4-9-2008

The Immunoepidemiology of Hookworm Infection in the Peruvian Amazon

Priya Shete

The Immunoepidemiology of Hookworm Infection in the Peruvian Amazon

Priya B. Shete
Yale University School of Medicine

Thesis Requirement for the Degree of
Doctor of Medicine

2007

Abstract

Hookworm infection caused by *Ancylostoma duodenale* or *Necator americanus* is a significant global health threat, causing chronic anemia, malnutrition, developmental delay, enteritis, and increased susceptibility to non-parasitic diseases. One of the most prevalent of the "neglected" tropical diseases, hookworm infection affects almost 1 billion people worldwide, particularly in developing countries. Current methods for diagnosis and treatment of hookworm infection are largely the same as they have been for the past century. However, several recent advances in the molecular characterization of hookworm virulence factors now provide researchers with an improved understanding of disease pathogenesis, potential targets for treatment and novel antigens for vaccine development. In order to better understand hookworm pathophysiology and immunology in human populations, a comprehensive, cross-sectional immunoepidemiologic survey of approximately 200 villagers in a remote area of the Peruvian Amazon was conducted. Hookworm prevalence rates were found to approach 40% by microscopic diagnosis. Additionally, molecular speciation techniques showed that both *A. duodenale* and *N. americanus* are endemic to this region. Reagents from a laboratory model of hookworm disease were then utilized to characterize human immune responses to hookworm specific antigens. By studying the immunoepidemiology of an endemic community we have found that a laboratory strain of hookworm, *Anyclostoma ceylanicum*, is a useful tool for describing species specific immune responses to disease. This work lays the foundation for future development of improved hookworm diagnostic techniques by molecular and immunologic methods.

Table of Contents

Acknowledgements

This work would not have been possible without the support of many generous individuals. First and foremost, I would like to thank Michael Cappello, M.D., for his mentorship and patience in guiding me through the world of hookworm infection. His dedication to addressing global health problems with thoughtful research in a collaborative environment exemplifies the finest qualities of any Physician-Scientist. Above all, I appreciate his absolute honesty of spirit. Over the years, Dr. Cappello has become a role model and inspiration for me, and hopefully, a friend as well.

I would also like to thank Lisa Harrison, for her perserverance and expertise in molecular biology. She is undoubtedly the best French-Canadian sister a girl could ever have and her friendship has made my time in lab feel like time spent at home. Thanks to Rick Bungiro for always taking the time to explain the intricacies of hookworm immunology and showing me the merit of meticulousness. I would also like to thank the entire laboratory (Jon, Blaise, Lisa D., Dan, Isaac, Keke and Wadim) for their friendship, support, and most of all the LAUGHS. It is so rare to find so many kindred spirits in one place and for that, I will always feel lucky.

Special thanks to Jake Kurtis, M.D., Ph.D. for assistance with statistical analysis, Diane McMahon-Pratt, Ph.D. for reviewing the work, and Margaret Kosek, M.D., Pablo P. Yori, and Cesar Banda, for supporting me during the fieldwork phase of this study. A very special thanks to Charles, my fearless field-worker and friend, whose patience and machete saved me many times over. Thank you to my friends, family and most of all to the people of the Amazon, who inadvertently showed me my way in life by allowing me to become a part of theirs.

Statement of Research Contribution

Although many individuals contributed to the progress of this research, the majority of the work of this thesis represents my own primary contribution. Under the supervision of Margaret Kosek, M.D., I designed and implemented all field based work related to this study. I spent 4 months in the Peruvian Amazon personally developing logistics, conducting epidemiologic surveys and collecting subject samples while supervising one fieldworker who assisted me in this endeavor. In the laboratory in Iquitos, Peru I diagnosed all parasites infections by fecal microscopy with the help of one laboratory technician. I set up the fecal cultures to rear third stage hookworm larvae for speciation by PCR and processed all samples. I acted as the sole primary care provider for the study site and provided medical care to all individuals of the community, including free anthelminthic therapy and anti-malarials for those who needed it. After shipping all of my samples and reagents back to Yale, I undertook the development of PCR based speciation of larval genomic DNA and fecal extractions and fecal gDNA based PCR speciation with the help of a laboratory colleague (LH) to assist in some assays. All immunoepidemiolgic work including Western blot analysis and screening enzyme linked immunosorbent assays of approximately 200 samples was performed solely by me. Finally, I performed all initial statistical analysis and referred to the expertise of a collaborator (JK) for more advanced analysis. All of this work was done under the supervision of Michael Cappello, M.D.

Financial Support

This work has been generously supported by a Wilbur Downs' International Health Research Fellowship, a Medical Student Summer Scholarship from the Infectious Disease Society of America, and grants from the Office of Student Research at Yale University School of Medicine.

Background

The majority of human hookworm disease is caused by two major bloodfeeding parasites, *Ancylostoma duodenale* and *Necator americanus*, which along with *Ascaris lumbricoides* and *Trichuris trichiura* make up the main soil transmitted nematodes. Upon infection of an individual, hookworms reside in the small intestine of the host, attach to the gastrointestinal mucosa and feed on blood from lacerated superficial capillary beds in the gut. Hookworm disease is a leading cause of iron deficiency anemia, malnutrition, and inflammatory enteritis. Often overlooked, human hookworm infection is one of the most prevalent chronic infections in the world, affecting approximately 740 million people in developing countries (de Silva 2003).

Biology

Ancylostoma duodenale and *Necator americanus* are both members of the family Ancylostomaditae, as are several other species of hookworm. *Ancylostoma ceylanicum* infects cats, dogs and to a lesser extent, humans, causing minimal clinical pathology and blood loss in human hosts. *Ancylostoma brazilinese* is a major hookworm in cats and dogs and causes cutaneous larva migrans in humans. *Ancylostoma caninum*, the dog hookworm, is endemic worldwide but human cases of eosinophilic enteritis after infection with this species are limited to Australia. Historically, distinguishing between Old World hookworm, *A. duodenale*, and New World hookworm, *N. americanus*, relied on examining adult morphology by microscopy. *Ancylostoma* species have teeth along the buccal capsule while *Necator* has a cutting plate.

Figure 1. Buccal capsule of *N. americanus* with cutting plate. From http://www.cvm.okstate.edu/~users/jcfox/htdocs/Disk1/Images/Img0070a.jpg accessed 01.29.2007.

Clinically, the most important distinguishing characteristics between the two species relates to blood loss and tendency to predispose to anemia in the host. *A. duodenale* causes 5 times as much blood loss as *Necator* (Hotez, 2001). Other distinguishing characteristics are summarized below.

Characteristic	*Necator americanus*	*Ancylostoma duodenale*
Male adult size (mm)	7 – 9	8 – 11
Female adult size (mm)	9 – 11	10 – 13
Rate of egg production (eggs per worm per day)	3000 – 6000	10000 – 20000
Life expectancy of infective larvae (days0	3 – 5	1
Life expectancy of adult worm (days)	3 – 10	1 – 10
Blood loss (mL/day per worm)	.03	.15
Lactogenic transmission	No	Yes
Oral transmission	No	Yes
Arrested development (larval hypobiosis)	No	Yes

Table 1. Comparative characteristics of human hookworm species. Adapted from Brooker 2004).

Critical to understanding the biology of hookworm is an appreciation of its unique life cycle (Figure 2). Infection typically occurs through contact with contaminated soil and penetration of L3 stage larvae through the skin. Once in the host, larvae continue development and migrate through the venous system and eventually arrive in the pulmonary vasculature. Once in the pulmonary capillary bed larvae rupture, enter the lung parenchyma and migrate up the respiratory tree until they reach the upper airway. The irritation caused by the hookworm larvae elicits a cough and thus the larvae are coughed up and swallowed by the host. Once larvae enter the gastrointestinal system they molt twice into their adult forms and develop into sexually mature male and female worms within 8 weeks of initial infection. Mating occurs in the gastrointestinal tract and adult females will typically release thousands of eggs per day. These eggs are then expelled in the feces of the host, where they hatch within 24-48 hours and develop into first stage larvae. These larvae molt twice to become infective third stage larvae, which may either infect a new host or 'hibernate' in order to conserve energy.

Although the life cycle of both *N. americanus* and *A. duodenale* are virtually the same, two important differences exist. First, *A. duodenale* can infect both cutaneously and orally, while *N. americanus* can only infect a host cutaneously. Also, while *N. americanus* larvae must infect a host within a short time after developing, *A. duodenale* larvae are more likely to enter a prolonged state of hypobiosis allowing infection to occur at various intervals and making the elimination of the disease and control of reinfection more difficult.

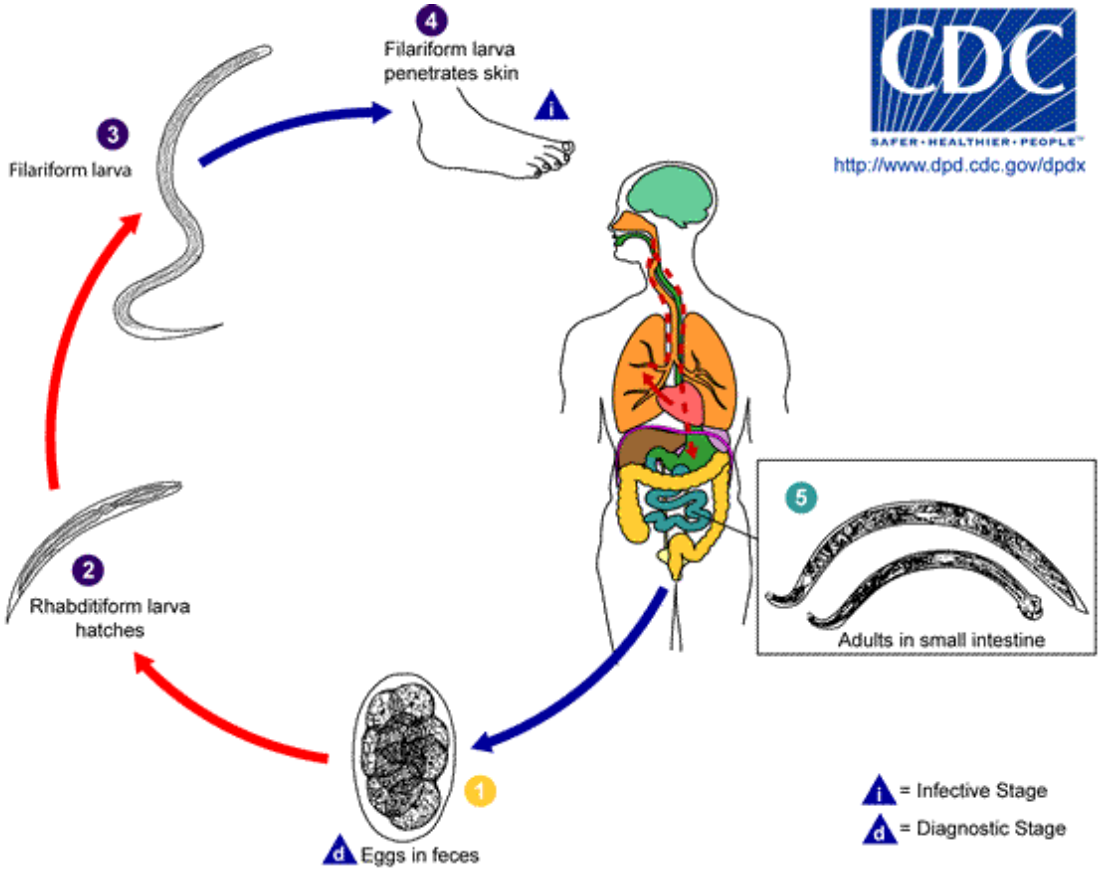

Figure 2. Life Cycle of hookworm. (Centers for Disease Control, Diagnostic Guide for Parasitic Diseases – DPDx accessed 11.15.2006).

Geographic Distribution

The geographic distribution of the two species of hookworm is distinct. *Necator americanus* is thought to be the most prevalent species of hookworm, with greatest geographic distribution. Endemic areas for *Necator* traditionally include South and Southwest China, South India, Southeast Asia, sub-Saharan Africa, and Central and South America. In contrast, *Ancylostoma duodenale* is thought to have a more limited geographic distribution, which includes South and West China, India, Egypt and Africa. Hibernation of L3 of *A. duodenale* is hypothesized to make the species extremely adaptive to temperate climates thus explaining the relatively greater prevalence of this species in Southern Europe, North India, China and other regions with long, dry winters (Brooker 2004). Geographic distribution is likely influenced by the close relationship between hookworm egg and larval viability and climate. In particular, humidity, temperature, ultraviolet light, rainfall, soil type and altitude all affect the viability of hookworm larvae (Chandler 1929).

Epidemiology

Based on estimates from 2001, over 740 million people worldwide are infected with hookworm (de Silva 2003). Although there has been a decline in hookworm prevalence over the past decade, the increase in overall global population has offset this improvement and the absolute number of hookworm infections has been increasing (Bungiro 2004).

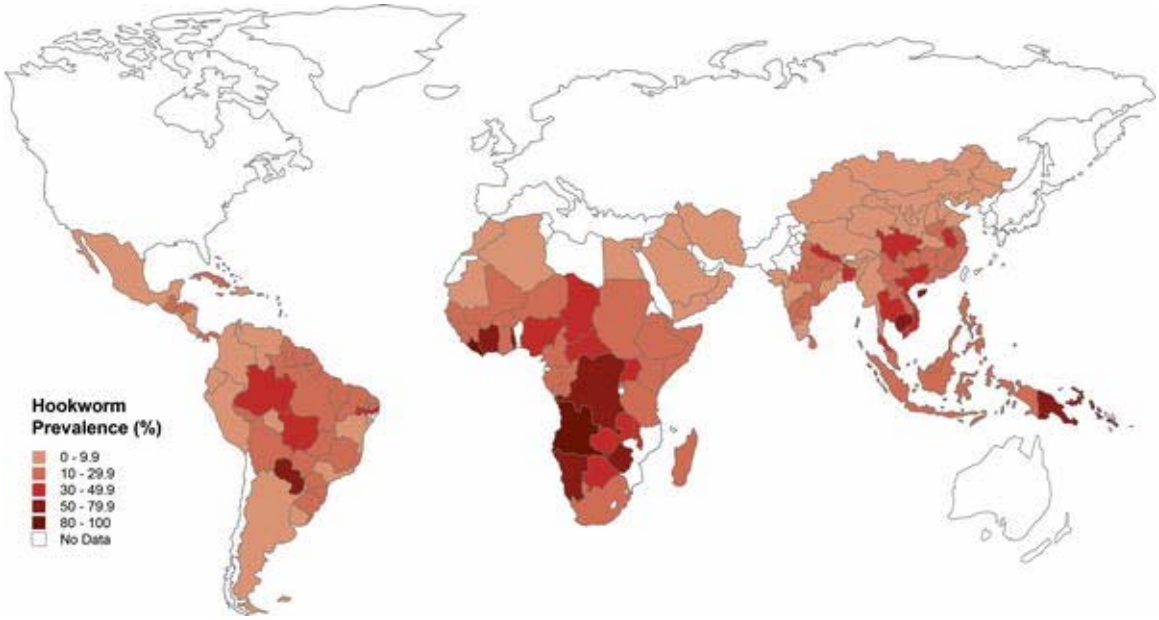

Figure 3. Worldwide hookworm prevalence. (Accessed from http://medicine.plosjournals.org/archive/1549-1676/2/3/figure/10.1371_journal.pmed.0020067.g001-M.jpg)

As with many other infectious diseases, prevalence patterns in hookworm infection can vary widely even within regions and countries, and even within microenvironments as specific as villages and households in endemic communities. One study of village schoolchildren living in Cote d'Ivoire showed that hookworm infection is extremely focal, occurring with higher prevalence and intensity in specific geographic areas (Utzinger 2003). The epidemiology of hookworm disease in regards to clinical manifestations reveals interesting patterns as well. First, several investigators have observed very high intensity and very low intensity infections occurring within the same endemic communities. Ten percent of infected individuals in a community excrete 70% of the eggs (Bundy 1995). Furthermore, the disease burden as measured by anemia seems to be associated with highest intensity infections. Investigation is ongoing for finding causes of extremely high worm burden in relatively few individuals and it is thought that

genetic susceptibility may be involved. Despite large geographic variation, the extent of the disease remains high despite modern advances in sanitation and health care.

A useful measure of the impact of hookworm disease, as with other more chronic diseases, is in terms of disability adjusted life years (DALYs) which uses both morbidity and mortality to calculate the impact of disease on a community. According to the World Health Organization (WHO 2002), hookworm disease is more burdensome than most all tropical diseases except for HIV, malaria, and tuberculosis, causing 22.1 million DALYs annually. Because of the distribution of the disease and its biologic mode of transmission, hookworm is known as one of the neglected tropical diseases that negatively affect the health and welfare of millions of impoverished people in the developing world.

Epidemiology of Hookworm Infection in South America and Latin America

Hookworm prevalence in Latin America and the Caribbean is strikingly high, with approximately 50 million individuals infected and another 340 million are at risk for infection (de Silva 2003, Brooker 2004). The 1996 Demographic Family Health Survey of Peru reported that 25% of deaths due to communicable diseases in children 1-4 years of age are due to intestinal infections, including intestinal parasitosis. Although not many studies on hookworm have been conducted South America, several studies exist that confirm the high prevalence of the disease. A 1984 study of four indigenous tribes of the Peruvian Amazon showed that of 165 villagers sampled, 92% had evidence of helminthic disease of which 72% were with hookworm (Bouree 1984). Egido and colleagues studying *Strongyloides* infection in the Southern Amazonian port city of Puerto

Maldonado report a 27% prevalence of hookworm infection in their cohort. However, no data on speciation is presently available (Egido 2001).

Brazilian studies indicate approximately a 30% prevalence of hookworm disease, primarily *Necator* species, in several recent studies (Miranda 1998). A study of hookworm prevalence in Paraguay describes overall hookworm prevalence at 59% of a population of 112 sampled. Speciation by L3 morphology identified a 73% prevalence of *Necator*, 15% of *Anyclostoma duodenale* and 12% with mixed infection among those infected. The majority of those infected with *A. duodenale* were under the age of 15 years (Labiano-Abello et al 1999).

In Peru, the only hookworm related studies to date have been examinations of risk factors for maternal-child morbidity and schoolage stunting among an extremely impoverished urban community Amazonian community of Belen, a neighborhood within Iquitos, Peru. These studies have examined the clinical outcomes and risk factors at baseline and after antiparasitic treatment, and have show the high burden of disease in this part of the Peruvian Amazon (Larocque 2005). Most experts believe that *Necator americanus* is the primary, if not sole, species responsible for hookworm infection in this part of the world. As of yet, however, no published evidence has demonstrated which species of hookworm exists in the Peruvian Amazon and no immunoepidemiological studies of hookworm infection have been done in this specific endemic region.

Clinical manifestations

Hookworm disease is typically a chronic illness that may lead to iron deficiency anemia, likely due to gastrointestinal blood loss caused by adult worm feeding. Excessive blood loss can also cause a moderate to severe hypoalbuminemia. Malnutrition due to impaired appetite and inflammatory enteritis, as well as malabsorption due to the effect of specific hookworm enzymes have been well documented (Chu 2004, Hotez 2004).

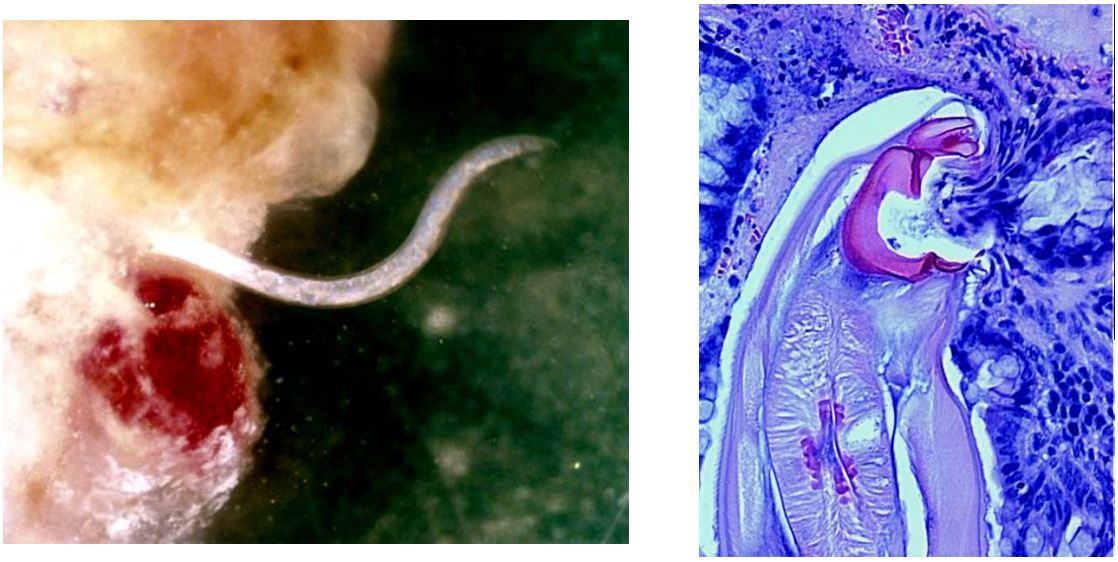

Figure 4. Left: Photomicrograph (2X) showing area of hemorrhage surrounding the site of adult hookworm attachment to the intestine. Right: H&E stained section (40X) of an adult *Ancylostoma ceylanicum* hookworm attached to the intestinal mucosa. Note plug of tissue within the worm's buccal capsule. (Images courtesy of R. Bungiro, Yale School of Medicine)

Additionally, hookworm has been implicated in a wide variety of non-intestinal syndromes. For example, repeated exposure to hookworm larvae can result in "ground itch," a local pruritic, erythematous popular rash most commonly found on the hands and feet where individuals are commonly exposed to soil and sand. Cutaneous larva migrans

is a syndrome associated with exposure to *A. braziliense* which includes the "creeping eruption" of a self-limited dermatologic condition caused by the migration of L3 larvae in the epidermis.

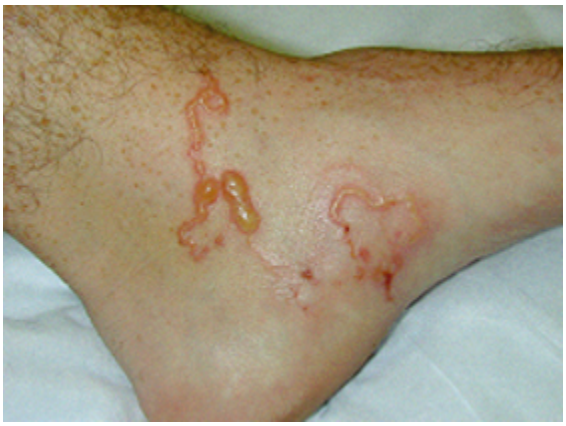

Figure 5. Cutaneous larva migrans. (from http://www.mjA.com.au/public/issues/177_04_190802/loo10413_fm-6.jpg Accessed 11.01.2006)

Clinical manifestations of hookworm disease progress in parallel with the life cycle of the worm. Once they have migrated to the lung, larvae can cause cough and sore throat. Pulmonary hookworm disease is similar to Loeffler's syndrome and is characterized by cough, sore throat, and eosinophilia. Individuals with cutaneous larva migrans may also develop a pneumonitis. *A. duodenale*, when orally ingested can cause Wakina disease, characterized by nasopharyngeal irritation, cough, dyspnea on exertion, hoarseness and elevated IgE (Hotez 2004).

As several studies have consistently indicated, the severity of morbidity depends in large part on the intensity of hookworm burden, typically measured by the number of adult worms expelled after treatment with an anti-helminthic or by quantitative egg counts from the feces of infected individuals. Additionally, the baseline health of the host also affects the actual morbidity associated with hookworm disease. Those individuals harboring other infections, such as HIV, malaria and other parasites may

suffer additional morbidity with concomitant hookworm infection than with either infection alone. In the developing world the combination of poverty and tropical diseases endemic to those areas make hookworm an even more important public health problem.

Diagnosis

Diagnostic determination of hookworm infection has remained the same over the past several decades, relying on direct microscopy and sedimentation for initial diagnosis. Direct microscopy relies on the timely processing of fecal samples by a trained technician adept at distinguishing intestinal parasites based on egg morphologies. For example, hookworm eggs are transparent, thin and ovoid, measuring approximately 60 microns (μm) by 40 μm. Of note, eggs of *Strongyloides stercoralis* are morphologically indistinguishable from hookworm eggs and require identification based on adult mouth morphology.

Figure 6. Hookworm egg under light microscopy. Accessed from Diagnostic Parasitology DPDx 11.16.2006).

For quantification of parasite burden, the Kato-Katz method, Stoll method and McMaster method are all used. The Kato-Katz technique is considered the gold standard for quantitative hookworm diagnosis. However, this method requires rapid processing of delicate samples, making its use in field situations cumbersome. Recently, several molecular methods have been developed in order to expedite the diagnostic process and improve specificity and sensitivity of detection methods.

Because the two main species of human hookworm have different but overlapping geographical distribution, advances in understanding epidemiology could be improved by accurate identification of the species of hookworm endemic in a given area. Speciation of hookworm can be done by examining the buccal capsule of adult worms. Although the gold standard, this method of corporal examination has many limitations. Culturing larvae from hookworm eggs is a time-consuming, inefficient, and delicate procedure and examination of the larvae requires well-trained personnel.

Recently, DNA-based techniques for molecular diagnosis of hookworm have been reported by several groups using primers designed to amplify species specific sequences within the cytochrome oxidase (COX) gene and the internal transcribed spacer (ITS) rDNA (Hawdon 1996, Zhan 2001, Monti 1998). The Cappello laboratory has begun the use of ELISA based assays for specific antigen detection of the hookworm infection (Bungiro 2005). Although these studies have been effective in controlled laboratory studies, no comparison has been made yet to the Kato-Katz method in the field.

Pathogenesis

Hookworm pathogenesis incorporates several key features that have recently been elaborated using animal models. Hookworms secrete a variety of molecules that contribute to disease pathogenesis at every level. For example, proteins involved in the initial stages of intestinal pathogenesis such as attachment, penetration, mucosal inflammation, and bloodfeeding, have been defined. These molecules are now being considered for potential roles in pathogenesis. Some of these proteins have been identified as critical hookworm virulence factors, namely anticoagulants, platelet inhibitors, secreted proteases and protease inhibitors (Bungiro 2004, Jones 2004). Characterization and identification of these virulence factors have provided researchers with potential targets for treatment and potential antigens for vaccine development with the goal of preventing disease caused by the intestinal stages of hookworm disease.

Other secretory proteins to contribute to an immunomodulatory effect of the hookworm on the host immune response. We theorize that these proteins will be useful in elucidating the immunology of hookworm infection in endemic communities. One aspect of hookworm pathogenesis that sets it apart from other intestinal parasites is the complex host-parasite interaction. The multiple stages of hookworm within the host as well as the ensuing exposure of the host to a variety of antigens make the immune response to hookworm a complex phenomenon. Using hookworm antigens developed from either whole worm soluble extracts (adult and larval stage), as well as recombinant proteins developed in the hamster model, we have selected several *A. ceylanicum* and *N. americanus* antigens to use in investigating human hookworm immunoepidemiology.

Risk Factors

Based on our knowledge of hookworm pathogenesis and mode of transmission, several risk factors for infection have been identified. Lack of sanitation, including poor personal hygiene, absence of a latrine, lack of shoe wearing, and manual/field labor are considered major risk factors for hookworm infection (Brooker 2004). Common to all of these risk factors is low socioeconomic status; although, in comparison to other indicators, the relationship between socioeconomic status and helminth infection is equivocal. In one review of the literature, Hotez and colleagues reported that while some studies have shown no correlation between income level and latrine access with hookworm infection, all such studies have corroborated a strong relationship between agricultural lifestyle and disease (Brooker 2004).

Socioeconomics of Hookworm Disease

In addition to the high prevalence of disease and its effect on the health of the individual, helminthic disease has serious socioeconomic effects. Identified as one of thirteen "neglected tropical diseases," hookworm disease joins the ranks of diseases such as schistosomiasis, lymphatic filariasis, onchocerciasis and trachoma. These diseases have been shown via mathematical modeling to be "poverty-promoting" which is to say that they in fact contribute to maintaing the conditions that perpetuate poverty in disease endemic communities. As one of the "poverty promoting" diseases, hookworm disease affects child health and development, maternal health and worker productivity and represents a significant public health concern (Hotez 2006).

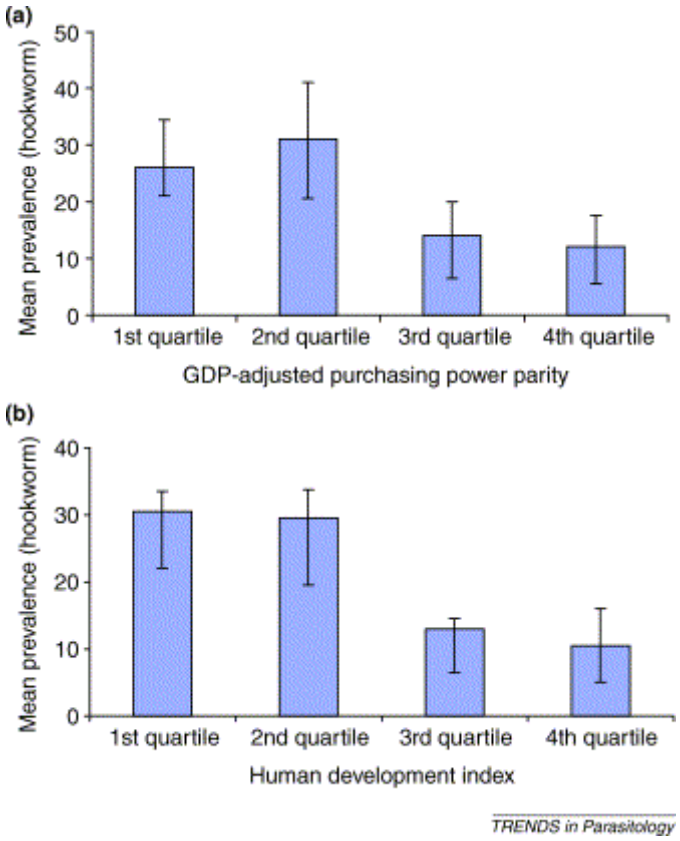

Figure 7. Relationship between hookworm infection and poverty. Measures of economic prosperity and development such as purchasing power parity and the human development index are highest in areas of low hookworm prevalence. Second quartile pucharsing power parity may have higher hookworm prevalence due to the type of work that such individuals partake in as opposed to those who are unemployed or have extremely low purchasing power parity. Of note, the large overlap between groups shows that such measures are trends only and thus socioeconomic status cannot be the only relevant contributor to hookworm infection rates. From de Silva 2003.

Treatment and Prevention

As a disease stemming from poverty and its associated characteristics, particularly in developing communities, it has long been recognized that planned urbanization, improved social infrastructures and poverty reduction are the most long lasting mechanisms for preventing hookworm disease. However, these systemic changes are extremely difficult to achieve and mechanisms for addressing component issues such as

shoe wearing and improved sanitation are often equivocal in their effect on disease burden (Hotez 2006).

Since the 1960s, when thiabendazole became the first benzimidazole licensed for human use, the treatment of hookworm disease by chemotherapeutics has been largely unchanged. The current drug regimen for hookworm infection as recommended by the World Health Organization is a single dose of a benzimidazole agent, e.g. 400mg of albendazole. Benzimidazoles remove adult worms from the gastrointestinal tract of the host and are relatively inexpensive for widespread use in developing countries. The World Health Organization has recommended de-worming of school-age children, aiming for 75% de-worming by 2010. Although this initiative represents a significant public health endeavor with many potential benefits, the fact that hookworm disease typically is concentrated in a very few individuals and the high burden of disease in adults as opposed to children may make school-based de-worming campaigns less efficacious in reducing hookworm infection rates than expected.

As with many of drugs of our era, the reliance on one class of drugs, e.g. benzimidazoles, for treatment of hookworm infection is wrought with potential hazards. Benzimidazoles act by interrupting microtubulin function in the parasite, usually as a result of glucose pathway interference. The high rates of re-infection with hookworm, even immediately after de-worming, suggest that benzimidazole therapies are not effective long term solutions. Some studies indicate that these drugs become less effective after repetitive use, perhaps suggesting the emergence of drug resistance (Hotez 2006).

New agents under development for the treatment of hookworm infection include tribendimidine (400mg single use) (Xiao 2005) and Cry5B, a nematicidal crystal toxin isolated from the soil bacterium *Bacillus thuringiensis* (Cappello 2006). Although the use of new agents in combination with or as an alternative to benzimidazole therapies may delay the emergence of drug resistance in the short term, any chemotherapeutic agent is unlikely to change the high rates of re-infection that typically occur soon after anti-hookworm therapy. In response to this, more appropriate prevention technologies, such as vaccine development have been recommended. (Hotez 2006)

Vaccination, though an extremely powerful preventative tool for control of infectious diseases, has not been very successful against hookworm thus far for a variety of reasons. First, eukaryotic pathogens such as hookworm are more difficult to create vaccines for because investigators are often unable to use standard vaccine technology. In particular, worms and other parasites require special eukaryotic expression vectors and animal models that make high throughput reverse vaccine technology impossible. Secondly, and perhaps more distressing, is that the lack of fruitful commercial markets for these vaccines in the developed world make their development by drug companies less likely. Fortunately, organizations such as the Bill and Melinda Gates Foundation and the Clinton Foundation are now showing an interest in "neglected tropical diseases" and are beginning to use their resources to leverage drug and vaccine development. (Hotez 2006) Despite these obstacles, one anti-hookworm vaccine specific to *N.* americanus is in human clinical trials. This experimental vaccine uses *Ancylostoma* secreted protein 2 (ASP-2), which is an antigen secreted by all species of hookworm larvae. *In vitro* studies

of anti ASP-2 antibodies show that they seem to prevent larval stage invasion. The effect of this vaccine on humans is still under investigation.

Specific Aims

This study is based on the known body of work regarding hookworm immunoepidemiology in the developing world and our expertise in animal models of hookworm infection. The aims of this project were as follows:

- To conduct an immunoepidemiologic survey of hookworm infection in an endemic community by first characterizing prevalence of hookworm infection and incidence of co-infections.
- To identify the species distribution of hookworm in this endemic community using molecular methods.
- To use laboratory reagents developed in an animal model to characterize anti-hookworm immune responses in an endemic community.
- To utilize immunoepidemiologic data to begin developing novel diagnostic assays for hookworm infection.

Materials and Methods

Study Design. We conducted a cross-sectional, two part immunoepidemiologic study focusing first on descriptive field-based assessment of community health status and hookworm disease burden at a macro-level and secondly on laboratory based assays for characterizing immune responses to infection. Field work was conducted between July-October 2005. This study was approved by the Ministry of Health of the Department of Loreto, Peru, the Internal Review Board of Asociacion Benéfica Prisma laboratory (Lima, Peru) and Johns Hopkins University, and the Human Investigations Committee of Yale University School of Medicine (HIC# 0507000390).

Study site. Tarapoto, a village located in the rural Amazon approximately 20km from the urban center of Iquitos, Peru was initially chosen as a study site due to its centrally located population and remote geographic location.

Figure 8. Map of the Iquitos, Peru and the surrounding area. From (http://encarta.msn.com/map_701513362/Iquitos.html**accessed on 11.26.2006**

Study participants and recruitment. Initially, a small community meeting in the village of Tarapoto was conducted to discuss the study objectives, importance of the study, and to address any questions and concerns members of the community may have with participation. An initial comprehensive epidemiologic survey and census of the village population was conducted. Information including basic demographics, past medical history, history of parasitic disease, and socioeconomic indicators as related to parasitic infection were collected. All study materials were translated into Spanish, the predominant language of the area and all information was verbally read/explained to

study participants if they were illiterate. Informed consent was obtained from each study participant, and risks and benefits of all study components and interventions were described.

Of approximately 235 inhabitants who were considered residents of the village if they resided within a 4 mile radius of the village center and spent greater than 5 days/week in the village, 190 were surveyed. The second component of the study involved collection of stool and blood samples from each subject. Of the 190 village residents interviewed, 176 agreed to participate and supplied a stool sample, while 164 also provided a 3-5mL blood sample. All residents of the village were included in the study. There were no exclusion criteria except residence outside of the village. Common reasons for abstention from participation in the study included being away from home and embarrassment over providing stool samples.

Stool Collection. Stool sample collection cups were distributed to all participants at the time of the initial interview with subject code, name, and a pictorial representation for those who were illiterate to assist in careful stool collection. Instructions were given to the domestic head of the household, generally the mother/wife and particular emphasis was placed on early morning collection of stool and taking care not to mix soil with stool since the majority of participants used the jungle/fields for defecation. A follow-up visit was conducted the subsequent morning to collect stool samples from participants enrolled the day before. Stool was stored in an insulated cooler in the shade (30 °C) awaiting transport back to the laboratory in Iquitos for diagnosis of intestinal parasites. To prevent exposure to heat as much as possible due to the fragility of hookworm eggs, stool

samples were processed immediately upon arrival back to Iquitos, typically within 3 hours of initial collection and presumably within 8 hours of defecation. With the aid of an experienced laboratory technician (CB) two initial tests for diagnosis of intestinal parasitosis were employed: direct microscopy and sedimentation method (Melvin 1982). These tests provided rapid, same day diagnosis of intestinal parasitosis. In particular, specimens were assessed for infection by hookworm, *Strongyloides stercoralis*, *Trichuris trichiura*, *Ascaris lumbricoides*, *Giardia lamblia*, *Enterobius vermicularis*, and *Entamoeba* species. Additional diagnostic tests, the Agar plate method and a modified Baermann method, were employed and Stoll counts were performed on all samples regardless of initial diagnostic results according to the protocols outlined below (Melvin 1982). Any stool sample deemed to be positive for hookworm disease based on direct microscopy and sedimentation was cultured using a modified Petri Dish method and larvae were collected at day 2 and day 4.

Stool Diagnostic Laboratory Methods. Direct microscopy. One drop of fresh feces is smeared on microscope slide and examined at high power for parasite eggs.

Sedimentation. Sedimentation is a diagnostic method designed to concentrate the protozoa, larva and eggs of helminthes for detection by direct microscopy.

1. To a sedimentation cup (any container with a large orifice that can hold up to 300-500cc fluid) water is added at room temperature (30ºC).
2. A piece of gauze is placed in a small colander that sits on the orifice of the sedimentation cup. The bottom of the colander should be slightly immersed in water. Approximately 5g of fresh stool is placed on the gauze and spread evenly with a toothpick or other disposable device.
3. The stool is allowed to sit for 20 minutes in the apparatus after which the gauze and colander are removed.
4. Supernatant is decanted leaving approximately 5 mL of sediment in the bottom of the cup.

5. 300 mL of water at room temperature is added to the cup and allowed to sit for 30 minutes, cleaning the sample and allowing it to sediment again. Supernatant is decanted the entire process repeated.
6. After 2nd wash supernatant is decanted and 5 drops of sediment is placed on a microscope slide and examined for eggs, larvae or protozoa.

Modified Baermann method (Melvin 1982).

1. 45cc of warm water (40ºC) is placed in a 50cc conical tube covered by two pieces of gauze (the gauze should be in contact with the water).
2. 5g of stool is placed on the gauze and allowed to sit for 6 hours. The larva should sediment in the bottom of the tube. 5 drops of sediment is removed and examine under microscope for eggs/larvae.
3. The Baermann apparatus remains intact for 24 hours total and sediment examined again in 24 hours.

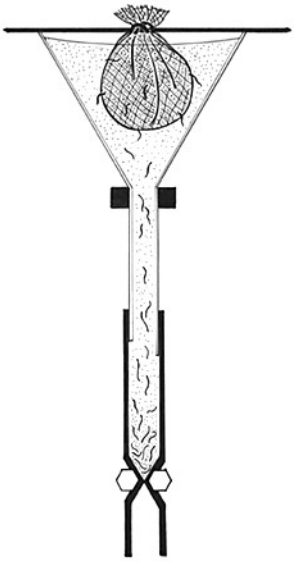

Figure 9. Schematic of a Baermann apparatus

Agar Plate Method. Yet another assay useful in the diagnosis of both hookworm and *Strongyloides* species is the Agar Plate Method, which relies on the motility of both species. One can observe the migration of the adult larvae since they create paths along the agar plate and more easily collect larvae for high power examination and corporal diagnosis. Agar plates are prepared using sterile water and agar (Fluconazole, a potent

anti-fungal, can be added to the agar plate in order to prevent fungal overgrowth that may inhibit the migration of the hookworm larvae) (Yori 2006).

1. One gram of stool is dropped in the center of the agar plate. One drop of water or normal saline is pipetted over the stool. This creates a humid, moist environment to encourage migration of the worms.
2. Specimens are thus cultured for 48 hours at 28ºC.
3. After two days plates are observed for tracks and checked under the microscope for larval diagnosis.

Stoll Method (Modified) for Hookworm Egg Quantification.

1. 40mL of purified, distilled water is placed in a 50mL conical tube.
2. A piece of gauze is placed over the opening of the tube and 1g of stool is smoothed onto the gauze to sit for one hour.
3. After 1hour the fluid in the top of the conical is poured off saving only 5 mL of the sedimentation found at the bottom of the conical.
4. The sedimentation is placed in a 5mL tube.
5. Two aliquots of the sediment, each aliquot of 50uL, is taken and placed on a glass slide for microscopic examination. All eggs seen in each aliquot are counted.
6. To calculate the amount of eggs in the sample as eggs per gram (epg) the number eggs detected in the each of the 2 aliquots were added and divided by 2. Then the average per 50uL is extrapolated to EPG by multiplying the eggs/50uL x 100 = the eggs per 5 mL = eggs per gram

After stool for all tests was removed from the initial sample, approximately 1.5 mL of stool from each participant was removed and stored in 1.5 mL Eppendorf tubes and frozen at -28°C for later transport to Yale. The remainder of the stool from hookworm positive subjects was cultured for larvae.

Culture. Culturing hookworm larvae was accomplished using a Modified Petri Dish Method (a technique developed by our collaborators Kosek and Yori in Iquitos for initial use as a culturing technique for *Strongyloides* larvae). Briefly, a small Petri dish placed inside a larger Petri dish. One part feces and one part bone charcoal are mixed

well using a wooden stick and placed in the smaller, internal Petri dish. Clean distilled water is placed in the larger Petri dish until the level of the water reaches the same depth as that of the internal Petri dish. The entire apparatus is placed in a refrigerator (as ambient temperature was warmer than this) with bottles of ice to regulate temperature to 28ºC for 4 days. As hookworms hatch from their eggs, the larvae, which are hydrophilic, effectively jump into the water ring surrounding the culture mixture. Water from the outer ring was collected at day 2, replaced with fresh distilled water and re-incubated for another two days at which time the final culture was collected and the stool mixture disposed. Water containing larvae was centrifuged, supernatant discarded and the pellet resuspended in clean distilled water and stored at room temperature awaiting shipment to Yale.

Treatment. Any study participant diagnosed with any form of intestinal parasite was offered standard treatment according to World Health Organization recommendations. Of note, treatment was provided after blood samples were obtained.

Parasite	**Treatment**
Hookworm (*A. duodenale* or *N. canus*)	Albendazole 400mg x 1 dose
Strongyloides stercoralis	Albendazole 400mg once daily x 3 days
Giardia lamblia	Albendazole 400mg once daily x 5 days
Ascaris lumbricoides	Albendazole 400mg twice daily x 3 days
Trichuris trichiura	Albendazole 400mg once daily x 5 days

Table 2. World Health Organization treatment recommendations for common intestinal parasitic diseases. Adapted from www.who.org. Accessed 07.07.2005.

As part of the agreement with the community to conduct this study, basic medical care was provided using a house-call system. Any villager requesting medical care was evaluated and treated (free of charge) regardless of their participation in the study. Appropriate referrals were made to the nearest health post in Santa Clara district (approximately 1 hour away by boat). Anyone complaining of fever was immediately tested for malaria using peripheral blood smear analysis (see below). Other routine laboratory tests were also provided for free based upon the patient's complaints. All medical care provided by the primary caregiver (PS) was discussed with a qualified medical professional (MK).

Serum Collection. Blood samples were obtained from study participants using a small gauge needle (23 ¼ or 25 ¼ gauge) and 5 cc syringe (Becton Dickinson, Franklin Lakes, NJ). Approximately 3-5 cc of blood was obtained from each participant and immediately placed in a vaccutainer tube. Tubes were immediately placed in a styrofoam cooler to prevent heat associated hemolysis and transported back to the laboratory in Iquitos for further processing (within 3-5 hours of phlebotomy). In the laboratory, hematocrits were obtained using a capillary tube method and centrifugation. In addition, a thick and thin peripheral blood smear from each study participant was evaluated for malaria using the methods outlined below (CDC 2006). According to recommendations made by the CDC, thick and thin smears were prepared on the same slide due to limited resources.

Thick smears.

1. Place a small drop of blood in the center of the pre-cleaned, labeled slide.

2. Using the corner of another slide or an applicator stick, spread the drop in a circular pattern until it is the size of a dime (1.5 cm^2).
3. A thick smear of proper density is one which, if placed (wet) over newsprint, allows you to barely read the words.
4. Lay the slides flat and allow the smears to dry thoroughly.
5. Examine slides by microscopy.

Thin smears
1. Place a small drop of blood on the pre-cleaned, labeled slide, near its frosted end.
2. Bring another slide at a 30-45° angle up to the drop, allowing the drop to spread along the contact line of the 2 slides.
3. Quickly push the upper (spreader) slide toward the unfrosted end of the lower slide.
4. Allow the thin smears to dry then fix the smears by dipping them in absolute methanol.
5. Examine by microscopy for species identification.

Any individual diagnosed with malaria was treated using World Health Organization recommended guidelines (WHO 2006). Finally, all blood samples were centrifuged at high speed for 5 minutes and serum phase removed and stored in 1.5mL Eppendorf tubes and frozen at -80 °C.

Frozen aliquots of stool and serum samples, as well as live larvae at room temperature, were transported from Iquitos to Lima, Peru via airplane and immediately taken to AB Prisma collaborating laboratories at Universidad Cayetano Herrida for refrigeration and processing for shipment back to the United States. It was assumed that the frozen human samples would be able to withstand a freeze-thaw cycle in this regard. Each individual sample tube was quickly wrapped in Parafilm® (SPI West Chester, PA) and, with the proper permission from the Government of Peru and the USDA, Department of Health and Human Services and State of Connecticut, all samples were shipped to the United States over 3 days. Frozen stool and serum were stored in dry ice for the duration of the journey. Upon arrival at Yale, all samples were appropriately stored in either freezers or at room temperature.

Serologic Studies. Serum aliquots were initially pooled into several groups depending on active infection status and pools were utilized in Western blot analysis. Naïve human serum from a donor never previously exposed to hookworm served as a negative control for all studies. To determine the value of Western blot in distinguishing species specific responses among a variety of endemic communities, an immunoblot of pooled sera from Peru was compared with that from Ecuador, where sera donations came from *Necator* infected individuals for total IgG responses to a small panel of our *A. ceylanicum* antigens.

In order to compare serum antibody levels with fecal antigen and egg count results, we utilized a recently developed indirect ELISA assay to measure immunoglobulin levels directed against pooled and specific hookworm proteins. The assay is both cost effective and requires only a small amount of patient sera. The pooled and specific recombinant antigens were derived from adult *Ancylostoma ceylanicum* hookworms that are maintained in Dr. Cappello's laboratory in the Yale Child Health Research Center (YCHRC). We have previously demonstrated that serum from subjects infected with hookworm will react with these antigens in an ELISA format (unpublished results). Each individual participant's sera was screened using this ELISA for total IgG, IgG4 and IgG1 immunoreactivity against hookworm antigens. Additionally, to further quantify and compare immune responses across the geographic regions described above, pooled banked serum from those locations was analyzed by ELISA methods.

Comparative serologic studies were performed on pooled, banked sera in the YCHRC from a variety of geographic locations, including Ecuador, Venezuela, and Guatemala as well as New Haven, CT. All serum banked from individuals in these

communities were obtained according to HIC approved protocols and with informed consent. Ecuadorian samples were collected from schoolchildren in the Pichincha Province. Guatemalan serum was obtained from a mass deworming project in the Lake Izabal region of Eastern Guatemala. Venezuelan serum came from an epidemiologic survey conducted in the Valle del Rio region of rural Venezuela. Naïve human serum was obtained from an unexposed individual in New Haven, CT.

Parasite Antigen Preparation. *Ancylostoma ceylanicum* adult worms and larvae were obtained from the ongoing hamster model for hookworm infection in the Cappello laboratory. Whole hookworm homogenates and larval homogenates (HEX, NEX and LEX) are routinely prepared using hookworms and larvae from this life cycle. Briefly, adult hookworms and/or larvae were harvested from a hamster, concentrated, and homogenized using sonication. Homogenates were stored in Tris-HCl. *Necator* soluble extract (NEX) was obtained from adult worms harvested from infected hamsters. Excretory-secretory product (ES) was prepared by incubating live freshly harvested adult worms in filter sterilized PBS. After 6-8 hours worms were removed and ES products removed after centrifugation (Bungiro 2002).

Western Blot. Western blot was used in order to test the reactivity of serum from endemic communities against a variety of *A. ceylanicum* antigens. Antigens taken from *A. ceylanicum* and *N. americanus* were run in a polyacrylamide gel at appropriate concentrations (LEX, HEX, ES, NEX were run at 2 mcg/well each, and rAceES-2 at 500ng/well as less of a recombinant protein is necessary for detecting signal). Gels were

run in replicate at 80V for 2-3 hours. Gels were immediately transferred at 4ºC for 1 hour at 100V. In order to minimize background interference that can become problematic when working with human samples, blots were blocked overnight in 5% Non-fat milk in phosphate buffered saline (PBS) with Tween (MPBS-Tween) at 4ºC. The next day blots were placed in a 1:1000 dilution of appropriate serum in 1% MPBS-Tween and incubated for 2-3 hours at room temperature while gently stirring. Blots were then washed vigorously 3 times for 10 minutes in PBS-Tween and then placed in secondary antibody for 1 hour at 37 ºC. In this case, the secondary antibody was a 1:5000 dilution of either goat α human IgG conjugated to or goat α hamster IgG conjugated to horseradish peroxidase (ICN Biomedicals, Aurora OH, Cappel #55402 lot# 03636 or # 55220 lot #03916, respectively). After vigorous washing 3 times for 10 minutes in PBS-Tween, blots were mounted onto glass plates, developed using SuperSignal chemiluminescent substance and exposed in the dark.

Enzyme Linked Immunosorbent Assay (ELISA). This assay has been optimized for human serum, using a chequerboard plate method to determine dilutions of primary and secondary antibodies. Antigens were uniformly coated at concentration of 2 μg/mL in sterile PBS and 100 μL per well was added to Immulon 2 HB 96-well plates (Dynex Techonologies, Chantilly, VA). To allow adequate time for antigens to adhere to the wells, plates were covered in saran wrap and incubated in 4ºC overnight. 300 μL of 1% MPBS was added to each well in a 96 well plate which was then incubated overnight in 4ºC. This blocking step is designed to prevent any non-specific epitopes from interfering with the primary antibody during the dilution steps.

To ensure precision, all samples were run in duplicate and mean values for each sample were used in analysis. Consistency within plates was ensured by calibrating OD values against blanks before means were taken. Consistency between plates was ensured by using two controls, a positive control made up of a pool serum from actively hookworm infected individuals from the study population and a negative "gringo" control comprised of serum from non-endemic, never before exposed serum from New Haven, CT.

Total IgG ELISA. On day 2, antigen coated plates were washed using an automated plate washer (ELx405 Bio-Tek) and blocked with 1% MPBS for 1 hour at 37ºC. During this time dilutions of serum were made using the dummy plate. After washing, 220 µL of MPBS-Tween were pipetted to into each well of every other row. To every other row, 220 µL of diluted serum at two times the appropriate concentration was added. For total IgG assays serum dilution of 1:200 was used. 100 µL of serum was pipetted into each well of test plate using multichannel pipetter, covered and incubated for 3 hours at room temperature. A 1:1000 solution of goat anti-human IgGt in MPBST was made. Plates were washed 4x using the automated plate washer as above. 100 µL of the secondary solution was pipetted into each well and plates were covered and incubated at 37ºC for 2 hours. 100 µL of streptavidin-HRP solution was pipetted into each well using the multichannel pipette. In the meantime, a citrate buffer (ph 5.0) based substrate solution was prepared (per plate: 500 µL ABTS, 10mL citrate buffer at room temperature, and 10 µL 30% H_2O_2). After secondary incubation plates were washed again 5 times and 100 µL of substrate added to each well. The absorbance (405nm) was

measured at 60 minutes (SpectraMax 190, Molecular Devices), and optical densities (OD) were recorded.

IgG subtypes and IgE ELISA. Methods for IgG1,IgG4 and IgE ELISA were more vigorous. Blocking with 1% MPBS occurred over 4 hours at 37ºC on Day 2. Serum concentrations were optimized using the chequerboard technique for IgG1 and IgG4 and serum dilution of 1:50 was chosen. 100 µL of serum was pipetted into each well of test plate using multichannel pipetter, covered and incubated overnight at 4ºC. The next day a 1:1000 solution of BD Pharmingen biotin-conjugated mouse anti-human IgG4 or IgG1 in MPBST was made. Plates were removed from the 4ºC fridge and washed 4x using automated plate washer as above. 100 µL of the secondary solution was pipetted into each well and plates were covered and incubated at 37ºC for 1 hour. A 1:1000 solution of streptavidin-HRP (ImmunoPure Streptavidin, HRP conjugated, Pierce 21126) was prepared. Plates incubating with secondary were washed 4x using plate washer and 100 µL of streptavidin-HRP solution was pipetted into each well using the multichannel pipette. Plates were then covered and incubated for 1 hour at 37ºC. After secondary incubation, plates were washed again 5 times and 100 uL of substrate added to each well. Spectrophotometry was performed at 60 minutes using (Spectra Max 190 M) and optical densities (405nm) were recorded.

Speciation. Because of the time consuming nature of morphologic examination to speciate between the different human hookworm species many molecular methods have been developed to facilitate hookworm diagnosis. Initially, DNA extractions from larvae of hookworm positive individuals were used in a PCR-based assay based on

sequence differences between the cytochrome oxidase (COX) genes of *N. americanus* and *A. duodenale*.

DNA Extraction and Amplification. Genomic DNA was isolated from L3 stage larvae that were cultured from the stool of hookworm positive individuals by modified Petri dish method. DNA was isolated using a modified proteinase K method.

1. 10-30 larvae were added to 40 µL of DNA extraction buffer (50mM KCL, 1.5mM MgCl2, 10mM Tris, pH 8.5, 0.01% gelatin, 0.45% NP-40, 0.45% Tween-20) with 20 µg/mL proteinase K.
2. Samples were incubated at 55°C for 18 hours.
3. Samples were incubated at 100°C for 10 minutes to inactivate proteinase K.
4. Genomic extracts were centrifuged at top speed for 5 minutes and then stored at -20°C.

Primers and conditions for amplification were based on those reported by Zhan and colleagues (Zhan 2001). To distinguish between *Ancylostoma duodenale* and *Necator americanus* two sets of species specific primers were used. (NaForward 5'-TTC GTT TGG AGT TGG CT and NaReverse 5'-TAG CTC CAG CCA AAA CT; AdForward 5'-TTC GTT TGG AGT TGG CA and AdReverse 5'-TGG CAC CAG CCA ATA CA). PCR reactions were carried out with 2µL of genomic extract as template DNA in a total reaction volume of 50 µL using Amplitaq® (Applied Biosystems, Roche). The following cycling conditions were used:

Preheat 94°C
94°C for 30" followed by 40 cycles of
94°C for 15"
55°C for 20"
72°C for 1' then
72°C for 10' followed by storage at 4°C.

Although this method has been useful in the past for speciating human hookworm (Zhan 2001, Difidele unpublished results), many difficulties in achieving reproducibility

were encountered with this technique. We hypothesize that the long period of time between larval harvesting and DNA extraction made this system sub-optimal. Additionally, with larvae from only 40 of 66 hookworm positive individuals an additional technique needed to be employed to create a more complete dataset.

Fecal PCR. To maximize our ability to speciate hookworm we used a fecal extraction method to obtain genomic DNA (gDNA) directly from the stool of each individual in the study population. A protocol based on the species specific differences in the ITS region of the hookworm genome was used. Feces were extracted using QIAamp® DNA Stool Mini Kit (Qiagen, Valencia CA) and approximately 200uL gDNA elution product stored at -20 C. PCR amplification of rDNA from products were accomplished a two-step procedure. PCR Reaction I: 4 µL template, 45 µL Supermix (Invitrogen Life Technologies, Carlsbad, CA), 3.3 µL MgCl2, 1 µL species specific forward primer, 1µL species specific reverse primer. PCR I employs NC2 (5'-TTA GTT TCT TTT CCT CCG CT) and NC5 (5'-GTA GGT GAA CCT GCG GAA GGA TCA TT) Cycling conditions were as follows:

Preheat 94°C
94°C for 5' followed by 40 cycles of
94°C for 15"
55°C for 20"
72°C for 30" then
72°C for 5' followed by storage at 4°C.

PCR Reaction II used all of the same reaction parameters except only 2 µL of the amplicon derived from PCR I were used as the template. Specificity for hookworm is achieved using species specific primers (NA 5'-CGT TAA CAT TGT ATA CCT GTA CAT AC and 5'-TGC GAA GTT CGC GTT TCG CTG AGC). The amplicon from

PCR II was then run on a 1% agarose gel and visualized with *Necator americanus* appearing at approximately 870 base pairs and *Ancylostoma duodenale* 690 base pairs.

DNA Sequencing. For quality assurance, several PCR products were sequenced and alignments compared with those of known *A. duodenale* and *N. americanus* sequences. Sequencing was done by the KECK facility at Yale University. Sequences were analyzed using NCBI's BLAST database and alignments done using DNAstar.

Statistical Analysis. All data were initially recorded on field forms and subjects were coded by number and letter. Numbers identified household and letters were specific to the individual. All data, including survey information and laboratory information were coded using this identification system. From field forms data were transferred to a Microsoft Access database (Microsoft, Redmond WA). Spectrophotometric data (OD values) were initially recorded in a Microsoft Excel database (Microsoft, Redmond WA). Basic statistical analysis including bivariate analysis was performed by SAS Software. Comparisons of antibody responses (anti- hookworm antigen total IgG and IgG4, IgG1 and IgE) were initially evaluated for normal distribution and IgG4 titers which were not normally distributed were then analyzed using non-parametric methods. P values of < 0.05 were considered statistically significant.

Results

Prevalence of intestinal parasites by fecal examination

The initial aim of this study was to characterize basic prevalence rates of hookworm infection in an endemic community. Based on the data from fecal examinations, we found that 98% of participants in this region of the Peruvian Amazon were infected with at least one intestinal parasite.

Parasite	**Prevalence % (n)**
Hookworm	38.82 (66)
Strongyloides stercoralis	14.12 (24)
Trichuris trichiura	41.18 (70)
Ascaris lumbricoides	75.88 (129)
Giardia lamblia	2.35 (4)
Enterobius vermicularis	0 (0)
Entamoeba coli	8.24 (14)
Entamoeba nana	0.59 (1)

Table 3. Prevalence of Intestinal Parasites in Study Cohort

In addition, co-infection with multiple intestinal parasites was also a common phenomenon among this group.

		Hookworm Positive (n=66)	**Hookworm Negative (n=104)**
Other parasitic infections			
Ascaris lumbricoides	Infected	82 % (54)	72% (75)
	Non-infected	18% (12)	28% (29)
Strongyloides stercoralis	Infected	23% (15)	9% (9)
	Non-infected	77% (51)	91% (95)
Trichura trichuris	Infected	47% (31)	38% (39)
	Non-infected	53% (35)	62% (65)
Giardia lamblia	Infected	3% (2)	2% (2)
	Non-infected	97% (64)	98% (102)

Table 4. Prevalence of Co-infection with Hookworm in Study Cohort. HW positive and negative status is determined by anyone of the six diagnostic methods described below unless otherwise stated.

Diagnostic Method	Number of HW positives detected	Number of HW negatives detected
Any Method	52	68
Direct Microscopy	12	108
Sedimentation	34	86
Agar Plate	25	95
Agar Plate with fluconazole	21	99
Modified Baermann 6 hours	4	116
Modified Baermann 24 hours	6	114

Table 5. Diagnostic Tests for Hookworm Detection. Not all samples were tested using all methods due to limitations in the amount of stool available and variability in testing. This table represents results form only 120 of 176 possible stool specimens.

Demographic Characteristics of Study Cohort

Basic demographic information from study participants was analyzed in relationship to hookworm infection status. The average age of study participants was 24.3 years. Males represented 56.32% (107) of the study population and females represented 43.68% (83). The mean ages of hookworm positive and negative subjects 27.28 and 23.02 years, respectively ($p < 0.05$). Although not statistically significant, a trend was noticed in the difference in distribution of hookworm disease and education level. A higher percentage of hookworm negative individuals were in the "under school age" category. Other results are summarized in the table below.

	HW positive (n=66)	**HW negative (n=104)**
Characteristic		
Age (years)	27.28	23.03
Education level		
Primary (n=71)	44% (29)	40.38% (42)
Secondary (8)	6% (4)	4.8% (4)
Higher education (1)	0 (0)	1.47% (1)
Under schoolage (21)	9% (6)	14.42% (15)
No schooling (17)	17% (11)	8.82% (6)

Table 6. Demographic characteristics of study population based on hookworm infection status.

Additional demographic data, including established risk factors for hookworm infection, were obtained from the study cohort. In particular, differences between hookworm positive and negative groups were noted for indicators such as where villagers typically defecated, profession, and shoe wearing practices. Hookworm positive individuals were less likely to have access to a latrine, and were thus more likely to defecate in an open field (92.5% versus 84.31% p=0.05), although open field defecation was high in both groups. Additionally, farming was found to be a more common occupation for hookworm positive individuals than uninfected individuals (32% versus 20.59%, p=0.02). Shoe wearing, long considered a fundamental risk factor for infection with hookworm was less common in the hookworm positive group (88% never wear shoes) than the negative group (76% never wear shoes) ($p<0.05$).

	HW Fecal Positive	HW Fecal Negative
Risk Factor	**% (n)**	**% (n)**
Excrement Elimination		
Latrine	5.97 (4)	9.80 (10)
Open field	92.54 (62)	84.31 (86)
River	1.49 (1)	5.88 (6)
Profession		
Fisherman	4 (2)	5.88 (4)
Farmer	32 (16)	20.59 (14)
Woodsmen	2 (1)	1.47 (1)
Housewife	20 (10)	23.53 (16)
Manual laborer	0 (0)	1.47 (1)
Student	24.00 (12)	17.65 (12)
Minor	12.00 (6)	25 (17)
Unemployed	4 (2)	3 (2)
Water source		
River	100 (50)	100 (68)
Well	0	0
Distance to water source (m)	93.58 (10-400)	95.15 (10-400)
Water treatment		
Boil	1.5 (1)	1 (1)
Chemical tx	9 (6)	4.9 (5)
None	90 (60)	94.12 (96)
Shoe Wearing		
Always	0 (0)	0 (0)
Almost always	4	7.35
Sometimes	8	14.71
Almost never	88	76.47
Never	0 (0)	1.47
Garbage disposal		
Open field	71.64 (48)	74.51 (76)
Burn	24.51 (25)	22.39 (15)
Bury	5.91 (4)	0.98 (1)
River	0 (0)	0 (0)

Table 7. Epidemiologic indicators for hookworm disease in the study cohort.

Although often asymptomatic, chronic hookworm infection may be associated with several non-specific gastrointestinal symptoms and general symptoms of anemia. In our study cohort, hookworm positive individuals tended to have more abdominal pain

then hookworm negative individuals (30% versus 19%, p=0.05). In addition, past medical histories pertaining to hookworm disease was taken from each individual. Hookworm positive individuals tended more towards the use of medicinal plants for treatment of common ailments including intestinal parasitosis. Use of non-steroidal anti-inflammatory agents including acetaminophen, was more common among hookworm negative individuals than hookworm positive individuals (22% versus 5%, p=0.02).

	HW Fecal Positive	**HW Fecal Negative**
Medical History		
Previous anti-parasitic treatment		
Albendazole/Mebendazole	76.47 (52)	72.00 (36)
Medicinal plant*	5 (3)	1.47 (1)
None	22 (11)	22 (15)
Last health care visit		
Never	2 (1)	0 (0)
> 1 year	86 (43)	82.35 (56)
1 month – 1 year	8 (4)	8.82 (6)
1 month	0 (0)	1.47 (1)
1 week	0 (0)	1.47 (1)
Current medication		
Anti-parasitic	0 (0)	0 (0)
Anti-malarial	8 (4)	11.76 (8)
Anti-inflammatory	5.08 (6)	22.06 (15)
Antibiotic	0 (0)	2.94 (2)
Medicinal plants	16.00 (8)	5.88 (4)
Symptoms of HW disease		
Weakness	8.00 (4)	10.29 (7)
Abdominal Pain	30.00 (15)	19.12 (13)
Both	2 (1)	0 (0)
None	60 (30)	70.59 (48)

Table 8. Past medical history and relationship to hookworm infection. Medicinal plants used for antiparasitic treatment include oje, salmarga, and retama.

Prevalence of Anemia in Study Population

Based on the pathophysiology of hookworm disease and in particular the blood-feeding component of infection, I hypothesized that infected individuals would have

lower blood hematocrits than uninfected individuals. Anemia in this population was defined as a hematocrit < 35 in males and < 33 in females based on parameters established by the Ministry of Health of the Department of Loreto, Peru. Gender did not affect hematocrit significantly.

Characteristic	HW fecal positive	SEM	P value
Sex			
Male	35.13	0.59	.450
Female	34.44	0.70	

Table 9. Hematocrits among men and women in hookworm infection groups.

Interestingly, there were no statistical differences between parasite infected and uninfected individuals in terms of hematocrit, not only in hookworm infection but even infection with any other intestinal parasites. However, when we compared hematocrits in individuals based on infection status for other intestinal parasites, we found that only trichuriasis correlated with a lower hematocrit (p=0.04).

		Hematocrit	SEM (+/-)	p value
Parasite				
Hookworm	Infected	34.76	0.54	.978
	Non-infected	34.74	0.58	
Ascaris lumbricoides	Infected	34.41	0.44	.114
	Non-infected	35.93	0.94	
Strongyloides stercoralis	Infected	34.10	0.93	.500
	Non-infected	34.86	0.44	
Trichura trichuris	Infected	33.80	0.52	.04
	Non-infected	35.46	0.61	
Giardia lamblia	Infected	32.33	1.45	.348
	Non-infected	34.80	0.41	

Table 10. Hematocrits among participants infected and uninfected for a variety of intestinal parasites.

Subjects with Malaria and Hookworm Co-infection

More than 12 % of study subjects (21/170) were found to be co-infected with hookworm and malaria. Among those individuals with malaria, the species distribution tended towards *P. falciparum* in hookworm uninfected individuals and *P. vivax* in hookworm infected individuals, although the sample size is too small to reach statistical significance.

	HW fecal positive (n=66)	**HW fecal negative (n=104)**
Malaria Positive	7 (10.6%)	14 (13.46%)
Malaria strain	Percent of Malaria Infected Individuals	
P. falciparum	29% (2)	78.57% (11)
P. vivax	57% (4)	21.42 (3)
Both	14.29%(1)	0 (0)

Table 11. Malaria prevalence and speciation in study cohort.

Speciation

PCR for speciation using COX-1 amplifying primers sets were initially used to distinguish between *A. duodenale* and *N. americanus* larvae cultured from stool of infected individuals. However, of 66 hookworm positive individuals, only 40 samples of cultured larvae were obtained due to technical reasons. After culture, the presence of larvae were confirmed by microscopy. In some cases, however, larvae were not detected. This could possibly have been due to a sampling problem when culturing larvae from lightly infected individuals who had not expelled many eggs. Additionally, temperature fluctuation in the field may have contributed to impaired culturing conditions.

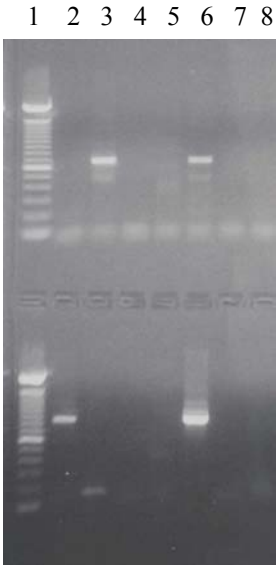

Figure 10. Representative gel after PCR from fecal extraction gDNA amplification. Upper panel shows *Anyclostoma duodenale* amplicons and lower panel shows *Necator americanus* amplicons. Lane 1: 1 KB Ladder Lane 2: *Necator americanus* (NA) positive and *Anyclostoma duodenale* (AD) negative sample. Lane 3: AD positive, NA negative. Lane 4: Negative for both species. Lane 5: Negative for both species. Lane 6: Mixed Infection. Lane 7 and 8: Negative for both species.

\

The results of speciation PCR by any of three methods is described below. Subjects were considered positive by PCR if amplicons wre detected using either larval gDNA or fecal gDNA as template. Results from PCR speciation show that both species of human hookworm are endemic to this part of the Peruvian Amazon in roughly equal amounts with 25% of individuals harboring mixed infections.

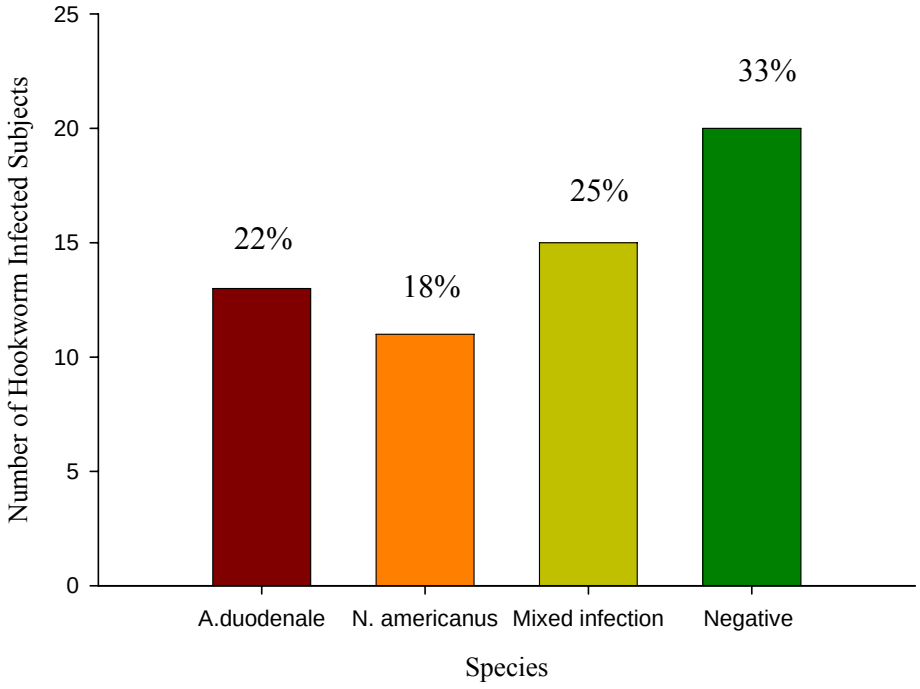

Figure 11. PCR results for speciation of hookworm fecal-microscopy positive individuals (n=62 total). Of note larvae were only cultured from stool of subjects found to be hookworm positive by microscopy. Fecal gDNA, however, was extracted from the stool of all study participants who provided samples.

Although we are still in the process of speciating all fecal gDNA samples for signs of *A. duodenale* infection, this data suggests that molecular methods may have a higher sensitivity for detecting and speciating infection in an endemic community. We have begun to analyze fecal gDNA from the microscopy negative samples and have found that of a pilot group of 26 subjects 7 were infected with *A. duodenale* only, none with *N. americanus* alone, 4 with mixed infection and 4 who were negative by PCR.

Nine individuals in this group had no fecal gDNA available for PCR and thus could not be assessed. These results indicate that microscopy may under-diagnose individuals, likely due to lack of adequate sample or misrepresentation of the total stool sample.

To confirm speciation results, several bands were taken from amplicons identified as either *A. duodenale* or *N. americanus* and sent for sequencing. The resulting alignments were compared against an NCBI database and compared to known sequences for a variety of nematodes.

Sequence Alignments

Results from these alignments show that PCR products amplified from fecal gDNA did in fact correspond to the correct species based on the sequence similarity to the ITS-1 and -2 regions of the genome. Several of our aligned sequences, however, exhibited interesting findings with a small, non-homologous region within the primer sequence likely indicative of a polymorphism.

```
Query  30   TTGCAAATAACAGAAACATCGTTGTTATACTAGCCACTGCCGAAACGTTCTAAAGTCGGT  89
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  221  TTGCAAATAACAGAAACATCGTTGTTATACTAGCCACTGCCGAAACGTTCTAAAGTCGGT  162

Query  90   AAACGATTCAGCAGCAACAACGAGTTTGCTGTCATTCAGCGCACGTTAGCAAACTAGCCA  149
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161  AAACGATTCAGCAGCAACAACGAGTTTGCTGTCATTCAGCGCACGTTAGCAAACTAGCCA  102

Query  150  GCCAACGTACATGTTGCAATATATTCTGATCTAGAACGGGAATCGCTAAAAGCAAGTGCC  209
            || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  101  GCTAACGTACATGTTGCAATATATTCTGATCTAGAACGGGAATCGCTAAAAGCAAGTGCC  42

Query  210  GTTCGACAAACAGTGCCACAAGCTACACTGTAGTAGATATA  250
            |||||||||||||||||||||||||||||||||||||||||
Sbjct  41   GTTCGACAAACAGTGCCACAAGCTACACTGTAGTAGATATA  1
```

Figure 12. Alignment of presumed A. duodenale PCR product with A. duodenale DNA for ITS-2. Showing 99% homology.

Figure 13. Alignment of ITS-2 Region for *N. americanus* with an amplified PCR product from our speciation showing 98% homology.

```
Query  1     CACGCCTGAGCTCAGGTTGCATTGCAAATGACACATCCACATGGCGAACATCGTTGTCCT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1055  CACGCCTGAGCTCAGGTTGCATTGCAAATGACACATCCACATGGCGAACATCGTTGTCCT  996

Query  61    TCACATTGTCTCCGTTCAACCACGCTCATAAGTCGCGAGAGCGATTAAACAGTGAACAAC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  995   TCACATTGTCTCCGTTCAACCACGCTCATAAGTCGCGAGAGCGATTAAACAGTGAACAAC  936

Query  121   GATATGTTCATGTCATACAGTATGCACCGCTATCATACGTTAGTAAACTAGCTAACTAAC  180
             |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  935   GATATGTTCATGTCATACAGTATGCACCGCTATCATACGTTAGTAAACTAGCCAACTAAC  876

Query  181   GTAGTGAATAACAGCGTGCACATGTTGCACATGTGTTCTTCACTTAAACGGGAATTGCTG  240
             |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  875   GTAGTGAATAACAGCGTGCACATGTTGCACGTGTGTTCTTCACTTAAACGGGAATTGCTG  816

Query  241   AACACAACACATACAATGCGTAGTACAGAGCAAGTACCGTTCGACAAACAGTGTTCAACA  300
             |||||     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  815   AACACGT---ATACAATGCGTAGTACAGAGCAAGTACCGTTCGACAAACAGTGTTCAACA  759

Query  301   ATACTCGGTGAGAGTACTGTCCACAAGCTACACTGTAGTATTATCGTTAACAACCCTGAA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  758   ATACTCGGTGAGAGTACTGTCCACAAGCTACACTGTAGTATTATCGTTAACAACCCTGAA  699

Query  361   CCAGACGTGCCGAAGGGAAAACCCAACGGCGCTATGCGTTCAAAATTTCACCACTCTAAG  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  698   CCAGACGTGCCGAAGGGAAAACCCAACGGCGCTATGCGTTCAAAATTTCACCACTCTAAG  639

Query  421   CGTCTGCAATTCGTGGTAAATAACGCAGCTAGCTGCGTTTTTCATCGATACGCGAATCGA  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  638   CGTCTGCAATTCGTGGTAAATAACGCAGCTAGCTGCGTTTTTCATCGATACGCGAATCGA  579

Query  481   CCGATCCATCGCTGAAGCTAGTCGAGTCTAATGTGACGACTAAGATGAAGTCACGATCAT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  578   CCGATCCATCGCTGAAGCTAGTCGAGTCTAATGTGACGACTAAGATGAAGTCACGATCAT  519

Query  541   CTGCAAACATCAAATGTAAAAAGTTAATATTTTGTGTTGGCGTCCACACATATTGTCCCA  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  518   CTGCAAACATCAAATGTAAAAAGTTAATATTTTGTGTTGGCGTCCACACATATTGTCCCA  459

Query  601   TCCACCATAACACGTAGCGCGCATTATTGGTTAACATGTGAAGGATCATTAAGGTTTCCT  660
             |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  458   TCCACCATAACACGTAGCGCGCATTATTGGTTAACATGTGAAGGATCATTAACGTTTCCT  399

Query  661   GATCACAAGAACAGGTACCACACCACACAAGTTATGTGTGTGTGTCTAACCACCAATACA  720
             |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  398   GATCACAAGAACAGGTACCACACCACACAAGTTATGTGTGTGTGTCCAACCACCAATACA  339

Query  721   AAAATTGAGGCGGCATTCAAGCAATGCTCATCAAGTCATAAGCTCAGCTGTATTCATGCG  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  338   AAAATTGAGGCGGCATTCAAGCAATGCTCATCAAGTCATAAGCTCAGCTGTATTCATGCG  279

Query  781   TATGTACAGGTAT  793
             |||||||||||||
```

Figure 14. Alignment of ITS-2 Region for *N. americanus* with an amplified PCR product from our speciation showing 98% homology. Highlighted in red is the insert corresponding to a possible polymorphism found in several of the sequences analyzed from our amplicons.

Immunoblot

We used standard immunoblot assays to characterize the antibody responses of pooled study subject sera from this endemic area of Peru against various hookworm antigen protein preparations, including whole worm extracts from *A. ceylanicum* adults (HEX) or larvae (LEX). *N. americanus* adults (NEX), and excretory/secretory proteins from adult *A. ceylanicum (*ES), as well as a recombinant *A. ceylanicum* ES protein, rAcES-2. We also subjected these proteins to SDS-PAGE and Coomassie staining in order to define the protein composition of each blot.

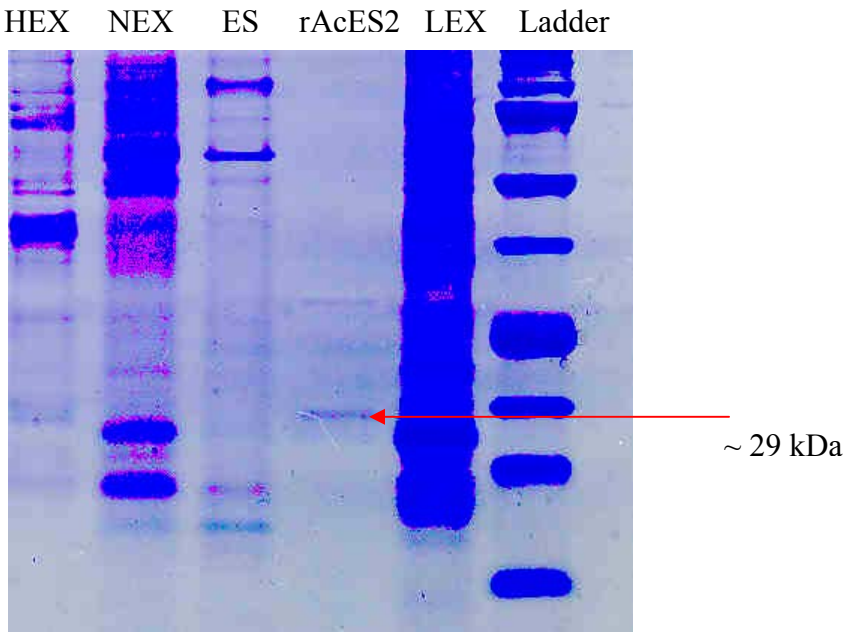

Figure 15. Coomassie Staining of Antigens used in immunoblot studies. Note the molecular weight of rAceEs-2 is approximately 29kDa.

Based on Coomassie results, all proteins of interest were present in appropriate quantity for signal detection. Following transfer to nitrocellulose, blots were probed with either Peruvian pooled serum or naïve human serum, with detection by chemiluminescence.

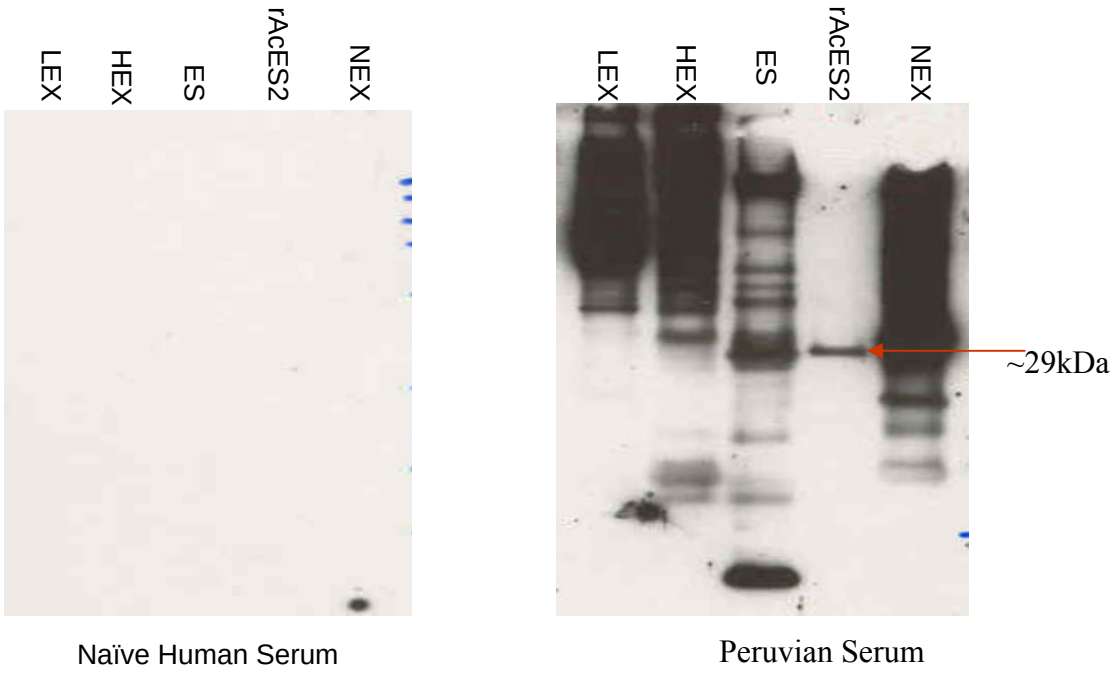

Figure 16. Western blot comparing immunoreactivity of naive human serum versus pooled Peruvian endemic serum against a variety of *A. ceylanicum* antigens. LEX (*A. ceylanicum* larval extract) HEX (*A. ceylanicum* adult whole worm extract), ES (*A. ceylanicum* excretory-secretory protein), rAcES-2 (recombinant *A. ceylanicum* excretory-secretory protein 2), NEX (*N. americanus* adult whole worm extract).

This immunoblot shows that pooled endemic sera recognizes components of each of the antigens tested, while sera from naïve (i.e. non-exposed) individuals does not. A limitation of these data is of course, that immune responses in the pooled sera may be a reflection of a few positive outliers as opposed to a common response had by all individuals in the community. Of note, the pooled sera represents a sample of serum from each individual in the community regardless of hookworm infection status. To explore whether this level of reactivity is reproducible with serum from other endemic areas we repeated the immunoblot and compared naïve human serum and Peruvian serum to pooled serum from *Necator* infected schoolchildren in Ecuador. We chose these samples because the major hookworm species identified to date in Ecuador is *N. americanus,* with no evidence of *Ancylostoma* available in the literature. The Ecuadorian blot is shown below.

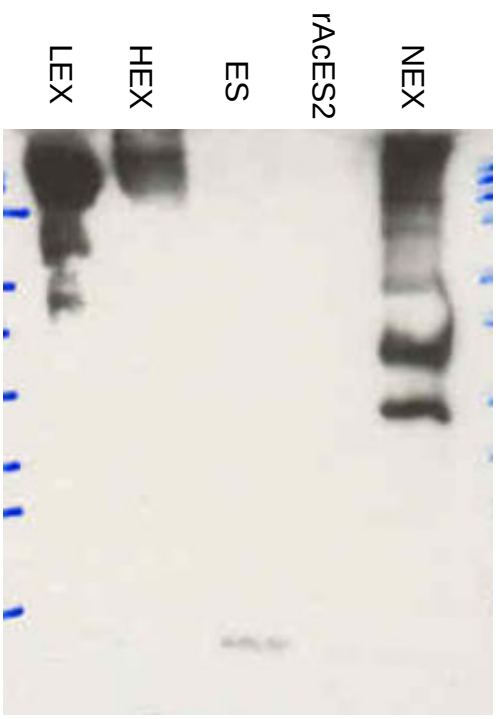

Figure 17. Western blot of *A. ceylanicum* antigens probed against Ecuadorian (*Necator* infected) endemic serum. Immunoreactivity is detected to HEX, LEX and NEX but not to ES or rAcES2.

These results show that while pooled sera from *Necator* endemic area recognizes (IgG) whole worm homogenates from both species (HEX, NEX, and LEX), there was no reactivity of Ecuadorian serum against *Ancylostoma ceylanicum* excretory-secretory products, in particular pure ES and rAceES-2. We further explored this result as a potential mode of distinguishing between endemic areas by hookworm species endemic to the area (see below).

ELISA Screening

One hundred and sixty four serum samples were screened individually using ELISA to quantify immunoreactivity against our panel of *A. ceylanicum* antigens. Below are schematic bar graphs representing each of the screening studies completed.

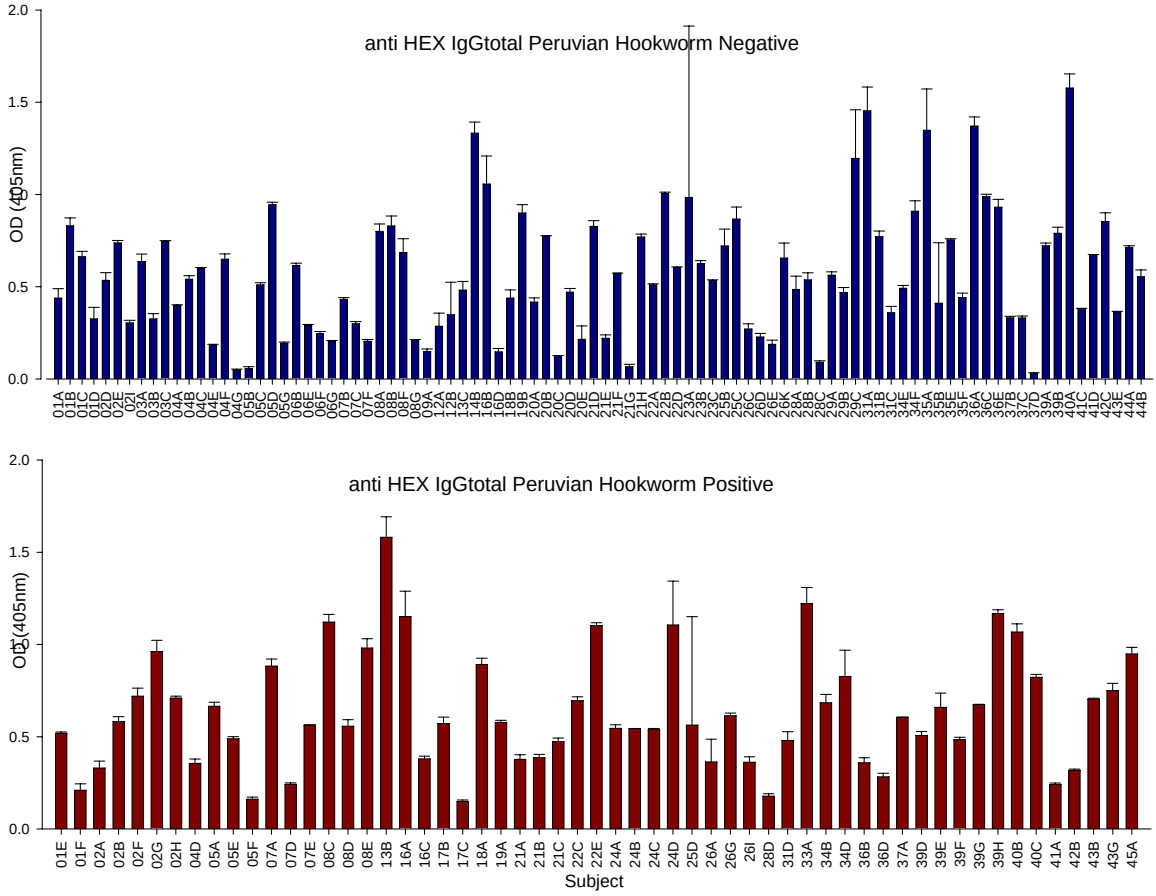

Figure 18. Screening ELISA to evaluate each individual subject's total IgG response to HEX antigen. Subjects are grouped by hookworm infection status based on fecal microscopy . X-axis corresponds to subject identification number and Y-axis to antibody response as measured by optical density (OD) at 405nm.

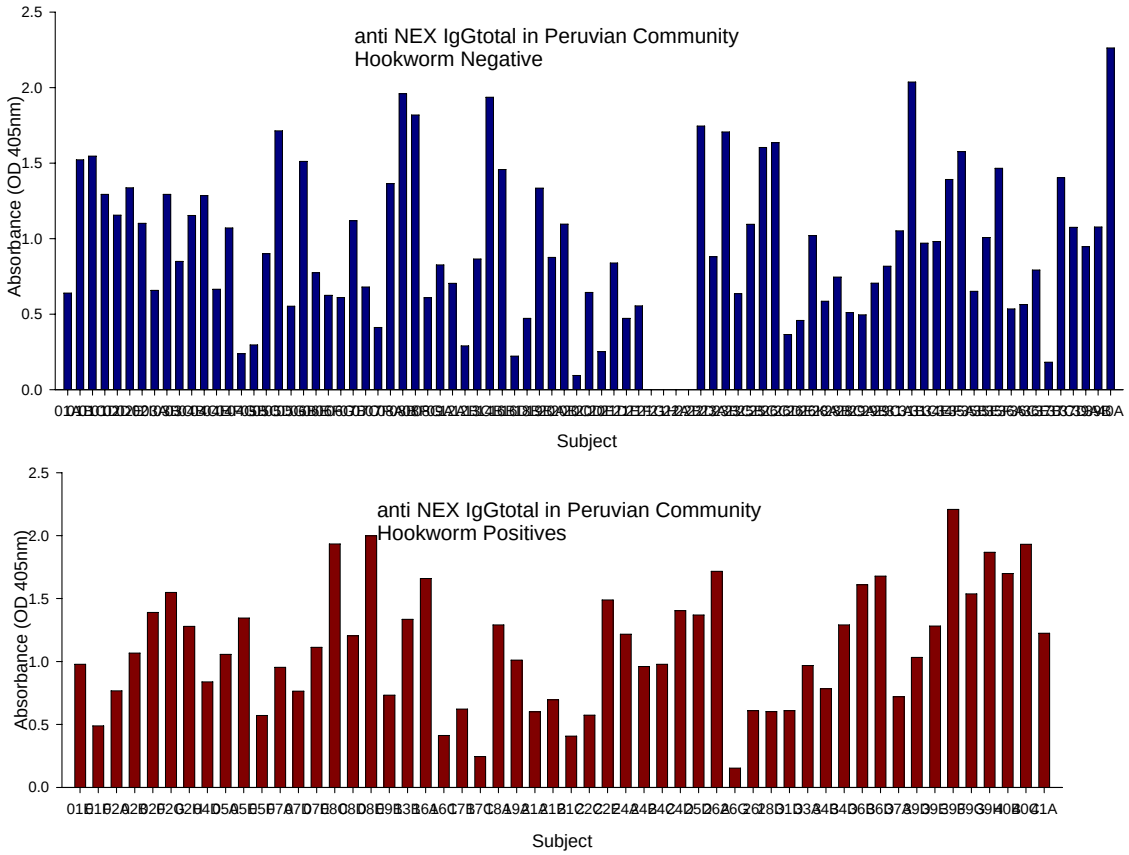

Figure 19. Screening ELISA to evaluate each individual subject's total IgG response to NEX antigen. Subjects are grouped by hookworm infection status based on fecal microscopy. X-axis corresponds to subject identification and Y-axis to antibody response as measured by optical density (OD) at 405 nm.

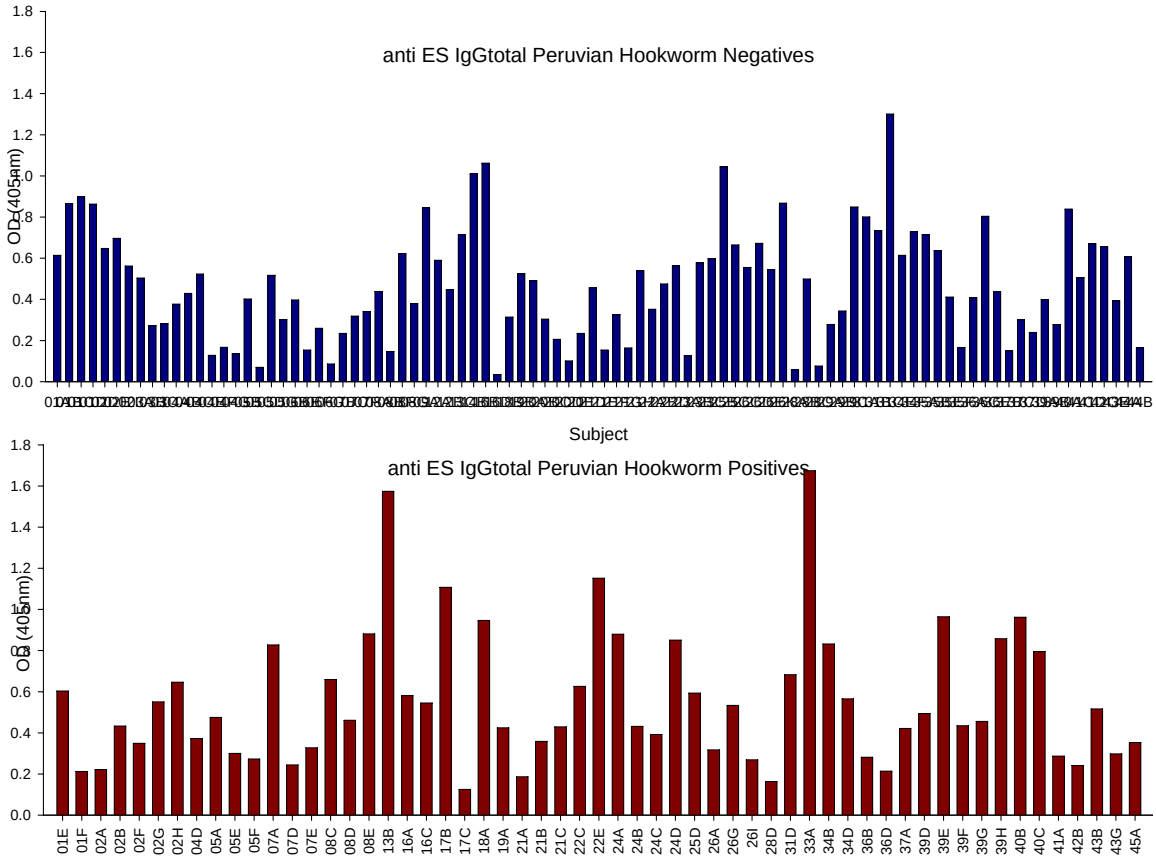

Figure 20. Screening ELISA to evaluate each individual subject's total IgG response to *A. ceylanicum* ES antigen. Subjects are grouped by hookworm infection status based on fecal microscopy. X-axis corresponds to subject identification and Y-axis to antibody response as measured by optical density (OD) at 405nm.

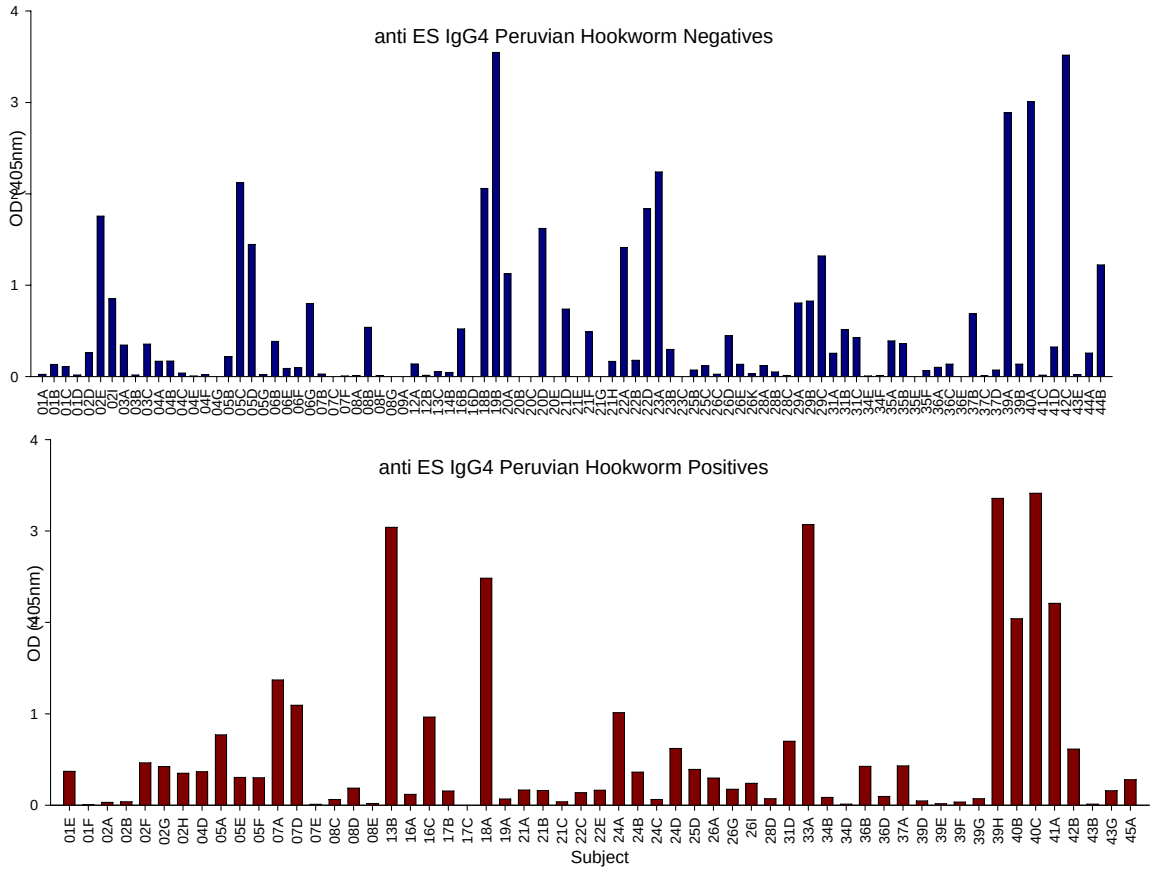

Figure 21. Screening ELISA to evaluate each individual subject's total IgG4 response to *A. ceylanicum* ES antigen. Subjects are grouped by hookworm infection status . X-axis corresponds to subject identification and Y-axis to antibody response as measured by optical density (OD) at 405nm.

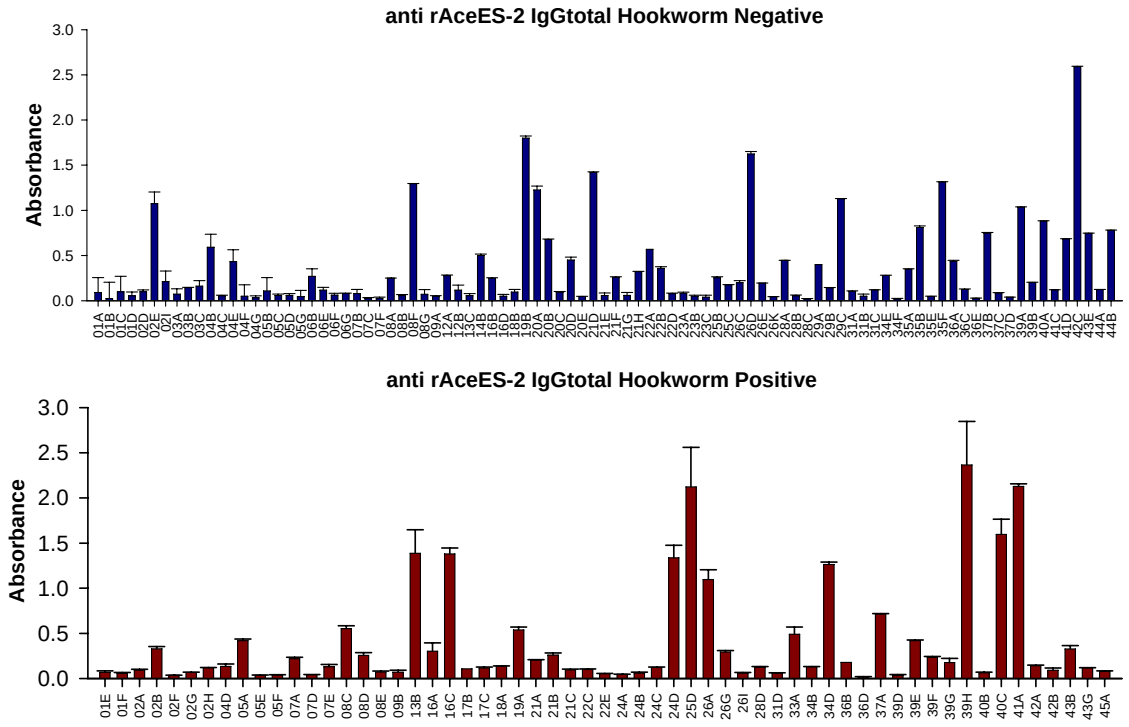

Figure 22. Screening ELISA to evaluate each individual subject's total IgG response to rAce-ES-2 antigen. Subjects are grouped by hookworm infection status . X-axis corresponds to subject identification and Y-axis to antibody response as measured by optical density (OD) at 405nm.

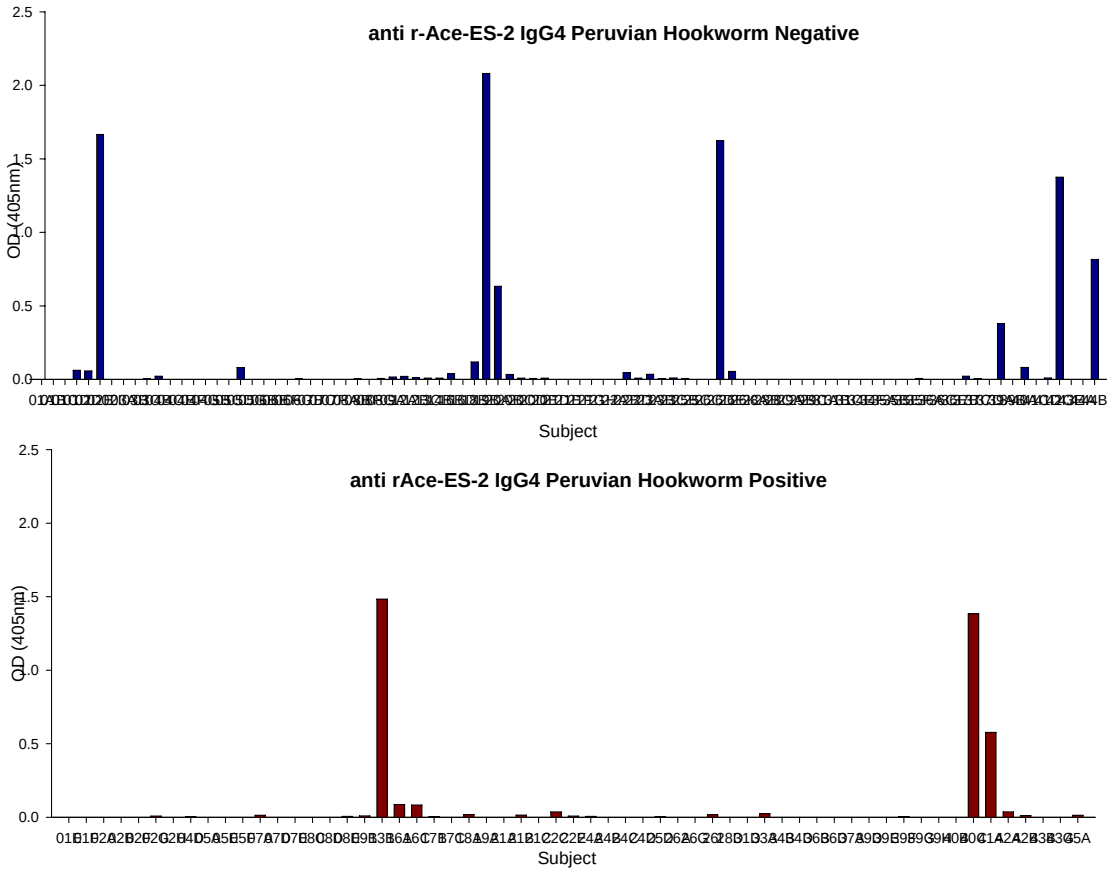

Figure 23. Screening ELISA to evaluate each individual subject's IgG4 response to rAce-ES-2 antigen. Subjects are grouped by hookworm infection status . X-axis corresponds to subject identification and Y-axis to antibody response as measured by optical density (OD) at 405nm.

These data demonstrate, as expected, a large range of antibody responses against hookworm antigens. We analyzed this ELISA data using bivariate analysis in order to identify study subject characteristics that correlated with antibody responses.

Bivariate Analysis of Antibody Responses (mean OD)

In terms of demographic data, a positive correlation between age and antibody responses to most antigens was found, meaning that immunoreactivity as measured by OD increased. However, no positive correlation was found between age and anti-rAceES-2 IgG4 or anti-ES IgGtotal responses.

Antibody	**Spearmann's Correlation Coefficient by Age**	**P value**
α HEX IgGt	0.34	0.0005
α NEX IgGt	0.204	0.0577
α ES IgGt	0.09	0.3313
α ES IgG4	0.344	0.0005
α rAcES-2 IgGt	0.3546	0.0003
α rAcES-2 IgG4	0.0944	0.3501

Table 12. Antibody response correlated to age using Spearmann's Correlation test.

We also used bivariate analysis to assess antibody response to our panel of antigens and hookworm infection status with the following results.

Antibody	**HW fecal positive (+/- SEM)**	**HW fecal negative (+/- SEM)**	**P value**
α HEX IgGt	0.62	0.57	0.65
α NEX IgGt	1.05 (.07)	1.02(.06)	0.089
α ES IgGt	0.52	0.48	0.45
α ES IgG4			0.25
α rAceES-2 IgGt	0.41	0.34	0.65
α rAceES-2 IgG4	0.66	1.00	0.80

Table 13. Antibody response analyzed as a function of hookworm infection status.

In addition, because IgG4 responses were not necessarily normally distributed, a logarithmic transformation was performed on these data, in particular α rAceES-2 IgG4 responses, and analyzed using parametric methodology. Hookworm negative individuals as determined solely by fecal sedimentation trended towards lower α rAceES-2 IgG4 responses than hookworm positive individuals (p= 0.1).

In order to find potentially synergistic relationships between immune responses to combinations of our antigens, we examined correlations between various immune responses.

Antibody 1	Antibody 2	Spearmann's Correlation Coefficient	P value
α HEX IgGt	α NEX IgGt	.4382	<0.0001
	α ES IgGt	.7775	<0.0001
	α ES IgG4	.3225	0.001
α NEX IgGt	α ES IgGt	.3488	0.0009
	α ES IgG4	.1680	0.1178
α ES IgGt	α ES IgG4	.3923	<0.0001
α rAcES-2 IgGt	α HEX IgGt	.2407	0.0153
	α NEX IgGt	.0976	0.3655
	α ES IgGt	.2082	0.0367
	α ES IgG4	.4640	<0.0001
	α rAcES-2 IgG4	.3551	.0003
α rAcES-2 IgG4	α HEX IgGt	.0335	.7397
	α NEX IgGt	.0089	.9345
	α ES IgGt	.0384	.7028
	α ES IgG4	.4220	<0.0001

Table 14. Spearmann correlation between two different antibodies.

This analysis revealed that antibody responses to whole worm or larval homogenates (HEX, NEX, and LEX) were positively correlated. Additionally, even total IgG responses to excretory-secretory (ES) protein was positively correlated to responses to HEX, LEX, and NEX. However, anti-ES IgG4 did not correlate to anti-NEX total IgG responses. Interestingly, analysis of responses to our recombinant protein shows that total IgG responses to this antigen did not correlate with response to *Necator* derived NEX, but did correlate to responses *Anyclostoma* derived HEX and ES (although the correlation to anti ES IgG4 was much more significant than to anti ES total IgG). Finally, anti-rAce-ES-2 IgG4 responses were only positively correlated to anti ES IgG4 responses.

Immunologic Crossreactivity

To assess potential immunologic cross-reactivity between serum from individuals infected with other parasites and their immune responses to hookworm antigens, we conducted simple bivariate analysis. We found that *Strongyloides* infected individuals exhibited a significantly higher anti ES IgGtotal response than negatives (p= 0.04).

Antibody	AL fecal positive (+/- SEM)	AL fecal negative (+/- SEM)	P value
α HEX IgGt	0.58 (.05)	0.61 (.03)	.688
α NEX IgGt	1.03 (.055)	1.05 (.091)	.902
α ES IgGt	0.49 (.03)	0.51 (.04)	.716

Table 15. Antibody responses by *Ascaris lumbricoides* infection status

Antibody	SS fecal positive (+/- SEM)	SS fecal negative (+/- SEM)	P value
α HEX IgGt	0.58 (.05)	0.61 (.03)	.688
α NEX IgGt	1.03 (.11)	1.03 (.05)	.976
α ES IgGt	0.61 (.09)	0.47 (.02)	.04

Table 16. Antibody responses by *Strongyloides stercoralis* infection status

Antibody	TT fecal positive (+/- SEM)	TT fecal negative (+/- SEM)	P value
α HEX IgGt	0.65 (.07)	0.58 (.03)	.381
α NEX IgGt	1.07 (.07)	0.99 (.06)	.400
α ES IgGt	0.47 (.04)	0.50 (.03)	.489

Table 17. Antibody responses by *Trichuris trichiura* infection status

Antibody	GL fecal positive (+/- SEM)	GL fecal negative (+/- SEM)	P value
α HEX IgGt	0.62 (.23)	0.59 (.03)	.858
α NEX IgGt	0.52 (.31)	1.05 (.05)	.07
α ES IgGt	0.50 (.15)	0.49 (.02)	.956

Table 18. Antibody responses by *Giardia lamblia* infection status

Species Specific Immune Responses

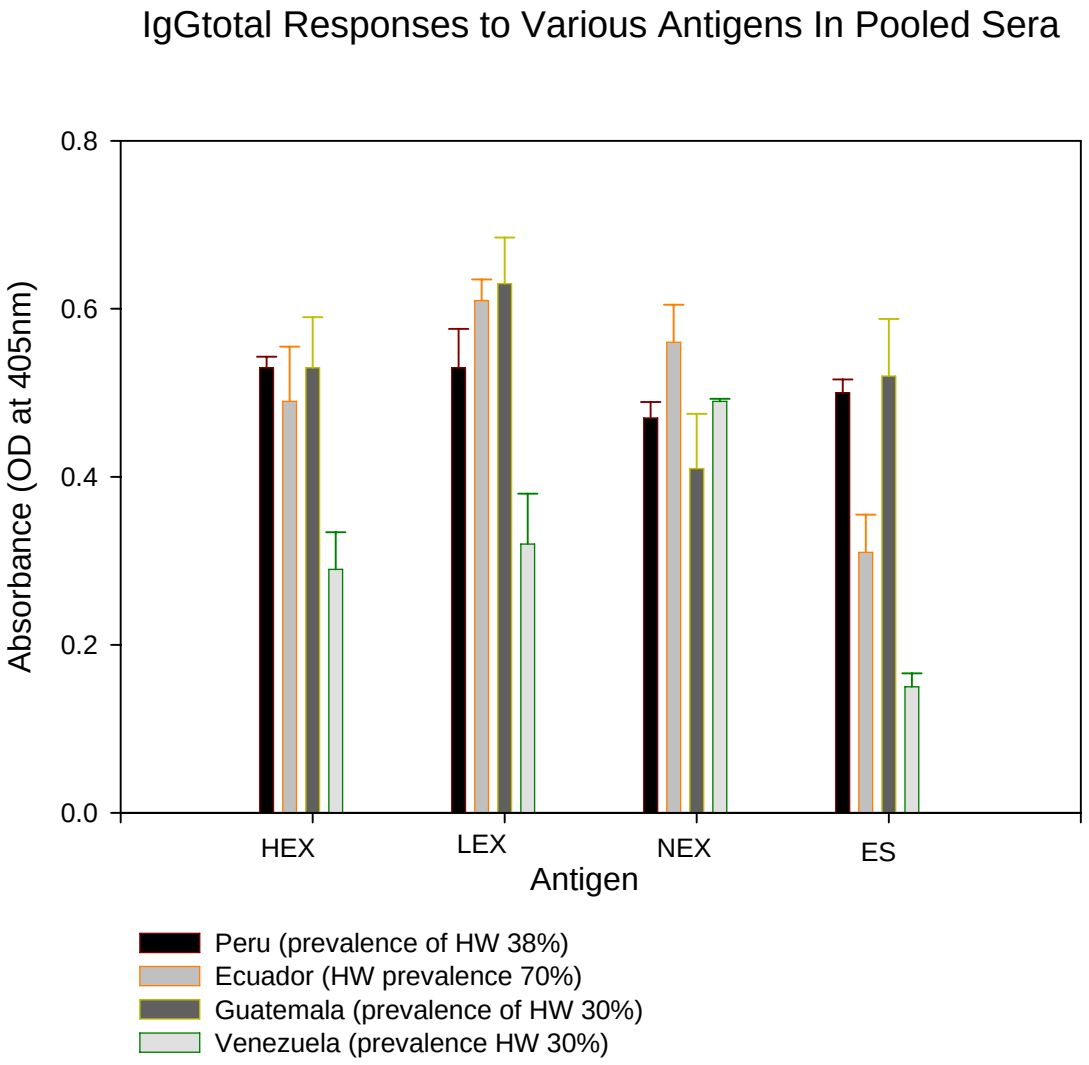

Figure 24. IgGtotal responses to various antigens in pooled sera from endemic communities. Prevalence rates for infection were ascertained by micrsocopy of stool samples at the time of stool collection. Estimated hookworm prevalence in each community is described in the legend.

In addition, to elucidate the relative immune responsiveness of pooled serum from other endemic areas, we carried out ELISAs against our panel of antigens. Immune responses to HEX, LEX, and NEX were no different among geographic groups with the exception of Venezuelan sera, which showed globally decreased antibody levels by

ELISA. The results show that the IgG4 immune responses to r-AceES-2 was higher in Peruvian serum than in other endemic areas. Among the communities from which pooled sera was tested, Peru is the only one in which *Ancylostoma duodenale* has been identified, with the other all being endemic for *Necator americanus*. These data suggest that the IgG4 responses to rAce-ES-2 are specific for *Anyclostoma* exposure.

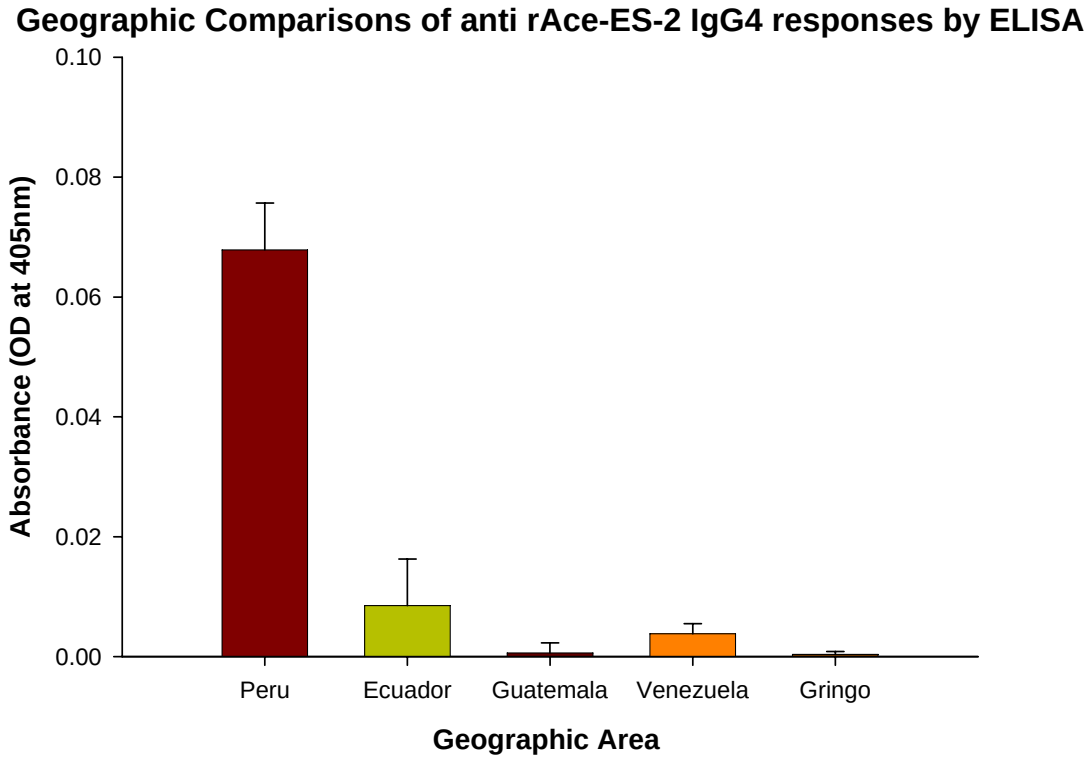

Table 25. Geographic comparisons of IgG4 responses to rAceES-2 between South American regions endemic for hookworm.

Discussion

The major findings of this project include:

1. This is the first report of *Ancylostoma duodenale* infection in the Peruvian Amazon.
2. Sera from hookworm infected subjects react strongly with protein antigens from the laboratory strain *A. ceylanicum*.
3. Serum IgG4 antibodies to the hookworm secretory protein AceES-2 are specific for *A. duodenale* infection.
4. Fecal PCR is more sensitive for detecting hookworm infection than standard microscopy (preliminary).

Epidemiology

One of the aims of this study was to characterize the prevalence of hookworm and other soil transmitted nematode (STN) infections in an isolated Peruvian community. Our survey and fecal microscopy data showed that 98% of the study subjects were infected with at least one intestinal parasite. As we hypothesized, infection with *Ascaris lumbricoides* was the most common, followed by *Trichuris trichiura* infection. Previous pilot studies in this particular region of the Peruvian Amazon have assumed hookworm prevalence rates of less than 10% (MSK, RG, personal communication). However, our data suggest that in this rural endemic area hookworm burden is higher than expected, with a prevalence rate approaching 40%. There are several possible reasons for the discrepancies in original thoughts about hookworm prevalence. First, many of these pilot studies were conducted in semi-rural or urban centers where periodic de-worming is

common and public health posts are more accessible. Risk factors such as socioeconomic status and profession in more central, urban areas do not favor infection. Finally, based on what is known about environmental conditions necessary for hookworm infection, the soil and water conditions surrounding the village of Tarapoto may be more conducive to hookworm development, creating a suitable microenvironment for infection. To better address the question of microenvironments, a larger scale study would need to be conducted, perhaps incorporating the use of Geographic Information System (GIS) technology.

Multiple parasitic infections were also found to be quite common in this population, as has been described for other STN endemic areas (Hotez 2001). In this study cohort approximately 80% of subjects were infected with at least two parasites. Additionally, virtually all hookworm positive individuals were co-infected with another parasite, the most common of which was *Ascaris*.

Risk Factors

The mean age of hookworm infected individuals, based on fecal microscopy, in this study cohort is 27.28 years as opposed to 23.03 years in hookworm negative individuals ($p < 0.05$). This significant difference corroborates previous epidemiologic work suggesting that hookworm prevalence increases with age (Bethony 2002). Many helminth infections show a straightforward relationship between infection status and age. For example, diseases such as ascariasis, trichuriasis, and schistosomiasis show peaks in intensity of infection during childhood. This may represent the development of protective immunity in childhood after early exposure to the parasite. In contrast,

hookworm intensity increases with age in many studies with a plateau in adulthood (Quinnell 2004). This finding has led to the hypotheses that hookworm may somehow modulate the immune response to prevent the development of protective immunity or may in fact may suppress the immune response completely (Maizels 2003). Our results corroborate this hypothesis, and work in Dr. Cappello's laboratory has demonstrated a significant suppression of cellular immune responses in animals infected with the hookworm *A. ceylanicium*.

Several measures of socioeconomic status have been used in epidemiologic studies which address soil transmitted nematode infection (Hotez 2001). As mentioned earlier, this category of risk factors include income, education, and occupation. In our study cohort, those who self-identified as farmers were more likely to be hookworm positive than hookworm negative ($p= 0.02$). There were no statistically significant differences between hookworm positive and negative groups in terms of educational background. However, there was a tendency for more hookworm negative individuals to be under school age (less than 5 years) than hookworm positive individuals. Although education may have a role in preventing hookworm infection by teaching proper sanitation and improving quality of life, it is more likely that this finding is simply another way of correlating age with hookworm infection.

Sanitation is an equivocal risk factor for hookworm infection when controlled for other factors such as socioeconomic status. However, our study shows that hookworm positive individuals had less access to a latrine and were more likely to use a neighboring field for excrement elimination compared to those in the hookworm negative group ($p = 0.05$). Although these results are similar to data from other endemic communities, there

are limitations in correlating demographic data at such as basic level with hookworm infection. First, it has been suggested that other measures of socioeconomic status are more useful indicators because they take into account the intermingling of a number of factors. Although other more descriptive factors, such as number of rooms in a home and purchasing power parity (PPP) are often good indicators of socioeconomic status, we did not collect these data but for this study.

Medical History and Clinical Correlates

As part of the epidemiologic and health survey administered to each study participant medical histories pertaining to intestinal parasite infection were taken. The most surprising result from analysis was the degree to which this group's access to health care is limited. The majority of the population (greater than 80%) had not been to see a health professional (doctor, nurse, public health worker or health post advocate) in over one year. The majority common chronic diseases went undiagnosed, although people were able to find nonspecific therapies such as anti-inflammatory agents and antibiotics for acute infections. Despite this lack of access to care, the majority of the population (approximately 70%) had previously received antihelminthic therapy in the form of albendazole or mebendazole. Although deworming campaigns as recommended by the World Health Organization do not reach this part of the Peruvian Amazon, I soon learned that several non-profit groups and missionary groups will typically bring treatment to this particular village and others like it once every 2 years.

In terms of hookworm associated clinical syndromes, although symptoms were often non-specific, a greater number of hookworm positive individuals complained of

abdominal pain and discomfort than those who were hookworm negative. In addition, hookworm positive individuals were more likely to use medicinal plants for treating common ailments such as presume parasitic infections. One possible explanation would be a higher association between those who do not have access to care (and take medicinal plants) could be those who have other risk factors for hookworm infection. However, multivariate analysis to assess the relationship between these variables did not show a significant association.

Anti-inflammatory medications, particularly ibuprofen and acetaminophen, are commonly used in this population for almost any condition. Twenty-two percent of hookworm fecal microscopy negative individuals were taking some form of a non-steroidal anti-inflammatory during the study, while only 5% of hookworm positive individuals were doing the same (p 0.02). While one explanation for this finding could be that those who are actively taking medication may have greater access to care and antihelminthic agents, the role of anti-inflammatory compounds and the immune response deserves some consideration.

The prevalence of anemia, as measured by blood hematocrit, was also evaluated in this study. We hypothesized that hookworm positive individuals would be more likely to be anemic than hookworm negative individuals based on the pathophysiology of the infection and the blood-feeding nature of the worms. However, there was no correlation between hookworm infection status and hematocrit. Infection with *Trichuris trichiura*, however, predisposed to a lower hematocrit in infected individuals. This finding is interesting as whipworm is known to cause colonic bleeding and inflammation/colitis, two potential mechanisms through which trichuriasis may cause anemia.

Hookworms and Co-infection

Because of the variety of effects of hookworm infection on the immune system, one natural sequelae of hookworm infection may be to effect the immune response other infections, notably HIV/AIDS, malaria, asthma, tuberculosis, bacterial infections and other helminthes. Positive associations between hookworm infection and other helminthes such as *Ascaris lumbricoides* and *Trichuris trichiura* have been widely described (Quinnell 2004). Our results support a positive association between *Ascaris lumbricoides* and hookworm infection, with 82% of those infected by hookworm also having *Ascaris* infection. Most immunoepidemiologists in the field believe that these effects are somewhat due to similar risk factors for co-infection, and some evidence exists to indicate that the immunosuppressive effects of hookworm infection may predispose to infection with other soil-transmitted nematodes (STNs).

Because of the high burden of disease and overlapping epidemiologic considerations especially in resource-poor settings, malaria and STN co-infection has become a major field of research interest. It is estimated that over 30% of the world's population is infected with malaria and/or parasitic helminthes (Mwangi 2006). Our results indicate that co-infection with malaria is extremely prevalent in this cohort. With more than 12% of the study population infected with malaria, co-infection with *Plasmodium* species may play a role in pathophysiology of disease. In particular, we noticed an interesting trend in the distribution of malaria species among our hookworm positive and negative groups. Hookworm negative individuals co-infected with malaria tended to be infected by *P. falciparum* while hookworm positive individuals co-infected with malaria tend to harbor *P. vivax* infection. In particular, the effects on infection by

one on immune responsiveness and infectivity of the other pathogen requires further study.

The literature in this field suggests that co-infection may be common because of similar risk factors and pathogen/vector geography. Immunoepidemiologic data on co-infection is equivocal (Mwangi 2006). Interestingly, co-infection with malaria and filarial nematodes appears to down-regulate the immune response to hookworm (Quinnell 2004). Based on competing upregulation of either Th1 or Th2 cell-mediated immune responses, it is hypothesized that most helminth infections suppress anti-malaria immune and that antihelminthic treatment may even increase the risk of contracting malaria (Mwangi 2006). However, the majority of these studies evaluate all soil transmitted nematodes without respect to species. As hookworm immunology advances to further explore the Th1 versus Th2 immunomodulatory patterns of hookworm infection, a better understanding of the mutual effect that malaria and hookworm have on each other will be possible.

Hookworm Speciation

As described earlier, accurate diagnosis of hookworm infection remains a critical component of effective epidemiologic surveillance. Current field based diagnostics rely on detection of nematode eggs in the feces but cannot differentiate between *N. americanus* and *A. duodenale* in this manner alone. Further copro-culture techniques are required to hatch L3 larvae from eggs in a controlled environment and then to further microscopically examine the larvae to distinguish between species. This method requires a skilled practitioner for identification of eggs in feces and is quite labor intensive. Given

that speciation is extremely important in conducting epidemiologic surveillance of hookworm distribution, the emergence of antihelminthic resistant organisms and clinical pathophysiologic correlates, the need for accurate and efficient means of distinguishing hookworm species within endemic communities is great.

Several groups have successfully developed polymerase chain reaction (PCR) based assays which use specific genetic markers to distinguish between species of hookworm. Cytochrome oxidase genes (Zhan 2001) and internal-transcribed spacer sequences (ITS-1 and ITS-2) from ribosomal DNA (rDNA) have proven useful markers for species specific identification of a variety of parasitic nematodes including hookworm (de Gruijter 2005). Even more useful than conducting PCR assays of larval DNA is to bypass corporo-culture and amplify genomic DNA from fecal extracts. Since all participants in this study and in other epidemiological cohorts provide stool samples for diagnosis this assay represents the most complete data set we have for speciation.

Using the ITS-1 and ITS-2 primers for gDNA amplification from fecal extracts, we were able to distinguish between *A. duodenale* and/or *N. americanus* in infected individuals within our study cohort. We confirm results of our previous pilot studies from this endemic environment to show that *A. duodenale* is present in the Peruvian Amazon in addition to the previously assumed presence of *N. americanus*. This result carries much importance in terms of assessing how we evaluate immunological responses to disease, design possible vaccines, and evaluate control mechanisms.

After gel electrophoresis, several amplicons corresponding in size to either *A. duodenale* or *N. americanus* were sequenced by the Keck facility at Yale University. Results from representative samples revealed that those amplicons thought to be *A.*

duodenale were 99-100% homologous to known ITS sequences and amplicons identified as *Necator americanus* were 98-100% homologous to known ITS sequences for that species. A few amplicons sent from hookworm positive samples were slightly less homologous within the ITS region and likely represent polymorphisms in the sequence among isolates from Peru. These polymorphisms suggest that sequence deviation may occur in even the smallest of endemic communities. Additionally, these sequence differences may be suggestive of inter- and intra-species variations that may have relevance to not only immunogenicity, but bendimidazole resistance. This finding provides a basis for future studies in population genetics within endemic communities and possible functions that such polymorphisms may have on species specific modes of disease pathogenesis and resistance to treatment.

Preliminary data, which is not included in this thesis, confirm that hookworm genomic DNA can be amplified from fecal samples from individuals who are negative for disease by conventional methods, i.e., microscopy. This result suggests that perhaps current detection methods not as sensitive as the molecular techniques. We are currently re-analyzing the demographic/epidemiologic data comparing infected versus non-infected individuals based on fecal PCR results. In addition, we are also comparing fecal PCR results with a novel hookworm antigen detection assay developed by Dr. Cappello's laboratory (Bungiro 2005).

Immunology of Hookworm Infection

This study shows that a laboratory hamster model for *A. ceylanicum* may be successfully used to study the immunoepidemiology of human hookworm infection. Data from western blot and ELISA analysis show that human serum from an endemic community recognize *A. ceylanicum* antigens. This finding is particularly significant because it is represents the first evidence that antigens from a repeatedly passaged laboratory parasite strain, *A. ceylanicum*, can be exploited to study the immunoepidemiology of human hookworm infection. The use of *A. ceylanicum* antigen based immunoassays thus provides great potential for carrying out larger scale studies in endemic communities. Moreover, the immunoepidemiologic data we present further validates the hamster model of *A. ceylanicum* as an effective means of characterizing human hookworm pathogenesis.

Review of Hookworm Immunology

Due to its life cycle and interactions with multiple organ systems including skin, lungs, and GI mucosa, hookworm infection elicits complex immune responses. A long history of hookworm immunology exists, with the earliest studies being observations of immunoprecipitates around the oral orifice of the hookworm (Sarles 1938, Otto 1942). Before beginning a discussion of the immunology of hookworm disease in particular, it will be useful to build our understanding of the immunology of parasitic diseases in general. A typical response to helminth parasites, and in particular those parasites that are too large to be phagocytosed includes a strong Th2 immune response with concomitant IgE and eosinophilia. This response is rooted in both traditional arms of the

immune system, namely the humoral system and the cell-mediated system. Experimental human hookworm studies, though limited, have shown similar findings. One study involving an adult human volunteer infected with 50 *Necator* L3 showed eosinophilia, and elevated hookworm specific IgE and IgG responses that gradually rose during primary infection and peaked after secondary re-infection (Wright 2005). Interestingly, as Behnke notes, despite its cultivation of a robust immune response upon initial infection, this response does not develop into protective immunity in humans (Behnke 1991).

Cell mediated immune responses

Recently cell-mediated immune responses in natural hookworm infection have been reviewed. This is a relatively new field of hookworm immunology and several surprising discoveries have been made. Proliferative lymphocyte responses to hookworm antigens have been shown in several experimental models and in naturally infected individuals. The intensity of the proliferative response in all studies is variable, although analysis of cytokine responses clearly show a mixed Th1/Th2 response as determined by upregulation of both Th1 and Th2 related cytokines (IFN-γ and IL-12 and IL-4, IL-5, and IL-13 respectively) (Quinnell 2004). This occurs in *Trichuris trichiura* infection as well, although most other intestinal nematodes exhibit a strongly Th2-biased response. Although our study did not address questions of cell-mediated immunity, the acquisition of human sera from Peru as well as banked sera from other parts of the world may be used in the future to examine cytokine profiles in our study cohort.

Humoral responses

Initial cutaneous infection with hookworm larvae provides the first contact between parasite and host and represents the first opportunity for the immune system to react. The larval sheath antigens present in larval sheath fluid have previously been shown to be highly immunogenic and stage specific. This fluid and its antigens are present in the cast off sheath the larvae leaves behind. Its immunogenicity is thought to be protective of the larvae itself as the immune system is drawn to the antigens in the old sheath while leaving the larvae itself alone. Immunoblot data from our study indicates that both actively hookworm infected and uninfected (but presumably previously exposed) individuals have total IgG immunoreactivity against larval protein extracts (LEX). We hypothesized the immune responsiveness to LEX is probably a non-specific marker of exposure but not a marker of disease and thus we did not pursue further quantitative analysis by ELISA.

Humoral responses to adult hookworm antigens are similarly robust. One study of individuals in Papua New Guinea showed an elevated IgG1, IgG4 and IgE response against Necator americanus adult ES (excretory-secretory extract) (Pritchard 1990). These results indicate that like immune responses to other parasites, anti-hookworm immune responses are dominated by Th2 mediated immunoglobulins affected by IL-4. This study further proposes that total IgG and IgE against adult Necator ES may be a good indicator of current infection and efficacy of chemotherapy (Pritchard 1995).

Several studies of hookworm infection in endemic communities show globally elevated responses from all immunoglobulin subclasses against adult hookworm antigens (Quinnell 2004). There is also established cross-reactivity between anti hookworm

antibodies and other helminthes (Loukas 1996). IgG4 and IgE responses have been shown to be more species specific although total polyclonal IgE is also globally elevated in helminth infection (Quinnell 2004). These studies guided our decision to screen sera from each individual for total IgG and IgG4 immune response to a panel of *A. ceylanicum* antigens. Work is currently underway to optimize the conditions for an IgE ELISA using these serum samples.

Immunomodulation

In order to survive for long periods of time in the host as is common for hookworm, they have developed several defense mechanisms including immunosuppression, immunomodulation, and immune inactivation. Several candidates for immunomodulatory function have been identified in hookworm, although the function of these molecules is still being determined. Much study has been done to evaluate the immunomodulatory and immunosuppressive effects of helminth infections on the host immune response. In particular, several findings have led to a rethinking of the hygiene hypothesis which has been used to describe the relationship between TH2 and TH1 responses. The hygiene hypothesis suggests that TH2 cell mediated allergic responses could be mitigated by exposure to TH1 inducing microorganisms. As stated this theory suggests that TH1 and TH2 responses are in competition with each other. In the case of helminth infections, it has been suggested that the strong TH2 responses they elicit may suppress pathological TH2 mediated inflammatory diseases (Maizels 2003).

Immunoepidemiology of Peruvian Subjects

Individual screening ELISA showed several interesting findings. First, as we hypothesized, total IgG responses to adult hookworm antigens were globally strong. Because total IgG is a non-specific marker of previous exposure to disease related antigens, we had hypothesized total IgG may not be different among hookworm positive and negative groups within endemic areas, but that IgG subtype analysis may show correlation between infection status and antibody levels. Our results show that total IgG responses to *Necator americanus* extracts (NEX*)* tended to be higher among hookworm positive individuals than negative individuals, although this result only trended towards significance ($p = 0.089$) (Table 14). After testing antibody levels for normal distribution among our cohort, we found that IgG4 levels tended to be non-parametric and thus evaluated them by Kruskal-Wallis analysis. Logarithmic transformation showed that hookworm negative individuals as determined by fecal microscopy trended towards lower anti rAceES-2 IgG4 levels than hookworm positive individuals. This suggests that IgG4 may be a useful marker for current infection.

By evaluating correlation between antibody levels and age, we found that age and antibody levels were typically positively correlated, which confirms previous studies showing that hookworm prevalence and intensity, as determined by fecal microscopy, increase with age. However, anti-rAceES-2 IgG4 and anti-ES total IgG levels were not correlated with age. Immune responses to helminth infections generally increase with age but plateau in early adulthood. This trend signifies the development of humoral immune responses in the face of repeated re-infection (Woolhouse 1992). In experimental infections eosinophilia increases initially after infection but decreases upon reinfection as

do lymphoproliferative/cell-mediated responses. In studies of endemic communities with *Necator* infection most IgG subtypes as well as IgE and eosinophilia increased with host age. However, in other studies IgG1, IgM, IgA, IgE and IgD decreased with host age suggesting some level of immunosuppression (Quinnell 2004).

One of the few immunoepidemiological studies of hookworm studied stage-specific antigens and the immune response to these antigens as a function of host age (Quinnell 1995). This study found a negative correlation between *Necator* anti-ES (Excretory Secretory protein) IgG levels and pretreatment worm burden. To indicate the presence of protective immunity antibody responses should increase with age corresponding to increasing levels of exposure to the parasite. In contrast, once a certain critical threshold of exposure and antibody production has been reached, a decline in worm burden with an increase in antibody levels may occur in adults, indicating protective immunity. Another possibility, however, given our understanding of potentially immunomodulatory effects of hookworm on the host, is that higher worm burdens with low antibody responses may be a reflection of an immunosuppressive effect. As Quinnell and colleagues speculate in their 1995 article, protective antibody titers may not necessarily increase over time if immunological memory is short. Our age related data suggests that antibody responses to *A. ceylanicum* ES and the recombinant AceES-2 may be indicative of immunosuppressive effects of these antigens on the host immune response in light of the lack of correlation with age. It is interesting to speculate that a function of AceES-2 may be to modulate the host immune response, which is consistent with data from the animal model (Bungiro 2004).

We tested correlations between antibody responses to our panel of *A. ceylanicum* antigens to assess synergistic relationships. Antibody responses to LEX, HEX, and NEX were positively correlated with each other. Total IgG responses to ES were also positively correlated to anti-LEX, HEX, and NEX IgG. However, anti-ES IgG4 levels did not correlate with anti-NEX IgG levels nor did immune responses to the recombinant AceES-2 antigen. Given that rAceES-2 is *Ancylostoma* derived and NEX is *Necator* derived, this lack of correlation may indicate that rAceES-2 is a species specific marker of infection. We are currently using molecular techniques to speciate all of our samples (including hookworm fecal microscopy negative) in order to assess whether or not individuals with current infection with either *A. duodenale* or *N. americanus* have different levels of anti-rAceES-2 total IgG, IgG4 or anti-NEX IgG. I hypothesize that given the high rates of reinfection known to exist in endemic communities only anti-rAceES-2 IgG4 may predict species specific infection.

Molecular Immuno-Diagnosis

Given our results suggesting that antibody responses to rAceES-2 may be useful as a marker to diagnose current infection and perhaps to distinguish between hookworm species in endemic communities, we evaluated this antigen in between endemic communities. Immunoblot analysis comparing pooled sera from Peru, where we have confirmed that both *Ancylostoma* and *Necator* species are endemic, with sera from Ecuador, where only *Necator* infection has been reported, show difference in immune response to rAceES-2. To more specifically gauge the species specificity of differential immune responses to rAceES-2, we undertook ELISAs comparing total IgG and IgG4

immune responses to a panel of *A. ceylanicum* antigens from a variety of South American regions endemic for hookworm. Total IgG levels against whole hookworm homogenates (LEX, HEX, NEX) and excretory-secretory protein (ES) were not significantly different in between endemic communities tested. IgG4 levels were virtually identical against the same panel of antigens. However, IgG4 responses to rAceES-2 were significantly higher in the Peruvian sera than in sera from Ecuador, Guatemala, or Venezuela. Based on field studies we know that hookworm prevalence in of these geographic regions is approximately the same, between 30-40%. However, speciation data from these sites indicate that only *Necator americanus* making the high IgG4 signal in Peruvian pooled serum indicative of a species-specific response ($p=0.02$).

The role of IgG4 in hookworm infection is potentially critical to developing new immunodiagnostic assays. IgG4 is not involved in complement fixation but does take an active role in mast cell sensitization and immediate hypersensitivity reactions. In hookworm infections, as described above, where mast cell degranulation and eosinophilia play a large role in fighting the parasite, IgG4 may be very important. IgG4 is also thought to be reflective of chronic infection, with alternatively activated macrophages activating a Th2 cellular pathway that allows for isotype switching from IgE formation to IgG4. In endemic communities thus, IgG4 may be a reflection of chronic hookworm exposure and infection.

A study of 120 individuals in rural Zimbabwae first assessed the role of antigen specific isotype responses as a marker for active hookworm infection (Palmer, DR et al 1996). This study used enzyme linked immunosorbant assays (ELISA) to characterize immune responses specific to whole hookworm (*Necator* species) homogenates (NOG)

and correlate these responses with age, sex and infection intensity of the study population. Results indicated a modest positive correlation between total IgG, IgG2, IgG3, IgG4 and IgE and egg counts. IgG4, in addition, was significantly associated with high intensity hookworm infection, making it a potential candidate for developing diagnostic techniques relevant to large scale epidemiological studies (Palmer 1996).

Conclusion

Several experts in the field of hookworm immunology agree (Loukas 2001) that novel developments in this field will likely occur with a better understanding of responses in endemic communities, considered the field of immunoepidemiology. Immunoepidemiology is a branch of immunology concerned with the distribution of immune responses, infections, and epidemiologic characteristics in populations and the relationship that each of these factors has on the others. In hookworm disease, the goals of immunoepidemiologic studies are to better understand the epidemiologic factors affecting the immune response and in particular the formation or lack of formation of protective immunity, immunosuppression, and impact on co-infections.

The ultimate goal of this work is to utilize reagents developed in a laboratory model of hookworm infection to characterize immunoepidemiologic aspects of human disease pathogenesis. Data from human immunoepidemiologic studies are often difficult to interpret due to confounding factors such as previous infection, co-infections, cross reaction with similar antigens or epitopes from other species of parasites, previous treatment history, and environmental factors. By demonstrating substantial overlapping immune responses that characterize human and animal hookworm infections, these data further validate the use of the hamster model as a valuable tool for investigating fundamental aspects of the host-parasite relationship.

Finally, our observation that anti-rAceES-2 IgG4 levels in hookworm positive individuals trended higher as compared to hookworm negative subjects, along with the significantly higher IgG4 immune response to rAceES-2 in areas endemic for *Ancylostoma* suggests rAceES-2 may have potential as a useful diagnostic reagent to

define species distribution in endemic communities. Future work will be directed at more fully characterizing the epidemiology of hookworm infection and disease using these novel molecular and immunologic methods.

References

Albonico M, Wright V, Bickle Q. Molecular analysis of the beta-tubulin gene of human hookworm as a basis for possible benzimidazole resistance on Pemba Island. Mol Biochem Parasitol. 2004;134(2):281-4.

Behnke JM. Immunology in Human parasitic diseases: hookworm infection (Gilles HM and Ball PAJ eds). 1991;93-95. Elsevier, Amsterdam, The Netherlands.

Bethony J, Chen J, Lin S, Xiao S, Zhan B, Li S, Xue H, Xing F, Humphries D, Yan W, Chen G, Foster V, Hawdon JM, Hotez PJ. Emerging patterns of hookworm infection: influence of aging on the intensity of Necator infection in Hainan Province, People's Republic of China. Clin Infect Dis. 2002;35(11):1336-44.

Bouree P, David P, Basset D, Coco O, Beavais B, David-Julien MC, Pouqnet *A.* [Epidemiological survey of intestinal parasitosis in Peruvian Amazonia]. Bull Soc Pathol Exot Filiales 1984;77(5):690-8 in French.

Brooker S, Bethony J, Hotez PJ. Human hookworm infection in the 21st century. Adv Parasitology 2004;58:17-288.

Bundy DAP. Epidemiology and transmission of intestinal helminthes. In: Farthing, MJG, Keusch GT, Wakelin D, eds. Enteric infection 2: intestinal helminthes. New York: Chapman & Hall Medical, 1995:5-24.

Bungiro RD, Harrison LM, Cappello MC. *Ancylostoma ceylanicum* Excretory-secretory protein 1 molecular cloning. Molecular and Biochemical Parasitology. 119:147-151 2002).

Bungiro RD, Solis CV, Harrison LM, Cappello MC. Purification and molecular cloning of and immunization with *Anyclostoma ceylanicum* excretory-secretory protein-2, an immunoreactive protein produced by adult hookworm. Infection and Immunity. 2004;42:2203-2213.

Bungiro RD, Cappello MC. Detection of excretory/secretory corproantigens in experimental hookworm infection. American Journal of Tropical Medicine and Hygiene. 2005;73:915-920.).

Cappello M, Bungiro RD, Harrison LM, Bischof LJ, Griffitts JS, Barrows BD, Aroian RV. A purified Bacillus thuringiensis crystal protein with therapeutic activity against the hookworm parasite Ancylostoma ceylanicum. Proc Natl Acad Sci U S A. 2006;103(41):15154-9.

Centers for Disease Control and Surveillance. Diagnostic Procedures for Parasitic Diseases DPDx.

http://www.dpd.cdc.gov/dpdx/html/DiagnosticProcedures.asp?body=Frames/DiagnosticProcedures/body_dp_bloodprocess.htm. Accessed 11.20.2006.

Chandler AC. Hookworm disease, its distribution, biology, epidemiology, pathology, diagnosis, treatment and control. New York: Macmiillan. 1929.

Chu D, Bungiro RD, Ibanez M, Harrison LM, Campodonico E, Jones BF, Mieszczanek J, Kuzmic P, Cappello M. Molecular characterization of Ancylostoma ceylanicum Kunitz-type serine protease inhibitor: evidence for a role in hookworm-associated growth delay. Infect Immun. 2004;72(4):2214-21.

Crompton DW. The public health importance of hookworm disease. Parasitology. 2000;121:S39-50.

de Gruijter JM, van Lieshout L, Gasser RB, Verweij JJ, Brienen EA, Ziem JB, Yelifari L, Polderman AM. Polymerase chain reaction-based differential diagnosis of Ancylostoma duodenale and Necator americanus infections in humans in northern Ghana. Trop Med Int Health 2005;10:574-580.

de Silva NR, Brooker S, Hotez PJ, Montresor A, Engels D, Savioli L. Soil-transmitted helminth infections: updating the global picture. Trends in Parasitology. 2003;19:547-551.

Egido JM, De Diego, et al. The prevalence of enteropathy due to strongyloidiasis in Puerto Maldonado (Peruvian Amazon). Braz J Infect Disease 2001;5:119-123.

Hawdon JM. Differentiation between the human hookworms *Anyclostoma duodenale* and *Necator americanus* using PCR-FRLP. J Parasitology 1996;82:642-647.

Hotez PJ, Bethony J, Bottazzi ME, Brooker S, Diemert D, Loukas A. New technologies for the control fo human hookworm infection. Trends in Parasitology. 2006;22:327-331.

Hotez PJ, Ferris MT. The anti-poverty vaccines. Vaccine 2006;24:5787-5799.

Hotez PJ, Brooker S, Bethony JM, Bottazzi ME, Loukas A, Xiao S. Hookworm infection. New England Journal of Medicine. 2004;351:799-807.

Jones BF, Hotez PJ. Molecular cloning and characterization of Ac-mep-1, a developmentally regulated gut luminal metalloendopeptidase from adult Ancylostoma caninum hookworms. Mol Biochem Parasitol. 2002 ;119(1):107-16.

Labiano-Abello N. Canese J, Velazquez ME, Hawdon JM, Wilson MI, Hotez PJ. Epidemiology of hookworm infection in Itagua, Paraguay: a cross sectional study. Mem Inst Oswaldo Cruz, Rio de Janeiro. 1999;94:583-586.

Larocque R, Casapia M, Gotuzzo E, Gyorkos TW. Relationship between intensity of soil-transmitted helminth infections and anemia during pregnancy. Am J Trop Med Hyg 2005;73:783-9.

Loukas A, Opdebeeck J, Croese J, Prociv P. Immunologlobulin G subclass antibodies against excretory/secretory antigens of *Ancylostoma caninum* in human enteric infections. American Journal of Tropical Medicine and hygiene. 1996;54:672-676.

Loukas A, Prociv P. Immune responses in hookworm infection. Clinical Microbiology reviews. 2001;14:689-703.

Maizels RM, Yazadanbaksh M. Immune regulation by helminth parasites: cellular and molecular mechanisms. Nature Rev Immunology 2003;3:733-744.).

Melvin DM, Brooke MM, et al "Laboratory procedures for the diagnosis of intestinal parasites. Atlanta, GA. 1982.

Miranda RA, Xavier FB, Menezes RC. [Intestinal parasitism in a Parakana indigenous community in southwestern Para State, Brazil] Cad Saude Publica. 1998 Jul;14(3):507-11. Portuguese.

Monti JR, Chilton NB, Qian BZ, Gasser RB. Specific amplification of Necator americanus or Ancylostoma duodenale DNA by PCR using markers in ITS-1 rDNA, and its implications. Mol Cell Probes. 1998;12(2):71-8.

Mwangi TW, Bethony JM, and Brooker S. malaria and helminth interactions in humans: an epidemiological viewpoint. Annals of Trop Med and Parasitology 2006;100:551-570.

Otto GF, Schugam NJ, Groover ME. A precipitin reaction resulting from *Necator americanus* larvae in serum from hookworm-infected individuals. Proc Helminthol Soc. Wash 1942;9:25-26.

Palmer DR, Bradley M, Bundy DA. IgG4 responses to antigens of adult Necator americanus: potential for use in large-scale epidemiological studies. Bull World Health Organ. 1996;74(4):381-6.

Pan American Sanitary Bureau. Health in the Americas. Washington D.C. 1998.

Pritchard DI, Quinnell RJ, Slater AF, McKean PG, Dale D, Raiko A, and Keymer AE. Epidemiology and immunology of *Necator americanus* infection in a community in Papua New Guinea: humoral responses to excretory-secretory and cuticular collagen antigens. Parasitology 1990;100:317-326.

Pritchard DI, Quinnell RJ, Walsh EA. Immunity in humans to *Necator americanus:* IgE, parasite weight and fecundity. Parasite Immunology 1995;17:71-75.

Quinnell RJ, Bethony J, Pritchard DI. The immunoepidemiology of human hookworm infection. Parasite Immunology. 2004;26:443-454.

Quinnell RJ, Pritchard I, Raiko A, Brown AP, Shaw M. Immune responses in human necatoriasis: association between interleukin-5 responses and resistance to reinfection. Journal of Infectious Diseases 2004;190:430-438.

Quinnell RJ, Woolhouse MEJ, Walsh EA, Pritchard DI. Immunoepidemiology of human necatoriasis: correlations between antibody response and parasite burdens. Parasite Immunology. 1995;17:313-318.

Sarles MP. The in vitro action of immune rat serum on the nematode, *Nippostrongylus muris.* JID. 1938;62:337-348.

Woolhouse MEJ. A theoretical framework for the immunoepidemiology of helminth infection. Parasite Immunology 1992;14:563-578).

World Health Organization Global Malaria Programme. WHO guidelines for the treatment of malaria. 2006. Geneva Switzerland).

Wright V, Bickle Q. Immune responses following experimental hookworm infection. Clinical and Experimental Immunology. 2005;142:398-403.

Utzinger J, Muller I, Vounatsou P, Singer BH, N'Goran EK, Tanner M. Random spatial distribution of Schistosoma mansoni and hookworm infections among schoolchildren within a single village. J Parasitology 2003 Aug;89(4):686-92.

Xiao SH, Hui-Ming W, Tanner M, Utzinger J, Chong W. Tribendimidine: a promising, safe and broad-spectrum anthelmintic agent from China. Acta Trop. 2005;94(1):1-14.

Yori PP, Kosek M, Gilman RH, et al. Seroepidemiology of Strongyloidiasis in the Peruvian Amazon. Am J Trop Med and Hygiene. 74(1):2006:97-102.

Zhan B, Li T, Xiao S, Zheng F, Hawdon JM. Species-specific identification of human hookworms by PCR of the mitochondrial cytochrome oxidase I gene. J Parasitology. 2001;87:1227-1229.