ANTHELMINTIC RESISTANCE IN PARASITIC NEMATODES

CONSORTIUM

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Executive Summary

Helminth infections are estimated to have a disease burden equivalent to 25% of that of HIV/AIDS and 50% of that of malaria. In recognition of the enormous health burden caused by these helminth parasites there is now an unprecedented effort, based on mass drug administration of very few anthelmintic drugs, to control these infections. However, reinfection after drug therapy is common and anthelmintic resistance is emerging and spreading as a problem. This proposal seeks to sequence the genomes and transcriptomes of anthelmintic resistant strains as an essential resource for better understanding of the genome diversity and mechanisms of resistance in human helminths. First, the genome sequences of anthelmintic susceptible and multiple-anthlemintic resistant strains of a model parasite *Telodorsagia circumcincta*) will enable identification of genetic and expression changes related to anthelmintic resistance. Second, sequencing the genomes of susceptible and clinical isolates resistant to helminth infections (human hookworm *Necator americanus*) will enable generation of resistance-associated variant maps and will provide resources to identify mutations and indels in genes subject to recent natural selection in the human parasites. Similarly, comparison of expression profiles will identify putative changes related to resistance.

The genomes of a susceptible *T. circumcincta* strain and the human hookworm *N. americanus* are underway (funded by NHGRI). Genomes of drug resistant isolates will provide the world community of helminth researchers with the resources needed to create a comprehensive map of genetic diversity in a model parasite and allow the utilization of this information in similar studies involving parasites of importance in humans. In the long run, the expected outcome will enable the potential mechanisms of resistance in human parasites to be correctly understood and genetic markers to be identified so that the development and spread of resistance can be monitored and managed.

1. Introduction

Infection of humans by nematodes results in substantial human mortality and morbidity, especially in tropical regions of Africa, Asia, and the Americas. The W.H.O. estimates 2.9 billion people are infected. In China alone 63% of the population (707 million people) harbor one or more parasitic nematode species [1]. Morbidity from nematodes is substantial and rivals diabetes and lung cancer in worldwide disability adjusted life year (DALY) measurements (Table 1)[2-3]. While mortality is low in proportion to the huge number of infections, deaths may still total over 500,000 annually. The most important parasites of this group include hookworms, Ascaris, and whipworm (>1 billion infections each) and the filarial worms that cause elephantiasis and African river blindness. Hookworms, probably the most significant public health threat of all nematodes, causes anemia by feeding directly on capillary blood. On top of the morbidity they cause directly, hookworms are the second largest contributor to the 26.7 million annual DALY's from iron-deficiency anemia (Table 1)[3]. Chronic anemia from hookworm infection is particularly devastating to children, who suffer from stunted growth and impaired intellectual development [4] and mothers who are at increased risk for anemia during pregnancy and childbirth.

Table 1. Major Nematode Parasites of Humans

Nematode species	Disease caused [a]	Individuals infected [a,b]	2000 DALY [d]
Ascaris lumbricoides	Ascariasis (large roundworm)	1.47 billion	1,252,000
Ancylostoma duodenale,	Ancylostomiasis (hookworm)	1.30 billion	1,829,000
Necator americanus			
Trichuris trichiura	Trichuriasis (whipworm)	1.05 billion	1,640,000
Enterobius vermicularis	Enterobiasis (pinworm)	~1 billion	ND
Wuchereria bancrofti, *Brugia malayi*, and others	Filariasis (elephantiasis)	120 million[a]	5,549,000
Strongyloides stercoralis	Strongyloidiasis (threadworm)	70 million [a] - 600 million	ND
Onchocerca volvulus	Onchocerciasis (river blindness)	18 million, >300,000 blind[a]	951,000
Dracunculus medinensis	Guinea worm disease	<80,000 [c]	ND
Trichinella spiralis	Trichinosis	ND	ND

[a] [5]; [b] [3, 6]; [c] [7]; [d] Disability adjusted Life Years Lost in 2000, W.H.O., 2001.; ND = not determined.

Needs in applied research and product development for nematode control in humans include diagnostics, vaccines, and a wider array of anthelmintic drugs. Diagnosis of nematode infection is labor intensive and often challenging, requiring the finding of worms or eggs in stool, blood, skin, or muscle [5, 8]. The development of reliable, rapid, and inexpensive diagnostic kits based on molecular markers is still limited [9-10]. Promising avenues for vaccine development include secreted antigens such as ASP-1 [11] and intestinal antigens such as H11 [12], but until recently the field has been limited to antigens that could be biochemically purified. The Bill and Melinda Gates Foundation is supporting a $16 million initiative to develop a hookworm vaccine [13]. Industry has largely ignored nematode control in humans except where partnerships with non-profit organizations such as the Carter Center and the W.H.O. have been established. Successful examples include Merck's donation of ivermectin for use in West African riverblindness control and Glaxo's donation of albendazole to the Global Alliance to Eliminate Lymphatic Filariasis. The dependence of control programs on a limited number of compounds makes drug resistance an even greater concern and the development of drugs acting by new modes of action an imperative. Applied research also relies upon continued progress in basic research characterizing aspects of parasite biology from host invasion [14] to nematode phylogenetics [15]. Genomic information on parasitic nematodes has already begun to accelerate research progress on vaccines, diagnostics, drugs (e.g. [16-17]), as well as basic research and this project intends to become the nematology community's major vehicle for advancing the studies towards a better understanding of genetic variations and anthelmintic resistance in human parasitic nematodes.

Resistance in parasitic nematodes is a growing problem that urgently increases the need for novel anthelmintics or vaccines. Conserving sensitivity to existing drug classes is imperative in the short term, and would be enhanced by the availability of genetic tests to detect steps of resistance selection prior to control failure.

2. The Challenge and Potential Impact

While public health measures have nearly eliminated one tropical nematode (the water-borne Guinea worm), and one other is on the way to elimination (lymphatic filariasis), cases of nematode infections in general have actually increased in recent decades. In these cases drug intervention, provided through foreign donations or purchased by those who can afford it, remains the major means of control. The high rate of re-infection after drug therapy means that vaccines remain the best hope for worm control in humans in the future. No vaccines are yet available, although promising avenues for vaccine development exist [11-13]. However, until safe and effective vaccines are developed, anthelmintics will continue to be used for treatment and control of nematode infections in both humans and domestic animals. The three major anthelmintic drug classes currently available are macrocyclic lactones (ML), benzimidazoles (BZ), and levamisole/pyrantel derivatives (LEV), all initially developed for use in livestock and pets then adapted for use in humans. Heavy use of drugs to control livestock parasites over several decades has led to nematode resistance to all drug classes [18-19]. The fact that many of the anthelmintic drugs have similar modes of action complicates matters, as the loss of sensitivity of the parasite to one drug is often accompanied by side resistance; that is, resistance to other drugs in the same class [18]. Anthelmintic treatment failures in humans have been observed [20-22], but widespread resistance monitoring is lacking [23]. The dependence of control programs on a limited number of compounds makes drug resistance an even greater concern and the careful management of those available is imperative.

The study of anthelmintic resistance has a long history, but has been focused almost exclusively on attempts to find associations between resistance and specific candidate genes. The best characterized anthelmintic resistant parasites are species of veterinary importance, and there is extensive literature describing candidate gene approaches in the study of resistance to BZ and MLs (reviewed in [24]). While specific mutations in the targeted genes have been linked to resistance [25-26], other mutations have also been reported [27-28], suggesting that more genes are involved than previously imagined [29]. Therefore, genome-wide studies are needed to better understand genetic mechanisms contributing to resistance. The core approach to identifying genes responsible for conferring resistance is to perform comparative pan-genome analysis of parasite populations that differ in their response to treatment. However, experience with attempts to assemble shotgun genomic sequence from *H. contortus*, and anecdotal reports from several investigators working with parasitic nematodes, indicate that **they are extraordinarily polymorphic and that any signal produced by a recent selective sweep generating anthelmintic resistance would be lost in the background noise.**

Therefore, the first challenge is to identify appropriate strain for these studies. What is required for a successful SNP-based approach (and variant detection in general) is a set of strains in which (a) the level of background polymorphism has been reduced several fold and (b) the resistance determinant(s) have been introgressed into the same genetic background as the determinants of susceptibility. Such a set of “near-isogenic” strains is not (and cannot be made) available in any human nematode parasite. However, such a set of strains, in a species of veterinary importance, has been made available by Drs. Grant & Bisset (members of the consortium). This set consists of two populations of *Teladorsagia circumcincta* one of which is susceptible to all classes of anthelmintic and has been through 2 generations of half sib matings. These matings were achieved by collecting the eggs from the uterus of a single female adult, and infecting a new host using the infective stages that grew from those eggs. This process was repeated, to generate a partially inbred strain susceptible to all classes of anthelmintic. This strain was then used in a cross with a *T. circumcincta* population resistant to the three major classes of anthelmintics: (BZ, ML and LEV). The F_2 progeny of this cross were selected for resistance to all three classes of anthelmintic (specifically oxfendazole, levamisole and ivermectin), followed by two further generations of backcrossing into the partially inbred susceptible population, with each generation again being selected for multiple-resistance. Thus five years later, two largely inbred populations of *T. circumcincta* were generated, one susceptible and one resistant, in which the genetic determinants for resistance to the three most important classes of anthelmintic have been introgressed into an otherwise susceptible genome. These are, therefore, “near isogenic” populations in which the principle phenotypic and genetic differences are related to anthelmintic resistance. Importantly, these anthelmintic resistances arose under field conditions (i.e. they were not selected from a laboratory population). Genome size is estimated to be 280 Mb (male) and 330 MB (female) using flow cytometry (e.g. [30]), and sequencing of the genome of this susceptible strain is supported by NHGRI (http://www.genome.gov/10002154). Dr. Bisset and Dr. Grant have also developed a mapping population from these strains, in which the F_2 progeny of the final generation of resistant X susceptible worms were selected for resistance to all three broad spectrum anthelmintic classes

and prepared for single worm PCR. Thus, there exists a “PCR ready” population in which resistance genes to each of the drug classes are segregating.

Based on the *C. elegans* genome sequencing, we determined that a comparable quality of the draft genome of the drug resistant *T. circumcincta* isolate will be ~20X coverage on the 454/Roche platform (12x titanium fragments, 6x 3kb pair-ends and 2x 8kb pair ends). Building on our past experience with manual annotation of ESTs using a multi-tiered approach, we will use the output from our automated gene prediction pipeline, and orthologous protein alignments to further validate start codons, length and conservation of final gene predictions in the genome. This standard is therefore acceptable for the model anthelmintic resistant genome.

The other major goal of the community is to use the generated data to underscore both i) the feasibility of creating comprehensive maps of genetic diversity in model parasitic species and then, ii) the transfer of this approach to human parasites, i.e. identify genetic variants (mutations, insertions and deletions) in genes subject to recent natural selection in human parasites. The sequencing of the 340MB human hookworm genome *Necator americanus* is underway (funds secured by NHGRI, http://www.genome.gov/10002154) thus this project will build on an existing experience studying parasitic helminths, but focus on generation of data related to a completely under-studied field, the genome-wide discovery and validation of anthelmintic-related mutations in clinical isolates. In addition, it will generate a resource to compare expression profiles of populations susceptible and resistant to anthelmintics and identification of expression changes putatively related to anthelmintic resistance.

3. Genetic variations and anthelmintic resistance in clinical isolates

Sequencing clinical isolates: Two drug classes, the BZs and MLs are the mainstay of several global programs which aim to control several human filarial and geohelminth parasites in developing countries. The success of these programs rests on the conservation of susceptibility to these drugs, which in turn requires sensitive methods to detect resistance selection. Studies of parasites of humans are inherently more difficult than those possible in animal models for human infections, for which experimental parameters can be tightly controlled and terminal euthanasia to accurately determine efficacy is possible. Importantly, the mechanisms of resistance to BZs are well conserved among nematode taxa, and there is no reason to expect this not to be the case with the other anthelmintic classes also. One would therefore expect to find similar principals that can be easily transferred across nematode taxa [31], even if the details differ between species. Furthermore, *T. circumcincta* and the human hookworm *N. americanus* belong to the same order (Strongylida; [15]), it is therefore expected that the SNP map generated using the “near-isogenic” lines of susceptible/resistant *T. circumcincta* will be a valuable resource for studying the genetic variation that allows the human hookworm *N. americanus* to overcome chemotherapeutic agents. Hookworm was chosen because, i) it infects over a billion people worldwide and is a major cause of anemia in children and pregnant women; ii) the genome sequencing of *N. americanus* is underway at the WUGC (supported by NHGRI, the genome sequencing will finish in 2010). Recent molecular analysis of previously reported point mutations related to BZ resistance in *N. americanus*, indicated that the mechanism is much more complex, possibly involving multiple genes and unidentified point mutations [21]. The massively parallel sequencing of clinical isolates will be obtained from treated and untreated patients (samples will be provided by Drs. Prichard and Bethony, members of the consortium) from different endemic areas. Furthermore, the *N. americanus* material that we will use is isolated from patients after repeated rounds of benzimidazole treatment and is phenotypically characterized so that a maximum number of resistance-related SNPs will be captured using this method. The subsequent analysis could focus on *N. americanus* genes that (a) are orthologous to genes of the *T. circumcincta* strain, and (b) contain experimentally confirmed SNPs to determine if the *N. americanus* genes are being selected for resistance in these populations. Six susceptible (for identification of the natural genetic variations) and twenty-six resistant *N. americanus* population will be sequenced (12 runs will provide us with ~36X coverage for each of the 32 genomes). Success in various portions of the project will be monitored. As noted above, the expected increase in the sequence throughput of the Solexa/Illumina technology may allow us to shift resources to sequence more clinical isolates, or to include other human parasites (i.e. the other two most prevalent geohelminths, Ascaris and Trichuris from which the material is also available).

Our preliminary results (unpublished observations) based on individual transcripts indicate that the expression level is altered in anthelmintic resistant populations; therefore, we plan to compare the expression level of adult populations that differ in resistance to anthelmintics using the RNA-seq approach. Digital expression measurements from sequencing of cDNA libraries (RNA-Seq) could be processed as follows. First, sequencing reads will be aligned to reference sequences. Samples exhibiting allele-specific coverage biases,

or excessive 5' or 3' coverage of cDNA sequences will be flagged for special consideration. Next, the processing pipeline will perform GC-correction and mapability-correction to obtain normalized read depths. The coverage breadth and depth of the transcriptome will be analyzed using our internal algorithm RefCov. 3' polydT-primed or randomly primed cDNA libraries will be considered/analyzed separately when we annotate and normalize across the expressed sequences to obtain gene-level expression data and identify genes with allele-specific expressions. Finally, the processing pipeline will apply the Significant Differences Among Groups algorithm to identify significantly expressed genes and allele-specific expression in resistant populations.

4. Integration and dissemination of the anthelmintic resistant genome data to the scientific community

This project will enable, i) a comprehensive analysis of the first anthelmintic drug resistant genomes and transcriptomes, with the focus on a multi-resistant model species followed by genomes of drug-resistant human parasites, and ii) a better understanding of how to create a high-throughput pipeline to acquire mutation data, structural variations, changes in expression, as well as the development of tools to support this process. We expect this project to set a foundation and become the major driver of genome-wide studies on genetic variations of parasitic nematodes. In the short run, it will deepen our understanding of genome-wide genetic changes involved in anthelmintic resistance and the potential mechanisms of resistance in human parasites, and in the long run will enable development of genetic markers so that the spread of resistance in human parasitic helminthes can be monitored and managed. Several hundred labs worldwide study a variety of human parasitic nematode species and their close relatives, including groups in the U.S., Europe, Asia, Australia and endemic countries.

All upcoming resources, including raw data, will be directly deposited to GenBank. To increase the interactions both on the data use level and data input level, we will provide documentation, including analysis summaries, results, and various software tools for community available via Nematode.net [32] and/or WormBase [33]. We will also create an extensive online User's Guide, an evolving Wiki site, and a list of Frequently Asked Questions, a Parasitic Worm Community Forum (a public bulletin board system for discussions on nematode research), and a Newsletter.

5. Estimated cost and time frame

Estimated total cost for this project would be $400k. The calculation is based on an average genome size of 330 Mb for the *T. circumcincta* and 340 Mb for *N. americanus*, and includes the following:

1. 22X coverage of the *T. circumcincta* on the 454 platform in a combination of fragments (12X), 3kb PE (6X) and 8kb PE (2X)
2. 36X Illumina coverage of the 32 N. americanus isolates (12 runs)
3. Genome assembly, annotation and data deposition
5. cDNA library construction and sequencing (0.5 run)

The project should be completed in 3 years.

6. Literature Cited

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