Molecular identification of hookworm isolates from stray dogs, humans and selected wildlife from KwaZulu-Natal and Mpumalanga provinces of South Africa

By

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ABSTRACT

Hookworms are nematodes that cause infections to the host via skin penetration of the third stage larvae and they are widely distributed in the tropical and subtropical regions. They parasitize a wide range of host species including humans and companion animals such as dogs and cats. An estimated 740 million people are infected worldwide, with sub-Saharan Africa having the highest documented prevalence. With the exception of the study conducted by Lamb *et al.* (2012), *Ancylostoma* species identification in Southern Africa was solely based on egg morphology and morphological characteristics of adult worms. This study therefore aimed at using molecular techniques to identify the hookworm species and their prevalence in stray dogs, school-going children from KwaZulu-Natal province, and selected wild canids and felids from Mpumalanga province of South Africa. A total of 356 faecal samples were collected and screened for the presence of hookworm eggs using coproscopy and coproculture, which yielded prevalence of 23.04% and 21.67%, respectively. Larvae derived from coproculture of a total of 55 samples were subjected to molecular analysis. DNA was isolated and subjected to PCR amplification, PCR-RFLP and sequencing of the nuclear ribosomal internal transcribed spacer (ITS1) and 5.8S rRNA region. PCR-RFLP showed an overall prevalence of 72.7% (40/55) for *A. caninum*, 12.7% (7/55) for a mixture of *A. caninum, A. ceylanicum*, and *A. braziliense*, 7.27% (4/55) for *A. caninum* and *A. ceylanicum* mixed infection, and 7. 27% (4/55) for *A. caninum* and unidentifiable species. These results are consistent with other studies which show that *A. caninum* is the most dominant hookworm species worldwide even though *A. braziliense* is regarded as a more important zoonotic species. Phylogenetic analyses of alignments based on the DNA sequences were also used to identify isolates which were sequenced. However, results of phylogenetic analysis were not consistent with results from PCR-RFLP analysis as none of the sequences matched with *A. ceylanicum*. Sequencing also showed a 0.68% prevalence for both *A. caninum* and *A. braziliense* (mixed infections) in dogs. Of great importance was the revelation that *A. caninum* can now cause a patent infection in South African (Ingwavuma area) school-aged children as all human isolates matched with *A. caninum* with a prevalence of 6%. For wildlife, a prevalence of 10% was recorded. However, due to the small sample size, these results cannot be regarded as a true reflection of the prevalence in wildlife. Thus there is a need for future studies that will increase sample sizes, broaden ranges as well as look into finding better detection methods, primers and/or restriction enzymes that are specific to hookworm species isolated in South Africa.

PREFACE

The experimental work described in this dissertation was carried out in the Discipline of Biological Sciences, School of Life Sciences, University of KwaZulu-Natal, Westville Campus, from March to November 2017, under the supervision of Professor S. Mukaratirwa and Prof. J. Lamb. The research was financially supported by the South African Medical Research Council and National Research Foundation (NRF).

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

DECLARATION - PLAGIARISM

I Philile Ignecious Ngcamphalala, declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
    a. Their words have been re-written but the general information attributed to them has been referenced.
    b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks and referenced.
5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed:  Date: 07/03/2019

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

Hookworms are soil-transmitted helminths (STHs) belonging to the family Ancylostomidae (Lamb *et al.*, 2012; Ngui *et al.*, 2013; Hasegawa *et al.*, 2017); and according to Liu *et al.* (2015), these strongyle nematode parasites have a wide range of host species which include humans and companion animals such as dogs and cats. Loukas *et al*. (2005) reported that hookworm infections are ranked among the most common and important tropical parasitic diseases in humans and companion animals worldwide, with high prevalence in humans, primarily in poverty stricken communities in developing countries (Shepherd *et al.*, 2018) which lack adequate water and sanitation (Brooker *et al.*, 2006). They cause severe protein malnutrition, iron-deficiency anaemia and intestinal blood loss through the haematophagous effect of the adult parasites as well as bleeding from the feeding sites in the intestines (Zhan *et al.*, 2001; Liu *et al.*, 2015; Xie *et al.*, 2017).

Most hookworm infections in humans are attributed to two species, *Ancylostoma duodenale* and *Necator americanus* (Hawdon, 1996; Zhan *et al.*, 2001; Phosuk *et al.*, 2013; Hasegawa *et al.*, 2014; Tun *et al.*, 2015; George *et al.*, 2016; Hasegawa *et al.*, 2017; Smout *et al.*, 2017), with *N. americanus* being the predominant species infecting humans and non-human primates (Hasegawa *et al.*, 2014; Hasegawa *et al.*, 2017). *Ancylostoma duodenale*, however, is regarded as the more pathogenic of the two, as it causes severe blood loss and subsequently anaemia (Zhan *et al.*, 2001). The common species that infect companion animals such as dogs include *A. caninum, A. ceylanicum, A. braziliense*, and *U. stenocephala*, whereas those that commonly infect cats include *A. braziliense, Ancylostoma tubaeforme*, *A. ceylanicum* and *Uncinaria stenocephala* (Taylor *et al.*, 2007; Bowman *et al.*, 2010; Liu *et al.*, 2015). However, *A. caninum, A. ceylanicum* (Taylor *et al.*, 2007; George *et al.*, 2016; Liu *et al.*, 2015) and *A. braziliense* may also infect humans and cause zoonotic diseases such as eosinophilic enteritis (EE) and cutaneous larva migrans (CLM) (Ngui *et al.*, 2012; Liu *et al.*, 2015).

Adult hookworms dwell in the small intestines of the host where they sexually reproduce and lay eggs that are passed out in faeces (Brooker *et al.*, 2006). In one to two days the eggs in the soil hatch to larvae under conducive conditions (Brooker *et al.*, 2006; Bowman *et al.*, 2010). Infection of the host is often via skin penetration by the third stage filariform larvae (Hotez *et al.*, 2004). This type of transmission occurs when soil contaminated with hookworm larvae comes into contact with exposed host skin, mostly on the hands and feet in humans and the feet of animals (Hotez *et al.*, 2004; Feldmeir and Schuster, 2012). Transmission of hookworms has also been reported to occur by the oral route (Landmann and Prociv, 2003; Hotez *et al.*, 2004) through accidental ingestion of soil-contaminated food, for example on fresh vegetables, or by drinking contaminated water (Landmann and Prociv,

2003). Transplacental and transmammary transmission routes have also been reported for some of the hookworm species, especially *A. caninum* (Taylor *et al.,* 2007; Bowman *et al*., 2010).

Hookworm infections are widely distributed in tropical and sub-tropical countries (de Silva *et al.,* 2003; Kaewthamasorn *et al.,* 2006; Bethony *et al.,* 2006; Uneke, 2010), particularly in places where conditions are favourable for their survival (warm and moist soil on which the parasite larvae thrive) (Traub *et al.,* 2004; Bethony *et al.,* 2006). Of all hookworm species reported globally, *A. caninum* is the most widespread (Landmann and Prociv 2003; Loukas *et al.,* 2005). An estimated 740 million people are infected with either *A. duodenale* or *N. americanus* hookworms worldwide, with sub-Saharan Africa having the highest documented prevalence (Hotez *et al.,* 2004; Bethony *et al.,* 2006; Hasegawa *et al.,* 2014; Hasegawa *et al.,* 2017). Hotez and Kamath (2009) reported that in sub-Saharan Africa, Nigeria and the Democratic Republic of Congo have the highest recorded number of human cases followed by Angola, Ethiopia, and Cote d'Ivoire. This then raises the necessity to assess the prevalence and distribution of hookworm infections, as well as to identify the species causing the infections in Southern Africa.

With regards to public health, knowledge and understanding of the prevalence and distribution of hookworms is important (Palmer *et al*., 2007). In the past, identification of hookworm species was based on morphological characteristics of the adult worms (post-mortem) or coprological examination of eggs recovered from faeces of infected hosts (Palmer *et al.,* 2007; Lamb *et al.,* 2012). Identification of *Ancylostoma* spp. based on morphological characters is labour intensive, time consuming and requires skilled personnel, but even then, there is always the possibility of overlooking mixed infections (Traub *et al*., 2004; Lamb *et al.,* 2012). Lamb *et al*. (2012), further reported that differentiating between *A. caninum* and *A. braziliense* in dogs using egg morphology is problematic.

Recently, species-specific molecular-based techniques for rapid differentiation of developmental stages of hookworms have been developed (Lamb et al., 2012). According to Lamb *et al.* (2012), these molecular based techniques had seldom been applied in the study of hookworms in Southern Africa. Thus, there is a paucity of information on the application of molecular tools for the identification of hookworm species in this region, more especially in humans, dogs and wild canids. Therefore, this study aimed at determining the hookworm species infecting humans (school-going children) and stray dogs from KwaZulu-Natal province and selected wild canids and felids from Mpumalanga province of South Africa, as well as their prevalence and phylogenetic relationships. Techniques applied included coproscopy, coproculture, polymerase chain reaction (PCR) amplification, PCR-Restriction fragment length polymorphism (RFLP) and sequencing of the nuclear ribosomal internal transcribed space (ITS1) and 5.8S rRNA region from the larval isolates.

1.2 Objectives

General objective

1. To identify the hookworm species of humans (school-going children) and stray dogs from KwaZulu-Natal province and selected wild canids and felids in nature reserves of Mpumalanga province, South Africa and to determine their prevalence and phylogenetic relationships.

Specific objectives

1. To determine the prevalence of hookworm infection in stray dogs and primary school children in selected areas of KwaZulu-Natal province and in felids and canids from game reserves in Mpumalanga province through coproscopy and coproculture.
2. To identify species of hookworms infecting stray dogs and humans in KwaZulu-Natal and wild canids and felids from game reserves in Mpumalanga province using PCR and PCR-RFLP.
3. To determine the genetic relatedness of hookworm species isolated from humans, stray dogs and wild canids and felids through phylogenetic analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Hookworms are soil-transmitted nematodes that cause infections through active skin-penetration of the host by third-stage larvae (L_3). They are widely distributed in the tropics and in subtropical regions (Bethony *et al.*, 2006), where environmental and climatic conditions including rainfall, surface temperature, altitude and soil temperatures are conducive to their transmission (Hotez *et al.*, 2008). According to Bethony *et al.* (2006), hookworm infections are ranked third in terms of disease burden after common roundworm (*Ascaris*) and whipworm (*Trichuris*) infections. Taylor *et al.* (2007) reported that hookworms of veterinary importance are from the superfamily Ancylostomatoidea, the important genera being *Ancylostoma*, *Uncinaria,* and *Bunostomum.* Genera of zoonotic importance are, however, *Ancylostoma* and *Necator* (Taylor *et al.*, 2007; Hossain and Bhuiyan, 2016). Even though hookworms are regarded as important pathogens, they remain largely neglected by the medical and international community mostly because they affect the world's most impoverished population (Bethony *et al.*, 2006). In developing countries, the risk of zoonotic infections in humans, associated with nematodes that parasitize dogs, increases with the ever-growing dog population (Mukaratirwa and Taruvinga, 1999).

2.2 Hookworm life cycle and modes of transmission

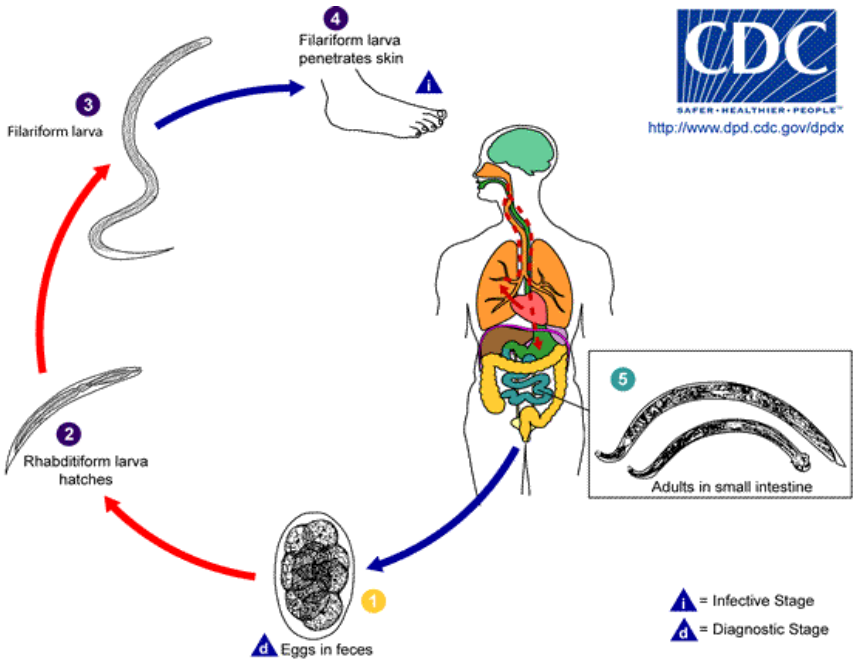

Figure 1: Hookworm life cycle in humans (Adapted from http://www.dpd.cdc.gov/dpdx).

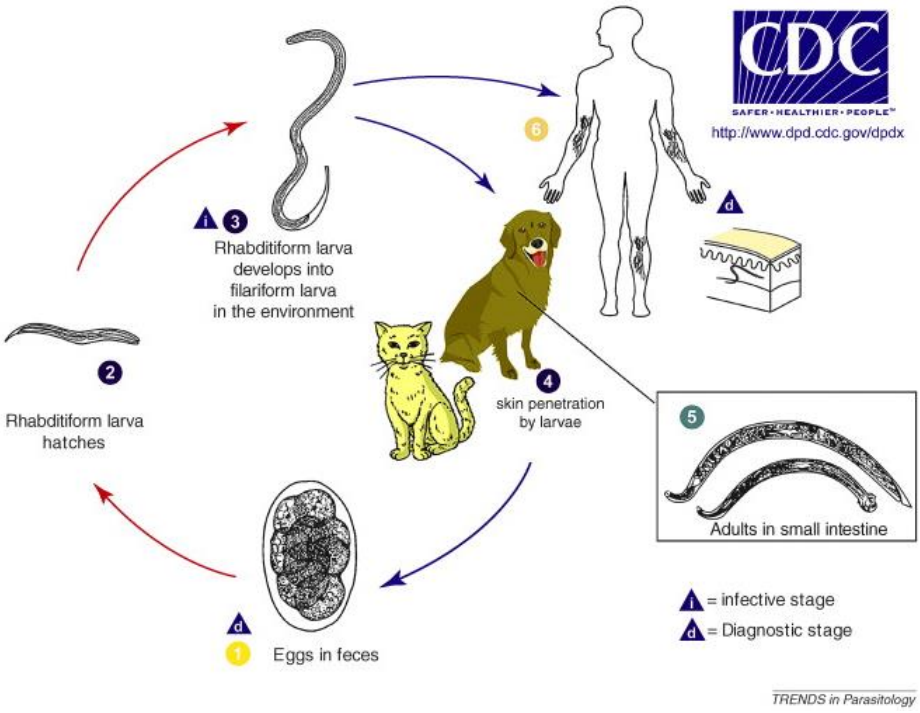

Figure 2: Hookworm life cycle and transmission in dogs and cats with humans accidentally infected causing cutaneous larva migrans (Adapted from Bowman *et al.*, 2010).

According to Liu *et al.* (2015), hookworm species have been reported to cause infections in a wide range of host species. *Ancylostoma caninum* is a dog, fox and occasionally a human parasite; *A. ceylanicum* infects dogs, cats, humans and wild felids; *A. tubaeforme* infects only cats; *A. braziliense* infects both dogs and cats; and *U. stenocephala* parasitizes not only cats, foxes and dogs but also other felids and canids. In addition, *U. stenocephala* can also use several mammals, for example rodents as paratenic hosts (Taylor *et al.*, 2007; Shepard *et al.*, 2018). *Ancylostoma braziliense* and *A. caninum* have been reported to cause cutaneous larva migrans (CLM) in humans (Figure 2) (Bowman *et al.*, 2010; Feldmeier and Schuster, 2012; Ngui *et al.*, 2012; Liu *et al.*, 2015). However, most hookworm infections in humans can be attributed to *N. americanus* and *A. braziliense* (Hawdon, 1996; Zhan *et al.*, 2001; Phosuk *et al.*, 2013; Hasegawa *et al.*, 2014; George *et al.*, 2016; Hasegawa *et al.*, 2017; Smout *et al.*, 2017).

Bowman *et al.* (2010) reported that the life cycle of hookworm spp. (Figure 1 and 2) begins with the passage of hookworm eggs in the host stool to the soil where, with favourable conditions, they hatch into the infective third stage (L_3) larvae within 5-10 days (Bowman *et al.*, 2010; Feldmeier and Schuster, 2012). According to Feldmeier and Schuster (2012), the larvae can remain infective for several months if conditions are favourable. The L_3 larvae enter the definitive host by the percutaneous route, then

migrate to the heart and lungs via blood (Taylor *et al.*, 2007; Bowman *et al.*, 2010; Hossain and Bhuiyan, 2016). In the bronchi and trachea, they moult into L_4 larvae, ascend to the pharynx, and then get swallowed and pass to the small intestines where they reside and mature into adult worms (Taylor *et al.*, 2007; Bowman *et al.*, 2010). Worms then attach to the intestinal wall and suck blood, then mature into male and female adults (Bowman *et al.*, 2010). However, hookworm infection in humans by either cat or dog hookworm larvae (*A. caninum*, *A. braziliense* and *A. ceylanicum*) results in cutaneous larvae migrans (CLM) due to larval migration under the host's skin (Landmann and Prociv, 2003; Bowman *et al.*, 2010; Mackenstedt *et al.*, 2015). Nonetheless, there have been several reports on *A. caninum* (Landmann and Prociv, 2003; Traub *et al.*, 2004; Palmer *et al.*, 2007; Brown and Copeman, 2008; Traub *et al.*, 2008; Bowman *et al.*, 2010; Ngui *et al.*, 2012; Liu *et al.*, 2015; Mackenstedt *et al.*, 2015; Tun *et al.*, 2015) and *A. ceylanicum* (Traub *et al.*, 2008; Bowman *et al.*, 2010; Ngui *et al.*, 2012; Smout *et al.*, 2017; Xie *et al.*, 2017) developing into adults in the human gut, causing eosinophilic enteritis (Bowman *et al.*, 2010).

In female dogs, some hookworm species larvae become arrested in skeletal muscles and tissue where they serve as a source of infection for young pups through transmammary and transplacental transmission routes (Taylor *et al.*, 2007; Bowman *et al.*, 2010). Transmammary transmission has however, been documented only in dogs infected with *A. caninum* (Taylor *et al.*, 2007; Bowman *et al.*, 2007). Hookworm spp. infecting canids may also be acquired through the ingestion of paratenic hosts such as infected rodents and cockroaches (Taylor *et al.*, 2007; Bowman *et al.*, 2010; Shepard *et al.*, 2018). Transmission can also be by oral infection, which occurs through accidental ingestion of larvae (*A. duodenale*) (Bethony *et al.*, 2006; Hossain and Bhuiyan, 2016). According to Hossain and Bhuiyan (2016), infective hookworm larvae may be transmitted through fomites when larvae creep onto clothes that are dried on the ground, leading to infestation when the clothes are worn.

2.3 Pathogenesis and clinical signs

Clinical signs of hookworm infections may be grouped as acute, manifested when the larvae migrate through the skin and viscera, and acute to chronic during infestation of the gastrointestinal tract by adult worms (Bethony *et al.*, 2006). A number of cutaneous syndromes may result from percutaneous penetration of larvae. For example, ground itch, a local erythematous and popular rash, may appear on the hands and feet from repeated exposure to *A. duodenale* and *N. americanus* (Bethony *et al.*, 2006). Infestation of humans by dog hookworms can also cause hookworm-related cutaneous larvae migrans (HrCLM) (Brown and Copeman, 2003; Brooker *et al.*, 2004; Bethony *et al.*, 2006; Hochedez and Caumes, 2007; Traub *et al.*, 2008; Bowman *et al.*, 2010, Feldmeier and Schuster, 2010; Mackenstedt *et al.*, 2015; Ng-Nguyen *et al.*, 2015), which is characterised by serpiginous tracks on the abdomen, feet and buttocks (Bethony *et al.*, 2006). *Ancylostoma braziliense* is however, the only hookworm species

that can cause creeping eruptions, with tracts in the skin persisting for over 100 days (Traub *et al.*, 2008; Ng-Nguyen *et al.*, 2015).

HrCLM disease can persist in the host body for months if left untreated. Signs and symptoms of HrCLM can be observed shortly after the larva penetrates the epidermis (Feldmeier and Schuster, 2012). For example, itching can be observed within an hour of the penetration of human skin by *A. braziliense*, whereas creeping eruption appears after a few days. An experimental infection using *U. stenocephala*, however, showed that papules become visible after 4-6 days and a track was observed two weeks after infestation (Feldmeier and Schuster, 2012). According to Feldmeier and Schuster (2012), manifestation and advancement of HrCLM depends on the hookworm species, host factors, as well as the natural history of the disease (Feldmeier and Schuster, 2012). Furthermore, animal hookworm larvae infections in humans may sometimes lead to erythema multiforme, eosinophilic pneumonitis, folliculitis, localized myositis, or ophthalmological manifestations (Bowman *et al.*, 2010). Oral ingestion of *A. duodenale* can lead to the manifestation of Wakana syndrome, which is characterised by nausea, vomiting, cough, pharyngeal irritation, hoarseness, and dyspnoea (Brooker *et al.*, 2004; Bethony *et al.*, 2006).

In the gastrointestinal tract of humans, acute and chronic manifestations are as a result of moderate to high intensity infestation by adult *N. americanus* and *A. duodenale* worms in the small intestines (Bethony *et al.*, 2006). Attachment of adult worms on the mucosa and submucosa of the small intestines results in extensive intestinal blood loss, which may lead to iron-deficiency anaemia if the blood loss exceeds the host's nutritional reserves (Hotez *et al.*, 2004; Bethony *et al.*, 2006). Bethony *et al.* (2006), reported that 40 or more adult worms are estimated to be sufficient to reduce haemoglobin reserves. The amount of blood loss, however, also depends on the hookworm species causing the anaemia as well as host iron reserves. For instance, single worms of *N. americanus* and *A. duodenale* have been estimated to cause loss of 0.03 and 0.15 ml of intestinal blood per day, respectively (Crompton, 2000). Hypoproteinaemia and anasarca have been reported to result from chronic protein loss (Brooker *et al.*, 2004; Bethony *et al.*, 2006). There have also been numerous reports on *A. ceylanicum* readily developing to adults in human hosts (Palmer *et al.*, 2007; Bowman *et al.*, 2010; Ngui *et al*, 2012; Phosuk *et al.*, 2013; Inpankaew *et al.*, 2014; Ng-Nguyen *et al.*, 2015; Mackenstedt *et al.*, 2015; Xie *et al.*, 2017; Shepard *et al.*, 2018), producing natural patent infections resulting in eosinophilic enteritis and anaemia (Ng-Nguyen *et al.*, 2015). Cases of similar transmission and cross infection have been documented for *A. caninum* (Brown and Copeman, 2003; Landmann and Prociv, 2003; Bowman *et al.*, 2010; Mackenstedt *et al*., 2015; Xie *et al.*, 2017; Shepard *et al.*, 2018) and for *A. braziliense* (Inpankaew *et al.*, 2014; Xie *et al.*, 2017). Landmann and Prociv (2003) reported that *A. caninum* infective larvae develop directly into adult worms in the human gut when orally ingested.

Most light hookworm infestations show little or no symptoms (Bethony *et al.,* 2006; Uneke, 2010). Severe infections have been shown to have adverse health and nutritional impacts on children (Bethony *et al*., 2006; Uneke, 2010; George *et al.,* 2016; Bradbury *et al.,* 2017) and women of reproductive age because of their low iron reserves (Brooker *et al.,* 2004; Hotez *et al.,* 2004; Bethony *et al.,* 2006; Hotez *et al.,* 2007; Brooker *et al.,* 2008; Bradbury *et al.,* 2017). These effects include iron deficiency anaemia, growth deficiencies (stunted growth), mental retardation (Brooker *et al.,* 2006; Uneke, 2010; Ngui *et al.,* 2012), protein energy malnutrition, listlessness, stomach pain, wasting and diminished concentration in class for schoolchildren (Uneke, 2010). Severe anaemia during pregnancy can have detrimental effects on both the mother and foetus as well as on the neonate (Bethony *et al.,* 2006). These effects include maternal mortality, infant mortality, low birth weight (Brooker *et al.,* 2008; Hotez *et al.,* 2008) and neonatal prematurity (Hotez *et al.,* 2008).

In companion animals, ancylostomosis caused by *A. ceylanicum A. caninum* and *A. tubaeforme* is characterised by blood loss and anaemia (Bowman *et al.,* 2010) as well as hypoproteinemia (Ng-Nguyen *et al.,* 2015) caused by parasitization of the intestines by the adult worms (Bowman *et al.,* 2010). According to Ng-Nguyen *et al.* (2015), in canines, *A. caninum* causes more blood loss per worm (0.08 - 0.2 ml/day) than *A. ceylanicum* (0.033 ml/day), *U. stenocephala* (0.0003 ml/day) and *A. braziliense* (0.002 ml/day) combined. It has been reported that blood loss in young kittens (Bowman *et al.,* 2010) and puppies is sometimes fatal (Taylor *et al.,* 2007; Bowman *et al.,* 2010) and can lead to neonatal mortality in pregnant cats and dogs (Seguel and Gottdenker, 2017). Puppies infected with *A. caninum* through transmammary transmission (suckled) may also experience bloody diarrhoea, which may contain mucus. Dogs may also be underweight, have a poor coat, pica and skin lesions (Taylor *et al.,* 2007). Hookworms such as *A. braziliense and U. stenocephala* cause negligible blood loss, but can sometimes induce disease (Bowman *et al.,* 2010). For example, because *A. braziliense* is not a blood sucker, it only causes mild digestive upset as well as occasional diarrhoea (Taylor *et al.,* 2007).

Taylor *et al.* (2007) reported that young pups and dogs under 1-year-old, especially those that were infected through the transmammary route (*A. caninum*), are most susceptible to acute or chronic haemorrhagic anaemia. Older dogs on the other hand experience a lighter infection, thus the anaemia is not as severe as in the pups. Ulceration at the site of percutaneous infection and most eczema can affect the skin of previously sensitised dogs. *Ancylostoma caninum* larvae have been shown to stay dormant in the infected dog skeletal muscle for months to years until they are triggered to recommence migration to the gastrointestinal tract, mature, and cause a new infection. Factors triggering this migration range from stress, severe illness or repeated large doses of corticosteroids (Taylor *et al.,* 2007).

2.4 Geographical distribution and prevalence

The geographical distribution of each hookworm species is imperfectly known (Bowman *et al.,* 2010). This can be attributed to overlapping distribution ranges and hosts harbouring mixed infections; only

U. stenocephala has large enough eggs to allow them to be morphometrically distinguished (Bowman *et al.,* 2010). There is also added difficulty in etiological and epidemiological sampling of wildlife (Xie *et al.,* 2017), mostly due to the modification of landscapes and climate change (Seguel and Gottdenker, 2017). According to Xie *et al.* (2017), *Ancylostoma* species are of greater medical and veterinary importance than species in the *Necator* genus. This is mainly due to their distribution, prevalence and the existence of species of zoonotic importance (Xie *et al.,* 2017). Shepard *et al.* (2018) noted that hookworm spp. under the family Ancylostomatidae and their hosts have overlapping distributions which also adds to their importance.

Human hookworms (*Ancylostoma duodenale* and *Necator americanus*) have been shown to have a sympatric distribution, which has led to mixed infections being commonly found in endemic areas (Zhan *et al.,* 2001). *Necator americanus* is most common and widespread in eastern and southeast Asia (especially in India and China), sub-Saharan Africa, and the western hemisphere (Ngui *et al.,* 2012; Phosuk *et al.,* 2013; Shepard *et al.,* 2018), whereas *A. duodenale* has been documented in the Middle East, India, Northern Africa, Europe (Ngui *et al,* 2012; Phosuk *et al.,* 2013), Australia (Ngui *et al.,* 2012) and America (Shepard *et al.,* 2018). There have also been reports of the occurrence of *A. duodenale* in *Crocuta crocuta* (Xie *et al.,* 2017) and of *N. americanus* using pigs as transport hosts (Hossain and Bhuiyan, 2016). Furthermore, based on phenetic characteristics, *N. americanus* has also been reported to parasitize other mammals such as pangolins and other primates including chimpanzees, gorillas and monkeys (Hasegawa *et al.,* 2014). Hasegawa *et al.* (2014) continued to report on a high prevalence of *Necator* species other than *N. americanus* in humans with close contact with wild gorillas (particularly gorilla PHP trackers and researchers). In sub-Saharan Africa, both *A. duodenale* and *N. americanus* occur in same areas (Mabaso *et al.,* 2003; Hotez and Kamath, 2009), the latter being the predominant species (Mabaso *et al.,* 2003; Brooker *et al.,* 2006; Hotez and Kamath, 2009). In 2003, *N. americanus* was reported as the dominant species in the KwaZulu-Natal province of South Africa, resulting in the most prevalent parasitic infections, particularly in the north-eastern part of the province (Mabaso *et al.,* 2003).

The dog hookworm, *A. caninum*, is common worldwide in tropical and sub-tropical countries (Taylor *et al.,* 2012; Shepard *et al.,* 2018) and sometimes in other countries in dogs imported from endemic regions (Taylor *et al.,* 2012). *Ancylostoma braziliense* and *A. caninum* have also been reported to parasitize cheetahs (*Acinonyx jubatus*) (Xie *et al.,* 2017). *Uncinaria stenocephala* infects dogs and cats in temperate and sub-arctic regions (Taylor *et al.,* 2007; Bowman *et al.,* 2010; Lamb *et al.,* 2012; Shepard *et al.,* 2018) of Europe, South America, North America, New Zealand, Australia and Asia (Bowman *et al,* 2010). *Ancylostoma braziliense*, also occurring in dogs and cats, is common in Africa (South Africa, Sierra Leone, Democratic Republic of Congo, and Somalia), Asia (India, Malaysia, Sri Lanka, the Philippines, Thailand, and Indonesia), South America (Brazil, Uruguay) and North America

(along the south-eastern Atlantic coast, Mexico and the Caribbean Sea) (Bowman *et al.*, 2010; Shepard *et al.*, 2018).

Ancylostoma tubaeforme has been documented only in cats worldwide (throughout the warmer ranges) (Taylor *et al.*, 2007; Bowman *et al.*, 2010; Shepard *et al.*, 2018). *Ancylostoma ceylanicum*, which occurs in cats, dogs, and wild felids, has been reported in Australia, Asia (India, Taiwan, Sri Lanka Thailand, Malaysia, Borneo, Indonesia) (Bowman *et al.*, 2010; Shepard *et al.*, 2018), and South America (Surinam) (Bowman *et al.*, 2010). Shepard *et al.* (2018) reported on the occurrence *Ancylostoma pluridentatum*, a hookworm of wild cats (jaguars and leopards), in Central and South America and *Ancylostoma kusimaense* in badgers in Japan. Hookworm species infecting wildlife reported in Africa include *Ancylostoma somaliense* (jackal), *A. genettae* (Genet), *A. protelesis* (Aardwolf) and *Necator gorillae* (gorilla) (Shepard *et al.*, 2018). Xie *et al.* (2017) reported a formerly unrecognised hookworm species that parasitizes wild giant pandas, *Ancylostoma ailuropodae* n. sp., found in Southwest China.

Cats and dogs are considered as the possible sources of infection for humans due to the close contact that humans have with these animals. This was confirmed when it was reported that humans that came into contact with infected dogs were three times likely to contract hookworm infections (Ngui *et al.*, 2012). According to Mabaso *et al.* (2003), approximately 3.5 billion people are at risk of infection with soil-transmitted helminths, predominantly in tropical and subtropical countries. Hookworm spp. are estimated to infect 600 million people globally and result in up to 135,000 deaths yearly (Ngui *et al.*, 2012). A total of 150 outbreak cases of human eosinophilic enteritis (EE) caused by *A. caninum* were reported in Australia between the years 1988 and 1992 (Ngui *et al.*, 2012). Similar cases have also been reported in South America, in the United States of America, Egypt, Israel, as well as in the Philippines (Ngui *et al.*, 2012). Hookworms are said to have a wider distribution on the African continent (Brooker *et al.*, 2006) and of the STHs reported in sub-Saharan Africa, hookworm species are said to be the most widespread (Bethony *et al.*, 2006). The highest hookworm infection prevalence has been documented in sub-Saharan Africa, followed by East and South Asia, the Pacific Islands, India, Latin America, and the Caribbean (Hotez *et al.*, 2007; Jiraanankul *et al.*, 2011).

Studies of the presence of gastrointestinal parasites in dogs in Africa showed *Ancylostoma* to be the most prevalent genus, with *A. caninum* being the dominant species (Mukaratirwa and Busayi, 1995; Minnaar *et al.*, 1999, Minnaar and Krecek, 2001; Lamb *et al.*, 2012; Ayinmode *et al.*, 2016). In Ibadan, Nigeria, a study conducted by Ayinmode *et al.* (2016) showed a 56.8% prevalence of *Ancylostoma* spp. and a low prevalence of 5.7% for *Uncinaria* species in dogs. In Zimbabwe, *Ancylostoma* has been reported in stray dogs (Mukaratirwa and Taruvinga, 1999); for instance, Mukaratirwa and Busayi (1995) reported a 38% prevalence of this hookworm species in stray dogs in the urban Bulawayo area of Zimbabwe. Minnaar and Krecek (2001) reported high prevalence of *Ancylostoma* spp. (88.4% *A. caninum* and 20.3% *A. braziliense*) in Boksburg, Gauteng, South Africa. A high prevalence of *Ancylostoma* species (93%) was also reported in Pretoria (Jericho and surrounding villages) and in

Zuurbekom, Johannesburg, South Africa (Minnaar *et al.,* 1999). A study conducted in the Durban metropole and coast showed a prevalence of 53.8% of *Ancylostoma* spp. (Mukaratirwa and Singh, 2010). Furthermore, Lamb *et al*. (2012) reported 95% prevalence of *A. caninum*, 26% of *A. braziliense* and 21% of *A. caninum* and *A. braziliense* mixed infection. According to Hotez and Kamath (2009), however, hookworm infections in other provinces of South Africa are less common than in the KwaZulu-Natal province.

Due to rapid urbanization and increased human-wildlife interaction (in conservation centres and zoological gardens for endangered and valuable animals), wildlife are emerging as significant helminthic zoonotic agents (Xie *et al.,* 2017). According to Seguel and Gottdenker (2017), wildlife prevalence has been documented mostly through necropsies, culled animals or animals incidentally found dead. Globally, at least 68 hookworm species have been reported in 9 orders, 22 families and 108 species of wild mammals (Seguel and Gottdenker, 2017). Hookworm species with the largest host range in wildlife include *A. tubaeforme, A. pluridentatum, A. braziliense* and the human nematode *N. americanus* (Seguel and Gottdenker, 2017). Out of 218 worldwide studies describing hookworm infections in wildlife hosts, only 16.4% were conducted in Africa (Seguel and Gottdenker, 2017).

2.5 Risk factors for hookworm transmission

Climatic and environmental factors play an important role in the transmission of hookworm infections (Bethony *et al.,* 2006) as they affect larval development and activity (Mabaso *et al.,* 2003). Higher incidence rates of hookworm infections in the humid subtropics and countries in warm temperate regions are due to the occurrence of conditions that favour skin penetration (Bowman *et al.,* 2010) and are ideal for the survival of larvae (Mabaso *et al.,* 2003; Bethony *et al*., 2006; Ngui *et al.,* 2012). These favourable conditions include wet surroundings as the larvae are susceptible to desiccation (Bowman *et al.,* 2010) and high temperatures (Hossain and Bhuiyan, 2016).

The essential environmental components supporting hookworm infection include climatic conditions and soil type groups (Mabaso *et al.,* 2003). In KwaZulu-Natal province of South Africa, the high prevalence of hookworm infections in humans was associated with areas at altitudes lower than 150 metres, containing well drained sandy soil coupled with warm humid coastal conditions and a low clay content of less than 15% (Mabaso *et al.,* 2003; Brooker *et al.,* 2004). The amount of moisture that is available to the larvae is dependent on soil texture. At low altitudes, sandy soil types are more advantageous to larval growth than clay soil because clay soil has a lower permeability due to smaller interstitial pores (Mabaso *et al.,* 2003). Thus, transmission in clay soil is inhibited as this tends to be wetter and less aerated than sandy soil when it is under similar climatic conditions (Mabaso *et al*., 2003). Rainfall and temperature are also important factors in the transmission of hookworm infections. The relationship between the two is that soil moisture is important for the survival of larvae during the

developmental stage whereas warmth is needed to speed up hatching and larvae development (Mabaso *et al.,* 2003).

Extremely high temperatures are not ideal for hookworm survival and high temperatures (above 35-40 ^{o}C) have been shown to inhibit larval development and in some cases even kill larvae (Mabaso *et al*., 2003; Hossain and Bhuiyan, 2016). Furthermore, even the briefest chilling temperatures have been shown to be fatal to larvae (Mabaso *et al*., 2003) although *A. duodenale* may survive harsh winters by undergoing larval arrest (Brooker *et al.,* 2006; Hossain and Bhuiyan, 2016). These extremities in climatic conditions may be the reason for the decrease of hookworm prevalence on the coastal plain of South Africa with increasing latitude from north to south as was observed by Mabaso *et al.* (2003).

The coastal areas of the Western Cape province experience cold and wet winters as well as warm and dry summer climates. The harsh summer conditions therefore lead to the desiccation of hookworm eggs and larvae while the winter climate hinders the development of eggs and kills the free-living stages (Mabaso *et al.,* 2003). According to Mabaso *et al.* (2003), the prevalence of hookworm infections in KwaZulu-Natal province also decreases with distance inland above 150 m a.s.l. westwards. This was attributed to steep climatic inclines created by topography, and more importantly, temperature. The change in climatic gradients may be the explanation for the absence of hookworm incidences in the south of the former Transkei, South Africa, in the time of the report by Mabaso *et al.* (2003).

Acquiring hookworm infection is directly related to exposure to filariform larvae in contaminated soil. This is most common in the tropics and subtropics in developing countries which are poverty stricken and lack adequate water supply and sanitation (Brooker *et al.,* 2006; Bethony *et al.,* 2006). Hookworm infection risk factors also include poor personal hygiene, open defecation practices, poor household sanitation (Brooker *et al.,* 2004; Jiraanankul *et al.,* 2011; Hossain and Bhuiyan, 2016), low education level and muddy house floors (Hossain and Bhuiyan, 2016). Ngui *et al.* (2012) reported that in Malaysian households that had latrines, they were either poorly maintained or they were mostly used by adults, while the children could defecate indiscriminately around the yard (Ngui *et al.,* 2012). Even adults in some instances would defecate openly, near river banks or in bushes found around their houses (Ngui *et al.,* 2012). The outdoor defecation of both animals and humans then lead to the widespread of contamination of soil by hookworms, increasing the risk of human and animal infections by larvae infesting hosts through percutaneous entry mostly via the feet (Ngui *et al.,* 2012). Walking barefoot (another common practice in poverty stricken communities) increases the risk of infection through skin penetration (Feldmeier and Schuster 2012; Ngui *et al.,* 2012; Hossain and Bhuiyan, 2016).

Another risk factor for infections with hookworms in humans is engaging in agricultural pursuits, particularly in areas where faeces are used as soil fertilizer (Brooker *et al.,* 2004; Hossain and Bhuiyan, 2016). High rates of infection can also be observed in farmers even when faeces are not used as fertiliser. For an example, in India, Sri Lanka and Bangladesh a high rate of infection was attributed to the use of

gardens and fields as defecating grounds (Brooker *et al.,* 2004) by people who then walked barefoot while working in the tea estates (Hossain and Bhuiyan, 2016).

2.6 Diagnosis and control/prevention

Diagnosis of hookworm infections can be done by examination of faecal samples from suspected individuals for strongylid-type eggs (Crompton, 2000; Zhan *et al.,* 2001), as each hookworm adult female produces thousands of eggs daily (Bethony *et al.,* 2006). Egg concentration techniques have been developed that can detect even the lightest of infections; an example is the formalin-ethyl acetate sedimentation technique (Bethony *et al.,* 2006). To measure the intensity of infection, the McMaster technique (Bethony *et al.,* 2006) and the Kato-Katz faecal-thick smear (Crompton, 2000; Brooker *et al.,* 2004; Bethony *et al.,* 2006; Levecke *et al.,* 2011; Hossain and Bhuiyan, 2016) can be used. The methods measure the intensity by estimating the number of eggs per gram of faeces (Bethony *et al.,* 2006). Other stool examination techniques include the ether concentration method, sodium acetate-acetic acid-formalin (SAF) solution, and the FLOTAC technique (Hossain and Bhuiyan, 2016).

Diagnosis is also based on morphological characterisation of adult hookworms (Zhan *et al.,* 2001; Traub *et al.,* 2004). This has been reported as impractical as the worms can only be recovered after treatment with anthelmintics (Zhan *et al.,* 2001), and differentiating between the adult worms is time consuming and requires skilled personnel (Traub *et al.,* 2004). Most commonly, subtle morphological features of the infective third stage larvae can be used to identify the hookworm species infecting individuals (Crompton, 2000; Zhan *et al.,* 2001). This is done microscopically by skilled personnel after culturing the eggs obtained from the faeces for one week (Zhan *et al.,* 2001).

Hookworm infections can also be diagnosed using molecular techniques when there is a need to differentiate between species (Brooker *et al.,* 2004). Molecular techniques produce rapid and reliable identification of hookworms at the different developmental stages (Zhan *et al.,* 2001; Traub *et al.,* 2004). These include DNA based techniques like PCR and PCR-RFLP (Zhan *et al.,* 2001; Traub *et al.,* 2004). Zhan *et al.* (2001) reported that PCR-RFLP showed different digestion patterns for numerous hookworm spp. using the ribosomal DNA (rDNA) genes and the 3' untranslated region of the cAMP-dependent protein kinase gene. The high rate of mitochondrial evolution has also prompted the use of the mitochondrial cytochrome oxidase subunit I gene (CO I) as a genetic marker for the assessment of genetic variations in various parasite species (Zhan *et al.,* 2001). In the identification of hookworm species, the nuclear ribosomal internal transcribed spacer (ITS)-1, 5.8S and ITS-2 regions have been successfully and continuously used as genetic makers (Traub *et al.,* 2004; Brooker *et al.,* 2004; Lamb *et al.,* 2012).

For treatment, HrCLM can be effectively cured with a single oral dose of ivermectin (200 μg per kg bodyweight) (Hochedez and Caumes, 2007; Feldmeier and Schuster, 2012). In countries where ivermectin is not available, oral administration of 400 mg of albendazole per day for 5-7 days can cure

HrCLM (Hochedez and Caumes, 2007; Feldmeier and Schuster, 2012). Hochedez and Caumes (2007) reported that for patients for whom oral albendazole and ivermectin are contraindicated (for example, light weight children), 10% albendazole ointment can be applied twice a day for 10 days. For removal of adult hookworms in the gastrointestinal tract, a single dose of 400 mg of albendazole is effective for both adults and children (Hotez *et al.,* 2004; Bethony *et al.,* 2006; Albonico *et al.,* 2008; Hossain and Bhuiyan, 2016). For children of 1-2 years however, the dosage of albendazole is reduced to 200 mg (Bethony *et al.,* 2006; Albonico *et al.,* 2008). Alternatively, administration of 100 mg of mebendazole twice a day for 3 days (Hotez *et al.,* 2004; Bethony *et al.,* 2006; Albonico *et al.,* 2008; Hotez *et al.,* 2007; Hossain and Bhuiyan, 2016), or of pyrantel pamoate (11 mg/kg, (maximum dose 1 g)) for 3 days or a single dose of 2 to 5 mg/kg of levamisole (repeat after 7 days in the case of heavy infection) can be used for hookworm treatment (Bethony *et al*., 2006; Hossain and Bhuiyan, 2016).

Presently, anthelmintics are also used for large scale disease reduction by mass treatment in endemic communities (Bethony *et al.,* 2006; George *et al.,* 2016). Existing efforts focus on the school age population as an estimated 25-35% of school going children are infected with one or more of the major STH (Brooker *et al.,* 2006). To significantly reduce and maintain the worm burden well below the threshold associated with disease (Bethony *et al.,* 2006), school-based deworming of all children irrespective of infection status is done (Bethony *et al.,* 2006; Brooker *et al.,* 2006; Levecke *et al.,* 2011; George *et al.,* 2016). Regular deworming also improves childrens' iron reserves, growth and physical fitness, school attendance and cognitive performance (Hotez *et al.,* 2004; Bethony *et al.,* 2006). According to Brooker *et al.* (2004), however, hookworm infections are more prevalent in adults than in school-going children, therefore school deworming programmes may be less effective in the control of hookworm illnesses. In endemic communities, after the first trimester, mass treatment of pregnant women with anthelmintic drugs is recommended to improve not only the mother's health but also that of the new-borns (Brooker *et al.,* 2004; Bethony *et al.,* 2006).

There are problems related to the widespread and frequent use of anthelmintics (Bethony *et al.,* 2006; Ngui *et al.,* 2012). These include reports of anthelminthic drug resistance and high re-infection rates that commonly occur in highly endemic regions (Brooker *et al.,* 2004; Ngui *et al.,* 2012). Ngui *et al.* (2012), reported that reinfection has been observed after as little as two-months post treatment. However, according to Brooker *et al.* (2006), because of the already available intervention strategies for the control of STH infections, it would be possible to develop new improved and effective approaches to helminth control.

Prevention of hookworm infections could also be achieved by increasing knowledge on soil transmitted helminths in rural areas (health education), and by promoting good hygiene and sanitation (Jiraanankul *et al.,* 2011; George *et al.,* 2016) through improved water (George *et al.,* 2016; Hossain and Bhuiyan, 2016) and socio-economic status (Hossain and Bhuiyan, 2016). High prevalence rates of hookworm infection can be reduced by the introduction of latrines, thus improving sanitation (Crompton, 2000;

Brooker *et al.*, 2004; Hossain and Bhuiyan, 2016). Risk of infestation with hookworms can be reduced by wearing protective footwear (Hochedez and Caumes, 2007; Feldmeier and Schuster 2012). Hookworm infections can also be reduced by raising public awareness. Smout *et al.* (2017), commented that it is important to highlight the importance of dogs in the transmission of hookworms to humans when developing public health protocols, since effective control and prevention of infection may necessitate better management and treatment of dogs. Hookworm transmission can also be reduced through boiling of drinking water (Hossain and Bhuiyan, 2016).

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample collection

A total of 356 faecal samples were collected from stray dogs housed at the Society for the Prevention of Cruelty to Animals (SPCA) in Durban metropole centres as follows; Kloof and Highway (n = 21), Springfield (n = 234), Amanzimtoti (n = 20), and Ballito (n = 10); and eight samples were also collected from the Westville veterinary clinic. Three samples from stray dogs were also collected from the Siyathuthuka community, Belfast, Mpumalanga province. Samples were also collected from two primary schools (Mbadleni primary school (n = 25) and Madeya intermediate primary school (n = 25)) in the Ingwavuma area, KwaZulu-Natal province of South Africa and from wild canids and felids in Mpumalanga game reserves. The game reserves from which samples were collected were; Cradle of life (n = 4 lions), Timbavati (n = 2 lions, n = 2 leopards and n = 1 wild dog) and Mjejane (n = 1 cheetah). Selection of schools was based on risk of infection from a previous study on soil-transmitted helminths of school-going children in the area (Kabuyaya *et al.,* 2017). The history of the stray dogs and wild animals was unknown and they were of mixed sex, different breeds and of varying ages. Faecal samples were collected in universal sample bottles and stored in a refrigerator (4 ^{o}C) until delivery to the laboratory for processing.

3.2 Faecal sample examination

The modified Wisconsin sugar flotation method was used to microscopically detect the presence of hookworm-like eggs (Pittman *et al.,* 2010). Faeces from each positive sample (faeces with hookworm-like eggs) were individually cultured and larvae recovered using the Baermann technique as described by Reiss *et al.* (2007). Culturing of faecal samples involved mixing 5 g of faeces with sterile semi-wet crushed charcoal in plastic 500 ml jars and incubating for 7 days at 27◦C (Reiss *et al.,* 2007). Third-stage larvae (L_3) were recuperated using the Baermann apparatus and preserved in 70% ethanol for molecular analysis.

3.3 DNA extraction

DNA was extracted from 4 pooled larvae from each coproculture positive sample using the Genomic DNA™ -Tissue MiniPrep (Zymo Research Corporation) following manufacturer's instructions. Pooled tissue samples of 4 larvae were microcentrifuged at 14,000 ×*g* for 5 minutes to facilitate the removal of alcohol. A solution of 95 µl of nuclease-free H_2O, 95 µl 2X digestion buffer and 10 µl proteinase K was added to the microcentrifuge tube containing samples. This mixture was then vortexed for 30 seconds and incubated at 55◦C for 2 hours. After incubation, 700 µl of genomic lysis buffer was added to the microcentrifuge tube; this was then mixed by vortexing for 30 seconds and centrifuged for 1 minute at

10,000 ×*g* to remove insoluble debris. The supernatant was transferred into a Zymo-Spin™ II Column in a collection tube and then centrifuged for 1 minute at 10,000 ×*g*. The Zymo-Spin™ column was then transferred into a new collection tube where 200 µl of DNA pre-wash buffer was added. This was centrifuged at 10,000 ×*g* for one minute. 400 µl of genomic DNA wash buffer was added to the spin column, which was then centrifuged for a minute at 10,000 ×*g*. The spin column was transferred into a clean microcentrifuge tube. To facilitate the elution of DNA from the column, 30 µl of elution buffer was added to the spin column; this was followed by 2-5 minutes of incubation at room temperature, and then centrifugation at 14, 000 ×g for 30 seconds. The eluted DNA was used for molecular analysis (PCR).

3.4 Molecular analysis

The extracted DNA was subjected to PCR targeting the internal transcribed spacer and 5.8S rRNA (ITS1-5.8S) region using primers AF (5'-CTTTGTCGGGAAGGTTGG-3') and AR (5'-TTCACCACTCTAAGCGTCT-3') designed by Liu *et al.* (2015). This primer pair is reported to amplify a 404 bp region of *A. ceylanicum*, a 404 bp region of *A. caninum*, a 408 bp region of *A. braziliense* and a 406 bp region of *U. stenocephala* DNA (Table 1). Each sample was amplified in reactions of 25 µl containing 12.5 µl DreamTaq PCR master mix (2X), 5 µl of DNA, 1µl of each primer (forward and reverse, concentration 10 µM) and 5.5 µl of nuclease-free H_2O. The PCR cycle parameters were: 1 cycle of 96◦C for 5 minutes to allow initial denaturation, followed by 35 cycles at 96◦C for 30 seconds, 54◦C for 30 seconds and 72◦C 50 seconds and 1 final extension cycle at 72◦C for 7 minutes. PCR products were separated by electrophoresing 4 µl of amplicons at 80v for 80 minutes in 2% agarose gels (in 0.5X TBE buffer). The electrophoresis buffer was 0.5X TBE, to which 100 µl 0.5 µg/ ml ethidium bromide was added to allow visualization of bands by UV transillumination. Restriction fragment length polymorphism (RFLP) analysis was performed by digesting 7 µl of each ITS PCR product with 0.2 µl of EcoRII in a total volume of 20 µl for 3 h at 37◦C. Using the same conditions, the PCR products were also digested directly with BsuRI. EcoRII digestion patters differentiate *A. ceylanicum* from *A. braziliense*; digestion with BsuRI differentiates *U. stenocephala* and *A. ceylanicum, A. braziliense,* and *A. caninum* (Table 1). Restriction fragments were separated by electrophoresis in 3% agarose stained with ethidium bromide under the conditions described above.

Table 1: Expected DNA fragment sizes for different hookworm species, presence/absence of cleavage sites for two restriction enzymes, and predicted fragment sizes after digestion with EcoRII and BsuRI (Liu *et al.*, 2015).

Species	PCR amplicons (bp)	Cleavage site		Predicted fragment size (bp)
		*Eco*RII	*Bsu*RI	
A. caninum	404	−	−	404
A. ceylanicum	404	+	−	76, 328
A. braziliense	408	++	−	76, 122, 210
U. stenocephala	406	−	+	87, 319

3.5 Sequencing and phylogenetic analysis

Thirty-four amplicons were randomly selected and sent for sequencing at Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa. Obtained sequences were BLAST searched on the NCBI Genbank database to identify the closest matches to each sequence. The sequences were downloaded, edited and aligned together with Genbank derived sequences of *A. caninum*, *A. braziliense*, *A. duodenale*, *A. ceylanicum*, *A. tubaeformae*, and *U. stenocephala* using the BioEdit Sequence Alignment Editor (Hall, 1999).

Trees were created using neighbor joining and maximum likelihood methods in PAUP 4.0b10 (Swofford, 2002) and Bayesian Inference as implemented in MrBayes version 3.0b4 (Huelsenbeck and Ronquist, 2001), based on the most likely model of nucleotide substitution (HKY) determined in MEGA 7 (Posada, 2008). In maximum likelihood analyses, starting trees were obtained by neighbor-joining followed by TBR branch swapping. One thousand bootstrap replicates were carried out in the neighbor joining and maximum likelihood analyses in order to assess node reliability. Bayesian analyses were run using four Markov chains and sampling every 100 generations until the standard deviation of the spit frequencies was less than 0.01. The chains were heated with the temperature scaling factor T = 0.02. The first 50,000 trees were discarded as burn-in, having checked in a preliminary run that this was more than sufficient to achieve stationarity. A 50% majority rule consensus tree was constructed from the remaining trees.

Individual pairwise genetic p-distances between the sequence haplotypes were determined using MEGA7. Between and within clade (species) genetic distances, based on the clades present in the phylogenetic analysis, were also calculated.

3.6 Data analysis

The number of samples positive for hookworm-like eggs were used in calculating the prevalence of infection (%) using the formula: Prevalence = (Total number of infected animals divided by the total number of animals examined) multiplied by 100.

CHAPTER 4

RESULTS

4.1 Faecal sample examination

Table 2 shows that, of the 356 faecal samples from humans and other animal species that were collected and screened by coproscopy for hookworm infection, 23.0% (82/356) were positive. Of the 82 positive samples, 21.1% (75/356) were found positive through coproculture. In the Durban metropole, hookworm prevalence by coproscopy was highest in stray dogs from the Kloof and Highway SPCA (47.6%), followed by Amanzimtoti (35%), Springfield (23.5%) and Ballito (20%), whereas the Westville clinic had the lowest prevalence (12.5%). Only three stray dogs were screened in the Siyathuthuka community, Mpumalanga province; they were all positive through coproscopy, whereas only 1 was positive through coproculture and was successfully PCR-amplified. One lion out of the 13 wildlife samples screened was positive for hookworm infection. Of the 75 samples that were deemed positive by coproculture, the ITS1-5.8S rRNA region of 55 was successfully amplified by PCR using the primers AF (5'-CTTTGTCGGGAAGGTTGG-3') and AR (5'-TTCACCACTCTAAGCGTCT-(3').

Table 2: Prevalence of hookworms in faecal samples of dogs, wild canids and humans from the Durban metropole and selected areas of Mpumalanga province, South Africa.

Origin	Species	N	% positive - coproscopy (number)	% positive - coproculture (number)	Number of samples amplified successfully for the ITS1/5.8SrRNA region
KwaZulu-Natal Province					
Ingwavuma	Human	50	6 (3)	6 (3)	3
Durban Metropole-SPCA					
Springfield	Dog	234	23.5 (55)	21.8 (51)	38
Kloof and Highway	Dog	21	47.6 (10)	47.6 (10)	5
Amanzimtoti	Dog	20	35 (7)	35 (7)	5
Ballito	Dog	10	20 (2)	10 (1)	1
Westville Veterinary Clinic	Dog	8	12.5 (1)	12.5 (1)	1
Mpumalanga Province					
Cradle of life (lions)	Lion	4	0 (0)	0 (0)	0
Siyathuthuka	Dog	3	100 (3)	33.3 (1)	1
Timbavati	Lion	2	50 (1)	50 (1)	1
	Leopard	2	0 (0)	0 (0)	0
	Wild dog	1	0 (0)	0 (0)	0
Mjejane	Cheetah	1	0 (0)	0 (0)	0
Total	-	356	23.0 (82)	21.1 (75)	55

4.2 Molecular detection

4.2.1 PCR amplifications

Agarose gel electrophoresis of PCR amplicons generated with primers AF and AR yielded single bands approximately 400 bp in length, corresponding with those reported by Liu *et al.* (2015) and as shown in Table 1, indicating that amplification was successful (Figure 3). However, these primers did not allow for easy differentiation between the species as they amplify regions that are of very similar sizes (404bp, *A. caninum*; 404bp, *A. ceylanicum*; 408bp, *A. braziliense*; 406bp, *U. stenocephala*). The amplicons were therefore subjected to PCR-RFLP using restriction endonucleases BsuRI and EcoRII (Figures 4 and 5) in order to allow differentiation between the species by size and number of restriction endonuclease cleavage products (as in Table 1).

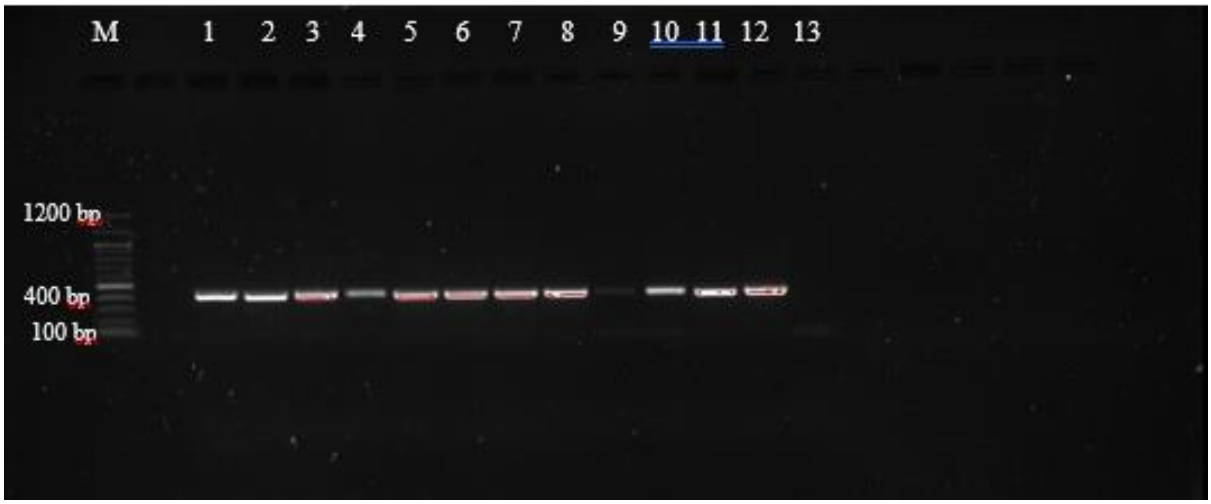

Figure 3: PCR amplification of the ITS1-5.8S region of L_3 larvae from selected hookworm spp. isolates from dogs and lion. (1) 8; (2) 39; (3) Lion; (4) NL1; (5) A10 (6) A11; (7) B24; (8) D22; (9) E2; (10) E16; (11) E17; (12) OBS2; (13) negative control

Figure 4: PCR-RFLP with BsuRI restriction endonuclease of selected third stage larvae (L_3) of hookworm spp. isolates from dogs. (1) A12; (2) B16; (3) C12; (4) D23; (5) E4; (6) Q4

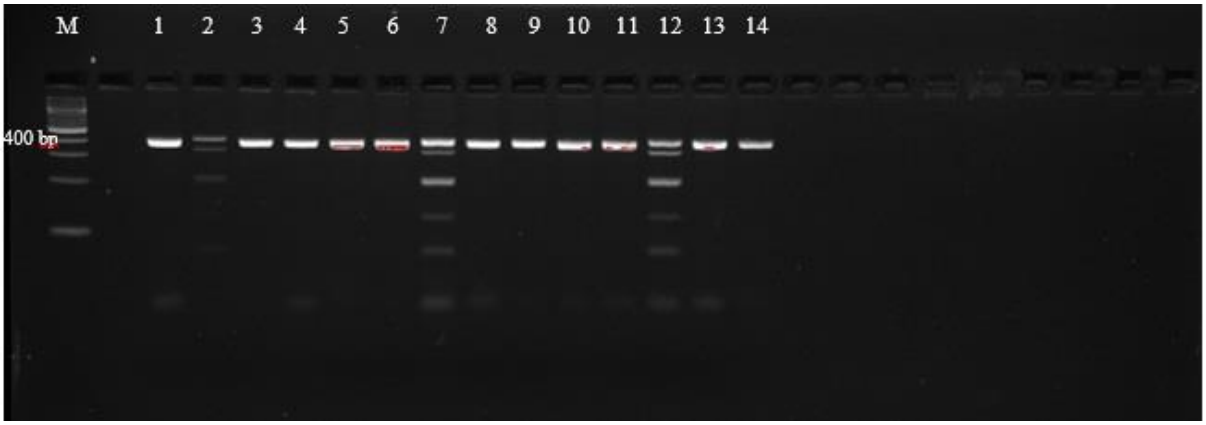

Figure 5: PCR-RFLP with EcoRII restriction endonuclease of selected third stage larvae (L_3) of hookworm spp. isolates from dogs. (1) C2; (2) 8; (3) B24; (4) D3; (5) Patc (6) A10; (7) A24; (8) T15; (9) E17; (10) A11; (11) A9; (12) E6; (13) T1-1; (14) Ett

4.2.2 PCR-RFLP analysis

An attempt was made to digest all 55 successfully amplified samples with the restriction endonucleases EcoRII and BsuRI. Forty of the samples failed to be digested by EcoRII, yielding a single band of 404 bp, consistent with an identification as *A. caninum* (Table 3) (Liu *et al.*, 2015). Fifteen samples were digested by EcoRII (Figure 5 and Table 3). These samples yielded complex banding patterns not consistent with those reported by Liu *et al.* (2015) for either *A. ceylanicum* only (two bands of 76 and 328 bp) or *A. braziliense* only (76, 122 and 210 bp, Table 1). Rather, 7 of the samples contained bands equivalent to 404, 328, 210, 122 and 76 bp, and an extremely small band likely to represent unincorporated primers (eg. samples 8, A24 and E6). This combination of bands is consistent with the presence in the sample of a mixture of *A. caninum* (404 bp), *A. ceylanicum* (76 and 328 bp) and *A. braziliense* (76, 122 and 210 bp) (Table 1). Probably, there were also mixtures of *A. caninum and A. ceylanicum* in four other samples. Four of the samples yielded bands that were consistent with *A. caninum* and one or two more band patterns that did not correspond with any of the bands that were expected, thus the samples were unidentifiable (unknown) (Table 3). Lastly, none of the samples were restricted by BsuRI (Figure 4 and Table 3), leading to the conclusion that *U. stenocephala* was not present in the sample set. The restriction results revealed an overall prevalence of 72.5% (40/55) of *A. caninum*,12.7% (7/55) mixed infection of *A. caninum, A. braziliense* and *A. ceylanicum*, 7.3% (4/55) *A. caninum* and *A. ceylanicum* mixed infection and 7.3% (4/55) *A. caninum* and unreadable bands.

Table 3: Species identification of hookworm isolates from faecal samples obtained from selected SPCAs and primary schools in KwaZulu-Natal and in game reserves from Mpumalanga province, South Africa using PCR-RFLP with EcoRII and BsuRI restriction endonucleases.

Origin	Sample	EcoRII	BsuRI	Hookworm species
Amanzimtoti	T1-1	404 bp	404 bp	*A. caninum*
	T15	404 bp	404 bp	*A. caninum*
	A8	404 bp	404 bp	*A. caninum*
	T17	404 bp	404 bp	*A. caninum*
	T14	404 bp	404 bp	*A. caninum*
Kloof	Patch	404 bp	404 bp	*A. caninum*
	Danger	404 bp	404 bp	*A. caninum*
	P41	404, 328, 210, 122, 72 bp	404 bp	*A. caninum, A. ceylanicum, A. braziliense*
	P39	404 bp	404 bp	*A. caninum*
	Qaphe	404 bp	404 bp	*A. caninum*
Springfield	E2	404 bp	404 bp	*A. caninum*
	B24	404 bp	404 bp	*A. caninum*
	A24	404, 328, 210, 122, 72 bp	404 bp	*A. caninum, A. ceylanicum A. braziliense*
	G11	404, 328, 212, 72 bp	404 bp	*A. caninum, A. ceylanicum*
	B16	404 bp	404 bp	*A. caninum*
	OBS2	404, 328, 212 bp	404 bp	*A. caninum,* Unknown
	E16	404, 328, 210, 122, 72 bp	404 bp	*A. caninum, A. ceylanicum, A. braziliense*
	E4	404 bp	404 bp	*A. caninum*
	C12	404 bp	404 bp	*A. caninum*
	A9	404 bp	404 bp	*A. caninum*
	A11	404 bp	404 bp	*A. caninum*
	A10	404 bp	404 bp	*A. caninum*
	E21	404, 328, 210, 72 bp	404 bp	*A. caninum, A. ceylanicum*
	C1	404 bp	404 bp	*A. caninum*
	B17	404, 328, 210, 122, 72 bp	404 bp	*A. caninum, A. ceylanicum, A. braziliense*
	B13	404 bp	404 bp	*A. caninum*
	E6	404, 328, 210, 122, 72 bp	404 bp	*A. caninum, A. ceylanicum, A. brazliense*
	B15	404 bp	404 bp	*A. caninum*
	E15	404, 328, 212, 72 bp	404 bp	*A. caninum, A. ceylanicum*
	D23	404, 328, 210, 122, 72 bp	404 bp	*A. caninum, A. ceylanicum, A. braziliense*
	B1	404 bp	404 bp	*A. caninum*
	D22	404 bp	404 bp	*A. caninum*
	OBS1	404 bp	404 bp	*A. caninum*
	A12	404, 122, 50 bp	404 bp	*A. caninum,* Unknown
	E17	404 bp	404 bp	*A. caninum*
	8	404, 328, 210, 122, 72 bp	404 bp	*A. caninum, A. ceylanicum A. braziliense*
	D3	404 bp	404 bp	*A. caninum*
	C2	404 bp	404 bp	*A. caninum*
	B2	404 bp	404 bp	*A. caninum*
	B3	404 bp	404 bp	*A. caninum*
	B8	404 bp	404 bp	*A. caninum*
	E1	404 bp	404 bp	*A. caninum*
	E9	404, 328, 122, 76 bp	404 bp	*A. caninum, A. ceylanicum*
	E18	404, 210 bp	404 bp	*A. caninum,* Unkown
	B20	404 bp	404 bp	*A. caninum*

	E5	404 bp	404 bp	*A. caninum*
	B25	404 bp	404 bp	*A. caninum*
	OBS11	404 bp	404 bp	*A. caninum*
Ballito	A3	404 bp	404 bp	*A. caninum*
Westville Veterinary Clinic	Ett	404 bp	404 bp	*A. caninum*
Pongola	MB23	404 bp	404 bp	*A. caninum*
	MI21	404 bp	404 bp	*A. caninum*
	MB18	404 bp	404 bp	*A. caninum*
Siyathuthuka	Dog1	404 bp	404 bp	*A. caninum*
Timbavati	Lion	404, 328, 212 bp	404 bp	*A. caninum*, Unknown

4.3 Sequencing and phylogenetic analysis

Thirty-four amplicons were randomly selected and sent for sequencing using only the forward primer (AF). Samples sent for sequencing included those that had more than one band after restriction with EcoRII, and which were suspected to be species other than *A. caninum*. However, only 31 of the sequences were readable and Genbank BLAST searches of obtained sequences showed that 28 of the samples matched with *A. caninum* while 3 were *A. braziliense*. After sequencing it was also revealed that of the 15 EcoRII restricted samples, 3 matched with *A. braziliense* while the remaining 12 were *A. caninum*, making the prevalence to be 94.4% (52/55) *A. caninum* and 5.45% (3/55) *A. braziliense* of the samples sequenced. Results of sequencing and phylogenetic analysis were therefore not consistent with the findings of the PCR-RFLP analysis.

For the sequenced sample isolates, the overall prevalence of hookworm infections recorded in all three-host species (dogs, humans and wildlife) was 8.7% (31/356). *Ancylostoma caninum* was the most prevalent hookworm species 7.4% (22/296) in dogs whereas only 1% (3/296) of the screened dog isolates were infected by *A. braziliense*. Mixed infections with both *A. braziliense* and *A. caninum* were found in 0.68% of the isolates. Wildlife and human samples had a prevalence of 10% (1/10) and 6% (3/50) respectively. *Ancylostoma braziliense* infections were only detected in dog samples from the Durban metropole (Table 4).

Table 4: Summary of hookworm species from dogs, humans and wildlife from the Durban metropole and Mpumalanga provinces identified through Blast searches after sequencing.

Infected host	**No. sequenced**	**Species identified**	**% positive**
Dogs	22	*A. caninum*	7.4
	3	*A. braziliense*	1
	2	*A. caninum and A. braziliense* (Mixed infection)	0.7
Wildlife	1	*A. caninum*	10
Humans	3	*A. caninum*	6
Total (%)	31		8.7

Sequenced samples were identified by their cladal affiliation with reference to other identified sequences downloaded from the NCBI Genbank and included with them in phylogenetic analyses of the nuclear ribosomal ITS1-5.8S DNA region (Table 5, Figure 6). All experimental samples grouped together to form a monophyletic clade (A) with reference to the outgroup represented by *U. stenocephala* (Figure 6). Clade A was however not supported. Clade B consisted of all haplotypes except haplotype 8 and was moderately supported (bootstrap value of 73% from neighbour joining analysis). Haplotype 8 represented sample A24. This sample was one of those with a complex banding pattern in the RFLP analysis (Figure 5), suggestive of a mixed infection by more than one, and possibly three hookworm species (*A. caninum, A. braziliense* and *A. ceylanicum).* It did not yield an identifiable sequence, possibly because it was a mixture of more than one species, and therefore would not sequence easily. Clade B was an *Ancylostoma* clade and included haplotypes identified as *A. caninum*, *A. duodenale*, *A. braziliense* and *A. ceylanicum*. The haplotypes further clustered to form clade D (*A. caninum, A. ceylanicum* and *A. duodenale*), which was sister to clade C (*A. braziliense*). There was little bootstrap support for the above clades, except for the *A. ceylanicum* clade, which was moderately supported.

Identification of experimental samples was based on cladal affiliation with samples of known identity downloaded from the Genbank, according to the phylogenetic species concept (Cracraft, 1983). Thus, experimental haplotypes 1, 2, 4, 5, 6 and 9 were identified as *A. caninum* based on their presence in a clade which contained Genebank derived haplotypes 11, 12, 13 and 14, identified as *A. caninum* (Table 5, Figure 6). Similarly, experimental haplotypes 3, 7 and 10 were identified as *A. braziliense* based on their co-occurrence in a clade (C) with three Genbank haplotypes identified as *A. braziliense*. The human experimental sample sequences did not cluster together to form a different clade on their own but formed a clade with the animal (dog) *A. caninum* Genbank haplotypes (Table 5).

Table 5: Haplotype status of hookworm species from experimental samples and the NCBI Genbank used in molecular identification of hookworms infecting dogs, humans and wildlife from the Durban metropole and Ingwavuma area of KwaZulu-Natal and Mpumalanga province of South Africa as shown in Fig. 6.

Haplotype	**No. of samples**	**Sample/Genbank Number**	**ID**	**Host (other than dog)**
Hap 1	13	E16, MB18, T15, OBS2, E1, B13, A12, MB23, A3, A9, A10, A11, A12	*A. caninum*	Human (MB18, MB23)
Hap 2	4	MI21, E15, C1, B16	*A. caninum*	Human (MI21)
Hap 3	1	Q4	*A. braziliense*	
Hap 4	1	P41	*A. caninum*	
Hap 5	3	C12, B24, Ett	*A. caninum*	
Hap 6	1	B15	*A. caninum*	
Hap 7	1	8	*A. braziliense*	
Hap 8	1	A24	unclear	
Hap 9	1	A8	*A. caninum*	
Hap 10	1	E6	*A. braziliense*	
Hap 11		JQ083587 (Haplotype E1), DQ438071	*A. caninum*	
Hap 12		JQ083588 (Haplotype E2)	*A. caninum*	
Hap 13		JQ083589 (Haplotype E3)	*A. caninum*	
Hap 14		JQ083590 (Haplotype E4)	*A. caninum*	
Hap 15		JQ083592 (Haplotype E6)	*A. braziliense*	
Hap 16		JQ083593 (Haplotype E7)	*A. braziliense*	
Hap 17		JQ083595 (Haplotype E8), DQ438054	*A. braziliense*	
Hap 18		DQ780009	*A. ceylanicum*	
Hap 19		LC036567.1, KF279132.1, DQ381541.2	*A. ceylanicum*	
Hap 20		EU344797	*A. duodenale*	
Hap 21		EU344797.1	*A. duodenale*	
Hap 22		HQ262167.1, HQ262166.1	*U. stenocephala*	

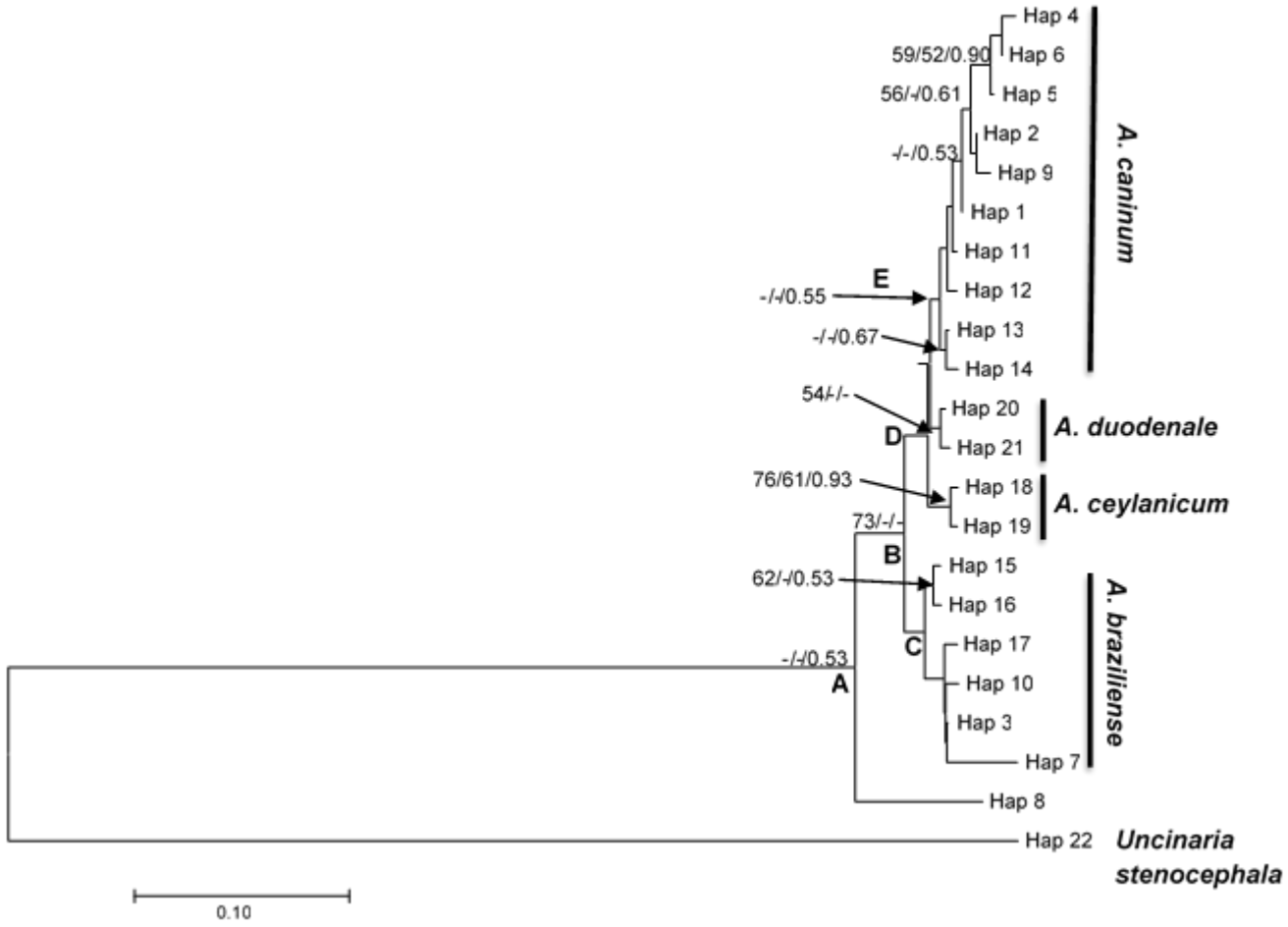

Figure 6: Neighbor-Joining tree based on haplotypes of the ITS1-5.8S rDNA region of experimental samples obtained from dog, human and selected wildlife samples (Hap 1 – Hap 10), Genbank derived haplotypes of the *Ancylostoma* species (*A. caninum*, *A. braziliense, A. ceylanicum* and *A. duodenale*) and Lamb *et al.* (2012) haplotypes of *A. caninum* and *A. braziliense* as the in-groups. GenBank derived *Uncinaria stenocephala* haplotypes were used as the outgroup (Hap 22). Support values indicated at the nodes are, in order, derived from Neighbor-joining (NJ) analyses and congruent Maximum likelihood (ML) and Bayesian inference analyses.

Various *Ancylostoma* clades are separated by shallow genetic distances and mean genetic p-distances within species clades ranged from 0.007 to 0.023 (Table 6, Table 7) and were smaller than those between species clades, which ranged from 0.024 to 0.062 substitutions per site, as might be expected according to the genetic species concept (Baker and Bradley, 2006). Overall, the mean within-*Ancylostoma*-species genetic distance was 0.016, whereas the between species distance was 0.040.

Table 6: p-distance matrix between hookworm species haplotypes as shown in figure 6.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	Hap 1	-																				
2	Hap 2	0.007	-																			
3	Hap 3	0.047	0.054	-																		
4	Hap 4	0.027	0.020	0.074	-																	
5	Hap 5	0.013	0.020	0.060	0.013	-																
6	Hap 6	0.020	0.013	0.067	0.007	0.007	-															
7	Hap 7	0.081	0.087	0.034	0.107	0.094	0.101	-														
8	Hap 8	0.114	0.121	0.081	0.141	0.128	0.134	0.101	-													
9	Hap 9	0.013	0.007	0.060	0.013	0.027	0.020	0.094	0.128	-												
10	Hap 10	0.054	0.060	0.007	0.067	0.054	0.060	0.040	0.087	0.067	-											
11	Hap 11	0.008	0.013	0.054	0.034	0.020	0.027	0.087	0.121	0.020	0.060	-										
12	Hap 12	0.013	0.020	0.047	0.040	0.027	0.034	0.081	0.114	0.027	0.054	0.007	-									
13	Hap 13	0.020	0.027	0.040	0.047	0.034	0.040	0.074	0.107	0.034	0.047	0.013	0.007	-								
14	Hap 14	0.027	0.034	0.034	0.054	0.040	0.047	0.067	0.101	0.040	0.040	0.020	0.013	0.007	-							
15	Hap 15	0.040	0.047	0.020	0.067	0.054	0.060	0.054	0.101	0.054	0.027	0.047	0.040	0.034	0.040	-						
16	Hap 16	0.034	0.040	0.027	0.060	0.047	0.054	0.060	0.107	0.047	0.034	0.040	0.034	0.040	0.047	0.007	-					
17	Hap 17	0.054	0.060	0.007	0.081	0.067	0.074	0.040	0.087	0.067	0.013	0.060	0.054	0.047	0.040	0.013	0.020	-				
18	Hap 18	0.027	0.034	0.047	0.054	0.040	0.047	0.081	0.114	0.040	0.054	0.020	0.027	0.020	0.027	0.054	0.060	0.054	-			
19	Hap 19	0.020	0.027	0.040	0.047	0.034	0.040	0.074	0.107	0.034	0.047	0.027	0.034	0.027	0.034	0.047	0.054	0.047	0.007	-		
20	Hap 20	0.020	0.027	0.040	0.047	0.034	0.040	0.074	0.107	0.034	0.047	0.013	0.020	0.013	0.020	0.034	0.040	0.047	0.020	0.027	-	
21	Hap 21	0.013	0.020	0.034	0.040	0.027	0.034	0.067	0.101	0.027	0.040	0.020	0.027	0.020	0.027	0.027	0.034	0.040	0.027	0.020	0.007	-
22	Hap 22	0.912	0.913	0.919	0.933	0.926	0.926	0.933	0.926	0.919	0.926	0.913	0.919	0.913	0.906	0.919	0.926	0.913	0.899	0.899	0.913	0.913

Table 7: Mean genetic p-distances within (shaded on diagonal) and between (below diagonal) *Ancylostoma* species.

	A. caninum	*A. braziliense*	*A. ceylanicum*	*A. duodenale*
A. caninum	0,023			
A. braziliense	0,062	0,027		
A. ceylanicum	0,048	0,053	0,007	
A. duodenale	0,030	0,050	0,024	0,007

CHAPTER 5

DISCUSSION

Through coproscopical examination of faecal samples, the overall hookworm prevalence in both KwaZulu-Natal and Mpumalanga provinces was found to be 23.0% (82/356); of those positive on coproscopy, 21.1% (75/356) were positive on coproculture. The decrease in sensitivity of coproculture was not expected as the method is considered to be more sensitive than coproscopy and may have been due to differences in intensity of infection in each sample; for instance, if the host was not heavily infected and the sample had a small amount of faeces with low numbers of hookworm eggs resulting in the portion of faeces cultured having few or no eggs.

Fifty-five of the 75 samples that were positive through coproscopy for hookworm larvae were PCR-amplified successfully using primers designed to amplify the nuclear ribosomal ITS1-5.8S rDNA region. The failure of 20 of the samples to amplify may have been due to low hookworm DNA concentrations, as the intensity of infection across the samples may not have been the same. The BsuRI enzyme, which distinguishes *U. stenocephala* from *A. caninum*, *A. ceylanicum* and *A. braziliense*, did not digest any of the samples, indicating the absence of *U. stenocephala* from the sample set. Forty of the samples, that were not digested by either BsuRI or EcoRII and produced products consistent with a size of 404bp, were deemed to be *A. caninum* (Liu *et al.,* 2015). Fifteen samples were digested by EcoRII: these digestion patterns did not correspond with the banding patterns reported by Liu *et al.* (2015) for pure samples of either *A. ceylanicum* or *A. braziliense*, as they presented more bands (up to 5) than could be explained by the presence of either of these species alone (2 or 3 bands respectively). The combination of bands produced was however, consistent with the presence of a mixture of *A. caninum, A. ceylanicum* and *A. braziliense*, or a mixed infection of *A. caninum* and *A. braziliense*. This came as a surprise as *A. ceylanicum* has not been documented in Africa but has been reported to be endemic in Asia (Bowman *et al.,* 2010; Liu *et al.*, 2015; Shepard *et al.,* 2018), Australia (Bowman *et al.*, 2010; Shepard *et al.*, 2018) and South America (Bowman *et al.,* 2010).

After sequencing, three of the samples (samples 8, A24, and E6) appeared to be *A. braziliense* whereas the other 12 were consistent with *A. caninum*. These sequencing results were more consistent with what was expected or predicted as there have been reports of *A. caninum* and *A. braziliense* infections in South African dogs (Minnaar and Krecek, 2001; *Lamb et al.*, 2012). However, in the case of mixed infections, it is likely that, if a sequence can be obtained, it will be that of the numerically dominant species present in the mixed infection, ie. either *A. braziliense* or *A. caninum*, as mentioned above. One would not expect to be able to identify more than one species from a single sequence electropherogram, as mixtures of equal amounts of more than one species of hookworm are likely to lead to confusing patterns. This might explain why, in these cases, the sequencing results identify one species only,

whereas the PCR-RFLP results identify mixtures of more than one *Ancylostoma* species. Further this might explain why the PCR-RFLP patterns for some samples are consistent with the presence of *A. ceylanicum* in a mixture, although it was not detected during sequencing. Further, examination of the lanes of the PCR-RFLP gels where multiple bands were observed shows that they were not all of equal intensity, consistent with the idea that some of the species were present in the mixture at higher concentrations than others.

The presence in this study of multiple bands in the EcoRII digests of certain samples, in contrast to the observations of (Liu *et al.,* 2015), might also be explained on the basis that their study was carried out in China, which is geographically distant and climatically different from South Africa, and might therefore have different patterns of prevalence of *Ancylostoma* species. It is also possible that the samples which produced multiple banding patterns were contaminated with nematode larvae of a different family. Furthermore, Liu *et al.* (2015), noted that *A. caninum* isolates from their study (China) were phylogenetically distinct from *A. caninum* isolates from the USA and Brazil. This was apparent on the neighbour-joining tree based on ITS1 and 5.8S rRNA sequences, in which *A. caninum* isolates from Brazil and the USA clustered to form a clade which was distinct from that formed by *A. caninum* isolated from China (Liu *et al.,* 2015). Such within-species diversity is also likely to be found in hookworm species other than *A. caninum*. Lamb *et al.* (2012) reported unique Southern African ITS haplotypes of hookworm species in dogs from Durban, South Africa which may also explain the differences in band patterns observed in our study.

All dogs (3 in total) from the Siyathuthuka community in Mpumalanga province were coproscopy-positive for hookworms, although isolates from only one of these was successfully amplified and shown to be *A. caninum*. By comparison, dogs from KwaZulu-Natal had a hookworm prevalence of 25.6% (75/293). Thus, there appears to be a higher hookworm prevalence in the Siyathuthuka community than in KwaZulu-Natal. This might be attributed to socio-economic differences, as Siyathuthuka is a rural area where there is poor veterinary care of animals, while dogs from Durban metropole, although deemed stray, were mostly lost and belonged to owners who took care of them, including regular deworming. Other studies have also found that rural areas or resource-poor communities tend to have higher hookworm prevalence when compared to urban areas where owners are most often capable of looking after their dogs (Minnaar and Krecek, 2001; Liu *et al.,* 2015). Furthermore, dog hookworm prevalence in the Durban metropole would be expected to be higher as human hookworms were reported to be more common in the KwaZulu-Natal province than in any other region of the country (Hotez and Kamath, 2009) due to the availability of conducive environmental conditions (favourable environment and soil types) that are ideal for hookworm survival and transmission; this is particularly true along the coastal plain of KwaZulu-Natal where Durban is situated (Mabaso *et al.,* 2003). The hookworm prevalence in dogs from Siyathuthuka community in Mpumalanga province should be interpreted with caution as the sample size was too small to reach a conclusion on the actual prevalence of hookworms

in this locality. For better comparison and validation of these results however, more samples should be collected from dogs and humans in resource poor and high-income areas of Mpumalanga and KwaZulu-Natal provinces.

Although there have been reports on the prevalence of dog hookworms in South Africa (Minnaar *et al.*, 1999, Minnaar and Krecek, 2001; Mukaratirwa and Singh, 2010) and on their molecular identification (Lamb *et al.*, 2012), this study is the first to report infection by the hookworm *A. caninum* in humans (school aged children in Ingwavuma area, KwaZulu-Natal province). All three human sample sequences were identified as *A. caninum* and neither of the two species known to infect humans (*N. americanus* and *A. duodenale*) (Hawdon, 1996; Zhan *et al.*, 2001; Mabaso *et al.*, 2003; Phosuk *et al.*, 2013; Hasegawa *et al.*, 2014; George *et al.*, 2016; Hasegawa *et al.*, 2017; Smout *et al.*, 2017) were detected, even though both species have been reported to be co-endemic in sub-Saharan Africa (Mabaso *et al.*, 2003; Hotez and Kamath, 2009). *Ancylostoma caninum* larvae have been reported to infect humans, manifesting as cutaneous larva migrans (Bowman *et al.*, 2010). There have also been reports of *A. caninum* readily developing into adults in the human gut and causing eosinophilic enteritis (Brown and Copeman, 2003; Landmann and Prociv, 2003; Bowman *et al.*, 2010; Mackenstedt *et al*., 2015; Xie *et al.*, 2017; Shepard *et al.*, 2018) even though Landmann and Prociv (2003) reported that eggs had never been detected in the faeces of patients. Reports on human infection by *A. caninum* have mainly come from Australia (Landmann and Prociv, 2003; Traub *et al.*, 2004; Palmer *et al.*, 2007; Ngui *et al.*, 2012), and reports on hookworm derived eosinophilic enteritis in humans are non-existent. The detection in this study of eggs of *A. caninum* in humans was a surprise, as this indicates that *A. caninum* is able to complete its cycle in humans; this constitutes the first report in South Africa of a patent *A. caninum* infection in humans with evidence of eggs in faeces. No human hookworm species apart from *A. caninum* were detected in Ingwavuma area of KwaZulu-Natal. The prevalence of *A. caninum* was 6 % (3/50), consistent with the report by Mabaso *et al.* (2003) that human hookworm prevalence in KwaZulu-Natal decreases with distance inland above 150 m a.s.l. westwards due to changes in topography and temperature.

Ancylostoma caninum was also detected in the lioness sample from Timbavati Private Nature and Game reserve. Results obtained for the prevalence of hookworm infection in the wildlife sampled are not a true reflection of the actual prevalence due to the small sample size in this study. The small sample size was due to difficulties related to safety of staff in these premises while attempting to obtain faecal samples from the animals. Xie *et al.* (2017) have also reported that obtaining wildlife samples from game reserves is still very difficult despite hookworm infections in wildlife (especially canids), posing a threat to human health. He further reported that rapid urbanization and increased human-wildlife interactions are increasing the importance of wildlife as helminthic zoonotic agents.

Phylogenetic analysis based on the nuclear ribosomal ITS (ITS1) region and 5.8S sequences indicated that all except four samples formed a clade with *A. caninum*. Results of this study are therefore,

consistent with finding from other studies that *A. caninum* is the most dominant hookworm species globally (Mukaratirwa and Busayi, 1995; Minnaar *et al.,* 1999; Minnaar and Krecek, 2001; Traub *et al.,* 2004; Palmer *et al.,* 2007; Lamb *et al.,* 2012; Liu *et al.,* 2015; Ayinmode *et al.,* 2016). Three experimental samples formed a group with *A. braziliense* while one (Hap 8) was an outlier, sister to the other *Ancylostoma* species (Clade B). Haplotype 8 had a p-distance ranging from 8.7% from *A. braziliense* to 14% *A. caninum* (Table 6). Experimental haplotype 8 therefore, was shown to be a unique haplotype, different from the unique Southern African haplotypes recorded by Lamb *et al.* (2012). The experimental haplotype 8 (A24) was sampled from a location different from Lamb *et al.* (2012)'s SPCA locations.

Sequencing of the samples was repeated to confirm the accuracy of some of the sequences that were obtained, especially those of human origin which were identified as *A. caninum*. DNA was re-isolated from each of the samples and PCR amplified before repeat sequencing. This resulted in contrasting results in the case of two samples (E6 and Q4). Sample E6 was identified *A. braziliense* on the first sequencing and then as *A. caninum* on the second, whereas Q4 appeared to be *A. caninum* on the first occasion and *A. braziliense* on the second. This could possibly be due to the presence of a mixed infection in these samples. However, this is not the standard way to detect mixed infections, therefore, there might be more samples with mixed infections that were overlooked as both *A. caninum* and *A. braziliense* have been reported in some of the same areas of study (Lamb *et al.,* 2012).

Future work could focus on the development of multiplex PCR assays with primers specific for the hookworm species of South Africa and a better method for the detection of mixed infections. Studies based on the mitochondrial cytochrome oxidase subunit I gene (CO I) might also give valuable complementary information on enabling identification of hookworm species. There is also a need for future studies on hookworm infections focusing on all provinces of South Africa by broadening the sampling sites in the various provinces of South Africa. There should also be a larger sample sizes especially wildlife and human samples to add on the scarce information.

CONCLUSION

The results of this study are consistent with other findings that *A. caninum* is the most dominant hookworm species globally. The finding of a presumably patent *A. caninum* infection in humans is of great importance and more studies are needed to determine the clinical presentation of this infection in school aged children and also the extent of eosinophilic enteritis due to this parasite in the affected communities. Future studies should look at broadening the range and increasing sample sizes within each South African province for validation and better comparisons of results. Additionally, having access to adult worms of the hookworm species would also help to improve results as they could be used as positive controls, ultimately reducing the chances of misidentification and increasing the validity of the results. This is the first study conducted on hookworms infecting wildlife in South Africa; a wider survey that includes all South African reserves and nature parks, a wider range of sampled animal taxa, and an increased number of samples is recommended in order to better understand the extent and severity of hookworm infections in South African wildlife. This would be vital as it would not only add to the already scarce information on hookworm species infecting wildlife in South Africa but also increase our understanding of the transmission of hookworms amongst the animals. There is also a need for primers and a PCR-RFLP test that can be confidently used in the identification of hookworm species infecting humans, dogs and wildlife and easily pick up mixed infections in Southern Africa as the *Ancylostoma* species have been shown to have unique ITS haplotypes.

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