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ABSTRACT

Genotyping *Ascaris* nematodes from diverse geographic locations

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Introduction:
There is some debate as to whether the pig intestinal giant roundworm, *Ascaris suum*, is a distinct species from *Ascaris lumbricoides*, the human intestinal giant roundworm. Over 800 million people world-wide are infected with this disease. The large amounts of morbidity and economic loss that this disease causes makes it important to continue to add evidence in respect to the speciation debate, as it will influence future public health interventions. The goal of this study is to identify the relationship between the ascaris genetic material (mitochondrial and nuclear) with the location or the host of infection (humans or pigs) in global ascaris populations.
Methods:
142 unique samples used in this survey of *Ascaris* spp. were collected by various programs and researchers. The samples had four main origins: Guatemala, Solomon Islands, Slovakia, and the state of Georgia. All of the samples were fecal specimens preserved in ethanol with the exception of the positive control, which was a whole worm preserved in ethanol. DNA extraction was used to isolate the genetic material, while a novel PCR assay developed after identifying descriptive loci in the genome of *Ascaris* spp. worms was used to amplify the selected targets. These targets include COXI 6A, COXI 7A, and TR3. Geneious was utilized to analyze the sequenced amplicons.
Results:
The dendrograms for the amplified targets CoxI 6A (76 sequences), CoxI 7A (23 sequences), and TR3 (18 sequences) were generated to show the clusters of samples of all geographic origins. In all three loci, different geographic areas seem to cluster more closely together than samples from different geographic areas. For example, when utilizing the COXI 6A locus all ten detected Solomon Island samples clustered closely together, as did the four detected Slovakian samples. In the COXI 7A locus, the Slovakian samples clustered together. While in the TR3 locus the Slovakian samples clustered together as did the Solomon Island samples. The samples from Georgia and Guatemala frequently were clustered together. In each loci, human and pig ascaris clustered closely together. For example, in the COXI 6A locus, a Georgian pig sample (P2) clustered with a large group of Solomon Island human samples. When utilizing the COXI 7A locus, one Guatemala (G66) human sample clustered with one Georgian pig sample (P3) as well as the pig positive control. In the analysis of the TR3 locus, it was found that a Georgian (P2) pig sample clustered with three Guatemala (G31, G38, G46) human samples as well as the pig positive control.
Discussion:
The results of this study suggest that this method can genotype soil-transmitted helminth infections. It also suggests that it can group them in accordance with their geography and by host, whether human or pig. While the clusters were helpful in illustrating the origin of the

infection, they also give evidence to the thought that there may be exchange of genetic information between certain populations. This is shown in the clustering of samples from different geographic locations together, as well as the clustering of samples from different hosts together.

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I. Literature review

Introduction

It has been debated by the wider scientific community whether *Ascaris lumbricoides*, commonly referred to as the giant intestinal roundworm, and the pig variant, *Ascaris suum*, are independent species (1). This parasitic worm is responsible for the infection of 800 million people globally, most of which are in low income countries (2, 3). There are no morphological differences between *A. lumbricoides* and *A. suum*, yet most genetic analyses show some subtle differences between the two worms (4). There are differing reports as to how different the genomes of these worms really are; however a significant number of reported genetic measures place them within the acceptable range to consider them as one species (4). The general consensus for researchers is to assume that these two organisms are different species until there is definitive proof that they are genetically related enough to propose a one species definition (4). Until this time, continuing acceptance of the Linnaean and Goeze names for the worms, *A. lumbricoides* in 1758 and *A. suum* in 1782 respectively (5), is recommended. Further complicating research on this soil-transmitted helminth (STH) is the fact that there are limited methods to accurately identify the genetic makeup of an infection (i.e., to genotype infections) (6). It is difficult to genotype infections because the recombination in sexual reproduction increases the genetic diversity in the population (6). The ability to reproduce sexually complicates the process of connecting one infection to another epidemiologically, as this type of reproduction increases genetic change from generation to generation (6).

The *Ascaris* life cycle

The *Ascaris* life cycle is similar to those of other STH diseases, meaning that there is no need for an intermediate host between the environment and the definitive host, whether it be a

pig, human, or another mammal (1). The basic lifecycle is that the definitive host ingests the infective eggs through the fecal-oral route of transmission; there is no intermediate host stage for this parasite. Once the eggs reach the small intestine, they hatch into the larval form of the organism. These larvae then penetrate the intestinal wall and travel to the heart and lungs, after which they migrate up the esophagus and are swallowed to mature in the host's stomach (1) (Figure 1). After maturation, the adult female worm has the ability to produce 27 million eggs during the course of an infection (1).

There is evidence to suggest that either human or pig ascaris may be able to infect either human or pig host. Experimental infections in the lab have shown the ability of ascaris eggs collected from human feces to infect pigs (2,7,8). Evidence has been collected that indicates that humans have been infected with pig-type ascaris (2,7,8). In settings with both human- and pig-type ascaris, infections of the pig variant in humans is extremely rare, which causes the question to be raised as to why that is (9). Each variant of human or pig ascaris may have specific adaptations that make them more adept at infecting their original host (9). This makes it possible for the human-type ascaris to outcompete the pig-type ascaris (9). In contrast, in situations where there is only pig-type ascaris, there are instances of human infection with the pig-type ascaris (9).

Figure 1: Ascaris life cycle

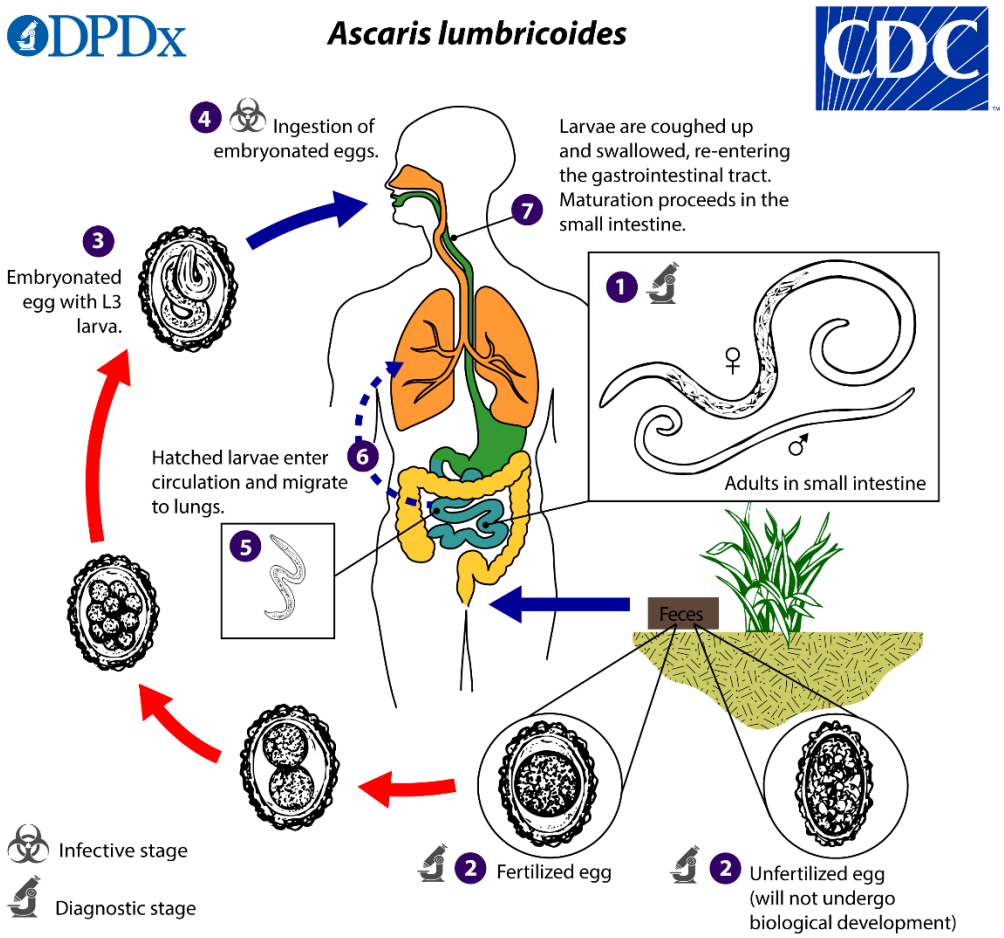

Image source: https://www.cdc.gov/dpdx/ascariasis/index.html

Ascariasis

The disease caused by *A. lumbricoides*, or ascariasis, is characterized by symptoms that include an obstructed and even perforated bowel, malnutrition, and mental development delays in humans (10). The severity of disease is burden-dependent, meaning the symptoms will become worse as the number of worms increase (1). As the number of worms increase, the likelihood of obstructed or perforated bowel becomes higher (1). It is thought that the migration of larva from the bowel to the heart and lungs damages the intestine and causes the malabsorption of nutrients, causing malnutrition, that eventually leads to developmental delays (1, 8, 11). The burden of disease is greatest in the five to fifteen-year-old age range, and infection is associated with both

physical and intellectual developmental stunting in this group (8, 11). The most pronounced effect of this disease is through the large amount of morbidity it causes, as opposed to mortality. It is estimated that there are 1.82 million disability-adjusted life-years (DALY's) lost every year due to *Ascaris* infections (12). There are an estimated 900 million children at high risk of contracting ascariasis, which would lead to massive loss of productivity and economic growth for their communities (3).

Diagnosis

Diagnosis of ascariasis is most commonly performed microscopically, by identifying eggs in feces (13). In some cases, adult worms may be passed per rectum and can be easily identified based on their distinct morphologic features and size, which may be up to 30 centimeters in length (14). Tests based on other methods such as the Polymerase Chain Reaction (15, 16) are also available, and these are discussed in the sections below. However, when it comes to differentiating *A. suum* from *A. lumbricoides*, molecular testing is required as these worms are thought to be morphologically identical. While differentiating between these two worms is inconsequential from a clinical perspective (i.e., treatment of the patient and management of the infection will not change), this is an important question from a public health perspective (discussed in greater depth in later sections of this thesis).

There are currently three explanations put forth in the debate regarding the true identity of these two worms: both *Ascaris suum* and *Ascaris lumbricoides* are valid species and there is limited (or no) exchange of genetics between the two; both are distinct species existing in host-specific parasitic niches with some cross-infections and occasionally hybridization; and finally, they are actually a single species (6). There have been several studies showing that after intervention and

monitoring, the human population that was cleared of infection becomes re-infected with pig-type *Ascaris*, which indicates some close relation between the two types of worms (8). Several attempts have been made to answer which explanation is correct utilizing genetic tests mentioned in detail later in this thesis, but the results and conclusions have varied from study to study (2, 5, 6, 17). The need for a genetic test that helps to answer the question of how related these two worms are. It will allow for better interventions and for improved diagnosis.

Available diagnostic tests

There are several different classes of diagnostic tests to diagnose ascariasis, which rely on antibody response, detection of parasitic structures such as eggs or larva, or detection of genetic material. Enzyme-linked immunosorbent assay tests detect antibodies generated by the host in response to an infection. As such, these tests are useful in identifying antibodies to an infection, but circulating antibodies are indicative of exposure and not necessarily an active infection (1) (Table 1). This is one of many reasons that these tests are used for populational studies, not individual diagnosis. Another reason that antibody-detecting tests are not suitable for this function is that the antibodies generated in response to a case of ascariasis are highly cross reactive with antibodies for other STH infections (1). For an understanding of whether an individual infection is current, microscopic examination of stool for evidence of the parasite (i.e., *Ascaris* eggs), or molecular tests that detect the parasites DNA are required. Microscopic detection of structures in feces is the current gold-standard for diagnosis of ascariasis (10). Diagnostic tests based on PCR are generally considered highly sensitive and specific and have been shown to be useful in genetic studies (18). The cost of utilizing such a test, which will limit the use of an assay developed around it, is a major

disadvantage of all PCR-based assays (Table 2). This is particularly important due to the fact that STH infections occur most frequently in low income settings (13).

Figure 2: Unstained wet-mounts and photograph of *Ascaris* life stages

Image source: https://www.cdc.gov/dpdx/ascariasis/index.html

From left to right: Fertilized egg with early stage embryos. Larva hatching from egg. Adult *Ascaris lumbricoides.*

Table 1: Available diagnostic tests for *Ascaris* characterization and detection

Type of Test	Test Name	Description
Microscopy	Kato-Katz	A thick smear preparation techniques that allows detection and counting of eggs. Due to its low costs and simplicity, WHO recommends as the gold standard of infection identification. However this test is suspected to be less effective at catching infections in breastfeeding infants due to watery stools as well as only catching 50% of infections that are low intensity.
	FLOTAC®	An egg flotation and counting technique that is more sensitive than the Kato-Katz test, but requires a centrifuge.
	Mini-FLOTAC®	An egg flotation and counting technique that is as sensitive as the Kato-Katz test, but more expensive. It does not require the expensive tests that the FLOTAC test does.
	McMaster Egg Counting Technique	An egg counting technique that is simple and gives an indication of drug efficacy. It, however, does not have the sensitivity of Kato-Katz
Serological	IgG and IGM antibody detection (i.e., ELISA)	Many commercial tests are on the market target these human antibodies, but the sensitivity of these tests and the duration after certain stages of the life cycle of *Ascaris* at which these antibodies can be detected are questioned. They may not be indicative of an active infection and could indicate a past exposure.
	Antigen (i.e., antigen capture ELISA)	This type of test is not very common for *Ascaris* spp, the targets are too similar to other helminths, and so cross-reactivity is an issue. Can be indicative of an active or recent infection as the antigens detected are produced by the living worm.
Molecular	Conventional PCR	Identifies the presence of *Ascaris* DNA. It is very specific and reproducible.
	qPCR	Also detects DNA but is a variation of PCR that allows users to quantify the infection. Extremely sensitive.
	Multiplex PCR	Allows for the identification of multiple parasites in one reaction, but issues with competitive inhibition in multiply infected samples can lead to poor sensitivity.

Note: Table describes each of the available test for detecting and evaluating *Ascaris* infections (10).

Table 2: Advantages and disadvantages of existing *Ascaris* tests

Test Type	Pros	Cons	Notes
Microscopy	Cost-efficient, quick, easily performed by trained volunteer	Requires relatively high burden of infection, differences in egg detection day to day, and non-random distribution of eggs in stool cause fluctuations in the egg counts	Will require a trained technician, which may be hard to find in certain areas of the world. This method is used to find both the egg and larval stage of the organism
Serology	Cheaper and faster than molecular methods. More sensitive that microscopy	*Ascaris* epitopes that are most commonly targeted for serology have a high frequency of cross reactivity and does not necessarily indicate current infection. This could lead to an over estimation of the number of people needing treatment	It is thought that antibody levels correlate with worm burden but has not gathered sufficient evidence.
Molecular	Highly sensitive and specific, with tests becoming more affordable. Can detect low intensities of infection, and rarely cross-react with other helminths	Costly, time-consuming, hard to do in field settings, and need a trained technician.	Although these tests are by far the most sensitive and specific, the cost of the equipment and reagents may make other tests more cost-effective on the population scale.

Note: Table shows the advantages and disadvantages of the broad classes of tests available for *Ascaris* spp. diagnosis (10).

Target markers for parasite detection

Targets used in parasite detection require several different characteristics when compared to those used for genotyping. This distinction must be considered when choosing sites to amplify by PCR. For parasite detection, a marker within the genome that has few polymorphisms and has

a low rate of evolution is preferred (15). These markers are sought out because they do not change substantially across individuals of the same species but do differentiate between highly related species. The use of such tests is to identify infections of specific species of parasites as opposed to classifying parasitic individuals within a species (19).

Mitochondrial genome

The mitochondrial Cytb gene was used as a target for diagnostic PCR, to identify *Ascaris* infections in samples, in conjunction with the nuclear marker of ITS-1. With the use of both nuclear and mitochondrial targets, the study by Leles et al. was able to identify 100% of all *Ascaris* positive samples (19). COX-1 has also been used in detection assays, as this mitochondrial locus is attractive to researchers for genetic studies since there are reportedly a limited number of haplotypes and high numbers of copies to amplify (15). This study's result indicates that this marker does have some diagnostic utility.

Nuclear genome

The Internal Transcribed Spacer 1 (ITS-1) locus, which can be found on the nuclear genome, has been extensively used as a diagnostic PCR target for *Ascaris lumbricoides* detection assays, with one study showing just two genotypes across a geographically distinct population in Brazil (19). As mentioned before, the study by Leles et al. found that their chosen markers identified 100% of *Ascaris* positive samples, with the ability to detect the DNA equivalent of one isolated egg or four eggs, when the genetic information is extracted from stool (19).

Target markers for genotyping

Mitochondrial genome

The mitochondrial (Mt) genome of both *Ascaris suum* and *Ascaris lumbricoides* are almost identical in sequence in structure, which strengthened the argument in favor of the one species hypothesis (5,7). However, parts of the Mt genome have a rapid evolutionary rate which is a feature that is conducive to differentiating strains within a population. Other features that make the Mt genome a good genotyping target are its known haploidy, putative selective neutrality, and the fact that it does not go through genetic recombination (5, 7, 20). A study comparing the analysis of nuclear and mitochondrial genomes showed that the latter is more reliable at differentiating between species with recent common ancestors (14). It should also be noted that choosing a target within the mitochondrial genome alone only reflects the genetic distribution of females within the population, not the whole population (6). This is due to the Mt genome being inherited maternally. In a recent study, researchers found that the two 'species' of *Ascaris* can differ by 2% in their mitochondrial genomes (5). This finding was interesting when compared to the result that the average difference between worms of the same species, but from different geographical areas, can differ by up to 1.5% (5). This difference of 2% was so small that the authors of this paper suggest that their data supports the one species hypothesis.

The loci within the mitochondrial genome most commonly used for genotyping include the cyclooxygenase 1 (COX-1) coding region and the NAD-1 coding region, which play several roles in the physiology and chemical pathways of the cell (7). The COX-1 gene is considered a reliable target to elucidate the relationship between *Ascaris suum* and *Ascaris lumbricoides* (5, 7, 20). NAD-1 has not been used as extensively. However, results from a study conducted in China show its potential as another genotyping target, as it has shown the ability to differentiate between highly related species (7).

It should be noted that many of the studies (6, 14) exploring the relationship between *Ascaris suum* and *Ascaris lumbricoides* have either included small numbers of targets, in some cases one (14), or small sample sizes (21) meaning that the results are less generalizable (6). A study done in China on the genetic variation within different geographical locations showed that there was very little gene flow between the human and swine populations of *Ascaris*. A re-analysis of the data from this study by the same research group showed that this initial assumption was incorrect and that there was indeed significant geneflow between *Ascaris* of different hosts (14). This illustrates the dangers of using one marker for the entire study. Incorrect interpretations of the data may lead to incorrect assumptions being made. In this case, the researchers originally analyzed the data from one locus, then reanalyzed the same data in conjunction with other genetic targets. The likelihood of false relationships getting identified goes drastically down as the number of targets go up.

Genotyping targets in the nuclear genome

Recently, the beta-tubulin gene has been used in studies examining the nuclear genome of these worms, but more traditionally, internal transcribed spacer (ITS) regions have been used (7, 8, , 14, 19, 20, 22). There is an ongoing search for more nuclear targets that are better suited for genotyping because there is a danger that markers like the beta-tubulin have a low frequency of polymorphisms (6). The beta-tubulin gene has risen in interest in studies seeking to examine the genetic diversity within *Ascaris* due to the thought that it is involved in the development of resistance to anthelmintic drugs (2). Zuccherato *et al*. conducted a study to assess the amount of resistance to anthelmintic drugs that existed in helminth populations in Brazil and used the beta-tubulin gene as their only marker (21). They found little variation in their study, perhaps due to small sample size or only assessing one target. Due to the minor variability seen in the beta-tubulin

marker, scientists have almost completely abandoned it for the purpose of genotyping. Another target is the ITS region of the nuclear genome that is considered "spacer genes" (7). These regions exist between the genes that encode components of the ribosomes for the cell, and includes the two regions: ITS1 and ITS2, which are most commonly used as nuclear genotyping targets (7). These regions are selected due to the fact that they are conserved enough for highly specific PCR primer design but are also thought to contain some single nucleotide polymorphisms that can differentiate populations. A study from Brazil analyzing the usefulness of utilizing the subregion ITS1 in differentiating *A. suum* and *A. lumbricoides* showed that the intra-individual variability was too great for this locus to be used in such a way (7, 14). Another study used the ITS1 marker as its sole point of interest and evaluated whether *Ascaris suum* and *Ascaris lumbricoides* are one species with host preferences or two interbreeding species. This study carried out by Iniguez et al. suggested that there was a clustering of worms by host using the ITS1 locus (14). There is some worry about ITS2 recently due to its lack of diversity between individuals (14). In separate worms, it was noticed that the ITS2 regions were identical, with absolutely no differences (14). This makes the marker less useful for differentiation of these two populations of worms. Ultimately, there were too many different versions or copies of this locus in a single individual to be able to accurately identify the relationships between populations of worms. Generally, there is no consensus on what the best nuclear targets are for genotyping of *Ascaris* spp.

Microsatellites

Microsatellites are a useful nuclear target that has been widely utilized for genotyping purposes. Microsatellites are essentially small repetitive units of DNA that are dispersed throughout the genome and vary between individuals and populations (7). These genetic markers vary in length between populations and so, can be used to differentiate between genetically isolated

groups. However, there are a few major drawbacks of this method. Microsatellite repeats tend to expand and contract between generations, and these changes can happen spontaneously. Consequently, there is a risk for members of distinct populations to possess an identical microsatellite repeat that is the same length yet arose spontaneously within the two populations. Therefore, common microsatellites can be shared even by distantly related individuals and if this happens, it can cause the estimates for diversity within the population to be underestimated (6). In several studies, these markers have added evidence to the one species position, showing hybridization between the two populations (7). The same study concluded that there seems to be a single interbreeding species with both genetic and physical differences on the population level due to the close contact of humans and pigs, the cross infections and hybridization that the researchers identified, and the molecular data (7). On the other hand, these repeats can mutate quickly, resulting in a target that is potentially too variable to link members of the same population (6).

Methods for assessing population genetic structure

There are many software tools and statistical tests that assess the genetic relationship between populations using a set of sequences from strategically selected genetic loci as input. Statistical/analytical methods for analyzing the genetic relationship between different populations are diverse. A study looking at the molecular epidemiology of *Ascaris* spp. in Brazil utilized the software GeneDoc and DAMBE to edit and analyze their sequences. Using this software, the nucleotide divergence and standard error were calculated using the Kimura-2 (K2P) model (14). The same study used the MEGA v.4 program to synthesize neighbor-joining genetic tree. The K2P model was used in conjunction with bootstrap analysis, only showing bootstrap values ≥50% and considering those ≥80% as strongly related. The Fisher's exact test was also utilized to evaluate the host specificity of the samples (14).

In a separate study conducted in Japan, the software ClustalW was used to align sequenced PCR products. These aligned sequences were then searched on NCBI using the nBLAST tool looking for homology (9). MEGA5 was then used to infer the best model to construct the dendrogram, which was the Neighbor-joining method. A second phylogenetic tree was generated by Bayesian analysis using the HKY-1 model in the BEAST software (9).

A third study looking at samples taken from China and Guatemala showed the analysis of the sequenced PCR products being analyzed through three separate statistical methods. The first method was used to calculate a pairwise analysis of the fixation index (Fst) among the populations and tested for differentiation between each pair of populations using the software Fstat v.2.9.3 (17). Bonferroni correction was applied and Fst values were standardized according to Meirmans (17). Additionally, a neighbor-joining tree using Phylip v.3.66 was used (17). The relationships were assessed using a bootstrap value of 1000. The authors of this paper noted that the first two methods (i.e., those based on the Fst fixation index) relied on *a priori* delineation of populations. The third method that this paper employed was using Bayesian clustering methods to identify populations for the evaluation of relatedness. This allowed for populations to be based upon only their genetic makeup. Two separate software were used to accomplish this, Structure v.2.1 and Baps v.4.14. While using the former, Markov chain Monte Carlo (MCMC) was utilized to produce a posterior probability (PP) (17). The PP was then utilized to construct a neighbor-joining tree using the Phylip v.3.66 software. Baps v.4.14 was utilized to construct a neighbor-joining tree and the Kulback-Leibler divergence matrix was used to estimate the genetic difference between identified clusters (17).

Potential public health impact of identifying the relationship between *A. suum* and *A. lumbricoides*

Interventions for ascariasis often fail due to the primary host (humans) being re-infected by ascaris post treatment. The tool that this thesis is centered around was designed to inform scientists and implementers of public health interventions on how much genetic exchange occurs between populations of worms, which can then be taken into account when designing interventions for this infection. The current World Health Organization (WHO) recommendation for interventions relating to helminth infections is to provide all suspected cases an anthelmintic drug, which removes worm burden but allows reinfection (1). The guidance on livestock treatment is less organized, and most commonly, the disease is caught in the slaughterhouses. In communities in which mass drug administration (MDA) has been carried out, it is thought that pigs may be re-infecting the humans after they have been cleared of the parasite (9, 14). A common public health intervention to reduce the incidence of STH's is the construction of sanitation facilities such as latrines and handwashing stations (1). This intervention does not do anything to contain or separate the other domestic carrier of *Ascaris*, pigs. A danger of the host swapping behavior of this parasite is that it may undergo hybridization, which may confer novel genes (such as resistance or virulence factors), lead to homogenization across the genomes of the entire population, or cause rapid genetic evolution (17). However, this may not be an issue if the two populations are genetically distinct and rarely mix. In this case, certain interventions may not be necessary. It is also a cause of massive economic loss, as it is a morbidity-causing disease (3). This causes those infected to be a drain on their families' or countries' resources and causes families who raise pigs to lose a form of wealth (14).

Study goals and aims

There is a need to develop an assay that can accurately evaluate the genetic diversity within and between populations of *Ascaris suum* and *Ascaris lumbricoides* throughout the globe. This

will allow for researchers to start gathering evidence as to whether pigs need to be included in any future *Ascaris* intervention. Understanding the diversity in and between populations of ascaris will help decide whether pig-type worms are, in fact, re-infecting treated human populations. Ideally, this tool would not require rely on *a priori* delineation of populations (i.e., as the Fst index does), as this information is often not available. The goal of this study is to identify the relationship between the genetic material (mitochondrial and nuclear) with the location or the host of infection (humans or pigs) in global ascaris populations. There are three aims associated with this goal. The first aim is to identify high heterogeneity sites in the genome of ascaris worms and design specific primers for those targets. The second aim is to understand the genetic relationship between *Ascaris* worms from different geographical locations. The third and final aim is to understand the genetic relationship between *Ascaris* worms from human and pig origin.

Significance

Many studies have attempted to assess the genetic relationship between swine and human-infecting populations of *Ascaris*, but this project attempts to build on these studies by increasing the number of targets used for genotyping (the PCR-based assay) to examine the genetic structure of different *Ascaris* populations. The information gleaned from this study will allow for the clustering of *Ascaris* spp. outbreaks without the need for epidemiologically-linked data. The data can further be used to show commonality between individual infections of *Ascaris* and indicate whether geneflow is occurring in the population. The achievement of the goal of this study will also illustrate a new method to characterize sexually reproducing parasites.

(II) Materials and methods

Ethics statement

Collection of fecal samples containing *Ascaris* sp. worms from the Solomon Islands was approved by the Atoifi Adventist Hospital Research Ethics Committee (Appendix A). The collection of fecal samples from individuals in Guatemala was approved by both the ethics committee of the Universidad del Valle de Guatemala and the National Health Ethics Committee of the Ministry of Public Health and Social Assistance of Guatemala (Appendix A). The samples collected from Georgia, Mississippi, and Slovakia did not need ethics approval due to the fact that these samples were collected from the ground and were non-human samples. These areas are already known to be heavily affected by STH organisms and therefor the researchers did not feel that the knowledge of the country of origin would negatively affect those within said countries. The Emory Internal Review Board (IRB) was consulted and it was determined that this project does not require IRB review because it does not meet the definition of research with "human subjects".

Samples

The *Ascaris suum* and *Ascaris lumbricoides* fecal samples were stored in ethanol at -80°C. These samples originated from Guatemala, Solomon Islands, Slovakia, and the state of Georgia in the United States. The samples collected from both Guatemala and the Solomon Islands were all human feces, while the samples from Slovakia were feces collected from dogs. These dog feces samples were collected from two adjacent villages in Central Slovakia where STH infections are known to be common (23). The samples from Georgia were infected pig feces collected from the ground on organic farms. The Slovakian dogs were thought to be passing *Ascaris* eggs following coprophagy of infected human feces. All informed consents were gathered and recorded from those providing the specimens, during which the participants were educated of their rights and what their samples were going to be used for.

PCR primer design

Primers were designed by first downloading two genomes of *A. Lumbricoides* and one genome of *A. suum* from the NCBI database (GB accession number: AMPH00000000.1, AEUI00000000.3, and JN801161.1). The genomic scaffolds were then aligned using the De Novo Alignment tool, which is included as part of the Geneious software package (www.geneious.com). Following alignment, the aligned *Ascaris* genomes were manually scanned using the Geneious alignment viewer for sites within the genomes possessing high heterogeneity. Sites with high SNP density that would fall within PCR amplicons of about 200 base pairs long were particularly sought after in both the nuclear and mitochondrial genomes. While heterogeneity within the amplicon was required so that the assay would have sufficient solving power, it was imperative that the priming sites had few to no SNPs within them. The priming sites had to have this attribute so that the primers would amplify DNA from all genotypes of *Ascaris*. The primers and their target loci can be found in Table 3. In this way, PCR primers were designed from the nuclear and Mt genomes. As by its very nature, the nuclear genome exists within an organism at a much lower copy number than the Mt genome. All nuclear genome targets were amplified using a nested polymerase chain reaction (PCR) as opposed to a traditional PCR. This means that these targets needed two rounds of amplifications, and therefore, two sets of primers as opposed to just one. Utilizing a nested PCR reaction increases the specificity as well as the sensitivity of the amplification. (Table 3)

Table 3: Primer information

Genome	Locus (Alias)	SNPs	Primer Name	Primer Sequence (5'-3')	Amplicon size (Sequence length)	Annealing Temperature (°C)	Amplification success (%)*
Mt	Cox1	6	F1Cox1	GCTAAACCTGGTCTTCTT	118	59	20
			R1Cox1	CAATTACCAAAACCACCAAT			
Mt	Cox1	6	F2Cox1	TGTTGCCTTTGATGTTGG	189	57	85
			R2Cox1	GCAAGATCAACCCTACCAC			
Mt	Cox1	7	F3Cox1	CGTAGTAGTTCTATTTCTTTGG	168	57	67
			R3Cox1	GGTTACCACCAGTCCTAG			
Mt	Cox1	6	F4Cox1	GGTGTGACTTTGTGGTGA	175	57	16
			R4Cox1	CAAACAGAATAAACATCAGG			
Nu	Undefined	8	F-TR1	GAGATTCAGTCCTCGCC	177	58	21
			R-TR1	CAGTTGAAATCTTTAATGCCAT			
			F-TR1-2	TGAGGCAGCGTTCTTG			
			R-TR1-2	TCTAAGAATACTGTGGTCGTG			
Nu	Undefined	5	F-TR2	TGATCACATCGTTGAAGC	162	58	30
			R-TR2	AGAGTATAAGCGGATGAACTA			
			F-TR2-2	CTCGATCTCATTATACTTCCATAG			
			R-TR2-2	CAAGGCACAGTAACGCT			
Nu	Undefined Nuclear region	9	F-TR3	AACTATCAGCTTCCTTAAATTCC	155	58	56
			R-TR3	GTTCCTGAATCGAAGCAAA			
			F-TR3-2	CTGTAGTGGATAGAGATATGAGG			
			R-TR3-2	ACTTGAAGTCATTGCGC			
Nu	Undefined Nuclear region	10	F-TR4	CTTTCGGTTTGATTGACAGA	186	58	53
			R-TR4	GAGATCAAAGTCGTCCCAT			
			F-TR4-2	ACCACGTCAACCTTCATTA			
			R-TR4-2	CACATTTCAGACCGCTTAG			
Nu	Undefined Nuclear region	8	F-TR5	GGATAAAGAGCCCGTATCATAC	171	58	22
			R-TR5	GGGACTTTATCATACCTCAAA			
			F-TR5-2	AGTCTTATTGATGCCATTGAA			
			R-TR5-2	CGCTGGATATCGGTCAAG			
Nu	Undefined Nuclear region	8	F-TR6	CAATCGACATTTAGAGGGGAATT	175	58	12
			R-TR6	TTAGGTTAGTAGGTAGGTTCAACT			
			F-TR6-2	ATGTCTCTTTATGTCTATACGGA			
			R-TR6-2	CGGTCAGGATAGTGGATTC			

*Amplification success is defined as the number of PCR reactions that produced the desired product divided by the number of times that those primers were used.

Isolation of *Ascaris* DNA

The isolation of DNA, both genomic and mitochondrial, from the utilized fecal samples was conducted using the slightly modified DNeasy PowerSoil kit Quick Start protocol. This protocol was modified by replacing the provided "powerbeads", with silica-zirconia beads. This was done due to the toughness of the outer shell of the *Ascaris* spp. eggs. The silica-zirconia beads are better suited to penetrate the shell of the eggs, thus exposing the genetic material. The protocol for the original kit can be found at www.qiagen.com. The extractions were performed on each fecal specimen separately. The same DNA extraction was performed on a whole adult *Ascaris* worm that was preserved in ethanol. This DNA was used as a positive control in the assay.

PCR annealing temperature optimization

Each primer set was entered into the Thermo-Fischer Multiple Primer Analyzer (www.thermofisher.com) to ensure that the primers had no primer-dimer or cross-reactivity to each other (i.e., so that the primers to not extend against each other). This same tool was utilized to determine an appropriate annealing temperature for the primer set. A PCR reaction was prepared using the reaction conditions and temperature cycling conditions described below, though with an annealing temperature gradient centered at the annealing temperature suggested by the Thermo-Fischer Multiple Primer Analyzer (Table 3), along with temperatures ranging at 6 degrees above and below this center, tested at intervals of 2 degrees Celsius. Reactions were spiked with DNA from a female *Ascaris* worm that was available at the Centers for Disease Control and Prevention (CDC) (DNA isolated as described above). Each run was also accompanied by a negative control that included water instead of DNA template. Each of these reactions was subjected to agarose gel electrophoresis on 2% agarose gels stained with ethidium

bromide before visualization of DNA bands. All gels included a 100 bp DNA ladder as a reference for the molecular weight of the resulting PCR products. The annealing temperatures that resulted in the cleanest and most defined bands in the correct position were selected for continued use.

Mitochondrial targets

The mitochondrial genetic sequences identified as good targets for genotyping, based on the number of SNPs within the region and the length of the amplicon, were all varying parts of the COXI gene. These were amplified using a traditional PCR, meaning that the target was amplified using one set of primers and one round of amplification. The reaction mixture and the reaction temperature cycling conditions can be found in Table 4. For quality control, each PCR was run alongside a positive control using extracted *Ascaris* DNA as template, and a negative control using autoclaved and de-ionized water instead of a template. The amplified PCR products were visualized alongside a 100 bp DNA ladder using 2% agarose gels that were stained with ethidium bromide as described above.

Nuclear targets

The nuclear targets have a lower number of copies in each cell compared to Mt targets because each cell possesses multiple mitochondria (which each has its own genome). But there is only one nucleus in each cell, possessing two copies of each gene, one inherited maternally and the other inherited paternally. Consequently, the assays targeting nuclear loci needed to be more sensitive than the assays amplifying the Mt targets. This meant a nested PCR was developed for each nuclear target, which requires that two separate PCR reactions were needed. The first PCR targeted a larger piece of DNA with primers flanking the true target sequence. Next, using the DNA amplified from the first reaction as template, a second reaction was performed targeting a

smaller portion of the DNA from within the DNA amplified during the first reaction. This process utilized two primer pairs. The exact reaction mixtures and temperature cycling conditions can be found in Table 4. For quality control, each PCR was run with a positive control using extracted *Ascaris* DNA as template and a negative control using autoclaved and de-ionized water instead of a template. The amplified PCR products were visualized with the Quick-Load® 100 bp DNA ladder (New England Biolabs, USA) using 2% agarose gels that were stained with ethidium bromide as described above.

Table 4: PCR reaction mixtures and conditions

	PCR ROUND	REACTION MIXTURE	THERMOCYCLER SETTINGS
MITOCHONDRIAL TARGETS (COXI#)	1st Round	2.5µl 10µM forward primer 2.5µl 10µM reverse primer 10µl of 5x reaction buffer 10µl of 5x reaction enhancer 4µl of dNTP's 1µl Taq polymerase 2µl of DNA template 18µl water	98°C for 30 seconds 98°C for 10 seconds 57°C for 10 seconds 72°C for 15 seconds 72°C for 2 minutes 4°C for ∞ (98°C for 10 seconds, 57°C for 10 seconds, 72°C for 15 seconds: 45 Cycles)
NUCLEAR TARGETS (TR#)	1st Round	1µl 10µM forward primer 1µl 10µM reverse primer 2µl of DNA 12.5µl HiFi Hotstart® Master mix 6.5µl water	98°C for 30 seconds 98°C for 10 seconds 61°C for 10 seconds 72°C for 15 seconds 72°C for 2 minutes 4°C for ∞ (98°C for 10 seconds, 61°C for 10 seconds, 72°C for 15 seconds: 25 Cycles)
	2nd Round	1.5µl 10µM forward primer 1.5µl 10µM reverse primer 5µl of 1st round product 12.5µl HiFi Hotstart® Master mix 4.5 µl water	98°C for 30 seconds 98°C for 10 seconds 57°C for 10 seconds 72°C for 15 seconds 72°C for 2 minutes 4°C for ∞ (98°C for 10 seconds, 57°C for 10 seconds, 72°C for 15 seconds: 35 Cycles)

Sequencing of amplified targets

Illumina library preparation

Ten to fifteen microliters of amplified PCR product were used to perform the library preparation. The NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (New England

Biolabs, USA) was used along with the NEBNext® Multiplex Oligos for Illumina® index kit (New England Biolabs, USA), the protocol of which is available at www.neb.com.

Sequencing

The sequencing reactions were prepared using the MiSeq reagent Nano Kit v.2 kit (PE250bp) and carried out on an Illumina® MiSeq (Illumina®).

Analysis of Illumina sequence data

The sequenced reads were analyzed in the software Geneious using a workflow developed for quality control, contig construction, and genotype analysis. The exact workflow was adapted from Barratt et al.'s workflow (under review in Cambridge Parasitology, 2019).

III. Results

319 individual PCR reactions were performed to collect all the data needed to investigate the genetic make up of the global *Ascaris* population. Samples were analyzed from humans infected with this helminth from Guatemala, the Solomon Islands, Slovakia, and infected pigs from the state of Georgia. The animals that the samples were collected from ranged from humans to pigs to dogs. The goal of this study is to identify the relationship between the ascaris genetic material (mitochondrial and nuclear) with the location or the host of infection (humans or pigs) in global ascaris populations.

Table 5: Summary of sequenced samples

ORIGIN	NUMBER OF SAMPLES	COXI6A	COXI7A	TR3
GUATEMALA	102	59	12	11
GEORGIA	6	3	2	2
SOLOMON ISLANDS	26	10	6	4
SLOVAKIA	8	4	3	1
TOTAL	142	76	23	18

Table 5 shows both the overall number of samples evaluated through the novel PCR assay and the number of sequences that were of high enough quality to sequence at each locus. It can be seen that the COXI6A locus has the highest sequencing rate out of the three loci illustrated. This includes all geographic locations and all hosts. Both the COXI 7A locus and the TR3 locus both had similar sequencing success to each other for each of the geographic locations and hosts. The COXI 7A had a slightly higher sequencing success than any of the TR3 sequencing, except the two loci had equal sequencing success for the Georgia samples.

COXI6A amplicon analysis

The COXI6A amplicon, which is an amplified fragment of the mitochondrial genome, was the reaction with highest sequencing success of all targets including COXI 7A and TR3 (76 sequenced/82 amplified). Using the sequence analysis and clustering approach described here, (24) it was observed that this locus could resolve some of the samples based on geography. As seen in Figure 3, all Solomon Island *Ascaris* clustered together. A specimen collected from a pig (P2) in Georgia also clustered with these samples indicating that the grouping by geography was not perfect. Additionally, the Slovakian samples clustered together using this locus, but were grouped along with a large contingent of Guatemala *Ascaris*. There also seems to be two main genotypes of Guatemalan samples, as shown in Figure 3. Also, two pig samples clustered together (positive control and P5) without any humans within the cluster. A third pig sample clustered with the Solomon Island samples showing the imperfection of this method in grouping by host. It was interesting that the rest of the human samples clustered with other worms of human origin, but no pig samples. Thus, this locus was able to group the sequenced samples by geography and by host accurately.

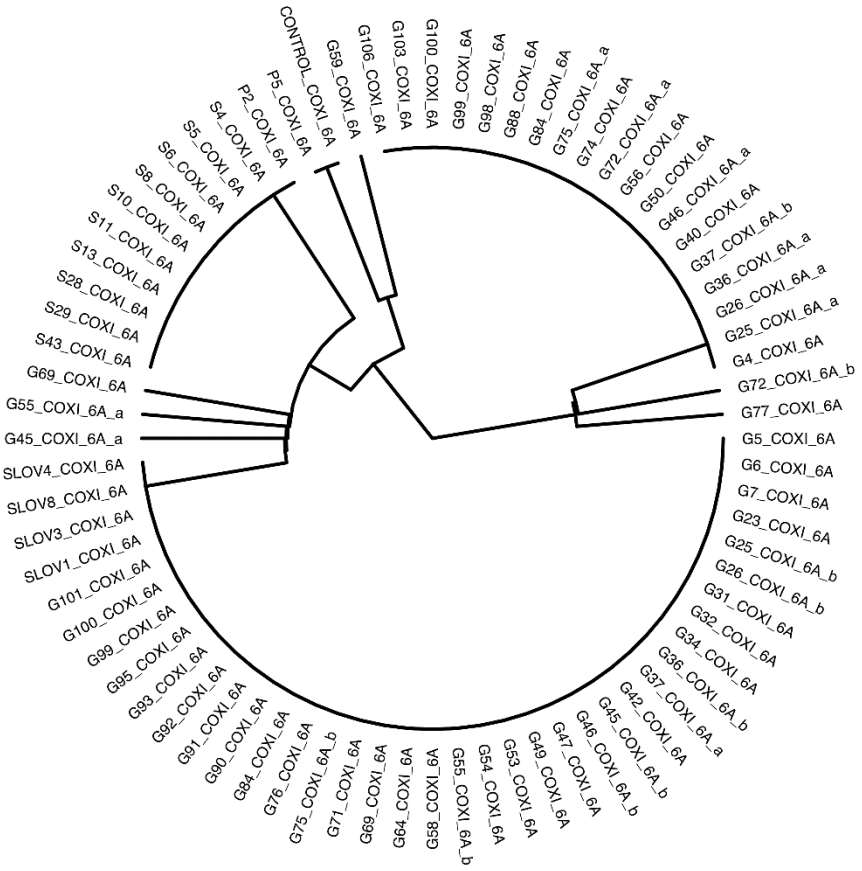

Figure 3: Dendrogram of *Ascaris spp.* utilizing the COXI6A amplicon. The dendrogram shows 76 COXI6A sequenced samples out of 82 amplified samples. These 82 samples were a subset of the original 142 samples analyzed through the PCR assay (Table 5). Guatemala human samples are denoted with a 'G#', Solomon Islands human sample with an 'S#', Slovakia dog samples with 'SLOV#', the Georgia pig samples with a 'P#', and the positive control with 'CONTROL'.

COXI7A amplicon genetic analysis

Utilizing the COXI7A locus, the Slovakian human *Ascaris* were shown to be distinct from any other group (Figure 4). These samples clustered together without sequences from other geographic areas. There was one major grouping of the Solomon Island human samples (S3, S6,

S10, S24) which had multiple Guatemala human samples (G90, G53, G23) with it. Only one Georgia pig sample (P3) of five had high enough quality data to undergo analysis, and therefore was not very illuminating. This lone Georgia pig sample clustered with the pig positive control and a human Guatemala sample (G66). It seems that this locus was also able to achieve part of the goal of this study as the geographic areas clustered together while using this locus.

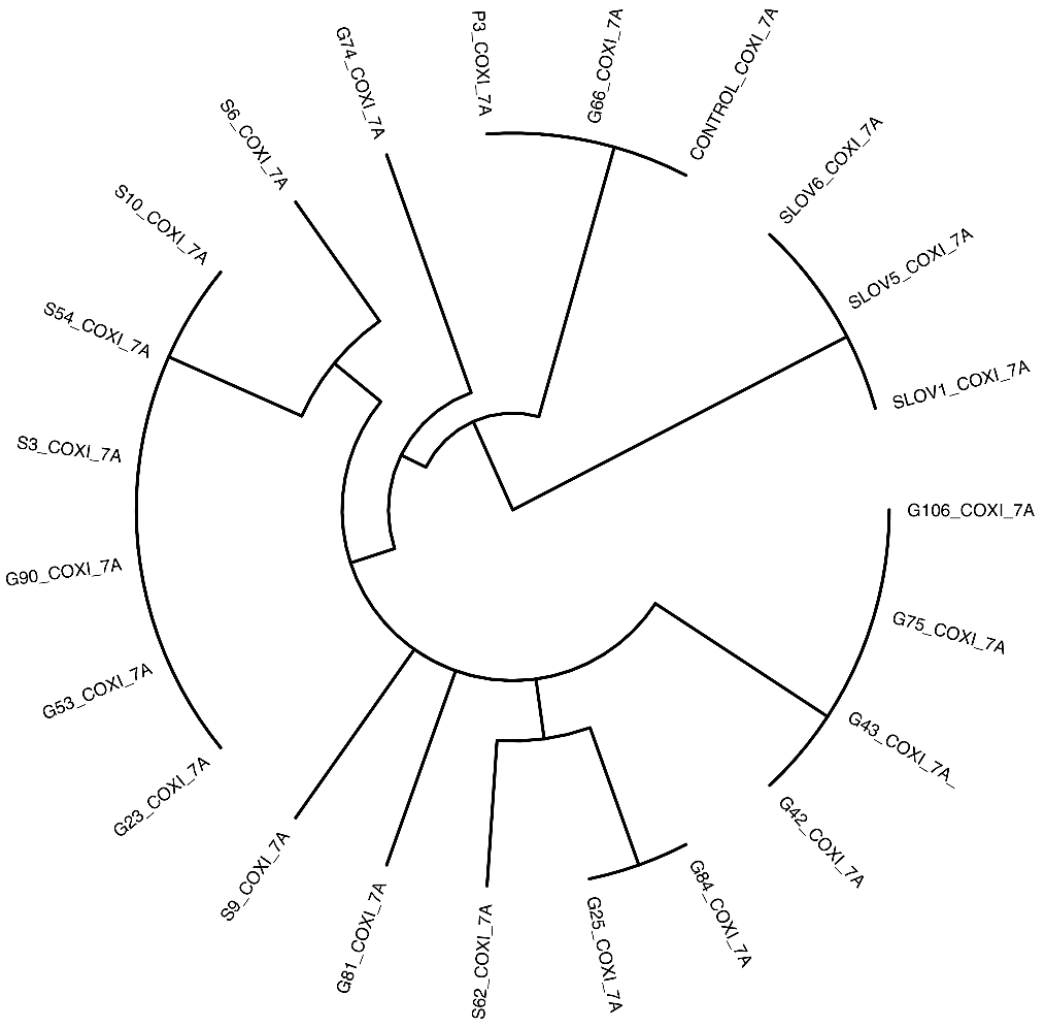

Figure 4: Dendrogram of *Ascaris spp.* utilizing the COXI7A amplicon. The dendrogram shows 23 COXI7A sequences of 115 successfully amplified samples. These 115 samples were a subset of the original 142 samples analyzed through the PCR assay (Table 5). Guatemala human

samples are denoted with a ‘G#’, Solomon Islands human sample with an ‘S#’, Slovakia dog samples with ‘SLOV#’, the Georgia pig samples with a ‘P#’, and the positive control with ‘CONTROL’.

TR3 amplicon analysis

The goal of the analysis of the TR3 amplicons was to evaluate this locus ability to group the samples by geography and original host. This marker was shown to isolate the human Solomon Island specimens into a cluster along with a single human Guatemala sample (G44). There also seems to be two main clustering’s of Guatemala human samples, one with the pig positive control and a pig sample from Georgia and the other with a single human Slovakian sample. There was only one human Slovakian sample and one Georgian pig sample (P2), which complicated interpretation at this locus of these locations. The grouping of the two pig-type ascaris samples together showed this target’s ability to group by host. This is also illustrated by the grouping of the majority of human samples separate from the pig samples.

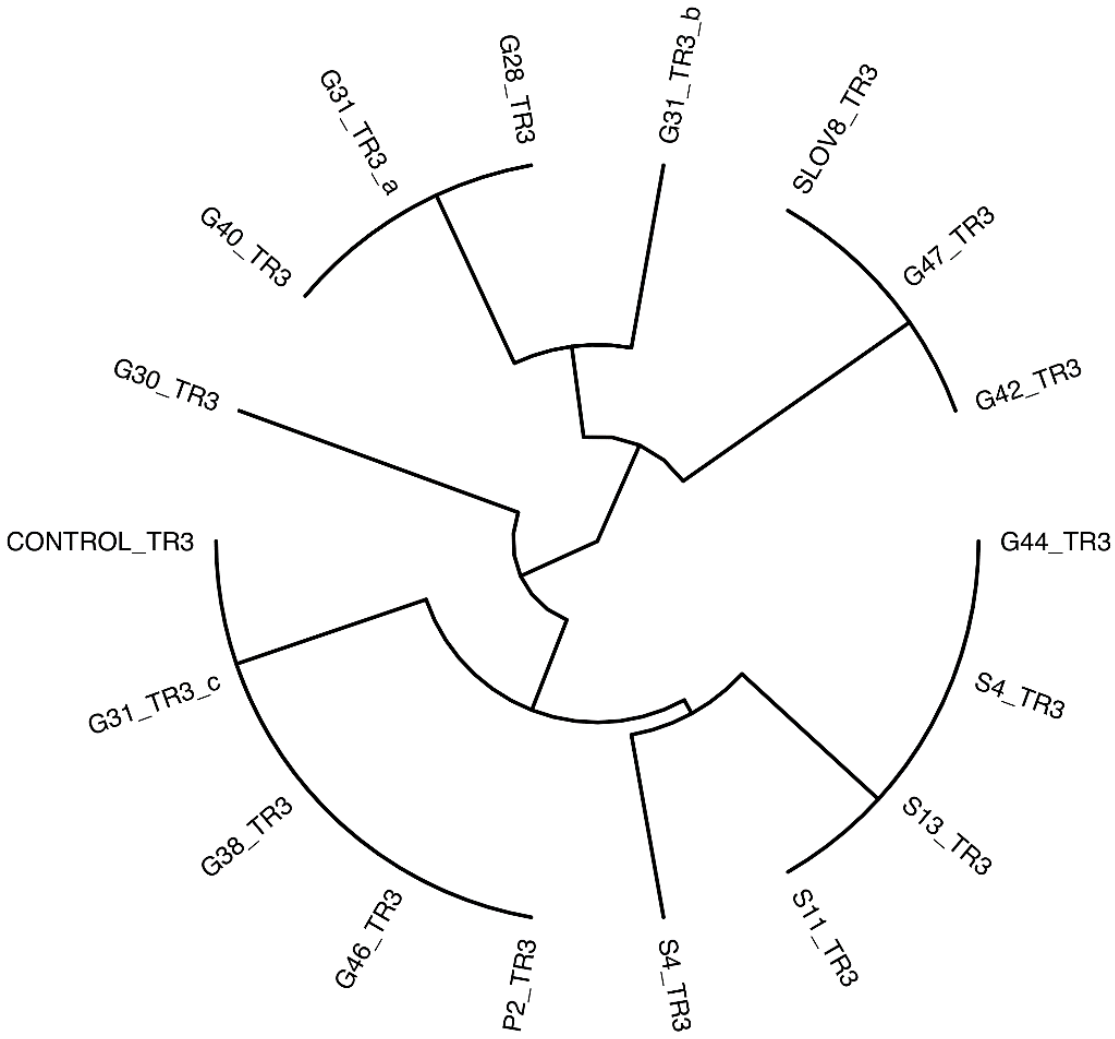

Figure 5: Cluster dendrogram of *Ascaris spp.* utilizing the TR3 amplicon. The dendrogram shows 18 TR3 sequences out of 21 amplified sequences. These 21 samples were a subset of the original 142 samples analyzed through the PCR assay (Table 5). Guatemala human samples are denoted with a 'G#', Solomon Islands human sample with an 'S#', Slovakia dog samples with 'SLOV#', the Georgia pig samples with a 'P#', and the positive control with 'CONTROL'.

IV. Discussion

The goal of this study is to identify the relationship between the genetic material (mitochondrial and nuclear) with the location or the host of infection (humans or pigs) in global ascaris populations. Analysis showed that nuclear and mitochondrial regions can be utilized to cluster *Ascaris* spp. worms according to their origin. Analysis also indicated that *Ascaris* that infects pigs clustered with *Ascaris* that infects humans across all three loci.

It was shown that the samples from the same geographical area clustered together far more than those that were not from the same area. While there were some outliers, most of the samples clustered within their own geography. This is consistent with the findings of several other studies looking at molecular tools and geographic clustering (6, 9). This may be due to the fact that each population of worms has been isolated long enough to evolve unique sequences at the loci that were targeted (14). This causes individuals with similar genetic sequences to interbreed more regularly, eventually giving rise to a widely dispersed haplotype throughout the geographic population of worms (14).

This concept is a widely accepted result of allopatric speciation (25), but it does not explain, for example, why the sample G44 clustered with the Solomon Island samples using the TR3 locus. Why would a worm from South America share such a genetic similarity with samples collected off of remote islands in the Pacific Ocean? The world is becoming increasingly globalized and through all the exchanging of ideas, materials, and the movement of people, it is possible that our parasites could be exchanged as well (8). There may have been an *Ascaris* spp. worm from the Solomon Islands that somehow arrived in Guatemala and interbred with the endemic population. All studies that were reviewed for this project, and that utilize genetic material as an indicator of relatedness, had samples that did not fit the overall trend (2, 5, 17, 21).

It was very interesting that the Georgia specimens, which were collected from pigs, consistently clustered with specimens that were collected from humans across different loci. This clustering by genetic information could be due to cross transmission between pig-infecting *Ascaris* and human-infecting *Ascaris* populations, which is supported by the findings of several other studies (2, 5, 17). The presence of both populations of worms (human and pig infecting) in the same area and common haplotypes between the two suggests that there is shared genetic sequences between worms that infect humans and worms that infect pigs. Due to the fact that there were few successful amplifications of the known *Ascaris* spp. worms from pigs, it was difficult to elucidate the relationship between the two populations of worms in humans and pigs. These preliminary relationships are based on limited data and must be further validated by futures studies.

There are several strengths and limitations that have been identified in this research project. The low number of pig type samples is a major limitation. Having such a small sample size of this prohibited any definitive data to be generated as to how closely the two populations of worms are related. A second limitation of this study is that some of the loci amplified had a small number of positive PCR reactions, and therefore a low number of sequences to analyze. Having large sample sizes in these studies is imperative to capture the diversity within the population. Alternatively, a strength of this study was the diversity of samples analyzed. Of the six inhabited continents, this study analyzed samples from four of them, which truly makes it a global survey of ascaris. A second strength was that both the mitochondrial and the nuclear genomes were targeted, increasing the strength of any results gathered. Future studies should aim to learn from both the limitations and strengths of this study.

This study, while not perfect, was informative and added to the knowledge regarding the genetics of *Ascaris* spp. The PCR assay and subsequent analysis of the generated sequences were able to cluster samples from the same geographical area fairly consistently. There was also some cursory evidence suggesting that there is shared genetic sequences between pig-infecting and human infecting *Ascaris* worms. This study could be improved by increasing the sample size for the pig samples and by identifying more successful regions to amplify.

V. **Public Health Implications**

- Since human and pig *Ascaris* show similar sequences at multiple targets, there may be gene flow between those populations.
- Pig variants of *Ascaris* and human variants of the worm shared genetic sequences at certain loci. If it is a result of geneflow between the populations of worms, then it should be explored whether swine should be treated in conjunction with humans during mass drug administration campaigns.
- This PCR assay was shown to be able to cluster samples by geographic origin, showing the efficacy of assays like the one used in this study. This type of assay may be useful if applied to other helminthic infections.
- The loci targeted in this study were informative, and able to differentiate between individuals within the same species. These sites may be useful in other genetic studies examining the relationship between individual worms, or worm populations.
- In all targets, the samples from Guatemala and Georgia clustered closer together than with samples from other regions. This shows that even at the continental level, populations are more closely related to other populations that are geographically near to each other.
- Certain loci were better at grouping samples from one geographic origin than grouping samples from other geographic origins. This result suggests that future studies evaluating the genetic relatedness of these worms should utilize multiple targets.

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Appendix A

Emory IRB determination

February 21, 2019

Travis Richins
Rollins School of Public Health

RE: Determination: No IRB Review Required
Title: *Eukaryotyping Ascaris*
Project Leader: Travis Richins

Dear Mr. Richins:

Thank you for requesting a determination from our office about the above-referenced project. Based on our review of the materials you provided, we have determined that it does not require IRB review because it does not meet the definition of research with "human subjects" as set forth in Emory policies and procedures and federal rules, if applicable. Specifically, in this project, you will utilize de-identified samples to develop a novel PCR based tool.

Please note that this determination does not mean that you cannot publish the results. This determination could be affected by substantive changes in the study design, subject populations, or identifiability of data. If the project changes in any substantive way, please contact our office for clarification.

Thank you for consulting the IRB.

Sincerely,

Amaree L. Lawrence, MS
IRB Analyst Assistant

Guatemala IRB determination

Memorandum

Date March 23, 2017

From Denise M. Marshall, BS
IRB Administrator, Human Research Protection Office

Subject CDC Approval of Continued Reliance on a Non-CDC IRB for CDC Protocol #5150: "A public health surveillance system for bacterial, parasitic, and viral causes of diarrhea, respiratory disease and unspecified febrile illness in Guatemala"

To Joe P. Bryan, MD
CGH/ DGHP

CDC's Human Research Protection Office has reviewed and approved the request to allow continued reliance on a non-CDC IRB for CDC protocol #5150 "A public health surveillance system for bacterial, parasitic, and viral causes of diarrhea, respiratory disease and unspecified febrile illness in Guatemala" in accordance with 45 CFR 46.114. The protocol has been reviewed and approved by the Universidad del Valle de Guatemala's Institutional Review Board and the IRB's **approval will expire on 03/12/2018.**

Please submit CDC form 0.1251, Request for Continuing Review of IRB-Approved Protocol, along with certification of current IRB review and approval at the relied-upon institution, approximately six weeks prior to the protocol's expiration date. If you do not yet have certification of continuation approval to include with your submission, please state on the 0.1251 that certification of continuation approval will be forwarded as soon as it is received.

Any problems of a serious nature should be brought to the immediate attention of the Human Research Protection Office.

If you have any questions, please contact your National Center Human Subjects Contact or the CDC Human Research Protection Office at (404) 639-7570 or via e-mail: huma@cdc.gov.

Solomon Islands IRB determination

01/08/2014

RE: Approval for Research Project No008 at Atoifi Adventist Hospital To

Whom It May Concern,

This is to confirm that Atoifi Adventist Hospital have approved the following research project No008 "Elimination of Soil Transmitted Helminths – One Village at a Time" through the Atoifi Adventist Hospital Research Ethics Committee.

Yours Sincerely

Peggy Kendall

Peggy Kendall CEO
Atoifi Adventist Hospital AAH