 2017), the occurrence of *A. caninum* was reported with higher frequency than *U. stenocephala* species. Moreover, the data also revealed mixed infections with other hookworm species such as *Ancylostoma ceylanicum* or *Ancylostoma braziliense*.

Hence, considering the above information and the impact of hookworm infections on veterinary and public health, it should be imperative to continuously monitor the prevalence of hookworms in dogs in Europe and Italy. In addition, the fact that there are few studies (Gorski et al., 2006 ; Strkolcova et al., 2022;) reporting the differentiation between hookworm species in dogs in Europe, but no study

conducted so far in Italy, demands future research to estimate and evaluate the zoonotic aspects of hookworm infections. Therefore, the present study aims to update the epidemiological scenario of hookworm infections in owned and stray dogs in southern Italy by performing a retrospective analysis of prevalence over ten years (2011-2021) and a molecular study to identify *A. caninum* and *U. stenocephala*.

3.3 Materials and Methods

3.3.1 Study design

The study design is summarized in Fig. 3.1. To update the epidemiological scenario of hookworm infections in dogs in southern Italy, two objectives were pursued. The first objective was to determine the prevalence and analyze the risk factors for hookworm infections in dogs in a Mediterranean area. For this purpose, a retrospective study was conducted analysing ten-years (January 2011-December 2021) of parasitological data from routine diagnostics in dogs from the Campania region (southern Italy). A total of 7,008 owned dogs (males= 4,030; females = 2,978) and 5,642 stray dogs (males= 3,059; females= 2,583) were referred to our laboratories (Parasitology Service of the Veterinary Teaching Hospital, University of Naples Federico II, Italy) for copromicroscopic examination. All fecal samples were analyzed using the FLOTAC technique (Cringoli et al., 2010, 2011). Moreover, data on dog's age, gender, lifestyle (stray/owned dogs) and breed size were collected.

The second objective was to identify hookworm species in dogs in the study area, by morphometric analysis of eggs and confirmation by molecular tests. To this end, all fecal samples collected in 2022 (total number= 1548) that tested positive for hookworms using the FLOTAC technique (N=72) were tested by molecular analyses, using two different protocols: i) protocol A as described by Traub et al., (2004), and ii) protocol B with some modifications of the previous protocol. In addition, a subsample of 40 of the 72 positive fecal samples was used for morphometric studies of hookworm eggs prior to molecular analyses.

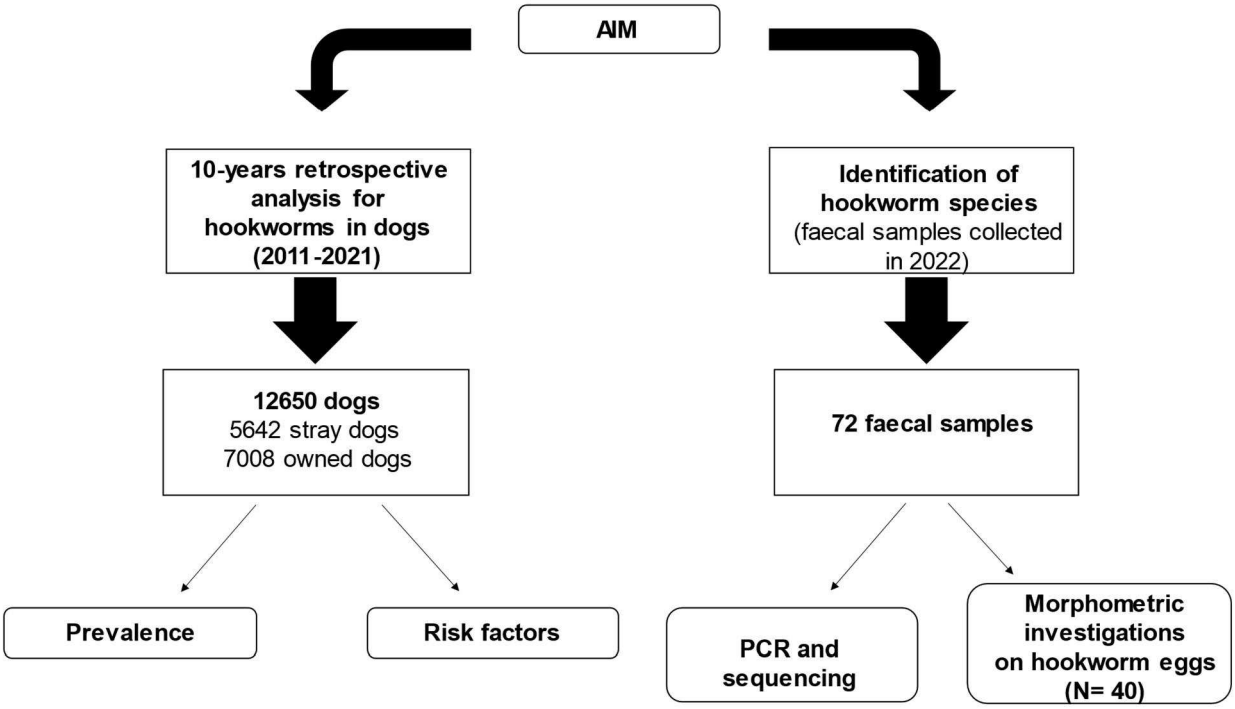

Figure 3.1. Study design.

3.3.2 Laboratory analysis

3.3.2.1 Coprological analyses

Each canine fecal sample (collection of three consecutive fecal samples per animal) was tested for intestinal parasites (helminths and protozoa) using the FLOTAC dual technique (Cringoli et al., 2010, 2011) with sodium chloride (specific gravity, s.g. = 1.20) and zinc sulphate (s.g. = 1.20) as flotation solutions. The detection limit (analytic sensitivity) was 2 eggs/oocysts/cysts/larvae per gram (EPG/OPG/CPG/LPG) of feces.

3.3.2.2 Morphometric analysis of hookworm eggs, DNA extraction and molecular analysis

All samples used for molecular analyses (N=72) were previously stored at -20°C. Therefore, morphometric analyses (major axis, minor axis and perimeter) of 20 hookworm eggs were performed for each of the 40 fecal samples using LAS X Leica software (version 5.0.2, 2021). In addition, the fecal samples that were morphometrically analysed and the ones (N=32) that were not possible to perform the morphometric tests (reduced amounts of fecal samples) were then subjected to molecular

analyses. Therefore, 72 fecal samples were subjected to DNA extraction using the Fast DNA stool kit (Qiagen, Germany) according to the manufacturer's instructions. Specific primers were used for amplification of the ITS1, 5.8S and ITS-2 regions according to Traub et al. (2004) as follows: forward primer RTGHF1 (5′-CGTGCTAGTCTTCAGGACTTTG-3′) and reverse primer RTGHR1 (5′-CGTTGTCATACTAGCCACTGC-3″) for the detection of *Ancylostoma* spp (680 bp region of *A. caninum* and *U. stenocepahala*). In order to confirm the amplification of *U. stenocepahala*, the purified PCR product of the adult was confirmed as *U. stenocephala* after sequencing the 680 bp region. Finally, the first PCR protocol (A) was performed according to Traub et al. (2004) and the second protocol (B) with some modifications of protocol A (Traub et al., 2004) as follows: in 25 µl volumes with the final mix containing 12.5 pmol of each primer, 1X buffer Mix (EmeralAmp® GT PCR Master Mix; Takara Bio Inc., Shiga, Japan), H_2O and 2 µl of DNA. Samples were heated at 96 °C for 10 min, 95 °C for 45 sec, 59 °C for 40 sec, 72 °C for 1 min for 10 cycles, then followed by 30 cycles of 95 °C for 45 sec, 58°C for 40 sec, 72 °C for 60 sec and 1 cycle of 72 °C for 7 min. The purified PCR products were sequenced in both forward and reverse directions and analyzed using Chromas 2.6.6 software. They were then compared with the NCBI/GenBank database using the Basic Local Alignment Search Tool (BLAST) and ClustalW software, for the discrimination between the two different hookworm species (*A.caninum* and *U. stenocephala*).

3.3.3 Statistical analyses

Statistical analyses were considered only for the dogs that resulted positive to the hookworm infections; other co-infections were excluded. Hence, dogs were classified into five age groups: puppies (up to 12 months); young dogs (13–36 months); adult dogs (37-72 months); old dogs (73–120 months); and very old dogs (> 120 months). In addition, dogs were classified into three groups (small, medium and large) based on the breed size and into five groups (A, B, C, D, E) based hookworms egg excretion (A= 2-50 EPG; B= 52-100 EPG; C=102-500 EPG; D= 502-1000 EPG; E ≥1002 EPG). Positivity for hookworms was analyzed in association with the above-mentioned variables (gender, age dog breed size and egg excretion) using univariate and logistic regression analysis. Data regarding the year of analysis were excluded from statistical analyses because the dog population of each year was variable. Moreover, data regarding previous antiparasitic treatment were very few and discontinuous so they were excluded from the statistical analyses as well.

Any association was considered significant at $P< 0.005$. The prevalence and the 95% confidence intervals (95 %CI) were calculated using the free online software «Sample Size Calculator» (Creative

Research Systems, CA, USA). All statistical analyses were performed using the SPSS® software (version 22,0, IBM Corporation, Armonk, USA).

3.4 Results

The results of the ten-year retrospective analysis (2011-2021) in southern Italy showed an overall prevalence of hookworm infection of 9.16% (1159/12650; 95% CI=8.67-9.68) in owned and stray dogs. More specifically, a prevalence of 5.1% (355/7008; 95% CI=4.57-5.61) was found in owned dogs with a mean egg shedding of 222.9 eggs per gram (EPG) of feces (2-6,440 EPG; SD=601.07) and 14.2% (804/5642; 95% CI= 13.35-15.20) in stray dogs with a mean EPG of 20.5 (2-556 EPG; SD=39.92). Prevalence values per year (2011-2021) of hookworm infection, both in owned and stray dogs are reported in Table 3.1. Co-infections with other helminths and protozoa (*Trichuris, Toxocara, Capillaria, Isospora, Giardia*) were also found (data not showed). Almost all dogs were apparently healthy (80%), while in a few cases abnormalities in fecal consistency such as diarrhea or the presence of blood (20%) were observed.

The results of the Chi-square test for the different variables considered (gender, age and dog breed size) are reported in Tables 3.2 and 3.3. Logistic regression revealed a significant association between positivity to hookworms and the variable "puppies" in both stray (13.84%; OR=2.4; 95%CI= 12.50-15.21; P= 0.004) and owned (7.07%; OR=2.2; 95%CI= 6.12-8.14 P= 0.000) dogs. Regarding the excretion of hookworm eggs in owned dogs, 193/355 (54.4%) were allocated in group A (2-50 EPG), 53/355 (14.9%) in group B (52-100 EPG), 69/355 (19.4%) in group C (102-500 EPG), 21/355 (5.9%) in group D (502-1000 EPG) and 19/355 (5.4%) in group E (≥ 1002 EPG); while, in stray dogs 759/804 (94.4%) were allocated in group A (P<0.005), 32/804 (3.9%) in group B, 11/804 (1.4%) in group C, 2/804 (0.3%) in group D and 0/804 in group E.

The results of molecular analyses showed that a total of 21/72 was confirmed with both protocols (A, B). Specifically, 6 samples were confirmed with protocol A and 19 with protocol B. In addition, only four samples were resulted positive at both PCR protocols (A, B) Sequencing revealed that 15 samples were identified as U. *stenocephala* (100% identity; MT345056) and 6 samples as *A. caninum* (100% identity; MT130933.1). Co-infections with the two hookworm species were not detected in any sample.

The results of morphometric analyses showed that 28/40 hookworm positive samples were similar to *U. stenocephala* (Fig. 3.2) (major axis of egg =80.532 ± 3.120 μm; minor axis=46.591 ± 3.691 μm; perimeter=214.477±3.703 μm) and 12/40 samples were similar to *A. caninum* (Fig. 3.3) (major axis

of eggs=66.305 ± 5.675 µm; minor axis=41.348 ± 4.033 µm; perimeter=175.375±6.029 µm) (Lucio-Forster et al., 2012).

Of the total of 40 fecal samples that were submitted to the morphometric analyses, only for 10 samples was possible to associate the result of molecular analysis, as follows:

2 samples with protocol A and 8 with protocol B. Finally, the molecular results of the samples confirmed by PCR and sequencing (N=10) agreed with the results of the morphometric analyses, i.e., *U. stenocephala* (n=9) and *A. caninum* (n=1).

Table 3.1 Results of prevalence (%) and 95 % confidence of interval (95 % CI) for hookworm infections in owned and stray dogs included in the study-period from 2011 until 2021.

Year of testing	Owned dogs		Stray dogs		Overall	
	Positive /total samples	Prevalence % (95% CI)	Positive /total samples	Prevalence % (95% CI)	Positive /total samples	Prevalence % (95% CI)
2011	13/171	7.60 (4.28-12.92)	104/848	12.26 (10.17-14.71)	117/1019	11.48 (9.62-13.64)
2012	9/270	3.33 (1.64-6.45)	88/624	14.10 (11.52-17.14)	97/894	10.85 (8.93-13.12)
2013	15/378	3.97 (2.32-6.60)	78/604	12.91 (10.40-15.91)	93/982	9.47 (7.75-11.52)
2014	13/472	2.75 (1.54-4.79)	96/630	15.24 (12.57-18.34)	109/1102	9.89 (8.22-11.84)
2015	22/707	3.11 (2.01-4.75)	40/266	15.04 (11.08-20.04)	62/973	6.37 (4.96-8.14)
2016	41/583	7.01 (5.15-9.50)	38/214	17.76 (13.02-23.69)	79/797	9.91 (7.97-12.25)
2017	130/1001	12.99 (11-15.26)	42/220	19.09 (14.25-25.04)	172/1221	14.09 (12.21-16.19)

2018	10/930	1.08 (0.55-2.04)	54/332	16.27 (12.55-20.78)	64/1262	5.07 (3.96-6.47)
2019	21/815	2.58 (1.64-3.98)	21/184	11.41 (7.37-17-13)	42/999	4.20 (3.08-5.69)
2020	36/522	6.90 (4.94-9.51)	118/817	14.44 (12.14-17.09)	154/1339	11.50 (9.87-13.36)
2021	45/1159	3.88 (2.88-5.20)	125/903	13.84 (11.69-16.31)	170/2062	8.24 (7.11-9.54)
Total	355/7008	5.1 (4.57-5.61)	804/5642	14.2 (13.35-15.20)	1159/12650	9.16 (8.67-9.68)

Table 3.2. Results of the positivity to hookworms (prevalence %, 95% confidence interval (95% CI), p-value) for the variables gender, age, breed size in all stray dogs included in the study (period 2011-2021).

Total stray dogs analysed =5642		**Hookworms**	
	No. analysed	**No. positive**	**Prevalence %, 95% CI, p-value**
Gender			
Male	2583	345	13.36 (12.08-14.74)
Female	3059	459	15 (13.77-16.33)
			P=0.078
Age			
Puppies (<12 months)	2428	336	13.84 (12.50-15.29)
Young (1–3 years)	1766	258	14.61 (13.01-16-36)
Adult (4–6 years)	745	124	16.64 (14.08-19.56)

Old (7–10 years)	577	72	12.48 (9.95-15.52)
Very old (>10 years)	126	14	11.11 (6.43-18.25)
			P = 0.004
Breed size			
Small	1457	206	14.14 (12.41-16.06)
Medium	3245	464	13.55 (12.43-14.75)
Large	940	134	14.26 (12.12-16.69)
			P=0.989

Table 3.3. Results of the positivity to hookworms (prevalence %, 95 % confidence interval (95% CI), p-value) for the variables gender, age, breed size in all owned dogs included in the study (2011-2021).

Total owned dogs analysed =7008		**Hookworms**	
	No. analysed	**No. positive**	**Prevalence %, 95 % CI, p-value**
Gender			
Male	2978	145	4.78 (4.14-5.72)
Female	4030	210	5.21 (4.55-5.95)
			P=0.519
Age			
Puppies (<12 months)	2576	182	7.07 (6.12-8.14)
Young (1–3 years)	1618	80	4.94 (3.96-6.15)
Adult (4–6 years)	2106	70	3.32 (2.62-4.20)
Old (7–10 years)	544	17	3.13 (1.89-5.06)
Very old (>10 years)	164	6	3.66 (1.50-8.15)
			P=0.000
Breed size			
Small	1440	84	5.83 (4.70-7.20)
Medium	5071	253	4.99 (4.41-5.63)

Large	497	18	3.62 (2.22- 5.7)
			P=0.137

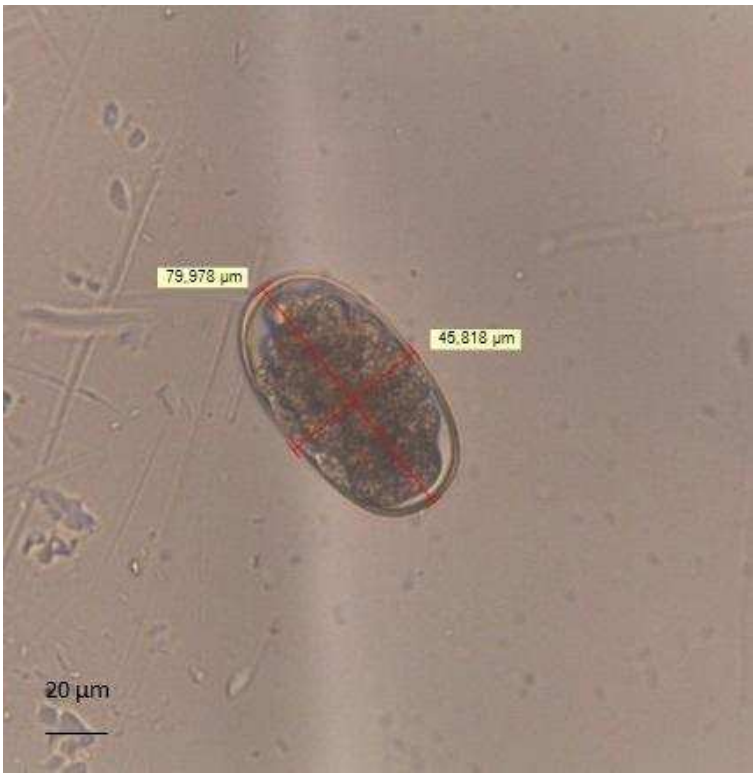

Figure 3.2. Egg of *U. stenocephala* (79 µm X 45 µm) with FLOTAC technique

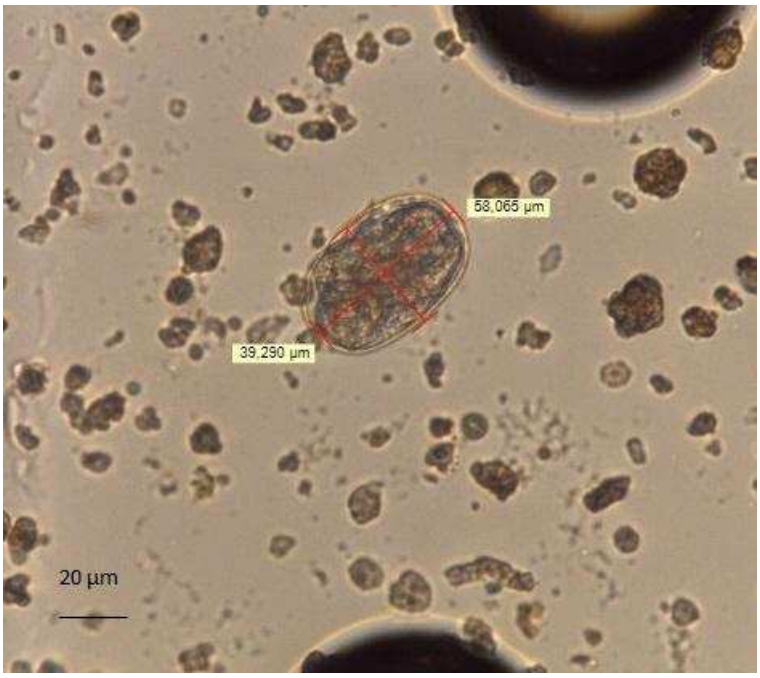

Figure 3.3. Egg of *A. caninum* (58 µm X 39 µm) with FLOTAC technique

3.5 Discussion and conclusions

Hookworms in dogs cause clinically relevant parasitic infections that are common worldwide, with prevalence rates varying by geographic area and dog population (Bajer et al., 2011; Lledò et al., 2015; Wright et al., 2016). In Asia, Africa, North America and Australia hookworm infection are widespread with different prevalence rates, e.g. 23-79% (Mahdy et al., 2012; Zibaei et al., 2020 ; Singh et al., 2022), 30-32 % (Mulinge et al., 2019; Merino-Tejedor et al., 2019; Idrissi et al., 2022), 10-91% (Kim et al., 2022) and 6-10% (Palmer et al., 2008; Massetti et al., 2022), respectively. The presence of hosts other than dogs, such as foxes and wolves, and climatic conditions favorable for larval development are important factors that could influence the distribution of hookworms in dog populations worldwide (Stronen et al., 2021). According to some studies conducted in Europe, the prevalence of hookworms ranges from 10 to 12% in foxes (Dybing et al., 2013; Lledò et al., 2015) and from 30% to 90% in wolves (Bindke et al., 2017; Al-Sabi et al., 2018), whereas a recent study on intestinal parasites in dogs in cities across Western Europe revealed a hookworm prevalence of 3.2% (Drake et al., 2022).

In Italy, data on the prevalence of hookworm infections in dogs vary widely from north to south and depend on the diagnostic method used, the study area (rural, urban, and suburban), the dogs' lifestyle, and the chemoprophylaxis regimes (Rinaldi et al., 2012; Simonato et al., 2015; Traversa et al., 2017; La Torre et al., 2018; Brianti et al., 2018; Simonato et al., 2020; Morelli et al., 2022). The data

obtained in the present study on the overall prevalence of hookworms (9.2%) in dogs in southern Italy is in line with the prevalence (11.6%) obtained in an harmonized survey recently conducted in Italy (Brianti et al., 2018). As expected, the prevalence was higher in stray dogs (14.2%) than in owned dogs (5.1%), confirming the data of previous studies from the same area (Rinaldi et al, 2012; Brianti et al., 2018). These findings showed that hookworms are still prevalent in dog populations in southern Italy, despite the increased awareness of veterinarians and owners promoted by the national and European guidelines of the European Scientific Counsel Companion Animal Parasites (ESCCAP) (ESCCAP, 2021).

Statistical results of the present study showed that positivity for hookworms was significantly related to the age of infected dogs with higher prevalence in puppies. According to Gates et al. (2009) (Gates et al., 2009), puppies are more likely to be infected through transmammary route during the lactation period in case of *A. caninum*. However, the possibility of transplacental transmission has not yet been described. In addition, the higher pathogenicity of hookworm species in dogs depending on the age of the dog must be considered. (Traversa et al., 2012). In fact, puppies affected by hookworm infection usually suffer from diarrhea and anemia and sometimes die in massive infections (Traversa et al., 2012).

The results of the present study showed variable values of hookworm prevalence per year (2011-2021), ranging from 1% to 13% in owned dogs and from 11% to 20% in stray dogs, but no temporal trend was observed. In contrast, retrospective studies conducted in the USA (in 2012-2018 and 2013-2017) (Drake et al., 2019; 2020) and in central Italy (in 2015-2020) (Morelli et al., 2022) showed an increasing trend in the prevalence of hookworm infections (2.02-2.96%, 1.17-2.77%, and 6.8-16.5%) over their study periods. This could be due to multiple factors such as: climatic conditions influencing parasite development in the environment, possible resistance to commercially available anthelmintics (Jimenez-Castro et al., 2019), the use of copromicroscopic techniques with different detection limits, and the different size of dog population used, all of which must be taken into account.

Although several studies on the prevalence of hookworms in dogs have been carried out in Italy, there are no data on the identification and discrimination of the different hookworm species based on molecular studies. In fact, PCR and sequencing are the only tools available so far for hookworm species identification (Strkolcova et al., 2022; De Silva et al., 2022). This is the first molecular identification of hookworm species in dogs in Italy showing that *U. stenocephala* is more prevalent than *A. caninum* in dogs in southern Italy. It is interesting to note that *U. stenocephala* occurs in regions where the climate is not optimal for its development (Strkolcova et al., 2022), such as the Mediterranean region. It is likely that our findings are due to both climatic changes and increasing animal movements as a result of globalization. It should also be noted that in the present study, not

all the fecal samples which were positive to the hookworm eggs with the FLOTAC technique (N=72), were also positive to the PCR protocols (A and B) (N=21/72) used (Traub et al., 2004). Moreover, the DNA used for the PCR analyses was extracted from the fecal samples naturally infected with Ancylostomidae eggs and the positive control was extracted from the *Uncinaria stenocephala* adult. However, there are other molecular studies that showed a high prevalence rates of hookworm infection (Ng-Nguyen et al., 2015; Oliveira-Arbex et al., 2017; Mulinge et al., 2019; Merino-Tejedor et al., 2019), using the same PCR protocol as described in the present study (Ng-Nguyen et al., 2015; Oliveira-Arbex et al., 2017; Mulinge et al., 2019; Merino-Tejedor et al., 2019;). This may suggest that either the PCR protocols used in the present study are less sensitive, or that a different substrate for DNA extraction should be considered, e.g., L3 larvae instead of eggs in the fecal samples, as shown in another study (George et al., 2016; Merino-Tejedor et al., 2019). In addition, the low prevalence rates of hookworm infections obtained with PCR analyses, reported herein, are similar with what reported in another study in which was performed a different PCR protocol, but using fecal samples naturally infected with high EPG of hookworms (Fu et al., 2019; De Silva et al., 2022) . The reduced detection limit of the PCR perfomed in the present study could be explained by two hypotheses: the type of matrix used for the DNA extraction, but perhaps, also the load of eggs excreted by the dogs naturally infected by hookworms. Therefore, further studies are needed to improve the sensitivity of the PCR protocol for hookworm detection, by investigating the detection limit and the best type of sample to use (i.e feces with eggs, floated suspension with eggs, L3 larvae). The morphometric results obtained in this study agree with previous studies (Lucio-Forster et al., 2012) and were also confirmed by the molecular analyses (Strkolcova et al., 2022). However, using only morphometric analyses of hookworm eggs, it is not possible to discriminate between different hookworm species. One of the limits of this study is that only a few numbers of samples were used for the morphometric analysis; in addition, all measurements were performed on samples naturally infected by hookworms without using a positive control from experimental infection. However, differentiation of different hookworm species such as *A. caninum* and *U. stenocephala* could also be possible by identification of L3 (Gibbs et al., 1961; Hill et al., 1985) which was not performed in the present study.

In conclusion, the findings of this study revealed a high prevalence of hookworm infections in dogs in southern Italy and updated the epidemiological scenario of the last decade. This study was the first to identify hookworm species (*A. caninum* and *U. stenocephala*) in dogs in Italy through molecular studies. Further studies are needed, especially to differentiate hookworm species and to develop increasingly sensitive, accurate and point-of-care diagnostics to provide more effective surveillance tools for the protection of human and animal health.

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Chapter 4

Comparison of different molecular protocols for the differentiation of *Uncinaria stenocephala* and *Ancylostoma caninum* infection in dogs

Illiano S, Ciuca L, Bosco A, Rinaldi L, Maurelli MP. Comparison of different molecular protocols for the differentiation of *Uncinaria stenocephala* and *Ancylostoma caninum* infection in dogs. *Submitted to Veterinary Parasitology, 2024.*

4.1 Abstract

The present study aims to assess the performance of different molecular targets using various matrices of samples for differentiation of *Uncinaria stenocephala* and *Ancylostoma caninum* in hookworm infected dogs. To this end, the DNA extraction was performed on the following matrices of samples: (i) larvae of *U. stenocephala* obtained from experimentally infected dogs with *U. stenocephala* with different larvae counts per microliter (µl); (ii) pure *U. stenocephala* eggs suspension in distilled water with different egg counts per µl; (iii) spiked dog fecal samples with different *U. stenocephala* eggs per gram (EPG) of feces; (iv) feces from dogs naturally infected with hookworm eggs; (v) fecal suspension with hookworm eggs recovered from the FLOTAC apparatus. All the samples were tested with four different PCR protocols targeting specific regions for the detection of *A. caninum* and *U. stenocephala* as follows: Protocol A (ITS1, 5.8S, ITS2) and Protocol B (18S) for the detection of both species, Protocol C (ITS1) for the detection of *A. caninum* and Protocol D (ITS1) for the detection of *U. stenocephala*. The best results were obtained with DNA extracted from *U. stenocephala* larvae matrix obtained from experimentally infected dogs, showing a detection limit of 3.5 larvae/µl for the protocols A, B and D. A moderate correlation was found between the FLOTAC technique and PCR protocols B and D with respect to fecal samples from dogs naturally infected with hookworms. Indeed, PCR protocols B (18S) and D (ITS1) gave the best results for feces and fecal suspension from naturally infected dogs. However, all the PCR protocols used showed lower sensitivity than FLOTAC technique. In conclusion, our results showed that the choice of DNA extraction samples is crucial, as this affects the diagnostic sensitivity of the technique.

4.2 Introduction

The most common canine hookworms (Nematoda, Strongylidae) are *Ancylostoma caninum*, *Ancylostoma braziliense*, *Ancylostoma ceylanicum*, and *Uncinaria stenocephala*. (Shchelkanov et al., 2021; Merino-Tejedor et al., 2019). In Europe, *A. caninum* and *U. stenocephala* pose a growing concern to both canine health and public safety for their zoonotic risk (Bowman et al., 2010; Traversa et al., 2012; Del Giudice et al., 2019). Dogs and humans may become infected either orally or percutaneously, through the ingestion of the larvae (L3) in soil or via contaminated food or water (Bowman et al., 2019). Transmission by dogs that prey on paratenic hosts is also possible for *A. caninum* and *U. stenocephala*, while trans-mammary transmission in dogs only occurs for *A. caninum* (Bowman et al., 2019; Traversa et al., 2012; ESCCAP, 2021). Hookworms are distributed worldwide in dogs and wild carnivores with varying prevalence rates. Canine hookworm infection prevalence is extremely variable with range from 1.7% (Myskova et al., 2019) to 16.9% in Europe (Ilìc et al., 2021) and in Italy from 0.6% (La Torre et al., 2018) to 14.2% (Illiano et al., 2023), and dependent on the climatic region and dog population. Diagnosis of canine hookworm infections has traditionally relied on the identification of eggs isolated in fecal floatation and species-based identification by examining adult worms following necropsies or anthelmintic treatment (ESCCAP, 2021). In order to discriminate between the different species of hookworms, molecular investigations such as polymerase chain reaction (PCR) and sequencing are necessary (Traub et al., 2021). In fact, diagnosis of single and mixed infections with hookworms relies on molecular analyses through different protocols which amplify regions like 18S, ITS1, ITS2 in species including *A. caninum*, *U.stenocephala*, *A. braziliense*, *A. ceylanicum* in the canine population (Ngcamphalala et al., 2019; Wongwigkan et al., 2020; Massetti et al., 2020; Singh et al., 2022; Oliveira-Arbex et al., 2022). Moreover, in the detection of hookworm species, several molecular studies use different matrices for DNA extraction such as feces containing hookworm eggs or hookworm larvae from coproculture, with highly variable results despite the use of PCR protocols that amplify the same target region of DNA (Merino-Tejedor et al., 2019; Mulinge et al., 2019; Štrkolcová et al., 2022). However, in Europe there are few studies and in Italy there is only one that discriminate between the species of hookworms through molecular analysis, with different PCR protocols (Demkowska-Kutrzepa et al., 2018; Štrkolcová et al., 2022; Illiano et al., 2023) and where *U. stenocephala* is highly reported. Given the zoonotic significance of canine hookworm pathogens and the lack of accurate molecular tools to discriminate between the hookworm species, the evaluation of performance of molecular diagnostic tools is necessary not only for enhancing and updating epidemiological data, but also to achieve new

therapeutic and prophylactic strategies. Hence, the present study aims to assess the effectiveness of different molecular targets using various matrices of samples testing positive for hookworms for the differentiation between *U. stenocephala* and *A. caninum* infection in dogs.

4.3 Material and methods

4.3.1 Study design and preparation of matrices

This study was conducted from October 2022 to October 2023 and its experiment design is presented in Figure 4.1. Ethical approval for using fecal samples for research purposes was given by the ethics committee of the Department of Veterinary Medicine and Animal Production, University of Naples Federico II with file number 0010200.

The following matrices of samples were used to perform DNA extraction and PCR:

i) larvae of *U. stenocephala* obtained from experimentally infected dogs, using different numbers of larvae per microliter (µl) (**U. s1)**;
ii) pure *U. stenocephala* eggs suspension in distilled water, using different numbers of eggs per microliter (µl) (**U. s2)**;
iii) spiked dog fecal samples, using different *U. stenocephala* eggs per gram of (EPG) feces (**U. s3)**;
iv) feces from dogs naturally infected with hookworm eggs (**U. s4)**;
v) fecal suspension with hookworm eggs recovered from the FLOTAC apparatus (**U. s5).**

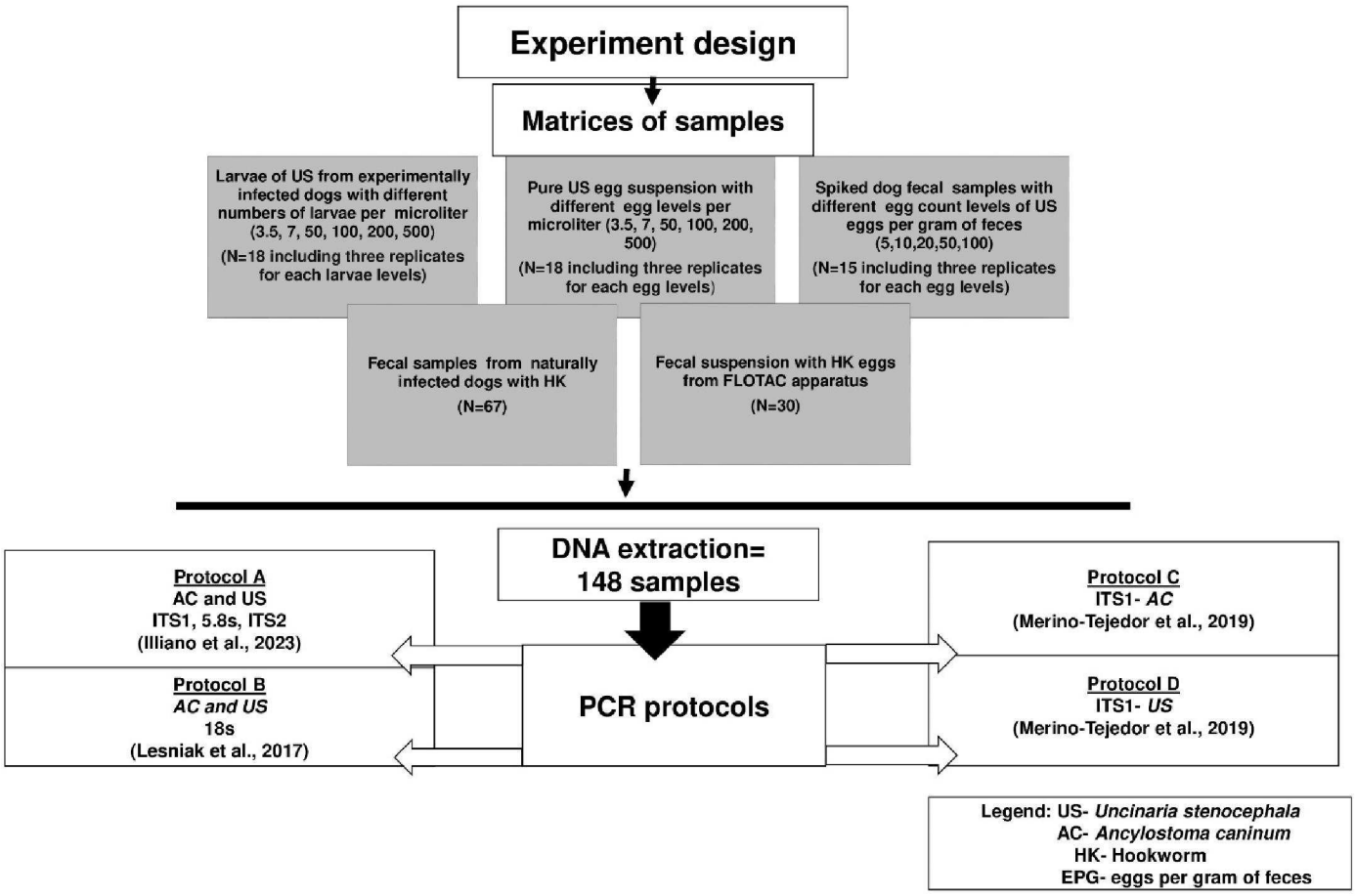

Figure 4.1. Experiment design.

4.3.1.1 Preparation of *U. stenocephala* larvae suspension from experimentally infected dogs (U. s1)

The samples of *U. stenocephala* larvae were obtained through experimental infection of dogs performed at the Clinvet International PTY LTD in South Africa. The samples were suspended in distilled water in a tube containing 5 ml and sent to the Department of Veterinary Medicine and Animal Production, University of Naples Federico II. The content of the tube was used to determine concentration levels by calculating the arithmetic mean of *U. stenocephala* larvae counts in 10 aliquots of 10 µl each (mean=26.87 larvae). Three replicates of each concentration (N=6) of larvae

suspended in distilled water (i.e., 3.5, 7, 50, 100, 200, 500) were performed. All the samples (No.=18) obtained were submitted to DNA extraction and PCR analyses.

4.3.1.2 Preparation of pure *U. stenocephala* eggs suspension from naturally infected dogs (U. s2)

The positive dogs naturally infected with *U. stenocephala* from a previous study (Illiano et al., 2023) were used as donors for the extraction of *U. stenocephala* eggs from feces, performing the egg recovery technique described by Bosco et al. (2018) with some modifications. In summary, 300 grams of fecal samples were diluted with tap water (ratio of 1:2) and then two sieves of different mesh sizes (63 and 38 μm) were employed to separate the eggs from the feces. The 38-μm sieve was washed with tap water to recover eggs and sedimented in a conical beaker for 4 min. The supernatant was eliminated, and the sediment obtained was a purified suspension of *U. stenocephala* eggs. The purified eggs obtained were suspended in distilled water in a tube containing 5 ml. The content of the tube was used to determine concentration levels by calculating the arithmetic mean of *U. stenocephala* egg counts in 10 aliquots of 10 μl each (mean=6.83 eggs). Three replicates of each concentration (N=6) of eggs suspended in distilled water (i.e., 3.5, 7, 50, 100, 200, 500) were performed. All the samples (No.=18) obtained were submitted to DNA extraction and PCR analyses.

4.3.1.3 Spiked dog fecal samples (U. s3)

Negative canine fecal samples (analysed 5 times by the FLOTAC basic technique to determine the absence of helminth eggs) were artificially spiked adding purified suspension of *U. stenocephala* eggs, prepared as mentioned above (paragraph 4.3.1.2), in order to achieve different egg count levels of 5, 10, 20, 50 and 100 EPG. After a thorough homogenization, three replicates of each EPG level were analysed using the FLOTAC basic technique (Cringoli et al., 2010, 2011) with a sodium chloride flotation solution (specific gravity=1.20). The detection limit (analytic sensitivity) was 1 egg per gram (EPG) of feces. The mean EPG and the percentage recovery of hookworm eggs were calculated for each EPG level to assess the accuracy of Fecal Egg Count (FEC), using the following formula: 100 - (true FEC - observed FEC) / true FEC * 100 (Bosco et al., 2018). All the fecal samples (No.=15) obtained were submitted to DNA extraction and PCR analyses.

4.3.1.4 Feces from dogs naturally infected with hookworm eggs (U. s4)

A total of 67 Australian cattle dogs bred in southern Italy were screened for the presence of hookworms using the FLOTAC basic technique (Cringoli et al., 2010, 2011), again using a sodium chloride flotation solution. The dogs were kept separately in single boxes and the fresh fecal samples were collected from the ground. Puppies under one month of age were excluded. All the fecal samples were submitted to DNA extraction and PCR analyses.

4.3.1.5 Recovery of fecal suspension with hookworm eggs from FLOTAC apparatus (U. s5)

For each hookworm-positive fecal sample from dogs naturally infected, after analysis as described in paragraph 4.3.1.4, the fecal suspension with eggs was recovered from both chambers of the FLOTAC apparatus (Cringoli et al., 2010, 2011). In summary, the FLOTAC chambers were cleaned with 1.5 ml of distilled water and the recovered suspension was transferred in a 15-ml tube. This step was repeated three times, as described by Maurelli et al., 2018. Subsequently, distilled water was added in the tube up to 14 ml and centrifuged at 2000 rpm for 15 min. The supernatant was discarded, the sediment was resuspended in 1 ml of distilled water and transferred to a 2-ml microtube, then 200 µl of this suspension was used for DNA extraction and PCR tests.

4.3.2 DNA extraction and molecular analysis

The DNA extraction was performed on a total of 148 samples obtained from different matrices as mentioned above (4.3.1.1-4.3.1.5), using the Fast DNA stool kit (Qiagen, Germany) as per the manufacturer's instructions and tested with each of the four different PCR protocols reported in Table 4.1. In particular, specific primers were used for amplification of the ITS1, 5.8S and ITS2 regions for the detection of *A. caninum* and *U. stenocephala* following the protocol outlined by Illiano et al. (2023) (protocol A). In addition, the 18S region was targeted for the detection of *A. caninum* and *U. stenocephala* in accordance with the methodology described by Lesniak et al. (2017) (protocol B). Furthermore, the ITS1 region was used for the detection of *A. caninum* following the protocol established by Merino-Tejedor et al., 2019 (protocol C). Similarly, the ITS1 region was employed for the detection of *U. stenocephala* as per the methodology described by Merino-Tejedor et al., 2019

(protocol D). Moreover, the following samples (obtained from dogs experimentally infected with *U. stenocephala* and *A. caninum* and performed at the Clinvet International PTY LTD in South Africa) containing: (i) 100 eggs of *U. stenocephala*, (ii) 100 larvae of *U. stenocephala* and (iii) 100 larvae of *A. caninum* were used as positive controls in all the PCR protocols (A, B, C, D) for all the matrices (Us.1-Us.5) included in the study to evaluate efficiency and specificity of the protocols.

The purified PCR products were sequenced in both forward and reverse directions and analyzed using Chromas 2.6.6 software. They were then compared with the NCBI/GenBank database using the Basic Local Alignment Search Tool (BLAST) and ClustalW software (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Table 4.1 PCR protocols (A, B, C, D) with corresponding amplified regions of DNA, primer sequences, thermal profile, hookworm (HK) species identified, dimensions of the target amplified and references.

Protocol	Amplified region of DNA	Primers	Thermal profile	Species of HK* detected	Length (bp)	References
A	ITS1, 5.8S and ITS2	RTGHF1 (5′-CGTGCTAGTCTTCAGGACTTTG-3′) RTGHR1 (5′-CGTTGTCATACTAGCCACTGC-3″)	96 °C,10 min, 95 °C 45 s 59 °C 40 s 72 °C 60 s for 10 cycles 95 °C 45 s, 58°C 40 s 72 °C 60 s for 30 cycles 72 °C 7 min.	*A. caninum* and *U. stenocephala*	680 bp	Illiano et al., 2023
B	18S	18S-F (5'-GGCGATCAGATACCGCCCTAGTT-3'), 18S-R (5'-TACAAAGGGCAGGGACGTAAT-3').	95 °C 10 min, 95 °C 30 s, 56 °C 30 s, 72 °C 60 s, for 40 cycles, 72 °C 10 min	*A. caninum* and *U. stenocephala*	620 bp	Lesniak et al., 2017
C	ITS1	Ancy-ITS-F (5'-CTTGTGTTGGTGGTTGAGCAT-3'), ITS-R (5'-CATTAGGCGGCAACGTCTGG-3').	95 °C 15 min, 95 °C 30 s, 60 °C 40 s, 72 °C 60 s, for 40 cycles, 72 °C 10 min	*A. caninum*	370 bp	Merino-Tejedor et al.,2019
D	ITS 1	ITS-F (5'-CATTAGGCGGCAACGTCTGG-3'), ITS-R (5'-CAAGTGCCGTTCGACAACA- 3').	95 °C 15 min, 95 °C 30 s, 58 °C 40 s, 72 °C 60 s, for 40 cycles, 72 °C 10 min	*U. stenocephala*	334 bp	Merino-Tejedor et al.,2019

*hookworm

4.3.3 Statistical analysis

The results were analyzed by univariate statistical analyses using 2x2 contingency tables, in order to assess the significant associations between the diagnostic techniques. The Kappa (κ) statistic was used to measure the concordance between the FLOTAC technique and each of the four PCR protocols (A, B, C, D) for all the samples included in the study, using the following criteria (Thrusfield, 2007): ≤0.2 = poor; 0.21-0.40 = fair; 0.41-0.60 = moderate, 0.61-0.80 = good and ≥0.80 = very good. Furthermore, the dogs were divided into four groups based on the egg shedding of hookworms (2-50 EPG; 52-100 EPG; 102-500 EPG; ≥502 EPG). The association was considered significant at $P< 0.05$. The prevalence and the 95 % confidence intervals (95 %CI) were calculated using the free online software «Sample Size Calculator» (Creative Research Systems, CA, USA). All statistical analyses were performed using the SPSS® software (version 22.0, IBM Corporation, Armonk, USA).

4.4 Results

4.4.1 U. s1 and U. s2

The results for matrices U.s1 and U. s2 are summarised in Table 4.2. The detection limit of protocols A and B was 7 eggs/µl of *U. stenocephala* and 3.5 larvae/µl of *U. stenocephala*. Conversely, the detection limit for protocol D was 3.5 eggs/larvae/µl of *U. stenocephala.* No positive samples were detected through PCR protocol C.

As regard the positive controls of the dog samples experimentally infected with *U. stenocephala* and *A. caninum*, the protocol B was able to detect all of them: larvae of *A. caninum* (AL), larvae (UL) and eggs (UE) of *U. stenocephala*, while protocol D only UL and UE, because it is specific for *U. stenocephala*. Protocol C detected only the larvae of *A. caninum* (AL) as expected, while Protocol A failed to detect UE. All the positive samples containing 500 eggs/larvae/µl from matrices U. s1 and U. s2 and all the positive controls were sequenced to confirm the specificity of results obtained. The positive samples were identified as U. *stenocephala* (100% identity, GenBank accession number: MT361102). The positive control samples UL and UE were identified as *U. stenocephala* (100% identity, GenBank accession number: MT361102) and AL control samples were identified as *A. caninum* (100% identity, GenBank accession number: MT130933.1).

Table 4.2 Results of PCR protocols A, B, C, and D from pure *U. stenocephala* larvae suspension (U. s1) and egg suspension (U. s2) at counts of 3.5, 7, 50, 100, 200, 500 larvae/eggs/µl

Counts of larvae/eggs/µl	**Protocol A**		**Protocol B**		**Protocol C**		**Protocol D**	
	Larvae	**Eggs**	**Larvae**	**Eggs**	**Larvae**	**Eggs**	**Larvae**	**Eggs**
3.5	POS	NEG	NEG	POS	NEG	NEG	POS	POS
7	POS	POS	POS	POS	NEG	NEG	POS	POS
50	POS	POS	POS	POS	NEG	NEG	POS	POS
100	POS	POS	POS	POS	NEG	NEG	POS	POS
200	POS	POS	POS	POS	NEG	NEG	POS	POS
500	POS	POS	POS	POS	NEG	NEG	POS	POS
[+]Positive controls UE/UL/AL	NEG/POS/POS		POS/POS/POS		NEG/NEG/POS		POS/POS/NEG	

Legend: larvae/eggs/µl- larvae/eggs per microliter; POS-positive; NEG-negative; [+]Positive controls UE/UL/AL (100µl eggs/larvae): positive controls of UE (eggs of *U. stenocephala*), UL (larvae of *U. stenocephala*), AL (larvae of *A. caninum*).

4.4.2 U. s3

Table 4.3 reports the outcome of the EPG levels of *U. stenocephala* detected with FLOTAC technique and the results of the A, B, C, D PCR protocols, using egg-spiked fecal samples at different EPG concentrations (5,10, 20, 50, 100). The results were expressed as mean EPG of the three replicates and as percentage of egg recovery. The egg-spiking test revealed that FLOTAC technique was able to recover the *U. stenocephala* eggs from dogs' feces with an accuracy range of 57.2- 86%. Moreover, the detection limit of the PCR protocols B and D was 10 EPG of feces (12/15; 80%; CI 95%= 51.37-94.69) while Protocol A was only capable of detecting 50 and 100 EPG levels (6/15; 40%; CI 95%= 17.46-67.11). No positive samples were detected through PCR protocol C, as expected. No sequencing was performed for these positive samples, because we used the pure suspension eggs U. s2, sequenced as described in paragraph 4.4.1.

Table 4.3 Results obtained for each PCR protocols A, B, C, and D based on *U. stenocephala* egg count levels of 5, 10, 20, 50, 100 EPG in spiked dog fecal samples. Detected EPG, % egg recovery obtained with FLOTAC were reported for each EPG level.

Egg count level	Detected EPG (mean)	% Egg recovery	Protocol A	Protocol B	Protocol C	Protocol D
5	4	80	NEG	NEG	NEG	NEG
10	8	80	NEG	POS	NEG	POS
20	17.2	86	NEG	POS	NEG	POS
50	38.6	77.2	POS	POS	NEG	POS
100	57.2	57.2	POS	POS	NEG	POS
+Positive controls UE/UL/AL (100µl eggs/larvae)			POS/POS /POS	POS/POS /POS	NEG/NEG /POS	POS/POS/ NEG

Legend: EPG: eggs per gram; POS-positive; NEG-negative

4.4.3 U. s4

Of a total of 67 dogs screened for hookworms, 30 (44.8%; CI 95%= 32.8-57.4) (21 females, 9 males, age range 4 months-16 years) resulted positive at FLOTAC technique. Furthermore, the hookworm-negative samples at FLOTAC were also confirmed by all PCR protocols (A, B, C, D). Fecal positive samples showed a range of EPG between 2 and 5280 (mean= 348.90 EPG). Based on EPG levels, the positive samples for hookworms were divided in five EPG levels: 2-50; 52-100; 102-500; ≥502 and PCR protocols were performed.

Table 4.4 reports the results of the FLOTAC technique based on the different categories of hookworm EPG and the PCRs outcome of all the 30 fecal samples. Briefly, 60% (18/30) of the total fecal samples which resulted positive at the FLOTAC technique were also confirmed with at least one of the PCR protocols used in the study. The protocol D recognized the highest number of positive samples (56.7%; 17/30), with good results already at low EPG level of 2-50 EPG (27.8%; 5/18). All the samples tested negative in PCR protocol C.

The overall κ concordance between FLOTAC tests and PCR protocols performed on feces was moderate (κ=0.495; p = 0.000) for both protocols B and D and fair (κ =0.332; p = 0.001) for protocol A.

All the positive samples (obtained from all the protocols) were sequenced and identified as *U. stenocephala* with high identity range (98.83-100%) with the following GenBank accession numbers: MT361112.1; MT361102.1; MT361110.1.

Table 4.4 Results of the FLOTAC technique compared with the positivity-outcome of the PCR protocols (A, B, C, D) included in the study regarding the feces samples of naturally infected dogs with hookworms.

		PCR protocols			
Hookworm EPG	**No. samples PCR-positive/total positive per burden category-FLOTAC (%) /(95%CI)**	**A No. positive sample (%) /(95%CI)**	**B No. positive sample (%)/(95% CI)**	**C No. positive sample (%) /(95%CI)**	**D No. positive sample (%) /(95%CI)**
2-50	6/18 (14.4)/(33.3- 58.9)	2 (11.1)/(1.9-36.1)	2 (11.1)/(1.9-36.1)	0	5 (27.8)/(10.7-53.6)
52-100	6/6 (100)/(51.7- 98.5)	3 (50)/(13.9-86.1)	4 (66.7)/(24.11-9)	0	6 (100)/(51.7-98.5)
102-500	5/5 (100)/(46.3-98.1)	4 (80)/(29.9-98.9)	5 (100)/(46.3-98.1)	0	5 (100)/(46.3 - 98.1)
≥502	1/1 (100)/(5.46- 89.22)	1 (100)/(5.5-89.2)	1 (100)/(5.5 - 89.2)	0	1 (100) (5.5- 89.2)
Total positive samples in PCR (No.=18)/total positive samples to FLOTAC (No.=30)	18/30 (60)/(40.75-76.78)	10/30 (33.3)/(17.9- 52.9)	12/30 (40)/(23.2-59.3)	0	17/30 (56.7)/(37.7- 74.0)
Total positive samples for each PCR protocol/ total positive samples in all PCRs (No.=18)		10/18 (55.6)/(31.4-77.6)	12/18 (66.7)/(41.2-85. 6)	0	17/18 (94.4)/(70.6-99.7)
[+]Positive controls UE/UL/AL (100µl eggs/larvae)		POS/POS/POS	POS/POS/POS	NEG/NEG/POS	POS/POS/NEG

Legend: HK-hookworms; EPG-eggs per gram

4.4.4 U. s5

Table 4.5 reports the results of the 30 fecal suspension samples positive to hookworms (mentioned in 4.4.3) recovered from the FLOTAC apparatus. A total of 50% (15/30) of the fecal samples which

resulted positive at the FLOTAC technique were also confirmed with PCR protocols B and D. Indeed, for the fecal suspension, the κ concordance between FLOTAC and PCR protocols was moderate (κ=0.442; p = 0.000) for both protocols B and D and poor (κ=0.186; p = 0.000) for protocol A. No statistically significant association ($p < 0.05$) was observed between the burden of the eggs shed and the positivity of PCR protocols A, B, and D.

Table 4.5 Results of the FLOTAC technique compared with the outcome of the PCR protocols (A, B, C, D) included in the study regarding the fecal suspension recovered from the FLOTAC apparatus from naturally infected dogs positive for hookworms.

		PCR protocols			
Hookworm EPG	**No. samples PCR-positive/total positive per burden category-FLOTAC (%) /(95%CI)**	**A (%) /(95%CI)**	**B (%)/(95 %CI)**	**C (%) /(95 %CI)**	**D (%) /(95%CI)**
2-50	4/18 (22.2)/(7.4-48.1)	1 (5.6)/(0.29-29.4)	4 (22.2)/7.37-48.1)	0	4 (22.2)/(7.4-48.1)
52-100	5/6 (83.3)/(36.5-99.1)	2 (33.3)/(6-75.89)	5 (83.3)/(36.48-99.12)	0	5 (83.3)/(36.5-99.1)
102-500	5/5 (100)/(46.3-98.1)	2 (40)/(7.26-82.96)	5 (100)/(46.29-98.13)	0	5 (100)/(46.3-98.1)
≥502	1/1 (100)/(5.5- 89.2)	1 (100)/(5.46-89.22)	1 (100)/(5.46- 89.22)	0	1 (100) (5.5- 89.2)
Total positive samples in all PCRs (No.=15)/total positive samples to FLOTAC (No.=30)	15/30 (50)/(31.7-68.3)	6/30 (20)/ (8.40-39.1)	15/30 (50)/(31.7-68.3)	0	15/30 (50)/(31.7-68.3)
Total positive for each PCR protocol/ total positive samples in all PCRs (No.=15)		6/15 (40)/ (17.5-67.1)	15/15 (100)/(74.7-99.4)	0	15/15 (100)/(74.7-99.4)
[+]Positive controls UE/UL/AL (100µl eggs/larvae)		**POS/POS/POS**	**POS/POS /POS**	**NEG /NEG/POS**	**POS/POS/ NEG**

Legend: HK-hookworms; EPG-eggs per gram

4.5 Discussion and conclusions

This study illustrates significant results regarding the molecular diagnosis of hookworms, evaluating the performance of different molecular targets and using different matrices of infected samples,

including pure larvae, pure eggs, feces and fecal suspension. The results obtained showed a highly variable output for each of the PCR protocols employed in the study.

Indeed, regarding the matrices, protocols A, B and D showed a low limit of detection (3.5 larvae/µl) when using DNA samples extracted from *U. stenocephala* larvae obtained from experimentally infected dogs. When DNA samples were extracted from *U. stenocephala* eggs from naturally infected dogs, only PCR protocol D showed a limit of detection of 3.5 eggs/µl, while protocols A and B showed a limit of detection of 7 eggs/µl. This might lead us to speculate that perhaps *U. stenocephala* larvae represent a better DNA extraction matrix than eggs that could improve DNA yield, increasing the sensitivity of molecular diagnosis for *U. stenocephala* in dogs. These results agree with other authors that showed a higher sensitivity of PCR analysis when DNA extraction was performed from larvae of hookworms (Ngcamphalala et al., 2019; Merino-Tejedor et al., 2019; Singh et al., 2022). However, it must be considered that in laboratory practice obtaining hookworm larvae is a complex and lengthy process involving coproculture and identification of an accurate method for their recovery, while also requiring highly skilled and trained laboratory staff (Zhan et al., 2001).

Furthermore, the sensitivity of the PCRs for egg spiked fecal samples was lower than pure *U. stenocephala* eggs suspension (see Tables 4.2 and 4.3). One possible explanation is that during the process of extracting DNA from the eggs in the feces, egg losses or inhibitors in the feces or even improper egg dispersal and thus a low amount and quality of DNA may occur, especially in cases where the eggs are very few (Srirungruang et al., 2022).

In this study, it has also been confirmed that the FLOTAC technique is very useful for the diagnosis of hookworms (Cringoli et al., 2011) and allowed the recovery of *U. stenocephala* eggs at all EPG concentrations in egg-spiked fecal samples, achieving a >80% accuracy at low egg count level (>20 EPG). These findings were lower than in those reported in previous studies for gastrointestinal nematodes in cattle and sheep with Mini-FLOTAC (Bosco et al., 2018; Amadesi et al., 2020), but higher to recovery rate values of equine strongyle eggs (Noel et al., 2017; Napravnikova et al., 2019) and of liver and rumen flukes (Bosco et al., 2023). However, many factors may influence the recovery rates of a technique (i.e., differences in egg composition and its interaction with flotation solution, the procedure of egg isolation and feces contamination, etc.) (Amadesi et al., 2020)

In this study, a moderate concordance was found between the FLOTAC technique and the PCR protocols B and D that gave the higher number of positive samples for both matrices from dogs naturally infected with hookworms (fecal samples and fecal suspension). However, further studies

are needed to confirm the sensitivity and specificity of the PCR protocols that gave the best results, using samples with mixed infections of *A. caninum* and *U. stenocephala*.

In detail, the PCR protocols that showed the highest efficiency was protocol D (18S) (56.7%; 17/30 for fecal samples and 50%; 15/30 for fecal suspension), followed by protocol B (ITS1) (40%; 12/30 for fecal samples and 50%; 15/30 for fecal suspension), while PCR protocol A was only able to detect 33% (10/30 for fecal samples and 20%; 6/30 for fecal suspension) of samples tested positive at FLOTAC technique. Finally, the fact that all the samples from the present study were infected with *U. stenocephala* justifies the negative results obtained in the PCR protocol C (ITS1, *A. caninum*). These findings highlight that discordances between hookworm-positive samples in copromicroscopy and negative results in PCR could be dependent on the amount of hookworm DNA extracted from the feces, which may not be sufficient for successful amplification. These results are in line with what is described by Strocolkova et al. (2022) and by Illiano et al. (2023) that used the same Protocol A and showed a large discrepancy between the number of positive samples to copromicroscopic assays, and the number of samples detected with PCR. Another study conducted by Oliveira-Arbex et al. (2022) used a different PCR protocol which amplifies the same regions (ITS1, 5.8S and ITS2) as our protocol A and focused on 51 fecal samples positive to hookworms at copromicroscopic tests detecting also in this case only 26 positive in PCR (50.6%). On the other hand, in several recent studies (Merino-Tejedor et al., 2019; Rodpai et al., 2024), a greater number of samples consisting in hookworm larvae were detected by amplifying the same aforementioned regions. The increased detection rate may be due to the DNA extraction that was performed from the larvae, confirming what has been described above.

However, the overall output for the fecal samples from dogs naturally infected with hookworms revealed a correlation between high values of hookworm EPG and positivity in PCR. A possible explanation to this might be provided mainly by the fact that the fecal samples were extremely variable in consistency, appearing sometimes more diarrheic or more compact with the presence of foreign material such as soil or straw that could affect DNA yield and purity. Furthermore, the DNA samples with low hookworm EPGs could be damaged in the process of conservation (-20°C) and refreezing.

In the present study, it should be considered that the PCR protocols A and B showed lower sensitivity for fecal suspension recovered from the FLOTAC apparatus (20-50%) compared with the fecal samples (33.3-56.7%) in dogs naturally infected. This could be due to the loss of hookworm eggs during sample collection from the FLOTAC. Therefore, new studies are needed to determine any losses during the egg recovery process.

Finally, the results of sequencing obtained from dogs naturally infected in the present study showed the presence of only one species, i.e., *U. stenocephala.* The evidence of the infection with *U. stenocephala* was also reported recently in other studies conducted in Italy (Illiano et al., 2023) and in other parts of Europe (Demkowska-Kurtzepa et al., 2019; Strocolkova et al., 2022) revealing increased spread of the infection, perhaps caused by climate change and animal movements.

In conclusion, our results showed that the PCR protocols B (Lesniak et al., 2017) and D (Merino-Tejedor et al, 2019) are the most sensitive and specific molecular tests for the detection of *U. stenocephala* infection in dogs, but the choice of DNA extraction sample-matrix is crucial, as this affects the diagnostic sensitivity of the technique. According to our results, pure larvae suspension of *U. stenocephala* could be considered as the best matrix to perform DNA extraction. Given its zoonotic significance, further studies are needed to provide the scientific community with increasingly useful and efficient diagnostic tools that can support not only the epidemiological study but can also be useful in clinical practice and in the therapeutic and preventive management of these parasitic diseases which should not be neglected, as per the guidelines issued by the European Scientific Counsel Companion Animal Parasites (ESCCAP).

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Chapter 5

Overall discussion

5.1 Overall discussion

The present PhD thesis provides some updates into the epidemiology and diagnosis of hookworm and ascarid infections in dogs.

Significant findings were obtained with respect to: i) the update of the epidemiological scenario of ascarid infections (*T. canis* and *T. leonina*) in dogs in Southern Italy; ii) the advancement of epidemiological data about hookworms' infection in dogs in Southern Italy and the assesment through molecular investigations of the prevalence of hookworm species (*A. caninum* and *U. stenocephala*) in the same area: iii) the evaluation of the diagnostic accuracy of different molecular protocols for the detection of *A. caninum* and *U. stenocephala* in dogs using various matrices of samples.

The result of the five-year retrospective study on ascarid infections in dogs in southern Italy (Chapter 2) and of the ten-year retrospective study on hookworm infections in dogs in the same area (Chapter 3) suggest to us that geohelmintic infections, despite being often underestimated by the veterinary practitioners, cannot be neglected since their prevalence values are still considerable (9.2% for both *T. canis* and hookworms) and their zoonotic potential still remains high (Traversa et al., 2012; Traub et al., 2021; Maurelli et al., 2022; Illiano et al., 2023).

Moreover, in both study stray puppies demonstrated the major risk to become infected as well as the most common source of infection and dissemination of these parasitic infections both for other dogs and humans. The results obtained suggest that despite the wide use (sometimes abuse or even misuse) of broad-spectrum deworming treatments aimed at controlling these parasitic infections, optimal control has not been achieved so far. Therefore, control of the environment, more rational use of treatments only after a proper diagnosis obtained through accurate and effective methods are mandatory in order to achieve better management of these parasitic infections.

The molecular findings obtained in Chapter 3 give the first molecular identification of hookworm species in Italy. Moreover, the results obtained, as reported in other studies (Demkowska-Kutrzepa et al., 2018; Strkolcova et al. 2022), make evident a strong presence of *U. stenocephala*, despite the fact that its presence is more reported in northern Europe (colder climate). This could be due the increasing animal movements due to globalization and climatic changes that are affecting geographic distribution of several parasitic and non-parasitic infections (El-Sayed et al., 2020). Furthermore, the overall molecular outcome obtained in Chapter 3 suggests a high discrepancy between the sensitivity of copromicroscopic analysis and the sensitivity of PCR that seems to be lower. For these reasons, investigating the sensitivity and specificity of PCR protocols for the detection of hookworm species and the factors that influence them, was essential to better provide accurate and effective diagnostic tools (Illiano et al, 2023). Hence, the results of Chapter 4 revealed that the sensitivity of PCR protocols

varies in first analysis based on the molecular targets to be amplified. This could be explained by the fact that some regions of DNA are more conserved (ITS) than others that are more variable (18s) and this could affect PCR sensitivity (Illiano et al., submitted).

Moreover, it is reported that variability and conservatism of DNA regions can lead to misidentification of infecting species, making necessary additional effort for precise hookworms' species determination. In addition the outcomes of Chapter 4 showed that the sensitivity of PCR protocols also varies depending on which matrix of sample (eggs in feces, eggs in distilled water, larvae in distilled water) is used in order to perform the DNA extraction. As reported in other studies, larvae represent the best DNA extraction matrix in order to achieve good PCR sensitivity. But it should be considered that under natural conditions, obtaining this DNA extraction matrix would first require additional diagnostic steps such as coproculture which would not only lengthen the diagnostic time but also require skilled laboratory technicians. However, the overall output obtained comparing copromicroscopic and molecular results highlight also in this study a discrepancy between the two techniques, although revealing a correlation between high values of hookworm EPG (at copromicroscopic test) and positivity in PCR (Illiano et al., submitted). Hence, these results underscore the importance of two-step (copromicroscopic-molecular) identification of hookworm species in dogs. Moreover, this study confirms a high prevalence of *U. stenocephala* as reported previously (Illiano et al., 2023).

In conclusion, despite the wide availability of anthelmintic products, infections with hookworms and roundworms are still the *leitmotif* of the pet clinics. Promoting "good diagnostic practices" of such parasitic infections in pets is necessary for "good clinical practice" aimed at safeguarding the health and well-being of dogs through the use of broad-spectrum anthelmintic products that are effective, safe and easy to administer. Veterinary practitioners must have as their primary goal the health and well-being of their patients and consequently human health. For this reason, confining geohelmintic infections (e.g., hookworms and roundworms) to the role of the "neglected part" of the veterinary profession is not only to deny the evidence, but more importantly will further promote the spread of these parasitic infection. Hence, attention to the problem must be maintained constantly throughout the animal's life, through examinations (corpomicroscopic and molecular) with high standard levels, anthelmintic protocols that evaluate age, physiological status, environment, lifestyle, movements, and risk of infestation on a case-by-case basis and correct and timely information towards the owner (Genchi and Rinaldi, 2016; ESCCAP, 2021), promoting awareness among practitioners as well for protecting both animals and humans' health in a **One Health** perspective.

5.2 References

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