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# Epidemiology and molecular characterization of human and canine hookworm

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# EPIDEMIOLOGY AND MOLECULAR CHARACTERIZATION OF HUMAN AND CANINE HOOKWORM

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Dedicated to my sons,

Penjani, Tabiso and Aiden

# ACKNOWLEDGEMENTS

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# ABSTRACT

Among the soil-transmitted helminths (STH), hookworms are a worldwide problem in both humans and animals. They cause non-specific gastrointestinal symptoms, and in young children and animals, they can cause stunting, malnutrition and anemia. Canine hookworms have significant zoonotic potential as a cause of cutaneous larvae migrans and eosinophilic enteritis in humans.

To determine the ecological niche of human hookworm in Brazil, two risk models were developed based on the Growing Degree Day-Water Budget (GDD-WB) concept, one based on accumulation of monthly temperatures above a base temperature of 15$^{\circ}$C and threshold WB value >0.4. The second was based on a 'gradient index' of the product of monthly accumulated GDD and WB values. It was determined that both environmental temperature and moisture are important in the distribution of hookworm.  This study supports the validity of the GDD-WB concept for mapping risk of hookworm at a national scale.

A cross-sectional survey was conducted in human outpatients in Mutuípe municipality, Brazil, to determine prevalence of soil-transmitted helminths, including hookworm. Mutuípe falls within the permissive zone for the transmission of hookworm. A sucrose double centrifugation flotation technique was used for the concentration of helminth eggs in fecal samples. Hookworm infection was the most prevalent of the STH and the prevalence was highest in adults and males. PCR was then used to determine the species of hookworm present. *Necator americanus* was confirmed by PCR as the predominant hookworm species. A single case of *Ancylostoma ceylanicum* was identified.

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A study on the prevalence of hookworms and other gastrointestinal parasites in shelter dogs in south Louisiana and the anthelminthic protocols used in the shelters was conducted. Fecal samples examined by direct smear, flotation and sedimentation methods revealed that hookworm had the highest prevalence (53.6%) followed by *Trichuris vulpis* (28.7), *Cystoisospora ohioensis* (17.2%), *Giardia duodenalis* (12.0%), *C. canis* (7.7), *Toxocara canis* (6.2%), *Dipylidium caninum, Alaria* spp and *Capillaria* sp  A PCR-RFLP developed to differentiate *A. caninum* and *A. braziliense* revealed *A. caninum* as the only species found. Evaluation of the anthelminthic protocols used in nine shelters showed current methods were inadequate for control of hookworms in shelter dogs.

# 1    BACKGROUND

## 1.1    Introduction

Hookworm occurs worldwide and usually concurrently with other parasites. Hookworm
was the focus of this dissertation; however, in surveying populations for hookworm, it is
important to describe the occurrence of other parasites as the morbidity associated is
confounded by co-infections. Hookworm surveys normally involve examination of fecal
samples for eggs microscopically and sometimes the more modern molecular methods.
Spatial modeling is useful as a tool in planning surveys as it provides some insight into
the possible occurrence of parasites in hard to reach areas or where surveys have never
been done. Canine hookworm in Louisiana was studied in preparation for work with
human hookworm in Brazil and for its zoonotic capability. This chapter describes
background information on ecological niche modeling, human and canine hookworm as
well as parasites that normally occur with hookworm.

## 1.2    Ecological Niche Modeling

A geographic information system (GIS) is a computerized system which is used to
capture, store, manipulate and analyze georeferenced data (Bergquist and Rinaldi, 2010;
Jones, 2012). It is widely used in environmental epidemiology and public health as it
enables a visual presentation of results and disease forecasts (Jones, 2012). Remote
sensing is defined as obtaining information about an object without physical contact with
it (Campbell and Wynne, 2011). Earth orbiting satellites are used in remote sensing to
collect information about the earth's surface by electromagnetic radiation reflected or
emitted by the earth's surface. The Landsat satellite program was launched in 1972 by the
US government to capture satellite images of the earth and continues to provide

information about the surface of the earth with a spatial resolution of 15-60m (Campbell and Wynne, 2011). The Moderate-resolution Imaging Spectroradiometer (MODIS) is another sensor that orbits the earth onboard the Terra and Aqua Earth Orbiting System (EOS) satellites and captures data at 250m to 1km spatial resolutions. Other satellite sensors, often used in geospatial health applications include the Advanced Very- High Resolution Radiometer (AVHRR) and Advanced Space-borne Thermal Emission and reflection Radiometer (ASTER) (Campbell and Wynne, 2011). These sensors provide data on land and ocean surface temperature, snow coverage, land use, land cover, elevation, topography, soil moisture, rainfall, and other environmental features.

Normalized Difference Vegetative Index (NDVI) is a measure of the greenness or chlorophyll activity of vegetation. It is derived from the red and near infrared radiation readings corresponding to different levels of vegetation cover. Surface temperature is influenced by the presence of vegetation, as plants absorb visible red light during photosynthesis and reflect solar radiation in the near-infrared spectrum which results in a high NDVI value. NDVI is calculated as follows:

$$NDVI = \frac{NIR - R}{NIR + R}$$

Where *NIR* is *near infrared radiation* and *R* is in the *red* spectral range (Campbell and Wynne, 2011).

GIS provides the ability to bring remotely sensed and other geospatial data into one analytical framework, thereby enhancing the range of analytical products (Campbell and

Wynne, 2011). These products include mapping of urban infrastructure, floodplain mapping, and disease distribution mapping.

Maps for human and animal parasites and as well as prediction/risk maps for the diseases they cause have been developed in many regions of the world. This has been possible using the biological/ecological niche concept for parasites that have part or all their life cycle affected by environmental conditions. Environmental properties that contribute to the ecological niche of a particular parasite or vector have to be determined in order to be able to predict the distribution of the organism (Malone, 2005). In pursuing ecological analysis, the following steps should be followed (Bergquist and Rinaldi, 2010):

- Construction of a GIS for the area of interest using data layers delineating environmental and climate features;
- Construction of a frame of georeferenced areas;
- Construction of a database with environmental and parasitological data;
- Statistical analysis to detect environmental risk factors and/or develop prediction models.

Risk maps based on known biological requirements and climatic variables have been employed for a number of diseases including malaria (Thomson et al., 1997; Yang et al., 2010), schistosomiasis (Lindsay and Thomas, 1999; Malone et al., 2001), filariasis (Gyapong et al., 2002; Lindsay and Thomas, 1999), leishmaniasis (Nieto et al., 2006; Salahi-Moghaddam et al., 2010),  Chagas disease (Mischler et al., 2012) *Opisthorchis viverrini* (Suwannatrai et al., 2011), STHs (Pullan and Brooker, 2012) and others

(Bergquist and Rinaldi, 2010). Models such as these are useful in developing and managing intervention programs (Daash et al., 2009; Dongus et al., 2011).

There are two main approaches to developing GIS models for disease (Malone, 2005). The statistically-based approach is used when knowledge of the biological requirements of the organism of interest is limited and only survey data is available (Lindsay and Thomas, 1999; Pullan and Brooker, 2012; Thomson et al., 1997). The second approach, the biology-based model, is initiated with known environmental parameters that are critical to the survival of the organism, that have been determined from laboratory or field studies (Nieto et al., 2006; Valencia-López et al., 2012). Statistical analysis is however required for the model validation process using independent field data (Malone, 2005).

A parameter that has become useful in biology-based modeling is that of heat units, also known as Growing Degree Days (GDD). It was initially developed for and used in crop farming to determine timing of phenotypic events (McMaster and Wilhelm, 1997). It is calculated using climatic data for the study area of interest by obtaining the average daily temperature and subtracting the base temperature ($T_{base}$), below which the organism cannot develop in the environment (McMaster and Wilhelm, 1997).

$$GDD = \frac{(T_{max} - T_{min})}{2} - T_{base}$$

When applied to parasitology, GDD is used to determine the period of time an organism needs to develop from one stage to another in the environment, and to assess if a given environment is suitable enough to support such development and therefore the

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propagation and transmission of the parasite (Malone, 2005). In addition to GDD, water budget (WB) has also proved important in risk modeling. WB is the ratio of precipitation (*P*) and evapotranspiration (*ET*), i.e. *P/ET*. It is a measure of soil moisture saturation, with 0 representing aridity and 1 representing full soil saturation. Values >1 represent water run-off (Shuttleworth, 1983). The concept of GDD and WB has been used successfully to generate predictive models of fascioliasis in the United States, Ethiopia, Ecuador and southern Brazil (Dutra et al., 2010; Fuentes et al., 2005; Malone et al., 1987; Malone et al., 2001; Valencia-López et al., 2012), schistosomiasis in China and East Africa (Malone, 2005; Zhou et al., 2001) and visceral leishmaniasis in Bahia, Brazil (Nieto et al., 2006).

## 1.3    Soil-Transmitted Helminths

Soil-transmitted helminths (STH) are those nematodes that have a soil-based or environmental developmental stage in soil in order for them to become infective. These include hookworms (*Necator americanus* and *Ancylostoma duodenale*), *Ascaris lumbricoides* and *Trichuris trichiura*. STH occur worldwide and are particularly important in tropical and subtropical countries that have high poverty levels in Asia, sub-Saharan Africa, and the Americas (Bethony et al., 2006; Brooker et al., 2006a; de Silva et al., 2003; Horton, 2003). As a group they are considered among the neglected tropical diseases (NTDs) and have received considerable attention since the United Nations millennium declaration in 2000 (Hotez et al., 2008). The association between STH and poverty is widely accepted (de Silva et al., 2003; Hotez, 2008) and has been shown to be significant and complex, with each component perpetuating the other (de Silva et al., 2003). Reduced productivity of chronically infected people perpetuates poverty and the

poor economic growth in such countries means poor sanitation and resultant high of STH
(de Silva et al., 2003; King, 2010).

### 1.3.1   Biology of STHs

Soil transmitted helminths belong to the Kingdom Animalia, Phylum Nematoda and
Class Secernentea.

### 1.3.1.1   *Ascaris lumbricoides*

Order:          Ascaridida

Superfamily:  Ascaridoidea

Family:         Ascarididae

Subfamily:    Ascaridinae

*A. lumbricoides* eggs are released into the environment in feces and develop to $L_2$
infective larvae contained in the egg after 18 or more days, depending on environmental
moisture and temperature (Aaron, 1950; Anderson, 2000; Brown, 1927). When the
infective eggs are ingested, the larvae develop into $L_3$ before they hatch in the small
intestine and penetrate the mucosa. They are then transported via the venous system to
the heart and lungs from about six days post infection (Dold and Holland, 2011; Geenen
et al., 1999). The larvae develop further in the lungs for approximately 10-14 days. They
then penetrate the alveolar wall and travel up the bronchial tree and trachea and are
swallowed and then develop to adults in the small intestine (Figure 1.1) (Dold and
Holland, 2011). The adult worms are the largest intestinal nematodes infecting the human
intestine and measure between 15 to 35cm (Anderson, 2000) and can live up to 23 weeks
(Olsen et al., 1958). Adult worms mate and females lay up to 200,000 eggs per day. Eggs
measure 65-75 x 35-50 µm and are passed in feces unembryonated (Anderson, 2000).

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Infertile and undeveloped eggs are not infective until fully embryonated eggs, which can remain viable in the environment for up to five years under favorable conditions (Anderson, 2000).
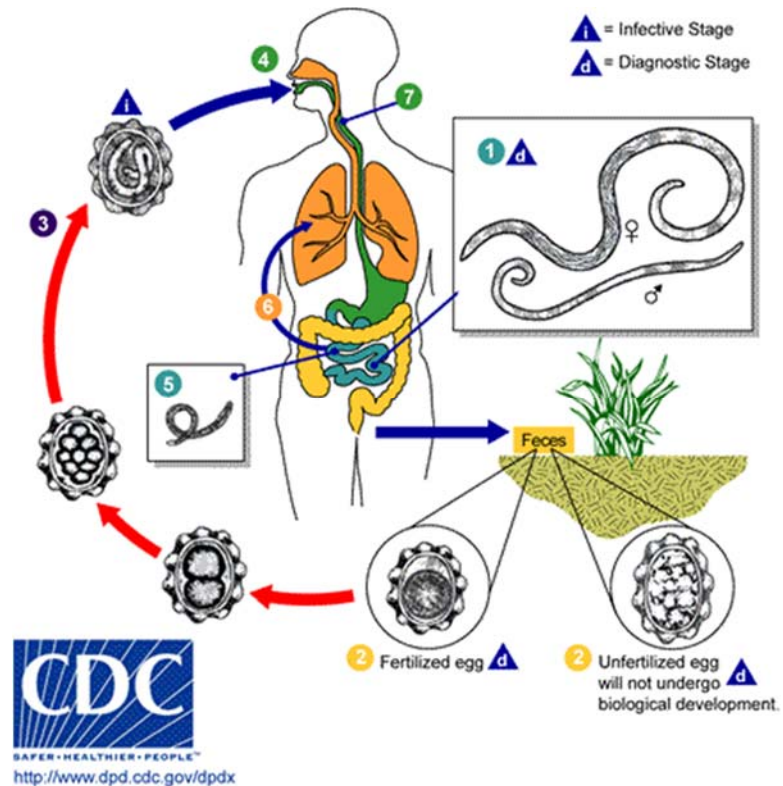


Figure 1.1: Lifecycle of *A. lumbricoides* (C.D.C., 2013a)

1.3.1.2  *Trichuris trichiura* (whipworm)

Order:          Enoplida

Superfamily:  Trichinelloidea

Family:          Trichuridae

Subfamily:    Trichurinae

Unembryonated eggs of *T. trichiura* are passed in feces and develop to infective $L_3$ larvae within the egg in 11 or more days depending on environmental temperature and moisture (Anderson, 2000; Brown, 1927). *T. trichiura* has a direct lifecycle, i.e. no tissue

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migratory phase. When the infective eggs are ingested they hatch in the small intestine and develop further into adult worms (Figure 1.2). The adult worms reside in the large intestine (cecum and colon) and thread their anterior ends into the mucosa, causing significant inflammation. Female worms start laying eggs 60-70 days after establishment and the unembryonated eggs are passed in feces. They can oviposit between 3,000 to 20,000 eggs per day (Anderson, 2000). Adult worms are thought to live an average of 1-2 years in the cecum/colon (Anderson, 2000).
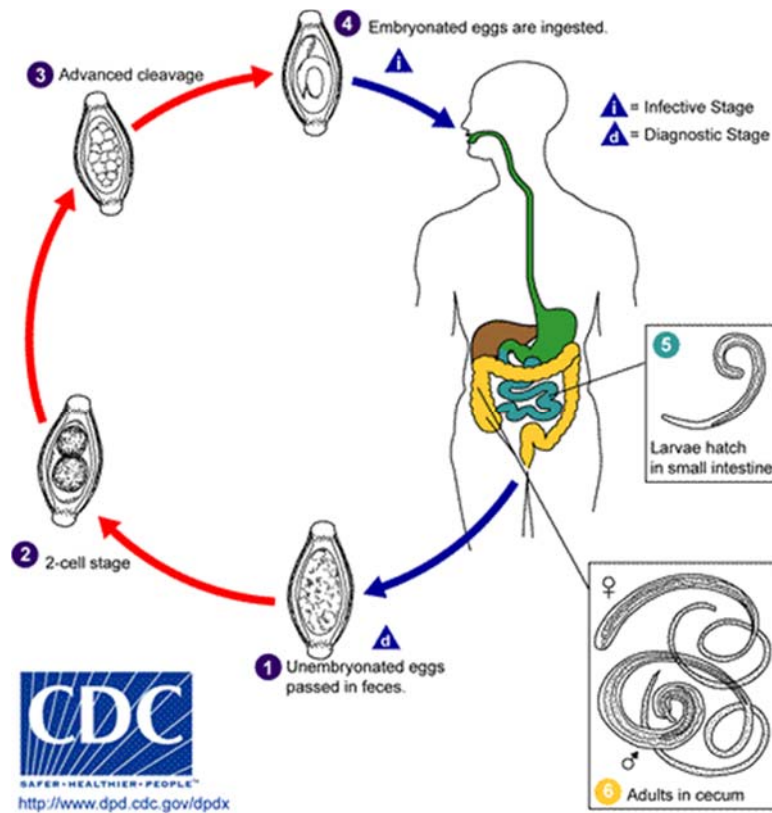


Figure 1.2: Lifecycle of *T. trichiura* (C.D.C., 2013c)

1.3.1.3   Hookworm

Order:          Strongylida

Superfamily:  Ancylostomatoidea

Family:        Ancylostomatidae

Subfamily:    Ancylostomatinae              Bunostominae

Genus:        *Ancylostoma*              *Necator*

*Ancylostoma duodenale* and *Necator americanus* are the two most important hookworms

infecting humans (Brooker et al., 2004). Their two to eight cell stage eggs, measuring 50-

80 x 36-42 μm, are passed in feces into the environment and develop to $L_1$ larvae within

1-2 days (Chandler, 1929) (Figure 1.3). The eggs do not develop in temperatures less

than 15°C (Udonsi and Atata, 1987) and the eggs of N. americanus die at temperatures

less than 7°C while those of A. duodenale survive (Anderson, 2000). The $L_1$ hatch and

develop to $L_2$ larvae which feed on bacteria in the soil and feces. The $L_2$ larvae develop to

$L_3$ which do not feed but retain the $L_2$ cuticle till they infect humans by the oral route (*A.*

*duodenale*) or skin penetration (both species) (Anderson, 2000; Looss, 1911). Infective

larvae (L3) develop in five or more days depending on environmental temperature and

moisture (Chandler, 1929) and can survive in moist soil for up to six weeks (Svensson,

1925; Udonsi and Atata, 1987). The optimum temperature for development of the free

living stages ranges from 21-27°C for *A. duodenale* and 25-28 for *N. americanus*

(Anderson, 2000; Crompton and Whitehead, 1993). On skin penetration, intense pruritus

is experienced at the site and is known as ground itch or hookworm dermatitis (Anderson,

2000; Schad and Warren, 1990). Larvae travel via the lymphatic and circulatory system

to the lungs (Looss, 1911) and, after penetrating into the alveoli, travel up the bronchial

tree to the pharynx and are swallowed. The pulmonary phase may induce episodes of

coughing and pharyngitis 7-14 days post infection (Nawalinski and Schad, 1974; Schad

and Warren, 1990). The larvae take up residence in the small intestine and develop into

L4 which feed on mucosa and blood and then into adults which attach to the mucosa and feed on blood (Pawlowski et al., 1991). *A. duodenale* females can produce 10,000 to 25,000 eggs per day and *N. americanus* 6,000 to 11,000 eggs per day (Anderson, 2000; Crompton and Whitehead, 1993). The prepatent period for *N. americanus* is 44 or more days (Crompton and Whitehead, 1993; Looss, 1911). *A. duodenale* is capable of hypobiosis, an arrested development stage, and the prepatent period can therefore be as little as 31 days up to eight months (Nawalinski and Schad, 1974). Life expectancy of the adult hookworms is 2-7 years (Anderson, 2000; Brooker et al., 2006b; Crompton and Whitehead, 1993). The eggs of the two common species of hookworm cannot be differentiated by microscopy; however, the infective larvae and adults can (Table 1.1).
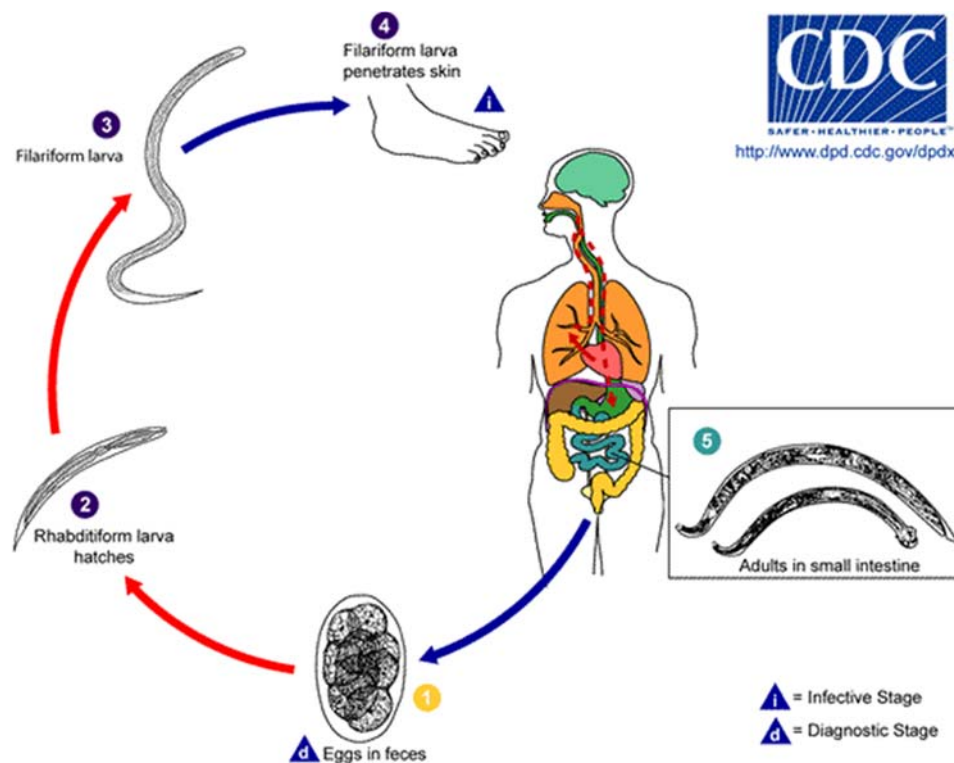
Figure 1.3: Hookworm lifecycle (C.D.C., 2013b)

Table 1.1. Morphological differences between *A. duodenale* and *N. americanus* (Chandler, 1929; Crompton and Whitehead, 1993; Looss, 1911; Nichols, 1956; Pawlowski et al., 1991; Svensson and Kessel, 1926; Wu and Peng, 1965).

| Stage | Features | *A. duodenale* | *N. americanus* |
|---|---|---|---|
| Infective larvae | Buccal cavity | | More prominent |
| | Genital primordium | | More anterior and smaller |
| | Esophagus | Narrower Continuous with intestine | Broader Constriction (gap) at the junction with intestine |
| | Intestine | Narrower than esophageal bulb at junction with esophagus | As wide as esophageal bulb at junction |
| | sheath | Striations inconspicuous | Conspicuous striations in sheath |
| | Body length (μm) | 600-700 | 500-600 |
| | Length of sheath (μm) | 720 | 660 |
| Adult | Buccal cavity | Two pairs of teeth | Two cutting plates |
| | Anterior end | Less distinct curve Less tapered | Distinct curve More slender and more finely tapered |
| | Size (mm) | Male: 8-11 Female: 10-13 | Male: 7-9 Female: 9-11 |

1.3.2   Public Health Significance

STH infections tend to cause chronic infections with low mortality rates. Symptoms associated with STH include diarrhea, reduced appetite and weight gain, impaired cognitive abilities, malnutrition, anemia, abdominal pain and stunted growth (Beaver, 1988; Bethony et al., 2006; Eppig et al., 2010; Ezeamama et al., 2005; Haque et al., 2010;

Jardim-Botelho et al., 2008; Stephenson et al., 1993; Yap et al., 2012). *A. lumbricoides* can cause life threatening intestinal obstruction, when the adult parasite is present in large numbers particularly in young children (Akgun, 1996; Villamizar et al., 1996) and rarely airway obstruction (Bailey and Warner, 2010). *A. lumbricoides* has also been associated with Vitamin A deficiency due to malabsorption. (Mahalanabis et al., 1976). In addition to the symptoms of STH infection above, *T. trichiura* causes dysentery and occasionally rectal prolapse in severe cases (Cooper et al., 1992; Haque et al., 2010). Trichuris may also cause anemia, although hookworms cause greater blood loss with resultant iron deficiency anemia (Stoltzfus et al., 1997). The iron deficiency anemia related to hookworm infection is more pronounced in moderate to high infections, particularly in children, but even low intensity infections cause lower hemoglobin levels in adults (Brooker, 2010).

The WHO uses Disability-adjusted life years (DALYs) to estimate the global burden of disease caused by any disease. DALYs are an estimate of the number of healthy life years lost due to disability or premature death caused by a particular disease and are derived by assigning disability 'weights' to different conditions and multiplying that weight by the number of years that an infected person is affected by that condition (Brown et al., 2009). Among the DALYs calculated for NTDs in 2010, STHs rank second with 5,184,000 DALYs after malaria (82,685,000 DALYs), with the highest impact being in sub-Saharan Africa (Murray et al., 2012). Ascariasis accounted for 1,315,000, trichuriasis 638,000 and hookworm disease had the highest with 3,231,000 DALYs (Murray et al., 2012). There was a 42.5% reduction in DALY impact by STH from 1990 to 2010, with the highest reduction being in ascariasis (68.8%) and the lowest (17.9%) in hookworm infections

(Murray et al., 2012). The great reduction in ascariasis may be due to the high efficacy of benzimidazoles against *A. lumbricoides* and improvements in sanitation, especially in China and Brazil (Fenghua et al., 1998; Pullan and Brooker, 2012).

1.3.3   <u>Prevalence</u>

It is estimated that over a billion of the world's population is infected with at least one of the STH (Bethony et al., 2006) and more than 300 million suffer from severe morbidity (WHO, 2010). The majority of STH are found in the tropics and subtropics (Figure 1.4) and WHO estimates that 130 countries are endemic (WHO, 2008). China, Southeast Asia, Central Africa, and the coastal regions of West Africa have the highest prevalence of *A. lumbricoides* infection (de Silva et al., 2003). *T. trichiura* prevalence is highest in Central Africa, southern India and Southeast Asia, while hookworm infections are common throughout most of sub-Saharan Africa, south China and Southeast Asia (de Silva et al., 2003).

Although there was a great decline in STH estimated prevalence in Latin America and the Caribbean (LAC) from 1994 to 2003, due to widespread control efforts, the risk of transmission remains high due to the highly permissive climate of this region (de Silva et al., 2003; Pullan and Brooker, 2012).  Prevalence estimates for LAC in 2003 were 16% for *A. lumbricoides*, 19% for *T. trichiura* and 10% for hookworm (de Silva et al., 2003). However, the prevalence varies within regions and countries based on socioeconomic factors, urbanization and sanitation (Pullan and Brooker, 2012). STH prevalence tends to be higher in rural areas due to lower socioeconomic levels, farming practices and poor sanitation (Brooker, 2010; Hotez et al., 2005). Economic development of rural areas has

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been linked to a reduction in STH prevalence, as seen in the Jiangsu province of China (Fenghua et al., 1998).
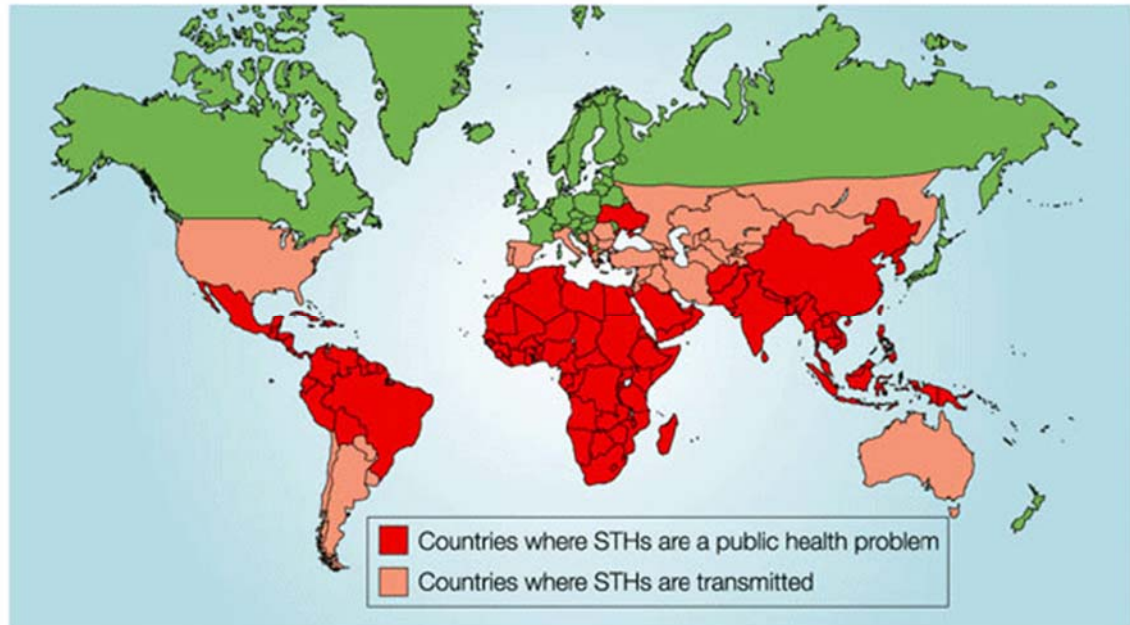


Figure 1.4: Global distribution of Soil-transmitted helminths. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology (Savioli and Albonico, 2004) (Appendix A).

1.3.4 <u>Diagnosis</u>

Since the symptoms of soil-transmitted helminthiasis are mostly non-specific, diagnosis of STH is primarily reliant on demonstration of helminth eggs in feces. The most commonly used method, and one that is recommended by WHO for surveillance of STH is the Kato-Katz method (Montresor et al., 1998). It was initially developed for the detection of *Schistosoma mansoni* in feces (Katz et al., 1972) but has since been adopted for use in detection of STH as well (Endris et al., 2013; Funk et al., 2013; WHA, 2001). The method is both quantitative and qualitative and has proved to be relatively robust and field-friendly as it does not require the use of expensive equipment like a centrifuge. However, hookworm eggs tend to collapse after approximately 30 minutes of the slide

being prepared and this could lead to erroneous results if there is a delay in examination of the slide (Booth et al., 2003). Another disadvantage is the loss of sensitivity in the test due to the small amount (41.7mg) of feces that is examined at a time (Booth et al., 2003; Endris et al., 2013). However, this may be overcome by the preparation of two or three slides per sample of feces and also repeated sampling for two to three consecutive days to overcome the day-to-day variation in egg shedding by adult worms (Booth et al., 2003), although it would be more time consuming and costly (Utzinger et al., 2008). Tarafder et al. (2010), used a Bayesian approach to determine the sensitivity and specificity of Kato-Katz method in the absence of a gold standard, and found that one stool sample was adequate with regard to detection of *Ascaris* and *Trichuris* but not hookworm eggs, and that two samples (from two days) gave better results.

The formalin ethyl acetate (FEAC) concentration/sedimentation method (Allen and Ridley, 1970; Young et al., 1979) is also used to detect STH eggs as well as many other parasite eggs, larvae, oocysts and cysts. Because of the formalin, the eggs are preserved and the sample can be examined long after collection (Baker, 2007). This is advantageous for samples that need to be transported for examination at a distant/referral facility. Drawbacks include the large amount of debris, the requirement of a centrifuge and safe disposal of reagents like ethyl acetate (Allen and Ridley, 1970; Baker, 2007). In a study in Côte d'Ivoire, FEAC yielded more *Trichuris* positives than the Kato-Katz method (Glinz et al., 2010). When compared to Kato-Katz, FEAC detected more hookworm positives and this was believed to be because of the larger quantity of feces that can be examined (Endris et al., 2013).

Spontaneous sedimentation, also known as the Hoffman-Pons-Janer method, is an easy and inexpensive method because it only utilizes water and a conical flask (Carvalho et al., 2012). However, it requires that the sample be examined the same day of the sedimentation or be refrigerated to prevent the hatching of hookworm eggs. If the sample is unpreserved, hookworm L1 larvae may be confused with *Stongyloides stercoralis* and need to be differentiated, since the treatment for the two is different (Garcia, 2001). In a comparison, the Kato-Katz method was found to perform better in detection of helminths than spontaneous sedimentation (Carvalho et al., 2012).

Flotation procedures which can be both quantitative and qualitative have not been used widely in human parasitology but are widely used in veterinary parasitology. The McMaster method (MAFF, 1986) typically uses sodium chloride solution as a flotation medium and is useful for the recovery of helminth eggs with the exception of operculated and schistosome eggs (Truant et al., 1981). In a study that compared the sensitivity of Kato-Katz method with that of the McMaster method, the results for the two were comparable except with regard to *Ascaris*, where Kato-Katz detected significantly more positive samples and higher egg counts (Levecke et al., 2011). In testing the McMaster method against an ether-based sedimentation method for detection of *Trichuris* in primate fecal samples, the McMaster test was less sensitive but detected significantly more eggs than the ether-based concentration method (Levecke et al., 2009).

Zinc sulfate solution (Specific gravity (SG) 1.18) (Faust et al., 1938) is another flotation medium that has been used extensively in veterinary parasitology (Dryden et al., 2005), with and without centrifugation, although centrifugation resulted in detection of more positive samples and higher egg counts (Dryden et al., 2005; Zajac et al., 2002). The

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centrifugal force created causes heavier fecal debris to sink to the bottom of the tube therefore prevents obscuring of parasite eggs (Dryden et al., 2005). When used on human fecal samples, zinc sulfate was more effective for the detection of hookworm eggs than FEAC, but not as effective for *Ascaris* and *Trichuris* eggs (Truant et al., 1981). Brandelli et al. (2012) used centrifugal flotation with zinc sulfate on human fecal samples in conjunction with other methods but no comparison of methods were reported. Another flotation medium that is widely used in veterinary parasitology but not in human parasitology is the sucrose (Sheather's) solution (SG 1.25-1.27). Sucrose has been used in a few human parasitology studies with success (Shakya et al., 2006) although information on how it performs in comparison with other helminth egg concentration methods is lacking. When compared with other flotation solutions, it was the best for gastrointestinal strongyle egg visualization and maintained good parasite egg quality for longer (Cringoli et al., 2004). Due to the higher SG, sucrose flotation results in higher fecal egg counts than zinc sulfate and, like zinc sulfate flotation, centrifugation enhances results (Cringoli et al., 2004; Dryden et al., 2005).

The FLOTAC method was developed more recently (Cringoli, 2006) that utilizes a FLOTAC® apparatus. The apparatus is used to perform flotation in a centrifuge and the retention of the upper portion of the floating suspension can then be visualized in the FLOTAC chamber under a microscope (Cringoli, 2006). FLOTAC can be both a quantitative and qualitative technique and utilizes up to a gram of feces. There is a choice as to the flotation medium that can be used in the apparatus (Cringoli et al., 2004) and this allows for flexibility. In comparison to triplicate Kato-Katz in two different STH endemic settings, the FLOTAC was found to be more sensitive in detection of STH eggs

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but yielded lower fecal egg counts (Glinz et al., 2010; Knopp et al., 2009). Depending on the flotation medium used, also allowed for detection of larvae, cysts and oocysts of protozoa (Cringoli, 2006). FLOTAC performed superior to FEAC in recent surveys (Glinz et al., 2010; Utzinger et al., 2008). When tested only for hookworm egg detection, FLOTAC was much more sensitive than Kato-Katz or FEAC, implying that there may be an underestimation of prevalence of human hookworm infections and probably other STHs and *S. mansoni* by routinely used methods (Kato-Katz and FEAC) (Utzinger et al., 2008).

Serology for antibody detection using enzyme linked immunosorbent assays (ELISA) have been developed and used to detect intestinal parasites such as *O. bifurcum* and *N. americanus* (Pit et al., 2001; Polderman et al., 1993). However, they are not very sensitive or specific and not able to differentiate current from past infection (Basuni et al., 2011; Verweij et al., 2001).

Coproculture is useful for differentiating nematode infective larvae for strongylids such as *N. americanus, Ancylostoma* sp, *O. bifurcum, Trichostrongylus* spp and *S. stercoralis* (Harada and Mori, 1955; Okazaki et al., 1988; Yong et al., 2007). However, coproculture is time consuming, taking six or more days, is labor intensive, and requires skilled personnel (Basuni et al., 2011; de Gruijter et al., 2005).

The development of PCR for the detection and identification of intestinal parasites in the last two decades has proved very helpful in parasitology (Verweij et al., 2001), and, together with the sequencing of the entire mitochondrial genomes of *N. americanus* and *A. duodenale*, study of the genetic variability in hookworms has become feasible (Chilton

and Gasser, 1999; Hu et al., 2002). Hookworm eggs are difficult to differentiate by microscopy because of their similarity in size.

Gasser et al. (1993) and Romstad et al. (1997) found the ribosomal (r)DNA region encompassing the internal transcribed spacer (ITS-1) , 5.8S, ITS-2 and part of the 28S rRNA gene as being ideal for PCR amplification as it was well conserved among strongylid nematodes but had adequate sequence difference between species for delineation (Figure 1.5). Genetic markers in the ITS-2 region were used develop species-specific primers to distinguish *N. americanus* from *O. bifurcum* (Romstad et al., 1997; Verweij et al., 2001) and to differentiate *N. americanus* eggs from those of *A. duodenale* in fecal samples using a semi-nested PCR technique (de Gruijter et al., 2005).
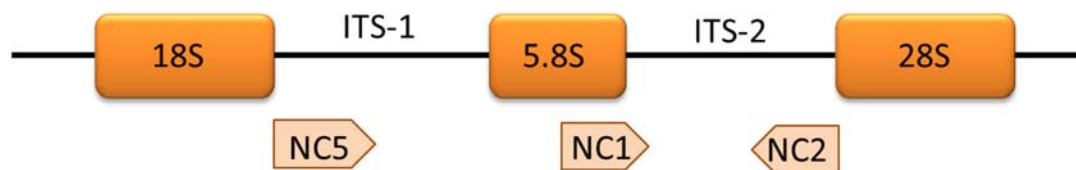


Figure 1.5. Schematic representation of part of the rDNA transcriptional unit and relative locations of primers NC5, NC1 and NC2 commonly used for hookworm species differential diagnosis. Adapted from (Romstad et al., 1997; Yong et al., 2007).

1.3.5    Treatment and Control

False negative results have been reported with PCR and have been attributed to the small amount of fecal sample used as compared to that used in coproculture or fecal concentration methods and also possible DNA degradation from long periods of storage (de Gruijter et al., 2005; Ngui et al., 2012b). There is a high degree of similarity between the ITS-2 regions of *A. duodenale* and *A. ceylanicum* (a zoonotic hookworm species) with only a few nucleotide differences, therefore, de Gruijter et al. (2005) found it necessary to recommend sequencing of the resulting PCR amplicons to differentiate the

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two species. Real-time PCR assays have also been developed for simultaneous diagnosis of hookworms and other species of intestinal parasites (Basuni et al., 2011). A PCR-linked restriction fragment length polymorphism (RFLP) was developed and used by Hawdon (1996) to differentiate *N. americanus* from *A. duodenale* using endonucleases and it was found that *Taq*1 worked best to differentiate single and mixed infections of hookworm.

With the growing international attention that neglected tropical diseases (NTD) were receiving in the 1990s, WHO developed a clear policy for the control of STH in endemic areas (Hotez et al., 2008; Montresor et al., 1998). The policy was based on prevalence and intensity of infection of the STH in a community (WHA, 2001). The Mass Drug Administration (MDA) concept was first applied to the control of lymphatic filariasis in China and was adopted by WHO for incorporation in control programs for STH and other NTDs (Hotez et al., 2007). MDA involves the distribution and administration of drugs to whole communities, rather than individuals infected or at risk of infection with preventable diseases that are endemic (Brown et al., 2009). In order to properly administer drugs for STH, WHO recommended that surveys be done to determine the prevalence and intensity of infection (Table 1.2) by STH in a community, and developed survey criteria guidelines (Montresor et al., 1998).

Table 1.2: Classes of intensity of infection. Adapted from Montresor et al. (1998)

|  | **Light intensity** | **Moderate intensity** | **Heavy intensity** |
|---|---|---|---|
| *A. lumbricoides* | 1-4999 epg | 5000-49999 epg | 50000 epg |
| *T. trichiura* | 1-999 epg | 1000-9999 epg | 10000 epg |
| Hookworms | 1-1999 epg | 2000-3999 epg | 4000 epg |

Community categories were developed as a guide for MDA and WHO recommends
MDA for communities with 50% or higher prevalence of STH, irrespective of intensity of
infection. However, with regard to hookworm, when the prevalence is over 20% and
anemia is prevalent, then MDA is recommended (Montresor et al., 1998).

The drugs that are commonly used for MDA are the benzimidazoles, albendazole and
mebendazole (WHA, 2001). Benzimidazole drugs bind selectively to β-tubulin of
nematodes, cestodes and trematodes and inhibit microtubule formation. This causes a
slow starvation of the parasite and inhibition of egg production (Martin, 1997).  In
addition to benzimidazoles, WHO also recommends praziquantel (tetrahydropyrimidine)
and levamisole (imidazothiazole) for STH (WHA, 2001). These drugs are nicotinic
agonists at acetylcholine receptors of nematode muscle and cause spastic paralysis
(Martin, 1997). In some cases, a combination of drugs resulted in better efficacy of
treatment than when a single drug was used. For the treatment of hookworm, a
combination of mebendazole and levamisole showed significantly higher efficacy
(Albonico et al., 2003) than when either drug was used alone. In other studies, the
addition of ivermectin to albendazole or mebendazole showed higher efficacy against *T.
trichiura* (Ismail and Jayakody, 1999; Knopp et al., 2010). Ivermectin causes paralysis of
the pharyngeal pumping of nematodes. With the reported relatively low efficacy of
benzimidazoles, and other drugs recommended by WHO against *T. trichiura* (Keiser and
Utzinger, 2008), oxantel pamoate, a nicotinic agonist like pyrantel, were used against *T.
trichiura* and found to have higher efficacy, particularly when combined with
mebendazole (Keiser et al., 2013). A differential anthelmithic effect by levamisole has
been reported among the human hookworm species, in which a greater efficacy was

observed against *A. duodenale* than *N. americanus* (Albonico et al., 2003; Albonico et al., 1998).

Concerns regarding the possible development of drug resistance against benzimidazoles has grown since the implementation of MDA in most STH endemic areas (Vercruysse et al., 2011). In investigating drug efficacy, fecal egg reduction (FER) rate is recommended, rather than cure rate, as it is a closer representation of worm burden (Albonico et al., 2004; Vercruysse et al., 2011). The egg hatch assay, which is useful for assessing hookworm resistance, is another test that can be used to determine drug efficacy, as hookworm eggs hatch within 1-2 days (Albonico et al., 2004). In light of reported FER rates of <90% in studies in Ghana (Humphries et al., 2013) and Zanzibar (Albonico et al., 2003), closer monitoring of MDA programs was called for in other regions as well. The association between the response to albendazole treatment and β-tubulin genotype frequencies was studied for STH from three regions of the world, and although *Ascaris* and hookworm did not show much modification in β-tubulin genotype frequencies, *Trichuris* showed evidence of resistance even in benzimidazole naïve populations (Diawara et al., 2013). This may explain the reduced efficacy of benzimidazoles in single drug administration protocols noted by other researchers.

Improved sanitation has had mixed reported effects on parasite infection. For the most part there have been reductions in prevalence and intensities of infections especially with ascariasis (Esrey et al., 1991; Jeffery, 1960; Ziegelbauer et al., 2012). However, the impact on hookworm infections has been less remarkable and in some cases lacking (Esrey et al., 1991; Gross et al., 1989). Although deworming remains the most effective way of controlling the spread of STH (de Silva et al., 2003; Hotez et al., 2006), it is

recommended that improvements to sanitation and hygiene education be included in control programs for STH in order to reduce infection incidence and re-infections (Ziegelbauer et al., 2012).

## 1.4   Human Hookworm Infection

*A. duodenale* and *N. americanus* are the most important hookworms in humans. *A. duodenale* is found mainly in subtropical climates up to 52ºN (Araújo et al., 1988), mostly in the Middle East, northern Africa, India, Australia and Europe (de Silva et al., 2003). *N. americanus* has a more tropical distribution and is endemic in South and Southwest China, South India, Southeast Asia, sub-Saharan Africa, and Central and South America (Brooker et al., 2004) (Figure 1.6). However, mixed infections do occur usually with prominence of one or the other (Araújo et al., 1988). There have also been a few unexpected findings with regard to distribution of hookworm species. For instance, Jonker et al. (2012) using PCR, in Malawi, a sub-Saharan country, unexpectedly found that *A. duodenale* was prevalent. In Malaysia, Ngui et al. (2012a) also using molecular techniques, unexpectedly found *A. ceylanicum* where initially they thought only have *N. americanus* was present in humans. In Ghana, *A. duodenale* was found in a population that had predominantly *N. americanus* (de Gruijter et al., 2005).

Mixed infections with other stongylids are also possible. Pit et al. (1999) found hookworm with *Oesophagostomum bifurcum* in Togo and Ghana and Sato et al. (2010) in Laos, found *Trichostrongylus colubriformis* as well as *N. americanus*, *A. duodenale* and *A. caninum* in human fecal samples using PCR. Since it is not possible to differentiate the eggs of human hookworm spp. from each other by light microscopy, coproculture or molecular methods are needed to definitively identify the species hookworm present.

Zoonotic hookworms, *A. ceylanicum* (Traub et al., 2008) and *A. caninum* (Landmann and Prociv, 2003; Prociv and Croese, 1996) have also been detected in Thailand, Laos and Australia. A. *ceylanicum* can cause patent infections (Knopp et al., 2010) but *A. caninum* does not.  Instead, *A. caninum* oral infection results in the development of an immature adult in the intestine with associated eosinophilia, intestinal ulceration and abdominal pain (Prociv and Croese, 1996).
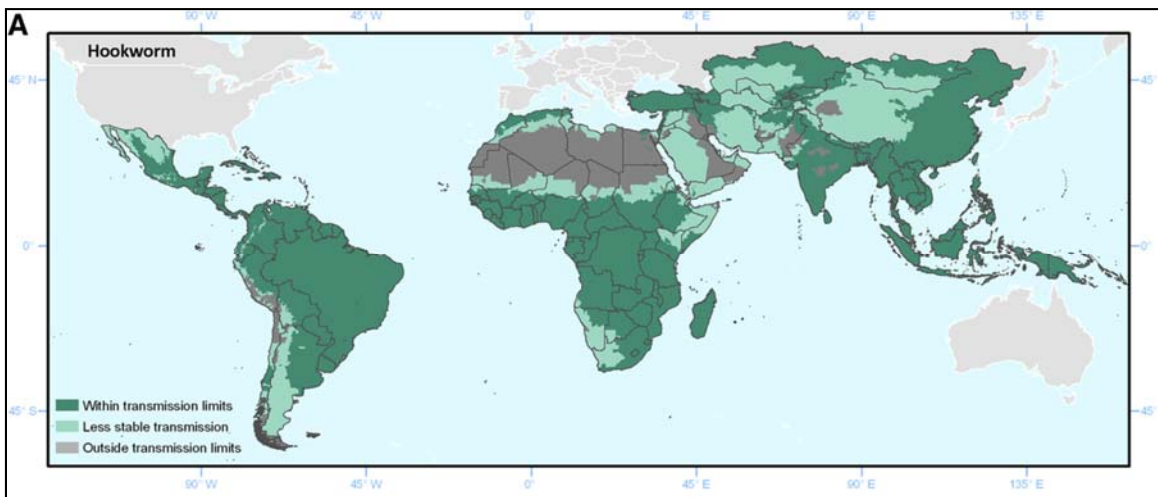


Figure 1.6: Climatic suitability for hookworm transmission defined by land surface temperature and aridity. Areas were defined as stable (dark green), unstable (light green, where infection prevalence <2%), or no risk (light grey). Adopted from Pullan and Brooker (2012).

Hookworm disease is characterized by iron deficiency anemia (Brooker et al., 2004). This anemia can be particularly severe in children and women of reproductive age who also have concurrent malnutrition (Brooker et al., 2004). However, even in adults without malnutrition, chronic infection can cause a reduction in hemoglobin (Santos et al., 2013). Iron deficiency anemia can result in low birth weight, premature birth and maternal death in pregnant women as well as low weight gain, reduced appetite and poor physical fitness in children (Crompton, 2000; Stephenson et al., 1993). The anemia is brought about by

the blood loss associated with the hematophagous $L_4$ and adult stages of the hookworm. Hookworms also produce anticoagulants which cause the intestinal mucosa to continue bleeding after the hookworm has stopped feeding. *A. duodenale* causes greater blood loss and it is estimated that the daily loss is 0.14 – 0.26 ml of blood per worm, while *N. americanus* is estimated at 0.02 – 0.07 ml per worm (Albonico et al., 1998; Pawlowski et al., 1991; Santos et al., 2013). Infections caused by *A. duodenale* have been associated with higher levels of iron deficiency and anemia in children (Albonico et al., 1998). Low intensity infections with multiple parasites are also likely to cause clinically significant anemia (Ezeamama et al., 2005).

### 1.4.1   Hookworm in Brazil

The tropical climate in Brazil is highly conducive for the development of free-living stages of hookworm in the environment (Mudenda et al., 2012). Socio-economic factors play an important role in facilitating or deterring the transmission of hookworm (Brooker et al., 1999). The prevalence of hookworm has been reported as being as low as 0.27% among higher income people (Santos et al., 2013) as opposed to rural and poor populations where it was found to be 9% among patients in the Amazon region (Araujo and Fernández, 2005).  In poor rural communities, the prevalence of hookworm has been reported to be as high as 80.2% (Gonçalves et al., 1990).

Few studies have gone on to determine the species of hookworm in Brazil. In one study in the state of Minas Gerais, only *N. americanus* was found (Fleming et al., 2006). In the northeastern region of Brazil, Gonçalves et al. (1990) found 70.9% of the hookworms were *N. americanus*, 2.1% were *A. duodenale* and the rest could not be identified Okazaki et al. (1988) reported similar findings in child out-patients in a farming

community in the same region, albeit with *A. duodenale* up to 12.5%. The Harada-Mori method of coproculture was used to identify larvae morphologically in both studies.

Prevalence of hookworm tends to be higher among males compared to females (Santos et al., 2013) and this has been attributed to activities that males may be involved in that put them at greater risk for infection. In a study among upper and middle class patients in Salvador, Brazil, the 30-50 year age group was the most commonly infected with hookworms (Santos et al., 2013) and there is general agreement among researchers that prevalence tends to increase with age and plateaus in adulthood (Fleming et al., 2006; Stoltzfus et al., 1997). However, others have found that neither age nor sex was correlated with prevalence where all family members were involved in agricultural activities (Barreto et al., 2000; Gonçalves et al., 1990; Okazaki et al., 1988).

## 1.5 Common Intestinal Parasites of Dogs

Intestinal parasites in dogs are widespread and are important because they cause morbidity and mortality in dogs as well as having a zoonotic potential for dog owners and those working with dogs (Blagburn et al., 1996). In dogs, intestinal parasites may cause enteritis, anemia, bloody diarrhea, weight loss, dermatitis and vomiting (Bowman, 2009) and contamination of the environment with dog feces containing eggs, oocysts, and cysts of parasites presents a public health risk. In addition to risk to humans, dog parasites can cause infection in other animals. For instance, *Toxocara canis* can cause visceral larval migrans (VLM) in cats and rabbits.

### 1.5.1 Biology and infection

#### 1.5.1.1 Hookworm

The species of hookworm found in dogs are *Ancylostoma caninum, A. braziliense, A. ceylanicum* and *Uncinaria stenocephala*. *A. caninum* occurs worldwide, *A. braziliense* is mostly in the southern US and central/ South America, *A. ceylanicum* is mostly found in Asia (Conlan et al., 2012; Smout et al., 2013; Traub et al., 2008) and *U. stenocephala* is found in the cooler climate of the northern America and Europe (Taylor et al., 2007; Traub et al., 2004). Canine hookworms have a zoonotic potential and cause cutaneous larvae migrans (CLM) or 'creeping eruption' when they penetrate human skin (Bowman et al., 2010). *A. braziliense* is the most common cause of CLM, while *A. caninum* has been reported to cause eosinophilic enteritis in humans in Australia (Prociv and Croese, 1996) and has been implicated as a cause for diffuse subacute neuroretinitis (Harto et al., 1999). In the cases of eosinophilic enteritis encountered, only a single adult worm was found. *A. ceylanicum* is the only canine hookworm capable of causing a patent infection in humans and it has been demonstrated experimentally as well as by natural infection (Ngui et al., 2012b; Traub et al., 2008).

The lifecycle of canine hookworm is similar to the human hookworm lifecycle. Canine hookworms also infect via skin penetration and follow the alveolar route of tissue migration before settling in the small intestine and maturing to hematophagous adults (Figure 1.7). However, the migrating larvae of canine hookworms may also undergo arrested development in the muscles and intestinal walls of the host and serve as source of trans-mammary infection (ingestion of $L_3$) to puppies (Bowman et al., 2010; Stone and Girardeau, 1968). Arrested *A. caninum* larvae tend to reactivate spontaneously, regardless

of whether the intestine has been evacuated of adults or with immune suppression (Schad and Page, 1982). Human infection by canine hookworms is also by skin penetration, but the larvae migrate in the epidermis forming erythematous tracts called cutaneous larva migrans (Bowman et al., 2010). Only *A. ceylanicum* is able to migrate to the intestine, mature, mate and produce eggs. The prepatent period for *A. caninum* via ingestion is 2-3 weeks and via skin penetration it is 4-5 weeks (Bowman, 2009). For *A. braziliense*, the prepatent period is reported as 13 or more days by either oral or percutaneous infection (Liotta et al., 2012a).
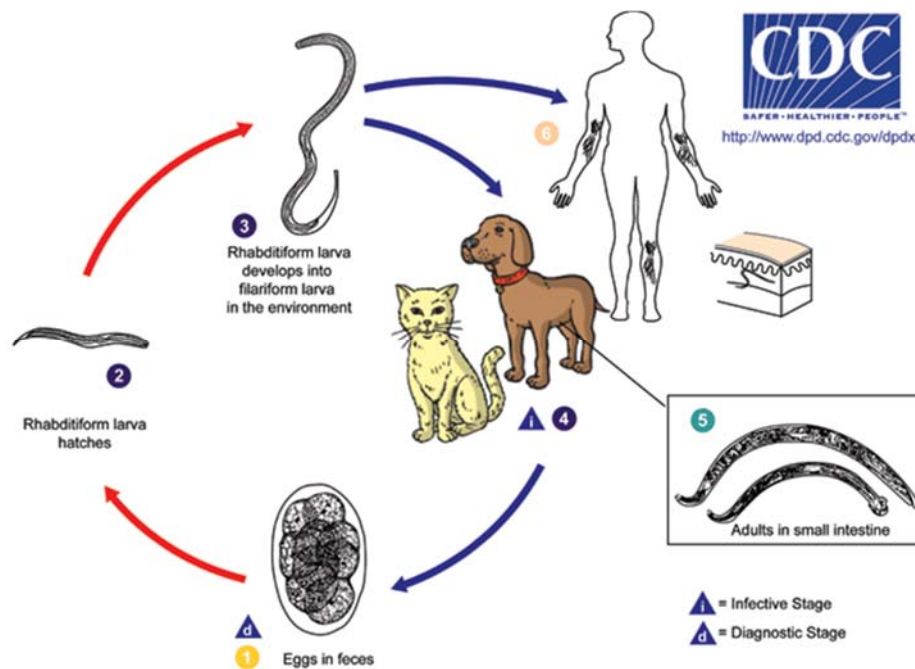


Figure 1.7. Zoonotic Hookworm (CDC, 2012)

Hookworms produce anticoagulants while feeding (Furmidge et al., 1996; Hotez and Cerami, 1983; Kasper et al., 2001; Stassens et al., 1996) and *A. caninum* causes blood loss of approximately 0.1ml per day per worm. This level of blood loss can cause severe and life threatening anemia in puppies. The $LD_{50}$ of *A caninum* in nursing puppies is

reported to be 75 worms per pound of body weight (Miller, 1971). The peak of the blood loss occurs even before eggs appear in the feces due to blood sucking by the $L_4$ stage, but it coincides with bloody diarrhea (Bowman, 2009). *A. caninum* is the most pathogenic of the canine hookworms because of the amount of blood loss associated with it.

1.5.1.2  *Toxocara canis* (roundworm)

*T. canis* is an ascarid similar to *A. lumbricoides* in humans. It is the largest nematode (10-15cm long) found in the digestive tract of dogs and other canids (Bowman, 2009). It has a worldwide distribution and the eggs are quite resistant to environmental conditions, able to survive for up to four years (Slifko et al., 2000). Eggs are unable to develop when exposed to temperatures less than -11ºC (Slifko et al., 2000) and more than 34ºC (Gamboa, 2005). Larvae appear in the eggs in 2-3 weeks depending on environmental conditions (Anderson, 2000) with higher humidity and temperature accelerating the development (Gamboa, 2005). Transmission is by ingestion of the infective egg, which contains infective larvae. The eggs hatch in the intestine and the larvae penetrate the intestinal mucosa and migrate through the hepato-tracheal route like *A. lumbricoides* (Beaver, 1969). They mature into adults in the small intestine, mate and lay eggs which are passed unembryonated in feces (Figure 1.8). The prepatent period is 2-4 weeks and one *T. canis* female can produce up to 85,000 eggs per day. Larvae are also capable of encysting in the intestinal walls and somatic tissues, particularly in adult immune dogs, where they become dormant for prolonged periods of time. In female dogs in late pregnancy, tissue larvae reactivate and infect puppies transplacentally or through the transmammary route, although the latter is considered a minor route of transmission (Bowman, 2009).  Heavy prenatal infection of puppies can cause severe abdominal

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distension and discomfort and life-threatening obstruction of intestines, bile and

pancreatic ducts may occur (Bowman, 2009). Infection can also occur by ingestion of

paratenic hosts (eg. small mammals) in which the larvae hatch and encyst in tissues

(Okulewicz et al., 2012). Ingestion of encysted larvae by dogs is followed by maturation

and oviposition by adults in the small intestine.
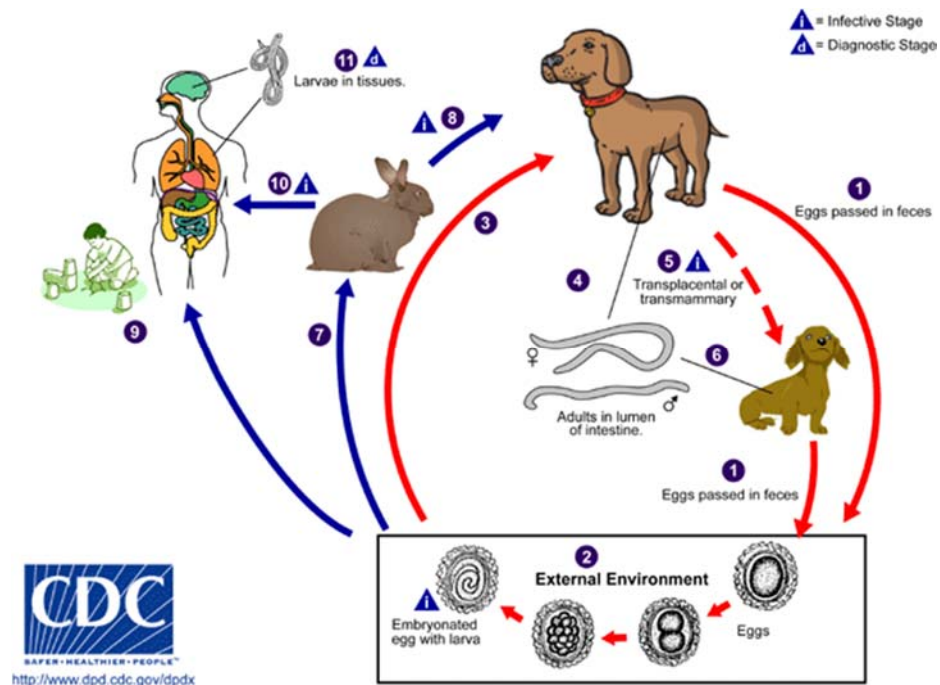


Figure 1.8. *Toxocara canis* life cycle (CDC, 2013)

If humans get infected accidentally by ingestion of infective eggs or encysted larvae in

paratenic hosts, larvae fail to mature and instead migrate extensively in the body causing

inflammation in various organs like the liver, brain, lungs, muscle and heart. This

condition is called visceral larva migrans (VLM) and if the migration is in ocular tissue,

ocular larva migrans (OLM) (Bowman, 2009). Seroprevalence of *T. canis* in a national

survey in humans in the USA was 14% and was associated with poverty and dog

ownership among other risk factors (Won et al., 2008).

*T. leonina* is an ascarid that occurs both in canids and felids (Okulewicz et al., 2012). It is similar in size, to *T. canis,* but they differ morphologically in that *T. leonina* males have a conical tail with no caudal alae while *T. canis* males have a digitiform appendage and caudal alae (Okulewicz et al., 2012). Although the eggs of the two species have similar dimensions, *T. canis* have the pits of the eggshell on the outer layer of the eggs, while *T. leonina* eggs are more translucent with a smooth shell, no striations or albuminous coat on the outer layer (González et al., 2007). Larvae of *T. leonina* develop to the infective stage in eggs in as few as three days at 30°C (Anderson, 2000). *T. leonina* is also more tolerant to cold temperature extremes with eggs being able to survive temperatures of -15°C for up to 40 days while those of *T. canis* do not (Okulewicz et al., 2012). *T. leonina* is sometimes referred to as the 'Northern roundworm' because it is most prevalent in cool climates. *T. leonina* has been reported to also cause VLM in humans although prevalence information is lacking (Bowman, 1987; Slifko et al., 2000).

The co-occurrence of *T. canis* and *T. leonina* is possible but one species tends to dominate. Age prevalence is also different in that *T. canis* is more prevalent in young dogs less than six months old while *T. leonina* tends to be more prevalent in older dogs (Okulewicz et al., 2012). This may be attributed to the somatic migration and encystation of larvae of *T. canis* which is minimal in puppies compared to older dogs and therefore puppies shed more eggs in feces. Due to the high prevalence of transplacental infection and development of patent adult infections of *T. canis* in puppies, contamination of the environment tends to be high and attributed to infection in puppies. Infection of older puppies and adult dogs mostly results in encystations of larvae.

### 1.5.1.3 *Trichuris vulpis* (whipworm)

*T. vulpis* is found worldwide in dogs and other canids e.g. foxes and cayotes. Unembryonated eggs measuring 70-80 x 30-40 μm are released into the environment in feces (Taylor et al., 2007). The eggs are highly resistant to desiccation and UV light and embryonate in 9-21 days depending on environmental conditions. Like *Toxocara*, they can also remain viable in the environment for years (Traversa, 2011). *T. vulpis* has a direct lifecycle like *T. trichiura* in humans. Infection occurs when the embryonated eggs with $L_3$ larvae are ingested and they hatch in the small intestine. The larvae penetrate the mucosa and develop further for another 2-15 days, emerge and proceed to the cecum or colon where they mature and live (Kirkova and Dinev, 2005; Traversa, 2011). Adult *T. vulpis* measure between 4.5 -7.5 cm and are shaped like a whip, with a thick posterior end and a thin long anterior end, hence the name 'whipworm' (Taylor et al., 2007). The adult worms possess an oral stylet with which to lacerate mucosa and capillaries as they thread their anterior ends into the superficial mucosa and feed on blood, fluids and mucosa, causing hemorrhagic typhilitis or colitis (Kirkova and Dinev, 2005; Traversa, 2011). In severe infections, bloody diarrhea, anemia, lethargy and dehydration can occur and may lead to death. The prepatent period for T. *vulpis* is 74-90 days and females lay, on average, 2,000 eggs per day (Bowman, 2009). There have been a few human cases where *T. vulpis* was implicated based on larger trichuroid egg size compared to *T. trichiura* and identification of partial adult worms, but was not identified definitively (Traversa, 2011). Therefore, *T. vulpis* is currently not considered zoonotic.

1.5.1.4  *Cystoisospora* spp.

Common coccidian parasites infecting dogs are *C. canis* and the *C. ohioensis*-complex (*C. ohioensis, C. neorivolta, and C. burrowsi*). Infection is by ingestion of sporulated oocysts. The sporozoites excyst in the small intestine and invade epithelial cells, where they multiply asexually (schizogony) and sexually (gametogony) and release unsporulated oocysts (Altreuther et al., 2011). The prepatent period is 8-12 days for *C. canis* and 4-10 days for *C. ohioensis* (Altreuther et al., 2011; Houk et al., 2013). In heavy infections, they cause stunting of villi and reduction in the absorptive area leading to diarrhea (Altreuther et al., 2011; Houk et al., 2013). This is more so in young puppies but infection is believed to be exacerbated by concurrent viral infection or other immunosuppressive conditions (Taylor et al., 2007). Oocysts found in feces need to be differentiated from other coccidian oocysts such as those of *Sarcocystis* spp and *Hammondia* sp. (Bowman, 2009). Oocysts of *C. canis* are the largest in the dog and measure 32-42 x 27-33 µm, while *C. ohioensis* oocysts measure 19-27 x 18-22 µm (Bowman, 2009; Houk et al., 2013). Sporulation occurs in two or more days (Altreuther et al., 2011; Houk et al., 2013). Crowding and lack of sanitation promote the spread of coccidiosis and as such should be avoided or corrected (Taylor et al., 2007).

1.5.1.5  *Dipylidium caninum*

*D. caninum* is the most common cestode (tapeworm) parasite of dogs, cats and other canids and is found worldwide (Molina et al., 2003; Taylor et al., 2007). The tapeworm sheds gravid proglottids that are elongate and resemble a grain of rice, with a pore opening on each margin. Eggs (oncospheres) which measure 25-50 µm contain a hexacanth embryo and are contained in egg packets with up to 30 eggs (Taylor et al.,

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2007). The eggs are released actively by the egg packet or on its disintegration. Fleas (*Ctenocephalides* spp, *Pulex irritans*) and lice (*Trichodectes canis*) serve as intermediate hosts.   The eggs are ingested by the intermediate host and in the hemocoel develop into cysticercoids. Development in the louse takes about 30 days while in the flea, it can take several months. The final host is infected upon ingestion of the intermediate host containing the cysticercoids and the prepatent period is approximately three weeks (Okulewicz et al., 2012). *D. caninum* has been found to infect man occasionally, especially young children that become infected by accidental ingestion of the intermediate host (Molina et al., 2003). The cysticercoid matures in the small intestine, attaches to the mucosa by its scolex and develops a neck which buds off immature segments that develop to mature and then gravid segments in a long strobila.  The adult tapeworm can measure up to 60 cm and is non-pathogenic, although the shed proglottids may cause discomfort as they crawl around the perineum (Taylor et al., 2007).

1.5.1.6  *Giardia duodenalis*

*G. duodenalis* is also known as *G. intestinalis or G. lamblia.* It is a flagellated protozoan parasite of dogs, cats and other mammals including humans (Taylor et al., 2007; Thompson, 2004). It is found worldwide and is transmitted fecal-orally. It has a simple and direct lifecycle, with trophozoites dividing by binary fission to form other trophozoites. Trophozoites encyst, are passed in feces and are fully infective (Taylor et al., 2007). The cysts measure 8-12 x 7-10 μm and are resistant to UV light, chlorine and iodine (Jarroll and Hoff, 1988). *G. duodenalis* may cause a variable semiformed mucoid, blood flecked to projectile diarrhea in dogs resulting in lethargy and weight loss. Species cross transmission is controversial as to whether dogs serve as a reservoir for human

infection (Bowman, 1987). Dogs become infected by direct fecal-oral contact from other

dogs or the environment. Water contamination has led to outbreaks in humans although

they can also get infected from inadvertently coming into contact with feces accompanied

by poor hand hygiene (Shields et al., 2008; Thompson, 2004). Human to human

transmission also occurs and it is quite common in child day-care facilities (Thompson,

2000).

1.5.2   Epidemiology of Canine Intestinal Parasites.

Intestinal parasites occur worldwide although there may be some differences in species

based on the region of the world. The susceptibility of a parasite to environmental

conditions may also limit its distribution. For instance among hookworm species, *U.*

*stenocephala* has higher tolerance for low temperatures than *A. caninum* (Balasingam,

1964). Intestinal parasites occur both in urban and rural areas, with prevalence in rural

areas tending to be generally higher (Bwalya et al., 2011; Dubná et al., 2007). However,

among the ascarids, *T. canis* was found to contaminate soil more in urban areas than in

rural areas, while prevalence was not significantly different, but prevalence with *T.*

*leonina* was higher in rural areas (Okulewicz et al., 2012). However, the greatest

influence on parasitism is how well cared for a dog is. Dogs that receive regular

veterinary care and live indoors are less likely to harbor intestinal parasites as opposed to

those that rarely receive veterinary care and are free roaming (Little et al., 2009). Studies

in shelter dogs have shown that parasitism is extensive in this population (Blagburn et al.,

1996; Papini et al., 2005). A big proportion of shelter dogs are owner surrendered,

implying that the environment they are coming from is highly contaminated with

parasites. Shelters may keep dogs for several months without adequate antiparasitic

therapy, thus allowing these dogs to continue shedding parasite eggs/oocysts/cysts in the premises of the animal shelter. Such dogs serve as source of infection or re-infection for newly acquired dogs in the shelter (Dubná et al., 2007). Overcrowding and poor sanitation are particularly important in enhancing the spread of coccidiosis and giardiasis (Dubná et al., 2007; Papini et al., 2005; Taylor et al., 2007; Thompson, 2004). Feeding of wet commercial food in a shelter was also found to be a contributing factor to high prevalence of giardiasis in Rome (Papini et al., 2005).

Within the USA, national surveys have been conducted on shelter dogs and owned (pet) dogs (Table 1.3) in the recent past. As has been seen in other parts of the world, shelter dogs in the USA have a high prevalence of intestinal parasites compared to pet dogs (Blagburn et al., 1996; Blagburn et al., 2012; Little et al., 2009). The most prevalent parasite in shelter dogs was hookworm, while in pet dogs, it was the protozoan parasites. This may be due to the fact that pet dogs receive anthelmenthic treatment often as part of the heartworm prevention, and this helps to keep the helminth infections in check. However, in the southeastern part of the country, hookworm was the most prevalent even among pet dogs, with protozoan parasites a close second. This situation may be attributed to the highly conducive tropical environmental conditions for free-living stages of hookworm to develop in the soil and remain infective longer than they would in the cooler and drier parts of the country. In a study that was conducted among dogs brought to the veterinary teaching hospital at Louisiana State University 30 years ago (Hoskins et al., 1982), the prevalence of parasites was high, comparable to the national study on shelter dogs in 2012 for the southeastern region (Blagburn et al., 2012). It is evident that pet dogs are in a much better state with regard to parasitism now than they were 30 years

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Table 1.3. Prevalence of common dog intestinal parasites from national and regional surveys in shelter and pet dogs.

| | Shelter dogs 1996 (Blagburn et al., 1996) | | Shelter dogs 2012 (Blagburn et al., 2012) | Pet dogs (Little et al., 2009) | | Owned dogs (Hoskins et al., 1982) |
|---|---|---|---|---|---|---|
| | National % | Southeastern % | National % | National % | Southeastern % | Louisiana % |
| Hookworm | 19.2 | 36.5 | 30.1 | 2.5 | 4.0 | 38.5 |
| *T. canis* | 14.5 | 17.7 | 12.5 | 2.2 | 1.6 | 8.5 |
| *T. vulpis* | 14.3 | 19.9 | 18.6 | 1.2 | 1.5 | 14.9 |
| *Cystoisospora spp.* | 4.8 | 5.7 | *C. canis* 4.8, *C. ohioensis* 8.3 | 4.4 | 3.0 | 2.8 |
| *Giardia spp.* | 0.6 | 0.7 | 3.4 | 4.0 | 2.3 | 0.8 |
| *D. caninum* | 0.1 | 0.2 | 0.9 | | | 2.2 |

ago and it can be attributed to better veterinary care, better anthelminthics, especially

since the discovery of ivermectin and its use as heartworm preventative (Bogan and

Duncan, 1984; Kreeger et al., 1990).  The national prevalence of parasites in shelter dogs

has not changed much in the last 20 years which highlights the fact that there is still a

population of dogs or dog owners that remains beyond the reach of the veterinarian, and

that calls for enhanced public education on the need for healthcare for dogs are warranted

(Blagburn et al., 2012).

### 1.5.3    Diagnosis of Canine Gastrointestinal Parasites

Canine intestinal parasites can be diagnosed using various flotation and sedimentation

methods. The most commonly used methods in the United States surveys are centrifugal

sucrose or zinc sulfate flotation (Blagburn et al., 1996; Dryden et al., 2005; Liotta et al.,

2012b; Little et al., 2009). These methods are good for diagnosis of nematode eggs,

protozoan oocysts and cysts but not for the heavier cestode and operculated trematode

eggs (Dryden et al., 2005; Liccioli et al., 2012). Zinc sulfate flotation is particularly

important for the demonstration of *Giardia* cysts. Direct smears are commonly used in

the clinical setting and are useful for detecting motile protozoa (Dryden et al., 2005).

Sedimentation methods are useful for the detection of cestode and trematode eggs

(Dryden et al., 2005) and when saline is used as a sedimentation medium, then

operculated eggs of some flukes with fully developed miracidia in eggs, such as

*Heterobilharzia americanum* and *Schistosoma* spp.,  are prevented from hatching (Baker,

2007). The Baermann sedimentation apparatus, which allow the release of larvae out of a

fecal sample and their collection in water, is useful for the isolation of lung worm

(*Angiostrongylus vasorum*) and *Strongyloides stercoralis* larvae as they exit the body via the gastrointestinal tract (Baker, 2007).

Preliminary identification of *D. caninum* may be made from fresh gravid proglottids, or adhered dried proglottids ('rice granules') are typically seen around the perineum so that diagnosis is often made on physical examination. The elongate shape, presence of two reproductive organs and shed egg packets can be seen under a light microscope by making 'squash preparations' of shed proglottids (Taylor et al., 2007). Commercial ELISA assays which are highly sensitive and specific are available for the diagnosis of *G. duodenalis* (Papini et al., 2005).

The diagnosis of CLM is by visualization of pruritic, erythematous tracts, 1-5cm long, most commonly found on parts of the body that have been in contact with moist soil, such as feet, hands, buttocks and the back as well as biopsy and histology with demonstration of migrating larvae in the epidermis (Bowman, 2009; Caumes et al., 2002; Jelinek et al., 1994). Diagnosis of VLM in humans is by aided by history of exposure to puppies, serology (antibodies to *T. canis*) and eosinophilia (Won et al., 2008).

Canine hookworm species eggs are difficult to differentiate microscopically except for *U. stenocephala* which has a notably bigger size (Traub et al., 2004). Differences in size and shape have been documented although they require a trained microscopist to detect them (Lucio-Forster et al., 2012). Molecular methods have been used to diagnose intestinal parasites in dogs, as well as to differentiate hookworm species (Gasser et al., 1996; Liotta et al., 2012b; Ngui et al., 2012b; Traub et al., 2004). PCR-RFLP by ITS fragment digestion using endonucleases was developed and used to differentiate *Ancylostoma* spp

of hookworm in dogs (e Silva et al., 2006; Liotta et al., 2012b; Traub et al., 2004). The performance of four endonucleases was compared for the differentiation of *A. braziliense*, *A. caninum* and *A. ceylanicum* and *Hinf*I produced the best profiles (e Silva et al., 2006).

1.5.4    Treatment and control

*T. canis* infection in puppies most often results in heavy oviposition and they are therefore considered a major source of environmental contamination and should be prevented from exposure to infection (Bowman, 2009). Hookworm infection is also most severe in puppies, commonly presenting with peracute disease due to severe anemia. CDC recommends that puppies be treated every two weeks for the first two to three months of life to clear intestinal infections before egg shedding occurs, thereby reducing public health dangers of VLM and OLM (Bowman, 2009; CAPC, 2013). In dogs where high levels of somatic larvae are likely, pregnant bitches can be cleared of tissue larvae by deworming daily with an effective dewormer like fenbendazole from 40 days of pregnancy until two weeks after whelping or by 3-4 treatments with elevated doses of ivermectin or other macrocyclic lactone drugs during pregnancy. (Bowman, 2009). *A. caninum* has been reported as being resistant to pyrantel in Australia (Kopp et al., 2007) and therefore attention must be paid to correct dosing of dogs and performing post treatment fecal exams in order to prevent and watch for the possible development of resistance in other parts of the world.

When dogs are acquired from shelters, they should be examined and treated for intestinal parasites and the treatment should continue for at least three months to get rid of reactivated or migratory larvae as they mature (Bowman, 2009; Traversa, 2011). It

should be noted adult dogs which are more commonly infected with *T. trichiura* than puppies should be treated as well (Traversa, 2011).

CLM in humans is treated with topical thiabendazole (Jelinek et al., 1994), ivermectin and/or albendazole (Caumes et al., 2002).

Tapeworm infections can be treated with praziquantel or epsiprantel and in the case of *D. caninum*, this therapy should be accompanied by use of insecticides to get rid of flea vectors and their immature stages both on the animal and in the environment (Taylor et al., 2007).

Coccidiosis can be treated with sulfonamides or toltrazural (Altreuther et al., 2011; Harder and Haberkorn, 1989). The infection can be prevented or controlled by implementing good sanitation practices, isolation of shedding dogs and avoiding crowding (Taylor et al., 2007). *G. duodenalis* can be treated with benzimidazoles like fenbendazole and nitroimidazoles like metronidazole (Taylor et al., 2007). Prompt removal of feces from the environment can aid in the control of intestinal parasites.

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# 2  MODELING THE ECOLOGICAL NICHE OF HOOKWORM IN BRAZIL BASED ON CLIMATE

## 2.1  Introduction

Hookworm infection is a worldwide public health problem, particularly in developing countries in Asia, Africa and Latin America, where poverty levels are high. An estimated 740 million people are infected (WHO, 2006). Of great concern in hookworm infection is the resultant iron deficiency anemia due to intestinal blood loss, especially in children and women of reproductive age and also the reduced cognitive ability of infected children (Hotez, 2008; Stoltzfus et al., 1996). Brazil introduced a Schistosomiasis Control Program in 1976, which is an active decentralized, federal program in known endemic areas (Amaral et al., 2006). Since the Kato-Katz method utilized in this program for the detection of *Schistosoma* eggs also shows the presence of many other helminth eggs, there is a bonus in using the results with respect to other helminth infections. Studies have shown that co-infection between *Schistosoma* sp. and soil transmitted helminths (STH) is common, including hookworm, *Ascaris lumbricoides* and *Trichuris trichiura* (Booth et al., 2003; Fleming et al., 2006).

Human hookworm infections are caused by *Necator americanus* and *Ancylostoma duodenale.* In Brazil, hookworm infections are predominantly caused by *N. americanus* (Fleming et al., 2006; Geiger et al., 2004). The eggs of this species do not develop at temperatures below 15ºC. The larvae prefer shady, moist areas with temperatures at or above 30ºC but are killed above 45ºC (Udonsi and Atata, 1987). Larvae fare best in sandy loam soils where they have been shown to migrate vertically to depths up to three feet (Palmer and Reeder, 2001) although most remain in the top 5 cm layer as long as the

[1]Adapted with permission from Geospatial Health
(Mudenda et al, 2012) (AppendixB)

soil is moist (Udonsi et al., 1980). Larvae have been shown to survive for up to six weeks although the yield becomes progressively lower over time (Beaver, 1953; Svensson, 1925; Udonsi and Atata, 1987). Other studies have shown that larvae survive for longer periods at lower temperatures (between 20 – 25ºC) than at temperatures above 30ºC (Svensson, 1925; Udonsi, 1988; Udonsi and Atata, 1987). When potential infectivity was assessed, it was found that larvae lost the ability to penetrate fresh rabbit skin in 11-15 days (Udonsi, 1988), apparently due to exhaustion of the worm energy stores. This evidence suggests that infectivity of hookworms in the environment is relatively short, on the order of a few weeks on average, and that the life cycle survival strategy of *N. americanus* is highly dependent on the 3-4 year longevity (Brooker et al., 2006b) of adult worms in infected human hosts.

Remote sensing and Geographic Information Systems (GIS) have been used to map the distribution of parasites and diseases and to develop prediction models of disease distribution in many regions of the world. An understanding of the environmental properties that contribute to the ecological niche of a particular parasite or vector is needed to be able to predict its distribution (Malone, 2005). Risk maps based on known biological requirements and climatic variables have been employed for a number of diseases including malaria (Yang et al., 2010), schistosomiasis (Lindsay and Thomas, 1999; Malone et al., 2001), filariasis (Lindsay and Thomas, 1999), leishmaniasis (Nieto et al., 2006; Salahi-Moghaddam et al., 2010) and others (Bergquist and Rinaldi, 2010). Such models have proven to be useful for formulating disease control programs in various parts of the world.

The concept of Growing Degree Days and Water Budget (GDD-WB) provides a measure of thermal-hydrological regime in relation to species specific knowledge of the base temperature (below which no development occurs), optimal temperature range for development, lethal upper limiting temperatures and limiting moisture regime in the context of the thermal units (GDD-WB) that must accumulate for a parasite-vector system to complete one or more generations. The GDD-WB concept has been used to generate predictive models of fascioliasis in the United States, Ethiopia, Ecuador and southern Brazil (Dutra et al., 2010; Fuentes et al., 2005; Malone et al., 1987; Malone et al., 2001), schistosomiasis in China and East Africa (Malone, 2005; Zhou et al., 2001) and visceral leishmaniasis in Bahia, Brazil (Nieto et al., 2006).

For hookworm, GIS and remote sensing methodologies have been used to study spatial distribution of the parasite and produce risk prediction models for the African continent (Brooker et al., 2006a), Kwazulu Natal in South Africa (Mabaso et al., 2003), and Ivory Coast (Raso et al., 2006). Risk maps based on biological data and climate regime have not yet been developed for hookworm infection in Brazil on a national scale.

In 2001 the World Health Assembly (WHA, 2001) World Health Organization's (WHO) member states agreed to implement or intensify control of schistosomiasis and STH infections. In 2009, the Directing Council of the Pan American Health Organization (PAHO) approved Resolution CD49.R19 urging member states to eliminate or drastically reduce, by 2015, the burden of 12 neglected infectious diseases including STH infections (PAHO, 2009). A study published in 2011 pointed out that important gaps of information on STH prevalence of infection at the first subnational level and below exist through-out the region including data of prevalence and intensity of infection by hookworm

(Schneider et al., 2011). Modeling the distribution and prevalence of STH infections could contribute to fill the existing gaps of epidemiological information.

The study reported here used hookworm prevalence data concurrently reported as part of surveillance and control programs for *S. mansoni* endemic areas in Brazil from 2000-2009. National scale risk maps on the distribution of hookworm infection were developed by applying known biological information on *N. americanus* to the climate envelope of conditions suitable for the survival, propagation and transmission of this hookworm species.

## 2.2    Materials and Methods

Brazil is located in South America at 10º South and 55º West (CIA, 2011). It has a mainly tropical climate with five major ecological zones (FAO, 2001). The hookworm prevalence data used was acquired from the Brazilian national notifiable diseases information system (Sistema Nacional de Agravos de Notificação – SINAN) and contained the proportion positive for hookworm at the municipality level from 2000 to 2009.  The methods of surveillance where not defined in terms of numbers and ages of people examined, although examination of about 30% of a population is recommended by WHO and the Schistosomiasis Control Program in Brazil (Machado, 1982), by regular surveillance of school children. Based on the low numbers of people examined in some municipalities compared to the population, the municipality data points with less than 100 people examined were excluded to avoid bias, resulting in a total of 975 municipality data points.

The mapping and modeling software used was ArcGIS 9.3 (Environmental Systems Research Institute Inc., Redlands, CA, USA). Boundary map shape files for political units in Brazil were obtained from a South American Minimum Medical GIS database resource (Malone et al., 2007b) using data downloaded from the DIVA-GIS website (http://diva.riu.cip.cgiar.org/index.php). The climate data was a long-term-normal (LTN) climate grid (18x18 km cell size) of South America (Corbett, 2005).  Each cell contained monthly long-term normal average data on rainfall, maximum daily temperature, minimum daily temperature, potential evapotranspiration, and the ratio of precipitation to potential evapotranspiration (PPE), also known as water budget (WB).

The Brazil administrative boundary map, prevalence data points, and climate grid data were imported into ArcGIS and re-projected to a geographic coordinate system (GCS), WGS 84 coordinate system format. The prevalence data point map and the climate grid attribute tables were joined using the polygon-to-point join function to produce a composite layer for GDD-WB analysis and calculation of the potential number of transmission cycles possible per year at hookworm positive municipalities. The values derived were used to extrapolate the model by calculating model variables for the entire national map, including municipalities where data was absent, using the layer properties/symbology function of ArcGIS.

## 2.2.1   GDD

Growing degree days (GDD) for hookworm were calculated by the field calculator function of ArcMap using a base temperature of 15°C for each month as follows (Malone et al., 2007a):

*(Mean monthly temperature – 15)\*Number of days in a particular month*

Months where mean temperature was lower than 15°C were considered as having zero GDD.

### 2.2.2  Moisture Adjusted GDD Model (MA-GDD)

The accumulated annual GDD was calculated after excluding the GDD accumulated in dry months, identified as a month when the water budget was less than 0.4. (Soil saturation or 'field capacity' is reached at a value of 1.0 and monthly values above 1.0 have surplus/runoff water.) This cut-off was used because below 0.4 the prevalence of hookworm in the 2000-2009 hookworm survey database from Brazil was found to be absent or low (<5%).

### 2.2.3  GDD-Water Budget Gradient Model (GDD X WB)

The product of accumulated GDD and WB for each data point was used to develop a 'gradient index' that relates temperature to a moisture gradient (water budget). The gradient index model was calculated for each month and the annual value was calculated by summing the monthly GDDxWB values.

### 2.2.4  Calculation of Mean Transmission Time and Potential Transmission Cycles per Year

The mean transmission time was defined as the time spent in the free-living state of the hookworm, from deposition of eggs into the environment to infection of the host by viable infective third stage larvae. It was calculated based on literature reports (Beaver, 1953; Svensson, 1925; Udonsi, 1988) by adding development time from egg to infective larva (14 days) and time as active infective larva (14 days).

*Transmission cycle GDD = (Mean transmission time) * Daily GDD*

Potential Transmission cycles per year values were calculated based on the number of

potential transmission cycles per annum by dividing annual MA-GDD by Transmission

cycle GDD (224). Models were then developed using WB, GDD and transmission cycle

values to predict potential risk zones for hookworm transmission in Brazil.

2.2.5   Model Validation

A literature search was conducted to review reports on independent surveys for

prevalence of hookworm and other STHs in Brazil. Results were used to validate the

MA-GDD and GDDxWB models.

2.2.6   Statistical Analysis

Statistical Analysis Software (SAS®, Cary, NC, USA), was used to determine

correlations between prevalence of hookworm and the variables MA-GDD and

GDDxWB using Pearson Correlation. The data were then $log_{10}$ transformed for

normalizing.  Analysis of variance (ANOVA) followed by Tukey's test was used to

compare MA-GDD means among low (1-4.9%), moderate (5-19.9%) and high (<20%)

categories of hookworm prevalence. These categories were adapted from WHO

guidelines for the control of STHs (WHO, 2006). The same was done for the GDDxWB

categories. Data points with prevalence less than 1% were assumed negligible due to

possible migration and were excluded from ANOVA, resulting in 564 data points of the

975 municipalities with data. Polynomial regression was used to determine the

relationship between mean transmission cycles and the GDDxWB index.

## 2.3 Results

The mean prevalence of hookworm among the municipalities in the data set was 4.99%

(range 0-70.22; Standard Deviation 8.93). The prevalence distribution at 975

municipality sites is shown in Table 2.1, categorized as very low, low, moderate and high

prevalence.

Table 2.1. Distribution of hookworm prevalence among the 975 municipalities surveyed
with corresponding MA-GDD and GDDxWB values.

| Categories | Hookworm Prevalence (%) | Number of municipalities | MA-GDD Mean (range; SEM) | GDDxWB Mean (range; SEM) |
|---|---|---|---|---|
| Very Low | <1 | 411 | 1672.2 (0-3586; 30.0) | 2029.9 (0-6618; 41.5) |
| Low | 1-4.9 | 303 | 1752.7 (540-3280; 29.2) | 2274.4 (666-7117; 60.5) |
| Moderate | 5-19.9 | 187 | 1891.0 (1088-3099; 28.0) | 2563.0 (1016-7041; 71.0) |
| High | >20 | 74 | 2057.7 (913-3014; 58.5) | 3493.8 (1134-7875; 207.0) |
|  | Total | 975 |  |  |

The Pearson correlation coefficients between hookworm prevalence and MA-GDD and

between hookworm prevalence and GDDxWB index were 0.24 (p<0.001) and 0.27

(p<0.001) respectively. The MA-GDD and GDDxWB $\log_{10}$ means in the three categories

of prevalence (low, moderate, high) were all significantly different (p<0.001) (i.e. the

MA-GDD $\log_{10}$ mean among the, low prevalence group was significantly different from

that in the moderate and high prevalence groups and that between the moderate

prevalence group was different from the high prevalence group). However, the MA-GDD

range in the high prevalence group overlapped with the moderate prevalence group range (Table 2.1).

The relationship between the mean transmission cycles and GDDxWB index was approximated by the formula $y=2.3+0.0031x-2E-07x^2$ ($R^2=0.522$) where $y$ is mean number of transmission cycles and $x$ is GDDxWB.

The MA-GDD model (Figure 2.1) and the GDDxWB model (Figure 2.2) both predicted a low hookworm prevalence of less than 5% in the northeastern inland (Caatinga) region and in the southern region of Brazil. The MA-GDD model (Figure 2.3) showed that where the transmission cycles were less than 5 per year, or more than 14 per year, the hookworm prevalence was low (<5%). The GDDxWB index model showed a low end cut off at 1300 (Figure 4) below which the prevalence of hookworm fell below 5%.

Thirty independent reports published between 1990 and 2010 by various authors were reviewed and used for the purpose of validating the climate risk prediction models developed in the current study (Aguiar et al., 2007; Alves et al., 2003; Bóia et al., 2009; Braga et al., 1998; Brooker et al., 2007; Buschini et al., 2007; Cabral et al., 2000; Carvalho-Costa et al., 2007; Cury et al., 1994; Ferrari et al., 1992; Ferreira et al., 1994; Geiger et al., 2004; Gomes et al., 2002; Gonçalves et al., 1990; Heukelbach et al., 2004; Lopes et al., 2006; Machado et al., 2008a; Machado et al., 2008b; Melo et al., 2010; Miranda et al., 1998; Miranda et al., 1999; Moraes and Cairncross, 2004; Oliveira et al., 2003; Rocha et al., 2000; Santos et al., 1995; Scolari et al., 2000; Silva and da Silva, 2010; Silva et al., 2007; Souza et al., 2007; Tsuyuoka et al., 1999). These articles covered 16 states out of Brazil's 26 states and hookworm prevalence ranged from 0 to 80.2% with

a mean of 20.3%. The number of people examined ranged from 54 to 13,279 and sampled

populations ranged from school children to whole communities. There were eight low, 11

moderate and 11 high hookworm prevalence sites. While the 30 data points available

from literature were limited there was a general agreement of survey data to the predicted

GDDxWB index risk map showing high hookworm prevalence risk in the amazon basin

(Figure 2.2). The Pearson correlation coefficient between the prevalence and GDDxWB

was 0.26 ($p = 0.158$) and between prevalence and MA-GDD was 0.15 ($p = 0.4116$).
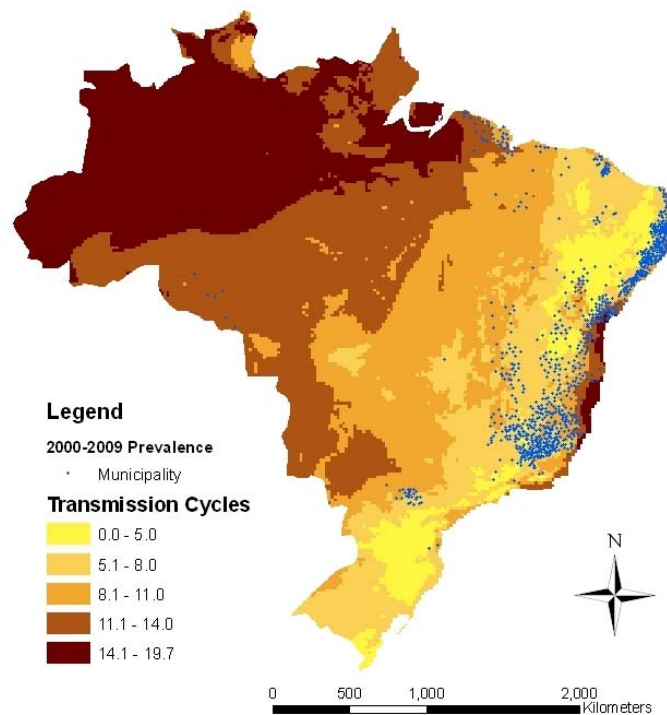


Figure 2.1. Risk Model for Hookworm using Moisture Adjusted GDD. Showing
moderate to high prevalence is most likely to occur between five and 14 transmission
cycles per year (yellow to dark brown). Areas with less than five transmission cycles per
year (yellow) are prohibitive to hookworm transmission. Blue dots represent municipality
data points.
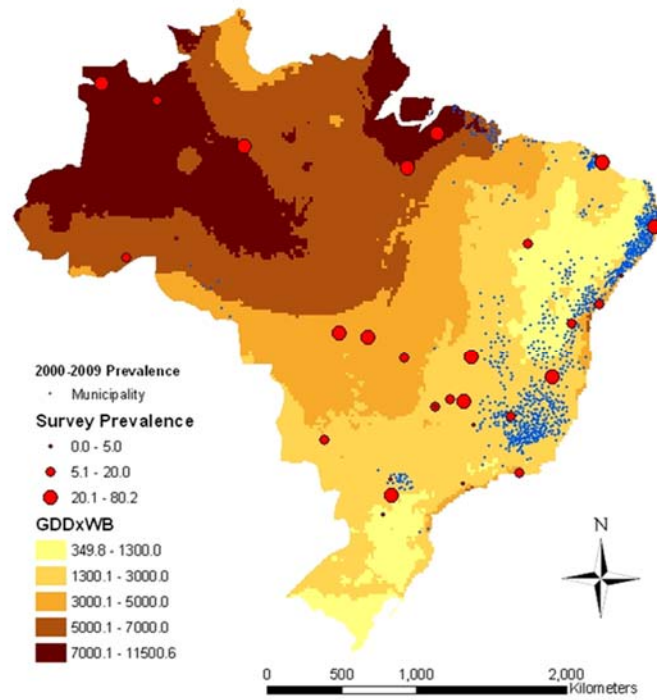
Figure 2.2.  Hookworm prevalence risk model using GDDxWB index. Areas of low (yellow) to high (dark brown) prevalence risk are shown. Blue dots represent municipality data points. Red graduated circles denote hookworm prevalence from independent survey reports.
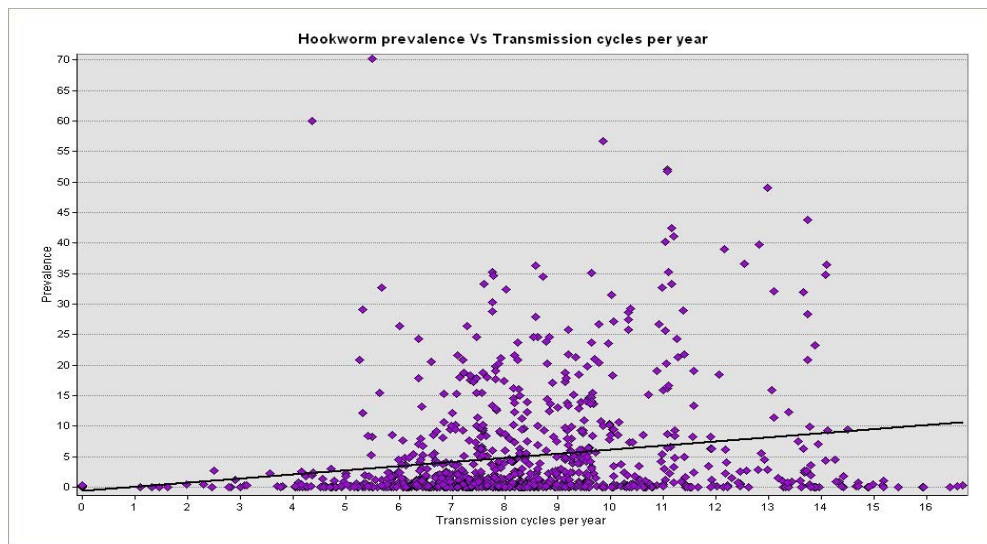


Figure 2.3. Scatter plot of hookworm prevalence from municipality data points against transmission cycles per year. The plot shows a lower end cut off at five transmission cycles below which the prevalence is less than 5% and an upper end cut off at about 14 transmission cycles above which the prevalence is less than 5%. Black line is a trend line ($y=0.9702x$).
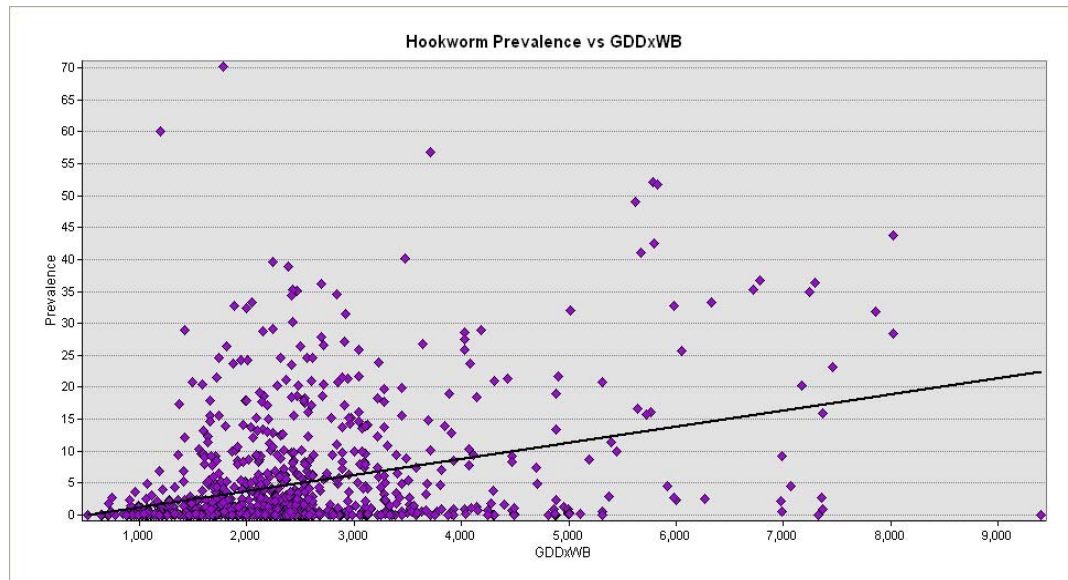
Figure 2.4. Scatter plot of Hookworm Prevalence against GDD-WB index. Prevalence levels increase dramatically above 1300 with no obvious upper limit. Black line is a trend line ($y=0.0031x$).

## 2.4    Discussion

In the current study on development of ecological niche models for hookworms in Brazil, there was a need for a standard measure of the developmental suitability of a site for propagation and transmission of the parasite based on climate and known biological parameters that can distinguish low, moderate and high risk conditions for hookworm disease.

Temperature and moisture environmental parameters are fundamental determinants of the distribution range and abundance of free-living species (Andrewartha and Birch, 1954) and suitable ranges are vital for hookworm eggs to hatch and for the larvae to survive and be transmitted to susceptible hosts (Udonsi and Atata, 1987). We developed and compare here two climate-based models to evaluate geographic risk of hookworm disease, the MA-GDD and GDDxWB indices, using a LTN climate grid (18 km $^2$) of Brazil. Both indices take into account environmental temperatures conducive to the development of

the free living stages of the parasite as well as prevailing moisture conditions. The MA-GDD index considers moisture above a given threshold of 0.4 and the GDDxWB index considers the entire range of a gradient of moisture.

### 2.4.1    MA-GDD Index and Transmission Cycles

The MA-GDD index reflects suitable days for development above the base temperature of $15^{\circ}$C, from which can be calculated the number of 'potential transmission cycles' that can be completed per year. Hookworm  infected  individuals have been reported to shed eggs continuously for long periods of time, leading to development of infective larvae ($L_3$) and potential year-round transmission. The developmental time of the hookworm from $L_3$ to egg producing adults in the host is fairly constant, with a mean of eight weeks (Beaver, 1988; Hoagland and Schad, 1978). The variation  in generation time (from egg to egg-producing adult) arises from the free-living stages of the parasite, here referred to as the mean transmission cycle period, which is highly susceptible to variation in environmental conditions.  Because of the high dependence on moisture of hookworms for movement, and to prevent desiccation, annual GDD was adjusted (MA-GDD) to exclude months where mean water budget was less than 0.4 as explained above. This result suggests that larvae development and $L_3$ transmission is impaired as soils get drier (Nwosu and Anya, 1980; Udonsi and Atata, 1987). Dry months were thus not included in the index calculations based on the assumption that the GDD from those months do not contribute to the annual accumulated GDD.

We propose use of the transmission cycle parameter as a biology-based criterion for regional risk modeling based on the GDD-WB concept. Literature reports indicate that the development time from egg to $L_3$ can be up to 14 days at temperatures less than $40^{\circ}$C

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(Brooker et al., 2006b; Schad and Warren, 1990), and the period of time that the majority of infective larva remain active in the environment ranges from one to four weeks (Beaver, 1953; Svensson, 1925; Udonsi, 1988). Therefore, taking two weeks for development to the $L_3$ stage and two weeks longevity (on average) in which $L_3$ must find a definitive host, the transmission time was estimated to be four weeks (28 days). This criterion was used as an indicator of biological suitability of different climate thermal-hydrological regimes for the purposes of this study. However, depending on the basis for calculation, which references are used, as well as availability of human hosts the transmission time can potentially range from 9 days (Palmer and Reeder, 2001) if infection occurs immediately after molting to $L_3$, to approximately 51 days (Palmer and Reeder, 2001; Svensson, 1925; Udonsi, 1988) if the larvae survive in the environment. Our estimation of the transmission cycle takes into account factors that may delay the embryonation and hatching of eggs (eg. low temperature) and also the period in which the $L_3$ are reported to be active in the environment prior to infection (Table 2.2).

The average annual mean temperature prevalent in Brazil is 23°C, a thermal regime expected to promote longevity of the larvae in the environment. *N. americanus*, unlike *A. duodenale*, does not exhibit hypobiosis (Loukas and Prociv, 2001) which could interrupt continuous egg shedding. Thus it is possible to use mean transmission cycles per year for describing *N. americanus'*s distribution and to generate risk map surfaces assuming adult populations are long lived (i.e. 4 years).

In the MA-GDD model, most areas with prevalence above 5% lay between index values of 1500 to 3000, allowing 5-14 mean transmission cycles per year (Figure 2.3). Areas with index values below this range lay in the Caatinga ecological zone, which is

Table 2.2. Important biological and climatic variables for the transmission of *N. americanus* used as baseline values for calculations based on the GDD-WB concept.

| Variables | Value | References |
|---|---|---|
| Base Temperature | 15 °C | (Udonsi and Atata, 1987) |
| Mean Annual Temperature | 23 °C | From Brazil climate data |
| Daily GDD | 8 | |
| Mean transmission cycle time (free-living phase) | 28 days (range 9-51) | (Beaver, 1953; Croll and Matthews, 1973; Palmer and Reeder, 2001; Udonsi, 1988; Udonsi and Atata, 1987) |
| Transmission cycle GDDs | 224 | |
| Mean Egg life expectancy | 13-14 days | (Schad and Warren, 1990) |
| Adult life span | 3-4 years | (Schad and Warren, 1990) |

characterized by deserts and xeric shrub and in the southern region of the country where mean temperatures are less than 21°C. Regions with the highest MA-GDD index lay in the Amazon region, the northern coast area and the Bahia coastal forests, which are characterized by moist broadleaf forests. Early work (Chandler, 1929) more recent literature reports suggest the Amazon region has high prevalence in indigenous populations (Bóia et al., 2009; Botero, 1981; Miranda et al., 1998). In the Bahia coastal forests of high annual rainfall the low prevalence suggests that hookworms do not survive long where there is frequent flooding and run-off and that larval stages do not survive well in water without suitable substrate (Schad and Warren, 1990).

2.4.2  GDDxWB Index

The GDDxWB index model had a higher correlation coefficient for hookworm prevalence as compared to MA-GDD (0.27 versus 0.24). This may be because MA-GDD did not have a completely linear relationship to hookworm prevalence, having an upper

end cut off (Figure 3).  Risk maps based on both the MA-GDD and GDDxWB indices

results suggest low temperature (mean temperatures less than 21°C)  inhibit hookworm

transmission in southern Brazil where climate is much cooler, with minimum

temperatures commonly falling below 15°C, the base temperature for egg hatching of

hookworm (Schad and Warren, 1990).

In contrast to the MA-GDD, the GDDxWB index provides the advantage of measuring a

moisture *gradient* of suitability but not the number of transmission cycles per year.

However, it is possible to relate transmission cycles and the GDDxWB index by use of a

quadratic equation (described above) to calculate the number of transmission cycles

expected at a given GDDxWB index value.  For both the MA-GDD and the GDDxWB

index there was a significant difference in $\log_{10}$ means of GDDxWB between areas of

low, moderate and high prevalence of hookworm (Figure 4).  At above a certain climatic

threshold, however, it is typical to observe considerable variation in actual versus

potential transmission levels at individual sites even in high risk climates because of

location-specific factors related to poverty, occupational-agricultural practices or local

environment, such as location along riverine wetland plains. Climate risk is therefore an

indicator of *potential* transmission related to mean transmission of individual sites in a

broader regional climatic envelope (Malone, 2005).

### 2.4.3   Data Validation

 A relatively limited number of 30 survey reports were available in the literature to

validate the climate risk models developed in the current study based on data collected as

part of the national schistosomiasis control program which focused on areas with known

or suspected schistosomiasis endemicity.   Results from the correlation coefficients

suggest the majority of the validation points fit better in the GDDxWB model (Figure 2) than the MA-GDD model.  The high hookworm prevalence predicted for the Amazon basin by both the GDD-WB and GDDxWB models is consistent with results reported  by Chandler (1929) and earlier reports that hookworm occurs in most of the country, including the Amazon, and  has lower prevalence in the drier areas found in the northeastern part of the country (DNERu, 1962).  In order to further validate the climate-based models produced in the current study, a comprehensive national survey for STH is needed, since hookworm often co-infects with other STH (Fleming et al., 2006).  A comprehensive survey should include areas of limited surveillance for schistosomiasis such as in the Amazon basin and the Caatinga region.

### 2.4.4   Other Environmental Factors

The objective of the current study was to develop climate based models that enable mapping of the climate envelop of hookworms in Brazil and did not take into account other factors relating to the prevalence of hookworm that can be used to further develop and  refine climate risk maps in future studies. Other environmental variables identified in literature reports as important in hookworm propagation and  transmission include soil type, slope, altitude and land surface temperature measurements derived from environmental satellites  (Brooker et al., 2006b; Mabaso et al., 2003; Udonsi et al., 1980). Social-economic status has long been recognized as an important variable relating to parasitic infections including hookworm (Hotez, 2008) and a similar trend has been shown in Brazil (Fleming et al., 2006).  Vegetation cover or shade have been shown to be important for the longevity of larvae (Udonsi and Atata, 1987), suggesting an impact of deforestation . Further studies are underway to refine maps based on climate suitability

alone and to validate the influence of these additional factors on the ecological niche of

*N. americanus*.

In summary, the prevalence of hookworm in Brazil appears to be strongly related to both

warmer and moister climatic conditions where the GDDxWB is above 1300 and where

the MA-GDD dictates a minimum number of five transmission cycles per year. Low

prevalence is predicted in areas that have mean monthly temperatures below 21ºC.  The

current study supports the validity of the GDD-WB concept for use in mapping risk areas

for hookworm. There is need for a systematic national survey for hookworm and other

STH in Brazil to elucidate STH disease risk outside the schistosomiasis endemic areas

where very limited surveillance exists. The models developed can be used to-identify

high hookworm prevalence risk areas for purposes of planning further surveillance and

control efforts in Brazil.

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# 3 HOOKWORM AND OTHER SOIL TRANSMITTED HELMINTHS AMONG PATIENTS IN MUTUÍPE, BRAZIL

## 3.1 Introduction

Soil transmitted helminths (STH) which include hookworm, *Ascaris lumbricoides* and *Trichuris trichiura* are found worldwide. It is estimated that more than 1.5 billion people are infected with at least one species of STH globally (Bethony et al., 2006; WHO, 2013). As a group, they represent the most prevalent parasitic infection of man and are most common in tropical and subtropical countries in East Asia, sub-Saharan Africa, and the Americas where they are associated with high levels of poverty (Brooker et al., 2006b). Co-infection with all three parasite species is also common (Bethony et al., 2006) and children infected with STH have been found to have physical and intellectual retardation, reduced cognitive ability and malnutrition (Bethony et al., 2006; Eppig et al., 2010; Jardim-Botelho et al., 2008; Stephenson et al., 1993; Yap et al., 2012). Other symptoms of infection include diarrhea, anemia, and abdominal pain (Beaver, 1975; Ezeamama et al., 2005).

Mass drug administration, which refers to the periodic distribution and administration of drugs to at-risk populations in endemic areas (WHA, 2001), is the main approach used in the control of STH (Hotez et al., 2007). The primary drugs used in the treatment and control of STH are benzimidazoles, particularly albendazole and mebendazole (Hotez et al., 2008). Control strategies are based on prevalence and intensity of infection in a community, with intensity of infection being estimated from fecal egg counts (Montresor et al., 1998).

Hookworm infection is the most important infection among the intestinal nematodes and ranks fourth among parasitic infections worldwide after malaria, leishmaniasis and lymphatic filariasis (Hotez et al., 2005) as determined by disability-affected life years (DALY) . It is estimated that approximately 740 million people worldwide are infected with hookworm (de Silva et al., 2003). Hookworm disease is often characterized by iron deficiency anemia which is of great concern in women of reproductive age and children (Brooker et al., 1999; Dreyfuss et al., 2000; Hotez et al., 2005).

The two human hookworms that are most prevalent are *Necator americanus*, occurring mostly in the tropics and *Ancylostoma duodenale*, found in more temperate climates. The few studies done so far in Brazil (Minas Gerais and São Paolo states) that have identified the hookworm as to species reported found predominantly *N. americanus* (Fleming et al., 2006; Geiger et al., 2004; Kobayashi et al., 1995).  However, a few A. *duodenale* infections were reported in Pernambuco state, in northeastern Brazil (Gonçalves et al., 1990; Okazaki et al., 1988). Because the two species have different epidemiological and ecological characteristics, it is important to differentiate them to be able to properly plan control measures (Brooker et al., 2006a), particularly with regard to public awareness and sanitation.

Most parasitological surveys for intestinal helminths utilize the Kato-Katz thick smear for quick diagnosis of parasite infection as recommended by WHO (Montresor et al., 1998). This method, though cost effective and technologically easy to perform lacks sensitivity and specificity for hookworm species detection (Basuni et al., 2011) when compared to time consuming classic morphological analysis or molecular methods like Polymerase Chain Reaction (PCR).

Levecke et al. (2011) compared the Kato-Katz method to the McMaster flotation method that is commonly used in veterinary parasitology and they found that Kato-Katz was more sensitive and resulted in higher fecal egg counts for *A. lumbricoides* than the McMaster method. However, there was no significant difference when used for *T. trichiura* and hookworm egg detection.

The use of PCR to differentiate hookworm species has proved useful because hookworm eggs are difficult to differentiate by microscopy as they are of similar size. Coproculture is useful for differentiating strongylid nematode infective larvae and may also be used to differentiate *N. americanus* from the *Ancylostoma* sp. However, it is time consuming and requires skilled personnel (de Gruijter et al., 2005). Using molecular methods, the internal transcribed spacer (ITS) region of ribosomal (r)DNA was identified as being ideal for amplification as it was a well conserved region in strongylid nematodes (Romstad et al., 1997) and had adequate sequence difference between species for delineation. Genetic markers in ITS-2 rDNA were used to distinguish *Necator americanus* from *Oesophagostomum bifurcum* (Romstad et al., 1997) and later de Gruijter et al. (2005) used it successfully to differentiate *N. americanus* and *A. duodenale* in fecal samples.

The tropical climate in Brazil is highly favorable to the development and transmission of hookworm and other STH and many independent studies have confirmed this (Mudenda et al., 2012). The hookworm prevalence varies based on environmental factors and socio-economic status and prevalence can range from less than 1% among city dwellers with good sanitation and nutrition (Santos et al., 2013) to over 80% (Gonçalves et al., 1990) in poorer rural communities.

The objectives of this study were to use a flotation method to determine the prevalence of soil transmitted nematodes in Mutuípe, a rural municipality in Bahia state, in Northeast Brazil and to determine the hookworm species present.

## 3.2 Materials and Methods

### 3.2.1 Study Area

Mutuípe municipality (latitude -13.206189, longitude -39.502773), located in Bahia state, Brazil, has a population of approximately 22000 people. Patients were referred to the Central Municipality Laboratory from six health posts scattered around the municipality namely: L'dio Santana, Teobaldo Pinheiro, Antonio Ribeiro leal, Centro (Moisés Gongalves I, Moisés Gongalves II) and Cajazeira. Mutuípe is a rural municipality with farming as the main occupation and contains a small city, Mutuípe, where the central health laboratory was located. The age, gender and location i.e. rural/urban were noted for each person. The study was done in tandem with other routine laboratory investigations of the Central Laboratory, Ministry of Health (Laboratório do centro de saúde, Ministério da Saúde). Authorization was obtained from the Health Secretary of the municipality and verbal consent of participants as part of the project "Recurrent Treatment: Effect on rural and urban *S. mansoni* population structure". (Exemption approval in appendix for IRB #EM-13-39) (Appendix C).

### 3.2.2 Parasitological Survey

The survey was carried out over a period of six days in July, 2012. All fecal samples that were submitted to the laboratory during that time were examined for STH eggs. Patients were routinely required to submit three fecal samples on consecutive days regardless of presenting complaint/symptoms. Fecal flotation was performed on 2g of feces using a the

double centrifugation technique (Baker, 2007; Sloss et al., 1994) with a sucrose solution

of 1.27 specific gravity. Two grams of feces were placed in a 15 ml conical tube and

filled with water up to the 10ml mark. The feces were homogenized with the water using

a small kebab wooden stick, then the tube was filled with water and a cap screwed on.

The tubes were centrifuged for 5 min at 2000 rpm using a fixed head, 80-2B Centribio

centrifuge (Curitiba, Brazil) and the supernatant was discarded. The tube was filled with

sucrose solution up to the 10ml mark and homogenized as before. The tube were then

centrifuged for 5 min at 2000 rpm and then placed upright in a rack. The tube was then

filled with sucrose solution till a positive meniscus formed and a cover slip was place on

top of the tube. The tube was left to stand for a minimum of 20 min before the cover slip

was removed, placed on a glass slide and examined by light microscope (Nikon YS100,

Nikon, Melville, NY). The fecal egg count was recorded and divided by two to obtain the

eggs per gram (epg) for each parasite species seen.

### 3.2.3    Larvae Culture and Examination

One gram of feces from each hookworm positive sample was cultured for six to nine days

using the Harada-Mori method (Harada and Mori, 1955)(Figure 3.1).  Briefly, a gram of

feces was smeared on a 1.5 x 11cm filter paper strip (medium porosity, slow flow rate, P5

grade, Fisher Scientific, Pittsburgh, PA); 4ml of filtered water was placed in a 15 ml tube

and the filter paper was inserted into the tube till the water was almost at the level of the

fecal smear but not touching it so that the filter paper did not reach the bottom of the

tube. The cap was then placed loosely on the tube. Cultures were done in triplicate at

room temperature (23-24ºC) for 1-6 days before they could be moved into a 29ºC

incubator at the Oswaldo Cruz (FIOCRUZ) laboratory in Salvador, Bahia. The culture

tubes were removed from the incubator and the filter paper carefully removed and discarded into a biohazard bag. The contents of the three tubes from each sample were combined into one tube and centrifuged at 2000rpm for 5 minutes to concentrate the larvae. The supernatant was aspirated with a plastic pipette leaving 1ml of water. The larvae pellet was resuspended and 0.1ml of the suspension was placed on a glass slide with a cover slip and examined under a light microscope at 10x power. The larvae present were measured and the species identified morphologically using published keys and descriptions (Nichols, 1956; Svensson and Kessel, 1926; Wu and Peng, 1965). The remainder of the suspension was placed in a well of a 24 well plate and the larvae were counted using an inverted microscope at 4x magnification, then placed in 1.5ml eppendorf tubes and stored at -20°C until DNA extraction was performed.



Figure 3.1: Harada-Mori culture. Infective hookworm larvae accumulate at the bottom of the tube.

3.2.4   Preparation and Storage of Fecal Samples

A 1-2 grams sample of individual hookworm positive fecal submissions were mixed thoroughly with 1% saline, strained through a sieve (tea strainer) and the filtrate was centrifuged at 1500rpm for 5 minutes. The supernatant was discarded and the pellet in the 15ml tube was stored at -20$^o$C until DNA extraction could be done. A random sample of 29 hookworm negative samples were similarly processed individually.

3.2.5   DNA Extraction from Infective Larvae and Fecal Samples

Eppendorf tubes (1.5ml) containing infective larvae in water were centrifuged at 2000 rpm for 5 minutes and the water removed leaving the last 0.1ml in the tube. The tubes were gently vortexed to resuspend the pellet.  DNA was extracted as per manufacturer's instructions using the QiaAmp® DNA Mini kit (Qiagen, Vinlo, Netherlands). A freeze-thaw cycle using dry ice and 56$^o$C was incorporated in the incubation part of the protocol to enhance cell lysis with Proteinase K. DNA was extracted from the frozen fecal samples using the QiaAmp® DNA Stool Mini kit (Qiagen, Vinlo, Netherlands) as per manufacturer's instructions. The DNA was stored at 4$^o$C until further examination.

3.2.6   PCR for *N. americanus* and *A. duodenale* Differentiation

 PCR reactions were performed in 25µl volumes using 10.5µl of fecal or hookworm larvae DNA sample, 12.5µl of 2X AmpliTaq Gold® PCR Master Mix (AmpliTaq Gold DNA Polymerase, 250U; GeneAmp PCR Gold Bugger, 30 mM Tris/HCl, pH 8.05, 100mM KCl; dNTP, 400 µM each; $MgCl_2$, 5mM, proprietary stabilizers) (Life Technologies, Coralville, IA) and 1µl (0.4µM) of each primer. A semi-nested PCR protocol was followed (de Gruijter et al., 2005) using the forward primer NC1 (5' - ACGTCTGGTTCAGGGTTGTT-3′) and reverse primer NC2 (5′-

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TTAGTTTCTTTTCCTCCGCT-3′) (Gasser et al., 1993) for the first round of amplification in a Bio-Rad PTC 0200 DNA Engine Thermal Cycler (Hercules, CA). In brief, amplification consisted of 94ºC for 5 min, 25 cycles at 94ºC for 30s; 55ºC for 30s; 72ºC for 30s, followed by a final extension step of 5 min at 72ºC. The amplicon (2µl) from the first round of amplification was used in the second amplification procedure using forward primer AD1 (5′-CGACTTTAGAACGTTTCGGC-3′) and NC2 as the reverse primer. The second round of amplification consisted of 94ºC for 5 min, 35 cycles at 94ºC for I min; 55ºC for 1 min, 72ºC for 1 min, and finally 72ºC for 5 min. Primers were obtained from Integrated DNA Technologies (Coralville, IA) . 5µl of each second-round amplicon was mixed with 1µl of 6X TrackIt™ Cyan/Yellow Loading Buffer (Invitrogen, Carlsbad CA) and examined on 1% agarose-TBE (IM Tris, 0.9M Boric acid, 0.01M EDTA, ph 8.4) (Invitrogen, Carlsbad, CA) gel with SYBR® Safe gel stain (TrackIt™, Invitrogen, Carlsbad, CA) with an Ultraviolet (UV) transilluminator (Fluorchem®Q, Alpha Innotech, San Leandro , CA).

PCR was performed first on the larvae DNA samples, and their amplicons were sequenced by the GenLab, School of Veterinary Medicine, Louisiana State University, using a 3130 Genetic Analyzer (Applied Biosystems®/Hitachi, Life Technologies, Coralville, IA). Sequence analysis was done using MEGA 5.2 software (Tamura et al., 2011) and similarity comparison was made against the GenBank nucleotide database using BLAST (NCBI). Those confirmed to be *N. americanus* were subsequently used as positive control samples for fecal DNA PCR. Molecular biology grade water was used a negative control. All amplicons that appeared dissimilar to the *N. americanus* expected

base pair size on the agarose gel were sequenced and searched against the GenBank database.

Statistical analysis was done using Statistical Analysis Software 9.3 (SAS, Cary, NC). Chi-square test and Fisher's Exact test were used to compare categorical data where appropriate. A $p$ value $< 0.05$ was considered significant.

## 3.3    Results

A total of 159 patients referred from the five health posts in Mutuípe municipality submitted fecal samples (Table 3.1). Of these, only 38 patients submitted three fecal samples during the survey period, therefore 277 fecal samples were collected and examined (Table 3.2). Patient age ranged from 3 months to 96 years old.

Table 3.1: Prevalence of parasites in out-patients from health posts of Mutuípe municipality.

| Health Post | Number of patients | Parasite positive (%) | Hookworm (%) | A. lumbricoides (%) | T. trichiura (%) |
|---|---|---|---|---|---|
| Centro | 51 | 11 (21.6) | 8 (15.7) | 4 (7.8) | 4 (7.8) |
| Cajazeira | 33 | 6 (18.1) | 4 (12.1) | 1 (3.1) | 2 (6.2) |
| A. R. Leal | 18 | 3 (16.7) | 2 (11.1) | 0 | 2 (11.1) |
| T. Pinheiro | 27 | 11 (40.7) | 10 (37.0) | 1 (3.8) | 1 (3.8) |
| L. Santana | 30 | 7 (23.3) | 7 (23.3) | 0 | 0 |
| **Total** | **159** | **38 (23.9)** | **31 (19.5)** | **6 (3.8)** | **9 (5.7)** |

There were 61 males and 98 females and 20 (32.8%) males and 18 (18.4%) females had parasites. There was no significant difference by gender among parasite infected people.

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Table 3.2: STH egg detection by number of fecal samples submitted by patients.

| Samples submitted | Number of patients | Hookworm | *A. lumbricoides* | *T. trichiura* |
|---|---|---|---|---|
| 1 | 79 | 19 | 3 | 3 |
| 2 | 40 | 6 | 1 | 2 |
| 3 | 38 | 6 | 2 | 4 |

Most of the infections were single parasite infections including one case with *E. vermicularis*. *A. lumbricoides* tended to occur with other parasites (Table 3.3).

Table 3.3: Occurrence of single and multi-parasite infections of STH in out-patients.

| Infections | Parasite | Number of patients |
|---|---|---|
| Single | Hookworm | 25 |
| | *A. lumbricoides* | 1 |
| | *T. trichiura* | 5 |
| Double | Hookworm, *A. lumbricoides* | 2 |
| | Hookworm, *T. trichiura* | 1 |
| | *T. trichiura, A. lumbricoides* | 0 |
| Triple | Hookworm, *A. lumbricoides, T. trichiura* | 3 |

The fecal egg counts of the STH in the patients were all of low intensity (Table 3.4) except for one child that had had an *A. lumbricoides* egg count of 8762/g.

Table 3.4: Fecal egg counts of STH in patients

| | Hookworm | *A. lumbricoides* | *T. trichiura* |
|---|---|---|---|
| Mean EPG | 113.8 | 2307.2 | 26.3 |
| Range | 1-633 | 1-8762 | 3-80 |

With regard to location, 63 (39.6%) of the patients came from urban Mutuípe and 96 (60.4%) came from a rural area (Figure 3.2). Although the number of parasitized patients was higher among rural dwellers (28/96; 29.2%) than those from the urban area (10/63; 15.9%), there was no significant difference between the two groups (*p*=0.054).

Hookworm had the highest prevalence (19.5%) of the nematode eggs seen. *Enterobius vermicularis* was found in one child by fecal examination.
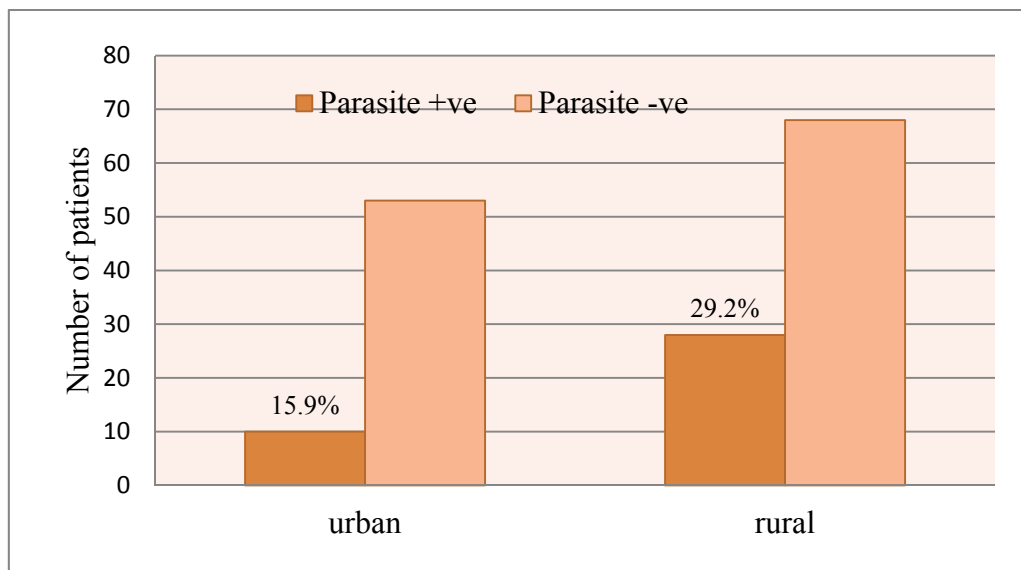


Figure 3.2: Proportions of parasitized and non-parasitized patients in urban and rural Mutuípe

3.3.1   Hookworm Infection

The ages of hookworm positive individuals ranged from 1.5 to 96 years old. The majority of these (70.97%) were adults over 20 years old. There was no significant difference in prevalence between patients less than 20 years old and those over 20 years old and there was very poor correlation between fecal egg count and age (R =0.065). Prevalence of hookworm increased with age, as did the mean epg and prevalence was highest in the >50 years age group but the mean epg was highest in the 36-50 years age group (Figure 3.3).

However, significantly more males (17/31; 54.8%) than females (14/31; 45.2%) were infected ($p = 0.035$). The mean hookworm fecal egg count was 110.3 (SD 152.4, Range 0.3-633) (Table 3.5).

There were 63 individuals from urban Mutuípe and 96 from the rural area of the municipality (Figure 3.4). Hookworm prevalence was higher among the rural dwellers (60.4%) compared to the urban dwellers (39.6%) although the difference was not significant ($p=0.08$).
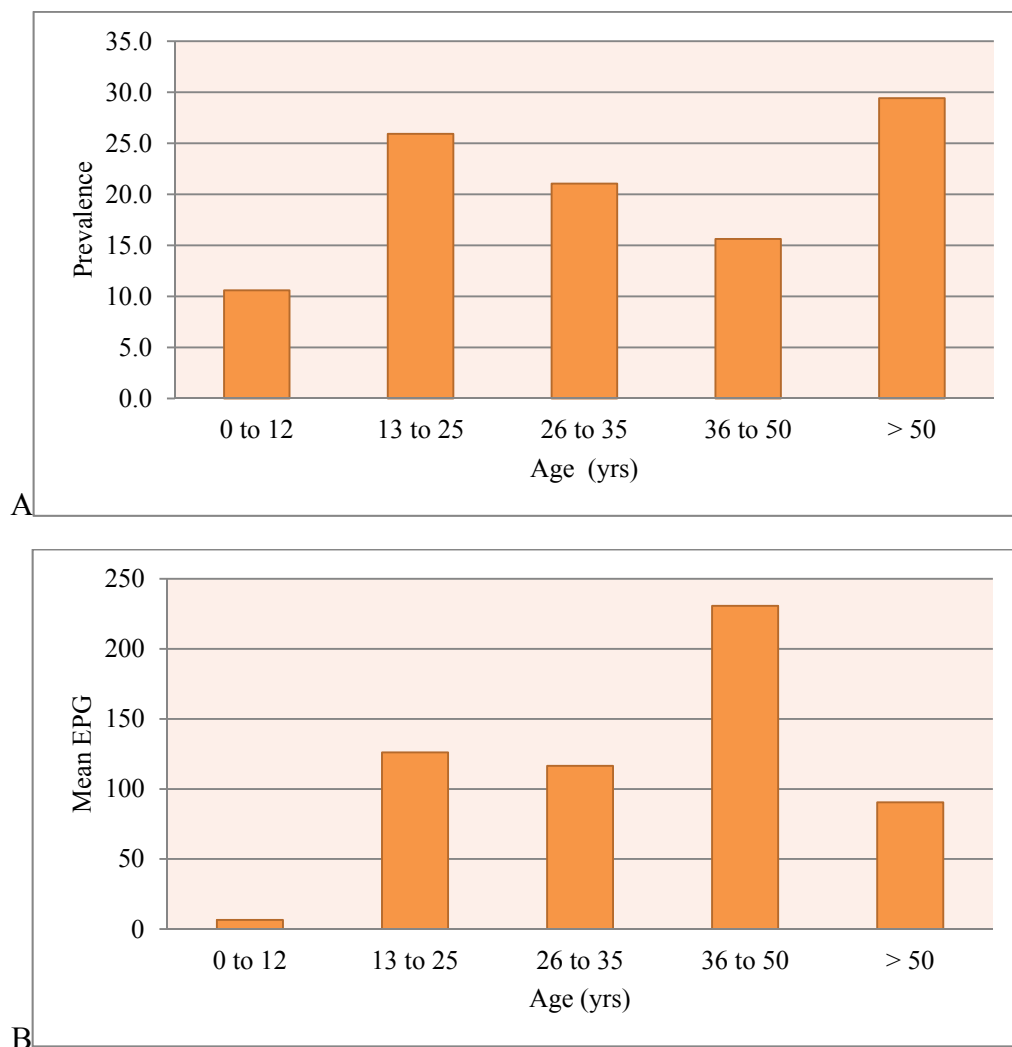
A

B

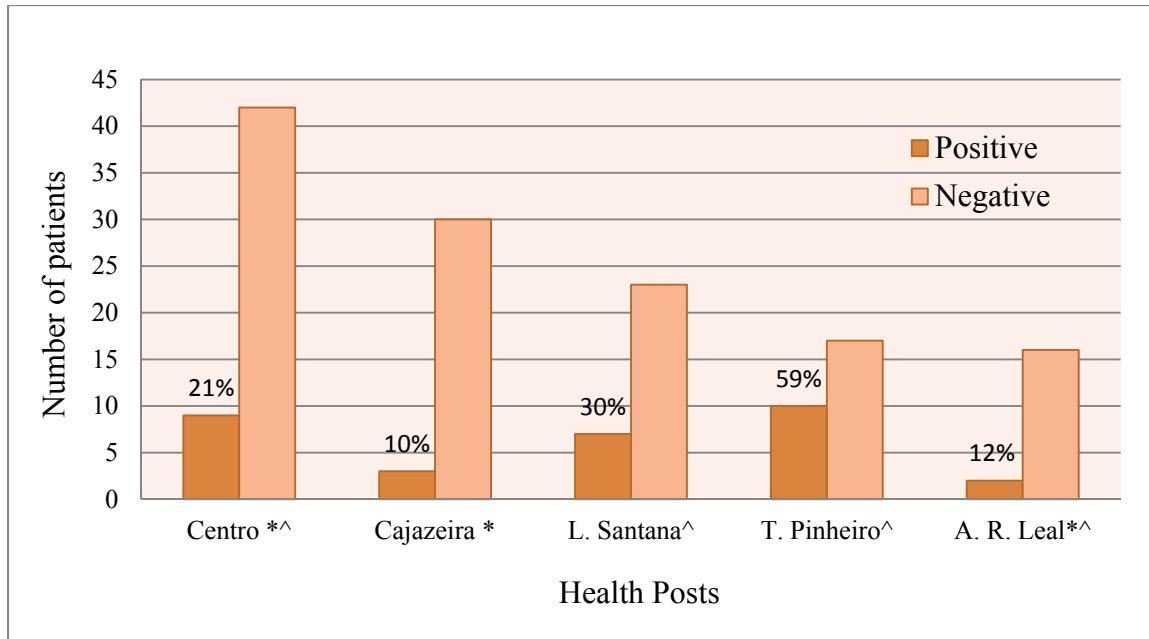Figure 3.3: Prevalence (A) and Mean hookworm EPG (B) among age groups.

Figure 3.4: Distribution of hookworm positive patients among health posts. *urban; ^rural

Infective larvae were recovered from 18 of 31 cultured fecal samples. The mean length was 667.4μm (SD 39.5, Range 571.8 – 738.7μm) and they matched the morphological characteristics of *N. americanus* (Table 3.5 and Figure 3.5).

Seventeen out of 18 of the hookworm larvae DNA samples were successfully identified by PCR and confirmed by sequencing. Sixteen samples had *N. americanus* and one had *A. ceylanicum*. PCR on the 31 hookworm positive fecal samples yielded 21 positive for *N. americanus* and one mixed infection with *N. americanus* and *A. ceylanicum*. The total number of samples identified using PCR as *N. americanus* was 23/31 with one sample also having *A. ceylanicum*. All of the fecal samples that were negative with microscopy were negative for hookworm using PCR. The *A. ceylanicum* sample was 99% identical (one nucleotide difference) with a query cover of 100% to GenBank accession number JQ673421.1, a sample originating from canine feces in Malaysia.

Table 3.5: Morphological characteristics of hookworm infective larvae in Mutuípe

| Characteristics | *Ancylostoma sp.* | *N. americanus* | **Mutuípe hookworm** (***N. americanus***) |
|---|---|---|---|
| Length of body | 660 µm | 590 µm | |
| Length of sheath | 720 µm | 660 µm | 667.4 µm |
| Anterior end | blunt | rounded | rounded |
| Intestine | Anterior end narrower than esophageal bulb No gap between esophagus and intestine | Anterior end as wide as esophageal bulb Gap between esophagus and intestine | Anterior end as wide as esophageal bulb. Gap between esophagus and intestine |
| Tail | Serrations not clear | Serrations clear | Serrations clear |



Figure 3.5. *N. americanus* infective larvae. Arrows point to the gap between esophagus and intestine.

## 3.4    Discussion

Mutuípe is located in a region where climatic conditions are highly conducive to the development of STH in the environment. Mutuípe falls within the permissive zone for hookworm development with regard to climatic temperature and moisture (Mudenda et al., 2012). The prevalence of parasites, particularly hookworm is comparable to what other researchers have found in similar environments in rural Brazil (Machado et al., 2008; Silva and da Silva, 2010) and in some cases, higher (Cury et al., 1994; Silva et al., 2007). One reason for the higher prevalence may be that this study was based on out-patients while most of the other studies were based on school children or whole communities. However, in comparison to what other studies have found in patients in various regions of Brazil (Santos et al., 2013), the prevalence of hookworm in this study is much higher. The convenience sampling in this study may be a source of bias and further studies are necessary to have a better understanding of the status of the community with regard to parasite prevalence. The method for STH  egg detection was also different from that employed by previous researchers in that a flotation technique was used that concentrated the nematode eggs in the quantity of feces examined (Baker, 2007; Dryden et al., 2005). The quantity of fecal sample used (2g) was also much more than what is used in the routine Kato-Katz method (41.7mg), even when three Kato-Katz slides are prepared. The double centrifugation flotation method therefore was more efficient at detecting low intensity infections, which may be more prevalent in areas where deworming is regularly practiced (Stoltzfus et al., 1997). The double centrifugation method used is also more conducive for detecting hookworm eggs that are quickly cleared in a Kato-Katz smear, as the eggs remain intact for hours after the slide is

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prepared. The flotation method is also less labor intensive with regard to the number of slides that need to be examined and eggs are easier to detect due to sedimentation of most fecal debris.

*N. americanus* was the predominant STH among patients in Mutuípe. Since most were low intensity infections (epg < 2000) (Montresor et al., 1998), anemia would be expected to be uncommon if present at all (Stoltzfus et al., 1996; Stoltzfus et al., 1997). Other researchers (Santos et al., 2013) found that low intensity infections may result in lower than normal hemoglobin content even in well-nourished individuals. If low intensity infections went undetected, patients would remain untreated, resulting in continued contamination of soils with hookworm eggs in populated areas. According to this study, the highest prevalence of hookworm and intensity of infection (epg) were found in adults over 35 years old and this finding is consistent with what other researchers have found (Santos et al., 2013; Stoltzfus et al., 1997). Therefore the use of anthelmenthics in all age groups in this region is justified. The current policy in the municipality indicates that all people who test positive for parasites with a Kato-Katz method, be treated for free (Jackson Costa, personal communication). However, the method used in the municipality was spontaneous sedimentation, which is not ideal for recovery of hookworm, and performed less efficiently than other methods (Inês et al., 2011). This underlines the need for an effective and sensitive method for detecting low intensity parasite infections. According to WHO recommendations, MDA should be used in areas where prevalence of STH and Schistosomiasis exceeds 50%. However, with regard to hookworm infection, if prevalence is over 20% and anemia is prevalent, then the whole community should be treated (Montresor et al., 1998). Future studies can therefore focus determining the

prevalence of hookworm and other STH in the general population, including adults and including assessment of the nutritional status and hemoglobin concentrations in order to determine the best approach to control.

Significantly more males than females were infected with hookworm, a finding comparable to other studies (Behnke et al., 2000; Santos et al., 2013) and has been attributed to males being involved in occupations or behaviors that put them at greater risk of infection, such as agriculture (Behnke et al., 2000; Stoltzfus et al., 1997).

Hookworm was found to be more prevalent in patients from rural areas than those living in urban Mutuípe, a finding although not statistically significant, is in agreement with what other researchers have found (Machado et al., 2008; Silva et al., 2007).

The use of a sucrose flotation method normally applied to veterinary parasitology proved to be adequate for the concentration of human intestinal nematode eggs. This method proved more sensitive than a semi-nested PCR procedure for hookworm egg identification. This may be because the amount of feces used in the flotation was much more than the amount from which DNA was extracted (2g and 200mg respectively). Also, the PCR on larvae was more successful (17/18 positive) than on fecal samples (21/31 positive) probably because the larvae samples were 'cleaner' with regard to PCR inhibitors normally found in feces. PCR on fecal samples proved to be faster and less tedious for identification of the species of hookworm than using the Harada-Mori culture method. The low recovery rate of infective larvae from the culture method may also be attributed to the low fecal egg count and the lesser number of culture days that some of

the samples were kept, since it is recommended culture be continued for at least seven days (Navitsky et al., 1998).

The low prevalence of *A. lumbricoides* and *T. trichiura* could be explained by their lifecycle and mode of transmission. They are transmitted orally rather than by skin penetration by *N. americanus*. Therefore good hand hygiene may be deterring the transmission even if they may be present in high numbers in the environment.

The identification of *N. americanus* as the hookworm species in Mutuípe adds to the body of knowledge on the distribution of hookworm species in Brazil. This is the first known report of *A. ceylanicum* in humans in Brazil, a zoonotic hookworm species that is most commonly found in Asia (Carroll and Grove, 1986; Traub et al., 2008).

## 3.4.1    <u>Conclusions</u>

- The prevalence of STH and hookworm among outpatients was found to be high.

- Adults and males had higher prevalence of hookworm infection compared to other groups.

- *N. americanus* was the predominant hookworm species and one case of *A. ceylanicum* was found.

- Sucrose double centrifugation method proved efficient for the concentration of STH and performed better than the Harada-Mori culture and PCR.

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# 4 GASTROINTESTINAL PARASITE SURVEY OF SHELTER DOGS IN SOUTHERN LOUISIANA

## 4.1 Introduction

Investigation of intestinal parasites in dogs is important in order to evaluate the risk to dog populations, estimate the level of environmental contamination and to assess the public health risk from zoonotic parasites. National surveys have shown that shelter dogs tend to be more parasitized than pet dogs (Blagburn et al., 2012; Little et al., 2009) due to inadequate veterinary care. Hookworm was found to be the most prevalent parasite in shelter dogs using flotation methods, particularly in the southeastern states of the USA. (Blagburn et al., 1996; Hoskins et al., 1982; Little et al., 2009). The last Louisiana survey was a retrospective study of Louisiana State University (LSU) Teaching Hospital records (Hoskins et al., 1982), therefore the level of parasitism in shelter dogs in Louisiana requires further investigation. For example, limitations in the egg/oocyst/larvae concentration methods used previously may have led to an underestimation of the prevalence of parasites such as tapeworms and *Giardia* sp. and, in order to enhance visibility and recovery of parasite eggs/oocysts, a double centrifugation flotation technique is now recommended (Liccioli et al., 2012; Sloss et al., 1994).

The most common species of canine hookworm in the US is *A. caninum*. *A. braziliense* which is the main cause of cutaneous larva migrans (CLM) in humans, has been found in Texas and Florida states in both dogs and cats (Liotta et al., 2012). However, the prevalence of *A. braziliense* in Louisiana dogs is unknown. Differentiating the eggs of hookworm species by microscopy is challenging as they are very similar in size, except for *Uncinaria stenocephala* which is larger and is more prevalent in the northern US

states (Blagburn et al., 1996; Lucio-Forster et al., 2012). Molecular methods developed for differentiating *Ancylostoma* spp. have proved useful and are less time consuming than microscopy (e Silva et al., 2006; Liotta et al., 2012).

The objectives of this study were to determine the prevalence of hookworm and other intestinal parasites in shelter dogs and to determine the relationship of sex, age and deworming status with parasite infection, as well as determine the species of hookworm in south Louisiana. The anthelminthic protocols used in animal shelters were also evaluated.

## 4.2   Methods

The study area was southern Louisiana and involved 10 animal shelters in nine (9) parishes, namely: Ascension, Acadia (Acadia, Crowley shelters), East Feliciana (Dixon Correctional Institute shelter), East Baton Rouge, Livingston (Walker Animal Control), Iberville, Tangipahoa, Pointe Coupee and St. Martin (Figure 1). Most of the dogs were kept in the shelters a minimum of five days with an average length of stay of two to three weeks, except in East Feliciana and Point Coupee shelters which had a 'no-kill' policy where animals may have been kept for years.

Fecal samples were collected before the daily cleaning from cages of individually housed dogs that had been acquired within the previous two weeks. Samples were placed in plastic containers and labeled with age, sex, breed and deworming status of the respective dog. The collection was opportunistic during the LSU Shelter Medicine course, at the time of routine visits of students and shelter medicine clinical faculty to animal shelters. Samples were submitted to the Louisiana Animal Disease Diagnostic Laboratory

(LADDL) and examined the same day. The age was determined from shelter records, where available, or estimated from examination of the dentition. The last known date of deworming was recorded if known.
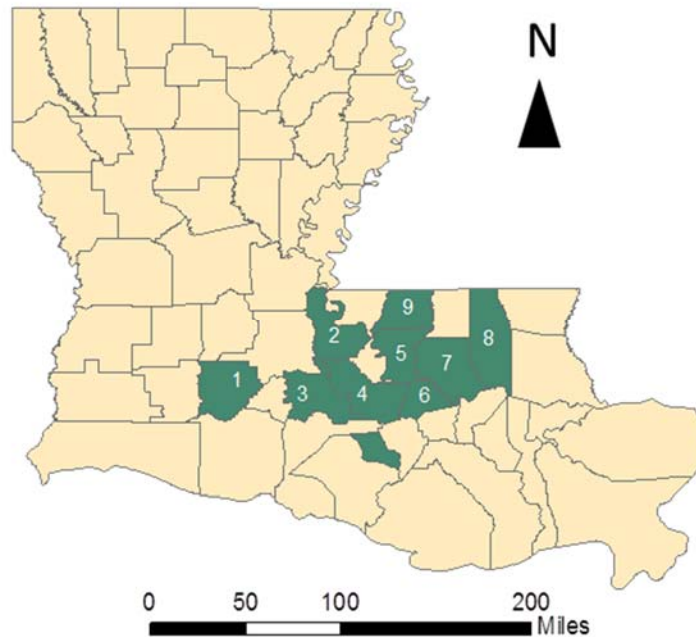


Figure 4.1. Map of Louisiana with the location of parishes in the study area in green. Acadia (1), Pointe Coupee (2), St. Martin (3), Iberville (4), East Baton Rouge (5), Ascension (6), Livingston (7), Tangipahoa (8), East Feliciana (9).

4.2.1    Parasitological  Examination:

A direct saline fecal smear, zinc sulfate flotation (SG = 1.18), sucrose flotation (SG = 1.25-1.27) and saline sedimentation (0.85%) (Baker, 2007) were used to process each sample. The modified Wisconsin double centrifugation method, which included an initial wash with water, was used for the flotation methods (Rehbein et al., 2011; Sloss et al., 1994). The saline sedimentation method involved three washes, allowing the tube stand for 20 minutes between washes. The supernatant was decanted, leaving the last 15mls of sediment before filling the tube with more saline. During the last decantation, a transfer

pipette was used and the sediment was then drawn into the pipette which was left upright

for five minutes to allow sedimentation in the pipette. The sediment was then placed on a

glass slide and examined under a light microscope. Slides were examined at 10x and 40x

for morphological identification of eggs/oocysts/larvae. A gram of feces was used for

each of the flotation and the sedimentation methods. Eggs per gram counts were noted for

helminth eggs found by the flotation methods.

### 4.2.2 PCR and Restriction Fragment Length Polymorphism (RFLP) for Hookworm Speciation

Hookworm positive fecal samples were cultured using the Harada-Mori technique

(Harada and Mori, 1955) and the resulting infective larvae were examined

microscopically to identify *Ancylostoma* spp. and then stored at -20$^{\circ}$C awaiting DNA

extraction. For samples that did not have sufficient fecal matter for culturing after the

parasitological fecal examinations were conducted, the sediment remaining from the

sedimentation method was placed in 1.5ml eppendorf tubes and frozen. DNA was

extracted from larvae using a QiaAmp® DNA Mini kit (Qiagen, Valencia, CA) using the

protocol for DNA extraction from tissue samples as per manufacturer's instructions. A

freeze-thaw cycle was incorporated during the incubation period with Proteinase K using

liquid nitrogen and 56$^{\circ}$C. DNA was extracted from fecal samples using a QiaAmp®

DNA Stool Mini kit (Qiagen, Valencia, CA) and stored at -20$^{\circ}$C until examined.

Two PCR protocols were performed using two sets of primers. In the first protocol, the

forward primer NC5 (5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and reverse

primer NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′) (Gasser et al., 1996) were used.

These primers span the ITS-1, 5.8S and ITS-2 regions of the ribosomal DNA of

*Ancylostoma* spp. resulting in a PCR product approximately 860 base pairs in size (e

Silva et al., 2006). PCR reactions were performed in 25µl volumes using 10.5µl of fecal

or hookworm larvae DNA sample, 12.5µl of 2X AmpliTaq Gold® PCR Master Mix

(AmpliTaq Gold DNA Polymerase, 250U; GeneAmp PCR Gold Bugger, 30 mM

Tris/HCl, pH 8.05, 100mM KCl; dNTP, 400 µM each; MgCl$_2$, 5mM, proprietary

stabilizers) (Life Technologies, Coralville, IA) and 1µl (0.4µM) of each primer. The

amplification protocol consisted of 95ºC for 5 min, 39 cycles of 95ºC for 30s; 50ºC for

30s; 72ºC for 1.5min and a final extension step of 8 min at 72ºC. RFLP was then

performed with 0.6µl endonuclease *Hinf*1 (New England BioLabs® Inc, Beverly, MA)

and 5µl of PCR amplicon DNA in a total volume of 10µl at 37ºC for an hour. The

reaction was stopped by incubation at 80ºC for 20 mins. 5µl of each PCR-RFLP product

was mixed with 1µl of 6X TrackIt™ Cyan/Yellow Loading Buffer (Invitrogen, Carlsbad

CA) and examined on 3% agarose-TBE (IM Tris, 0.9M Boric acid, 0.01M EDTA, ph 8.4)

gel (Invitrogen, Carlsbad, CA) with SYBR® Safe gel stain (TrackIt™, Invitrogen,

Carlsbad, CA). The gel was read with an Ultraviolet (UV) transilluminator

(Fluorchem®Q, Alpha Innotech, San Leandro, CA).

The second PCR protocol involved the use of forward primer NC1 (5´ -

ACGTCTGGTTCAGGGTTGTT-3´) and reverse primer NC2 (Gasser et al., 1993). The

reactions were performed in a 25µl volume as above with 1µl of each primer and 10.5µl

of DNA. The amplification protocol was as follows: 94ºC for 5 min, 35 cycles at 94ºC for

1 min; 55ºC for 1 min, 72ºC for 1 min, and finally 72ºC for 5 min (de Gruijter et al.,

2005). The PCR product from this reaction was digested as above with *Hinf*1

endonuclease.

The PCR products from the two kinds of PCR reactions were sequenced and the samples that were found to contain *A. caninum* were used as controls in the RFLP procedure. *A. braziliense* DNA from eggs was donated by Dr. Dwight Bowman, College of Veterinary Medicine, Cornell University, Ithaca, NY.

Statistical analysis software (SAS 9.3, Cary, NC) was used for statistical analysis. Chi-square or Fisher's exact tests for categorical data comparisons and student's t-test for difference in means were used. A 95% confidence interval was adopted and a *p* value <0.05 was considered significant.

## 4.3    Results

Of fecal samples from a total of 209 dogs examined, 163 (78%) were found to be parasitized (Table 1).  Multiparasitism was present in 79 (37.8%) dogs. The most prevalent parasites found were, in descending order, *Anyclostoma* sp. (53.6%), followed by *Trichuris vulpis,* coccidia of the *Cystoisospora ohioensis* complex, *Giardia duodenalis, Cystisospora canis, Toxocara canis, Dipylidium caninum, Alaria* spp., *Uncinaria stenocephala and Capillaria* spp. *U. stenocephalas* was found in one dog and diagnosed as a mixed infection with *Ancylostoma* spp.

### 4.3.1    Distribution of Parasites by Parish

Among helminth parasites, Hookworm and *T. vulpis* were recovered from dogs from all the parishes, and the other parasites were found in variable prevalence (Table 4.1). Point Coupee parish had the highest percentage of dogs with parasites (94.1%) and East Feliciana had the least (31.0%). The trend was similar for hookworm, but with regard to *T. vulpis*, Tangipahoa had the highest percentage (44.8%) and St. Martin the least (9.1%).

*T. canis* and *D. canimum* were found in dogs from five of the nine parishes, *Alaria* in three parishes, *Capillaria* in one parish. Among protozoan parasites, *Cystoisospora* sp. were found in dogs from all the parishes, but individual species differed in distribution, with *C. ohioensis* being most common. *G. duodenalis* was recovered from dogs in seven parishes.

### 4.3.2 Effect of Age on Prevalence

The age of dogs ranged from 1.5 months to 12 years. There were only 12 dogs over three years old. There were 39 dogs less than six months old and 87.2% of them had parasites compared with 75.9% of those aged six months or more (Table 4.2). There was no statistical difference ($p=0.48$) in prevalence between the two age groups, except the younger (< 6 month) age group had significantly more dogs with *T. canis* ($p = 0.0009$) and *Cystoisospora* sp ($p=0.001$) than the older age group.

Mixed parasite infections were found in 18 (52.9%) dogs less than six months and in 61 (47.3%) of dogs over six months (Table 4.3). Hookworm was the most frequently found single parasite infection (46/163) followed by *T. vulpis* and *Cystoisospora* spp. The most common combination of parasites was with hookworm and *T. vulpis* followed by hookworm and *Cystoisospora* spp. This trend was the same in the two age groups, however, with regard to proportions, the hookworm and *T. vulpis* combination was found more in older dogs (14.7%) as compared to younger dogs (11.8%). The hookworm and *Cystoisospora* spp. combination was more frequently found in younger dogs (8.8%) as compared to older dogs 5.4%. *Capillaria* spp. and *Alaria* spp. were found in mixed infections only in dogs over six months old.

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Table 4.1. Prevalence of parasites found in dogs from shelters in nine parishes of south Louisiana

| Parish | Acadia | Ascension | Iberville | Point Coupee | Tangipahoa | St. Martin | Livingston | East Baton Rouge | East Feliciana | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| Total Samples | 22 | 37 | 26 | 17 | 29 | 11 | 16 | 22 | 29 | 209 |
| Parasite positive (%) | 20 (90.9) | 29 (78.4) | 24 (92.3) | 16 (94.1) | 25 (86.2) | 8 (72.7) | 12 (75.0) | 20 (90.9) | 9 (31.0) | 163 (78.0) |
| Hookworm (%) | 13 (59.1) | 23 (62.2) | 17 (65.4) | 16 (94.1) | 21 (72.4) | 2 (18.2) | 5 (31.3) | 13 (59.1) | 2 (6.9) | 112 (53.6) |
| *Trichuris* (%) | 9 (40.9) | 9 (24.3) | 5 (19.2) | 2 (11.8) | 13 (44.8) | 1 (9.1) | 7 (43.8) | 9 (40.9) | 5 (17.2) | 60 (28.7) |
| *Toxocara* (%) | 5 (22.7) | 2 (5.4) | 2 (7.7) | 0 | 2 (6.9) | 2 (18.2) | 0 | 0 | 0 | 13 (6.2) |
| *Capillaria* (%) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 (4.5) | 0 | 1 (0.5) |
| *D. caninum* (%) | 0 | 2 (5.4) | 1 (3.8) | 0 | 2 (6.9) | 2 (18.2) | 0 | 2 (9.1) | 0 | 9 (4.3) |
| *Alaria* (%) | 1 (4.5) | 0 | 0 | 1 (5.9) | 0 | 0 | 1 (6.3) | 0 | 0 | 3 (1.4) |

Table 4.1. Continued

| Parish | Acadia | Ascension | Iberville | Point Coupee | Tangipahoa | St. Martin | Livingston | East Baton Rouge | East Feliciana | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| *Cystisospora. canis* (%) | 3 (13.6) | 1 (2.7) | 5 (19.2) | 0 | 0 | 2 (18.2) | 2 (12.5) | 3 (13.6) | 0 | 16 (7.7) |
| *Cystoisospora ohioensis complex* (%) | 3 (13.6) | 3 (8.1) | 7 (26.9) | 2 (11.8) | 8 (27.6) | 0 | 7 (43.8) | 3 (13.6) | 3 (10.3) | 36 (17.2) |
| *Giardia sp.* (%) | 3 (13.6) | 0 | 0 | 1 (5.9) | 1 (3.4) | 2 (18.2) | 2 (12.5) | 15 (68.2) | 1 (3.4) | 25 (12.0) |

Table 4.2. Parasite prevalence in dogs less than 6 months old and more than 6 months old.

| Age in months | Sample size | Parasite positive (%) | Hookworm (%) | *Trichuris* (%) | *Toxocara* (%) | *Cysto-isospora* (%) | *Giardia* (%) |
|---|---|---|---|---|---|---|---|
| < 6 | 39 | 34 (87.2) | 22 (56.4) | 10 (25.6) | 6 (15.4) | 16 (41.0) | 6 (15.4) |
| > 6 | 170 | 129 (75.9) | 90 (52.9) | 49 (28.8) | 7 (4.1) | 30 (17.7) | 23 (13.5) |
| *p* value | | 0.125 | 0.695 | 0.69 | 0.009 | 0.001 | 0.762 |

### 4.3.3   Effect of Sex on Prevalence

Fecal samples were obtained from 101 female and 106 male dogs as well as two sets of litters that were housed together.   The litters were not included in the analysis based on sex. More female dogs (79.2%) were parasitized compared to male dogs (76.4%), although this difference was not significant (p=0.629) (Table 4.4).   With regard to individual parasite species, there was no statistical difference between the sexes.

### 4.3.4   Deworming

Shelter records indicated that 125 of the dogs had been dewormed before the day of fecal collection. The anthelminthics used were pyrantel, fenbendazole or ivermectin, singly or in combination with each other or with heartworm medication. The majority of dogs admitted to the shelters were only treated once, usually upon intake. The time period from deworming to fecal collection ranged from a day to six months with a mean of 24 days. Only 49/125 records provided the date deworming was given.

Table 4.3. Occurrence of single species or combinations of parasite species found in dogs over 6 months and less than 6 months of age.

| Parasite | Number | <6months | >6months |
|---|---|---|---|
| Hookworm | 46 | 5 | 41 |
| *Trichuris* | 14 | 2 | 12 |
| *Toxocara* | 3 | 2 | 1 |
| *Dipylidium* | 1 | 1 | 0 |
| *Cystoisospora* | 13 | 6 | 7 |
| *Giardia* | 7 | 0 | 7 |
| hookworm + *Trichuris* | 23 | 4 | 19 |
| hookworm + *Toxocara* | 1 | 0 | 1 |
| hookworm + *Dipylidium* | 1 | 0 | 1 |
| hookworm + *Alaria* | 1 | 0 | 1 |
| *Toxocara + Dipylidium* | 1 | 0 | 1 |
| hookworm + *Cystoisospora* | 10 | 3 | 7 |
| *Trichuris + Cystoisospora* | 5 | 0 | 5 |
| hookworm + *Giardia* | 8 | 2 | 6 |
| *Trichuris + Giardia* | 1 | 0 | 1 |
| *Cystoisospora + Giardia* | 2 | 0 | 2 |
| hookworm + *Trichuris + Toxocara* | 2 | 1 | 1 |
| hookworm + *Trichuris + Dipylidium* | 2 | 1 | 1 |
| *Trichuris + Dipylidium + Capillaria* | 1 | 0 | 1 |
| hookworm + *Trichuris + Giardia* | 4 | 0 | 4 |
| hookworm + *Trichuris + Cystoisospora* | 3 | 0 | 3 |
| hookworm + *Cystoisospora + Toxocara* | 2 | 2 | 0 |
| *Trichuris + Cystoisospora + Toxocara* | 1 | 0 | 1 |
| hookworm + *Giardia + Toxocara* | 1 | 0 | 1 |
| hookworm + *Cystoisospora + Dipylidium* | 2 | 1 | 1 |
| hookworm + *Cystoisospora + Giardia* | 2 | 1 | 1 |
| *Toxocara + Cystoisospora + Giardia* | 2 | 1 | 1 |
| hookworm + *Trichuris + Giardia + Cystoisospora* | 1 | 1 | 0 |
| hookworm + *Trichuris + Cystoisospora + Alaria* | 2 | 0 | 2 |
| hookworm + *Trichuris + Giardia + Cystoisospora + Dipylidium* | 1 | 1 | 0 |
| Total number of dogs | 163 | 34 | 129 |

Table 4.4. Parasite prevalence in male and female dogs.

| Sex | Sample size | Parasite positive (%) | Hookworm (%) | *Trichuris* (%) | *Toxocara* (%) | *Cysto-isospora* (%) | *Giardia* (%) |
|---|---|---|---|---|---|---|---|
| Male | 106 | 81 (76.4) | 54 (50.9) | 28 (26.4) | 6 (5.7) | 21 (19.8) | 12 (11.3) |
| Female | 101 | 80 (79.2) | 55 (54.5) | 30 (29.7) | 7 (6.9) | 24 (23.7) | 15 (14.8) |
| *p* value | | | 0.629 | 0.613 | 0.598 | 0.706 | 0.491 | 0.451 |

Of the dogs that were dewormed, helminth eggs were found in 59.8% when compared to 79.8% of untreated dogs and this difference was statistically significant ($p$=0.0013) (Table 4.5). With regard to parasite species, dewormed dogs had significantly less hookworm positive diagnoses ($p$=0.0002) than untreated dogs. The difference in the hookworm prevalence was pronounced in dogs treated with pyrantel compared to untreated dogs ($p$=0.0002) (Table 4.6). Upon comparison of fecal egg counts for dogs treated with pyrantel and untreated dogs, there was no statistical difference, although the treated group had a lower mean EPG (Table 4.7). There was no statistical difference with regard to the other helminths.

Table 4.5. Parasitism in shelter dogs based on history of deworming.

| | Sample size | Helminth Positive (%) | Hookworm (%) | *Trichuris* (%) | *Toxocara* (%) | *D. caninum* (%) |
|---|---|---|---|---|---|---|
| Dewormed | 125 | 75 (59.8) | 54 (43.2) | 36 (28.8) | 6 (4.8) | 6 (66.7) |
| Not dewormed | 84 | 67 (79.8) | 58 (69.0) | 23 (27.4) | 7 (8.3) | 3 (33.3) |
| *p* value | | 0.0013 | 0.0002 | 0.823 | 0.300 | |

Table 4.6. Prevalence of parasites in dogs with a history of anthelminthic treatment at shelters.

| Anthelminthic | Dewormed dogs | Helminth positive (%) | Hookworm (%) | *T. vulpis* (%) | *T. canis* (%) | *D. caninum* (%) |
|---|---|---|---|---|---|---|
| Pyrantel | 84 | 57 (67.9) | 45 (53.6) | 31 (36.9) | 3 (3.6) | 4 (4.8) |
| Fenbendazole | 14 | 4 (28.6) | 2 (14.3) | 0 | 2 (14.3) | 2 (14.3) |
| Ivermectin | 1 | 1 (100.0) | 0 | 0 | 1 (100.0) | 0 |
| Combinations* | 26 | 10 (38.5) | 6 (23.1) | 5 (19.2) | 0 | 0 |
| Total | 125 | 72 (56.7) | 53 (41.7) | 36 (28.3) | 6 (4.7) | 6 (4.7) |

*Combinations included: pyrantel + ivermectin (9); pyrantel + moxidectin (Advantage multi®) (2); fenbendazole + ivermectin + moxidectin (Advantage multi®) (13); moxidectin (Advantage multi®) + ivermectin (2)

Table 4.7. Occurance of hookworm and *Trichuris* in dogs treated with pyrantel.

| | Samples | Helminths % | Hookworm % | *Trichuris* % | Mean HW epg |
|---|---|---|---|---|---|
| Dewormed | 84 | 50 (59.1) | 46 (54.8) | 31 (36.9) | 2034.1 |
| Untreated | 82 | 67 (81.7) | 58 (70.7) | 23 (28.1) | 4528.2 |
| *p* value | | 0.059 | 0.033 | 0.223 | 0.057 |

4.3.5   Speciation of Hookworm by PCR

DNA was successfully amplified with NC5/NC2 primers in 41 out of 88 (Table 4.8) samples and resulted in a ~860 base pair (bp) size amplicon (Figure 4.3). RFLP resulted in five main fragment sizes which matched those of *A. caninum* (e Silva et al., 2006). The

five fragments were ~ 222, 189, 146, 129 and 89 bp in size. Sequencing of the undigested

PCR product from seven samples with similar PCR-RFLP products confirmed that the

species was *A. caninum*. The rest of the samples tested resulted in non-specific

amplification.

PCR using the NC1/NC2 primer set resulted in an amplicon ~300 bp in size. Of the 88

samples that were tested, DNA was successfully amplified in 75 of them and RFLP

resulted in three main fragment sizes (128, 87 and 73 bp) (Figure 4.3) which matched *A.*

*caninum* control samples. The *A. braziliense* DNA was successfully amplified with both

primer sets and resulted in similar size amplicons as *A. caninum*. Digestion with

endonuclease *Hinf*1 of the PCR product from the NC5/NC2 primer set reaction, resulted

in five clear bands (396, 308, 222, 145 and 125 bp)(Figure 4.2) while digestion of the

NC1/NC2 PCR product resulted in two clear bands or fragments (176 and 127 bp)

(Figure 4.2 and 4.4). However, the PCR product from the NC5/NC2 primer set was not

always consistent both with *A. caninum* and *A. braziliense*, sometimes resulting in

additional bands (Figure 4.4). RFLP of the PCR product with additional bands using

NC5/NC2 primers produced different results (Figure 4.4) than when a single band

amplicon was digested.

Table 4.8. Comparison of PCR-RFLP protocols for speciation of *A. caninum* from 88
samples by size of PCR amplicon and number of fragments generated from endonuclease
digestion.

| Primer sets | Amplicon (bp) | Positive samples (%) | *Hinf*1-RFLP fragments |
|---|---|---|---|
| NC5/NC2 | ~860 | 41 (46.6) | 5 |
| NC1/NC2 | ~280 | 75 (85.2) | 3 |
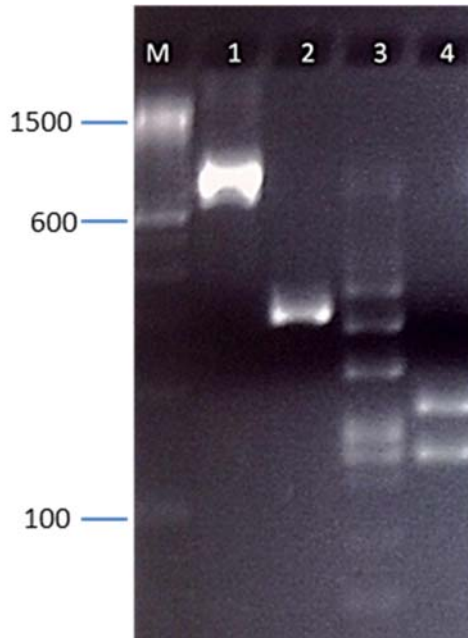
Figure 4.2. PCR-RFLP results for the *A. braziliense* using primer sets NC1/NC2 and NC5/NC2. Lane 1, PCR product using primer set NC5/NC2; Lane 2, PCR product using primer set NC1/NC2; Lane 3 RFLP product from primer set NC5/NC2; Lane 4, RFLP product from primer set NC1/NC2; Lane M, 100bp DNA ladder.
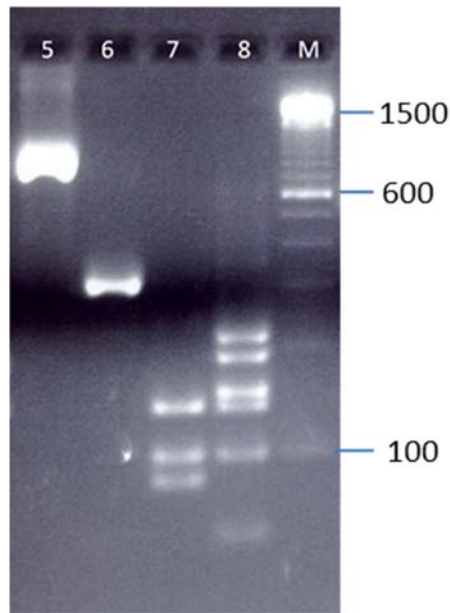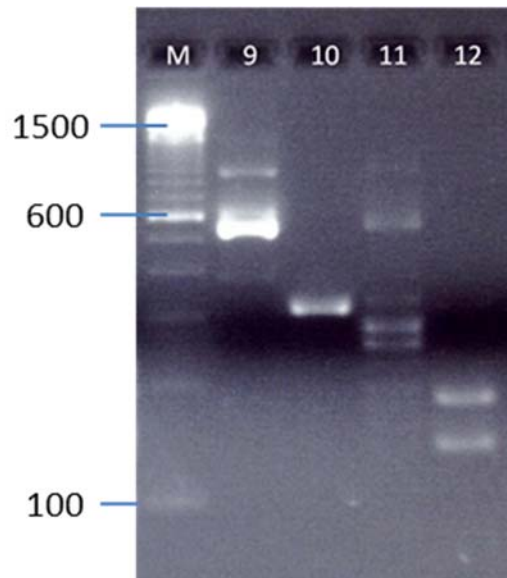


Figure 4.3. PCR-RFLP results for the *A. caninum* using primer sets NC1/NC2 and NC5/NC2. Lane 5, PCR product using primer set NC5/NC2; Lane 2, PCR product using primer set NC1/NC2; Lane 3 RFLP product from primer set NC1/NC2; Lane 4, RFLP product from primer set NC5/NC2; Lane M, 100bp DNA ladder.

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Figure 4.4. PCR-RFLP results for *A. braziliense* showing non-specific amplification using NC5/NC2 primer set in Lane 9; Lane 10, PCR product using primer set NC1/NC2; Lane 11, RFLP product with unrecognizable fragment sizes from PCR product of primer set NC5/NC2; Lane 12, RFLP result from primer set NC1/NC2; Lane M, 100bp DNA ladder.

## 4.4   Discussion

The prevalence of parasites in South Louisiana shelter dogs was higher than what has

been reported in the past in both the 1982 Louisiana survey and a 1996 national survey of

shelter dogs (Blagburn et al., 1996; Hoskins et al., 1982). However the trend of finding

hookworm in the highest prevalence, followed by *T. vulpis,* was comparable to previous

reports. The high prevalence of parasites raises concern for the risk of infection or re-

infection of pets and humans due to environmental contamination, particularly from dogs

adopted from shelters but also in the homes from which the shelter animals were

surrendered. The lack of a significant difference in parasitism between dewormed and

untreated dogs indicates that the deworming protocols currently used by the shelters were

inadequate and ineffective. This calls for a change in how the deworming is done or the

drugs used. Most shelters only treated once and often only adoptable dogs were treated. It

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is advisable that the deworming be repeated as per manufacturer's instructions for the medication to be effective i.e. treatment with pyrantel should be repeated after 10-14 days in order to be effective against newly matured adult worms in the intestine. Helminths with migratory larvae or larvae that undergo hypobiosis are a challenge to treat because commonly used dewormers, such as pyrantel, have no effect on larvae stages and require that the dog be retreated 2-4 weeks after the first treatment to eliminate all adult worms (Bowman, 2009).

A previous Louisiana study (Hoskins et al., 1982) found that hookworm prevalence was significantly higher in male dogs but this difference was not observed in this study. The difference between young and older dogs with regard to *T. canis* and *Cystoisospora* spp. was expected considering the life cycle of *T. canis* where infection can be acquired transplacentally and possible acquired immunity to *Cystoisospora* spp. with age. This difference has been observed in other studies (Blagburn et al., 1996; Hoskins et al., 1982; Little et al., 2009).

There was a significant difference in hookworm prevalence between the pyrantel-treated group and the untreated dogs. However, the fecal egg counts were not significantly different but highly suggestive that pyrantel may have been effective. It has been observed in controlled experiments on efficacy against *A. caninum* that when adult worms occur in large numbers, they produce fewer eggs than when adults are in lower numbers in the intestine (Kopp et al., 2007) and it was suggested that a reduction of adult hookworm in the intestines would thus be a better indication of treatment efficacy. Kopp et al. (2007) in Australia also observed an increase in anthelminthic resistance to pyrantel

pamoate by *A. caninum*, a finding that requires confirmatory investigation in other

regions of the world.

*Alaria* spp. were found in three dogs from three parishes. Diagnosis requires

demonstration of eggs by sedimentation examination because the eggs, like many other

trematode eggs, do not float in common fecal flotation solutions. *Alaria* is acquired

through ingestion of intermediate or paratenic hosts like snails and frogs (Taylor et al.,

2007).  Adults of *Alaria* live in the intestine attached to the mucosa but causes little

pathology or clinical signs, and is therefore usually an incidental finding at necropsy.

Human infection has been reported and a fatal case of *Alaria* in man was reported due to

possible consumption of undercooked frog legs (Fernandes et al., 1976; Freeman et al.,

1976). *Alaria* mesocercaria, the infective stage for mammals, have previously been

recovered from snakes and frogs in Louisiana and from experimentally infected alligators

(Shoop and Corkum, 1981). Therefore precautions should be taken when handling raw

meat from wildlife sources and in food preparation using meat from these species, since

the potential for human infection does exist.

The prevalence of parasites in the all shelters studied were over 72% except for East

Feliciana parish for which only 31% of the dogs were parasitized. This can be attributed

to the high level of care that animals in that shelter received as well as being housed in

very sanitary conditions. The East Feliciana shelter was also a 'no-kill' shelter and

administered a combination of anthelminthics, including heartworm preventatives,

regularly. They also performed *Giardia* ELISA tests on site. The other shelter (Point

Coupee) with a 'no-kill' policy had a very high prevalence of parasites even though they

too administered a combination of anthelminthics regularly. Dogs in this shelter were let

out in a dirt run where feces were never picked up and this could explain the high number of dogs with hookworm. The St. Martin shelter, which reported good management practices and use of fenbendazole on all the dogs, had a low prevalence of hookworm (18.2%). By contrast, five of the shelters reported deworming only puppies or adoptable dogs, and only upon intake, thereby putting them at risk of infection from dogs that were not dewormed. Other than in the East Feliciana shelter, treatment for coccidiosis or *Giardia* was only given upon diagnosis by the visiting veterinarian. Although overcrowding was not a major finding among the shelters, it can contribute to the prevalence of coccidiosis and *Giardia* infections particularly among puppies and should therefore be avoided. Findings from these shelters indicate that a combination of appropriate and regular deworming as well as maintaining sanitary conditions, like picking up feces in common areas, are essential in preventing intestinal parasite infection in dogs kept in shelters.

It is essential and highly recommended that dogs are dewormed on adoption and the adopting individual be advised to take the dog for a full examination, including a fecal exam, by a veterinarian as soon as possible to prevent parasitic infections from posing a risk to humans and other pets. It was not possible to properly determine drug efficacy as this was not a controlled study, and there was great variation in period between deworming and sample collection as well as inconsistencies in dosage. Further studies are therefore required to determine if drug efficacy is a factor in the high level of parasitism found in shelter dogs. In order to prevent possible development of anthelminthic resistance, it is advisable to deworm based on fecal exams and give appropriate anthelminthic in the recommended dosage (Palmer et al., 2007).

*A. caninum* was the only hookworm species found in this study by analysis of the 75 of 88 DNA samples that were successfully amplified with the NC5/NC2 primer set. The NC5/NC2 primer set was only able to amplify *Ancylostoma* specific DNA in 41 samples and resulted in much non-specific amplification. DNA amplification may have failed in the other samples due to possible DNA degradation or presence of PCR inhibitors, since the DNA was from eggs in fecal samples. In the samples that did have amplification of the ~860bp target sequence when using the NC5/NC2 primer set, many of them also had additional amplifications that distorted the results of the RFLP, although other researchers have not reported this problem. The results from the NC1/NC2 primer set PCR were consistent and the resultant RFLP fragments, three for *A. caninum* and two for *A. braziliense*, were also consistent, suggesting this method of differentiating *A. caninum* and *A. braziliense* is more optimal. Further investigation should be done to determine if the NC1/NC2 PCR-RFLP can be used to differentiate other *Ancylostoma* species.

4.4.1   Conclusion

- The prevalence of gastrointestinal parasites in Louisiana shelter dogs is higher than what has been reported previously.

- Hookworm is the most prevalent intestinal parasite and *A. caninum* is the predominant hookworm species in shelter dogs.

- *T. canis* and *Cystoisospora* spp. prevalence were significantly higher in dogs <6 months old.

- *Alaria* spp. was reported in dogs from three parishes.

- Anthelminthic protocols for shelters were found to be inadequate and needed to be revised to prevent the spread of gastrointestinal parasites.

- PCR-RFLP using primer set NC1/NC2 was more reliable and efficient than using primer set NC5/NC2.

## 4.5    References

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# APPENDIX A

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# APPENDIX B

Ntombi Mudenda
PhD Candidate
Department of Pathobiological Sciences
School of Veterinary Medicine
Louisiana State University
Skip Bertman Drive
Baton Rouge, LA 70802
USA

Dear Dr. Mudenda, I am writing to confirm our authorization from Geospatial Health journal to use your publication titled "Ecological niche modelling of hookworm in Brazil based on climate" (Vol 6 No. 3, Sept 2012, pg 111-123) as part of your dissertation.


Best regards

Laura Rinaldi, *Associate Editor Geospatial Health*

# APPENDIX C

**University Hospitals**
Case Medical Center

CENTER FOR CLINICAL RESEARCH
AND TECHNOLOGY
11100 Euclid Avenue, Lakeside 1400
Cleveland, Ohio 44106

Exemption approval for IRB# EM-13-39                    October 14, 2013

Ronald E Blanton,

Upon administrative review of your project entitled "Hookworm and Gastrointestinal Nematodes Among Patients in Mutuipe Municipality in Brazil" (IRB # EM-13-39), the IRB has determined that this protocol meets the criteria for exemption under Federal regulation 45 CFR 46.101 (b) (4).

Please note the expiration date for this exemption approval is **10/14/2019**.

Continuing review is not required for research for your exempt project. However, if the research is ongoing for more than six years, you must resubmit the project for re-review as a new submission.

Please see the UHCMC IRB policy regarding Exempt Research for more information:
http://www.uhhospitals.org/Portals/6/docs/research/irb/forms/May_07/IRB_Policy _Exempt_Human_Research_5_2007.doc

Please feel free to contact the Office of Institutional Review at (216) 844-1529 if you have questions.

Thank You,

UHCMC IRB Chairperson
(Signature was applied by the IRB Administration Office)

*The UHCMC IRB operates under the HHS Federal Wide Assurance of Compliance number 00003937 and IRB registration numbers 00000684 and 00001691*

*p. 1 of 1 for Ronald E Blanton iRIS Reference # 029135*

# THE VITA

Ntombi B. Mudenda was born and raised in Zambia. She graduated from the University of Zambia, School of Veterinary Medicine with a Bachelor of Veterinary Medicine. She worked for the School of Veterinary Medicine as a house surgeon before pursuing a Master of Science degree in wild animal health at the Royal Veterinary College in London, UK under the sponsorship of the Beit Trust. Ntombi then returned to Zambia and lectured at the Veterinary School. She became a Fulbright foreign exchange student and joined the doctoral program at Louisiana State University, School of Veterinary Medicine in 2010. Ntombi is anticipating graduation with a Doctor of Philosophy in parasitology in December 2013.