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Gene expression profiles associated with the transition to parasitism in *Ancylostoma caninum* larvae

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Abstract

Ancylostoma caninum is a common canine parasite responsible for anemia and death in infected dogs. Gene expression profiling was used to investigate molecular differences between two different forms of the third larval stage (L3s): infective free-living larvae and in vitro serum-stimulated larvae that mimic the initial stages of parasitism of a host. We developed an *A. caninum* cDNA microarray consisting of 4191 EST clones, and used it to identify a set of 113 genes that are differentially regulated between infective and parasitic larval stages. Real-time RT-PCR was used to confirm the expression differences of a subset of the genes. Of the genes repressed upon serum stimulation, seven encode members of the 'Ancylostoma secreted protein' ASP family, while another transcript encoding a 24 kDa excretory protein with similarity to ASP was up-regulated in serum-stimulated L3s. This suggests that different members of a protein family that has important implications for the hookworm's parasitic lifestyle are regulated in a complementary manner in response to serum stimulation. Comparison of two strains of *A. caninum* from North Carolina and Maryland only identified a single gene, one of the members of the ASP family, that was differentially repressed upon serum stimulation.

Keywords: ASP; Parasitic transition; Hookworm; Mixed model ANOVA; Microarray

1. Introduction

The canine hookworm, *Ancylostoma caninum* is a common blood-feeding intestinal parasite that can cause anemia and death, especially in severely infected puppies. As a close relative of the human hookworms *A. duodenale* and *Necator americanus*, *A. caninum* is also a good model for understanding the pathogenesis of these species, which along with other soil transmitted parasitic nematodes, infect up to one-third of the world's population [1,2]. The infective form of *A. caninum* is a free-living third-stage larva (L3) that remains non-feeding and developmentally arrested in the environment until it enters a dog, at which point it typically resumes maturation to reach adulthood.

Numerous studies have focused attention on the different genes and pathways that might be involved in the transition from developmentally arrested to reactivated stages of hookworm L3s [3–5]. To date, secreted proteins such as the 'Ancylostoma secreted proteins' or ASPs, encompass the majority of characterized molecules associated with this transition. In addition, metalloproteases and cysteine proteases may help the invading larvae to evade the host immune system [6–8]. Superoxide dismutases represent a group of antioxidants also found in the excretory–secretory products of hookworms that function to defend against free radicals produced by the host immune system [9,10]. Some of these molecules have been evaluated as vaccine candidates but show varying levels of efficacy [8,11], supporting a need for a more comprehensive, transcriptome-wide view of the transition to parasitism.

There is much interest in understanding the genetic mechanisms behind larval development in nematodes, as it may be the key to the identification of products associated with

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particular life stage transitions and parasitism. Given their close phylogenetic relatedness [12,13], studies designed to uncover differential expression levels during development in the free-living *Caenorhabditis elegans* soil nematode have been used as a model for arrested infective larval stages of parasites such as *A. caninum* [14,15]. Some researchers liken the infective L3 (iL3) stage of parasites to the dauer phase of the free-living nematode, *C. elegans*, although a strong case for a common dauer pathway has yet to be made [16]. Other studies are trying to identify transcripts that are associated with parasitism in particular nematode species [17].

This study uses cDNA microarrays for gene expression profiling of the dynamic transformation from non-feeding, developmentally arrested, infective iL3 to in vitro serum-stimulated feeding ssL3 of *A. caninum*. To account for strain variation and address potential factors to be considered in vaccine development, two isolates of *A. caninum* were assessed, one derived from North Carolina and propagated in laboratory Beagles since 2000 and the second derived from Maryland and propagated in steroid immuno-suppressed laboratory dogs since the early 1970s. These two isolates were chosen because they were readily available to the investigators, and a difference in observed infectivity rates (P. Arasu, unpublished data) may suggest some underlying genetic component. Moreover, sequence analysis suggests substantial DNA level variation at two loci between the isolates (Moser and Gibson, in preparation). Utilizing a statistically intensive approach to the analysis of microarray data [18,19] that partitions multiple sources of variance, and starting with a well-structured experimental design, small fold-changes in gene expression levels of up to 700 ESTs are shown to be significant for the effect of serum stimulation, but very few strain differences are detected.

2. Materials and methods

2.1. Microarrays

Approximately 10,000 individual expressed sequence tags (ESTs) from an *A. caninum* Shanghai strain iL3 cDNA library in pBluescript vector were obtained from the Parasitic Nematode Sequencing Project, Genome Sequencing Center, Washington University [20]. By clustering with BLASTCLUST software [21], 4191 individual ESTs were chosen for printing. The clones were picked robotically from the original plates and re-arrayed into new 384-well plates. Because of multiple rounds of rearraying both by hand and robot at Washington University and after receipt of the clones, ambiguity was introduced into the annotation, and the identity of all clones is not certain. The clones were grown up from bacterial cultures with Terrific Broth and plasmid DNA was prepared [22]. cDNA clones were PCR amplified with either of the following primer sets (AcFor 5′-GTTTTCCCAGTCACGACGTTG-3′ and AcRev 5′-TGAGCGGATAACAATTTCACACAG-3′, or T3 5′-CAATTAACCCTCACTAAAGG-3′ and T7 5′-GTAATACGACTCACTATAGGG-3′) in 100 μl reactions under standard conditions [21]. The PCR reactions consisted of an initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 60 s, and a final extension of 10 min at 72 °C. All PCR products were tested for length and purity on 0.8% agarose gels stained with ethidium bromide. PCR products were cleaned up with Millipore 96-well vacuum filtration plates following the manufacturer's specifications (Product #MANU3010). The purified PCR products were then diluted with DMSO to a final concentration of 50%, and the solution was printed in duplicate onto amino-silane coated slides (Corning #40003) with a GMS-417 pin-loop arrayer at the NCSU Genome Research Laboratory. The spotted DNA was air-dried, UV cross-linked to the slide, and the slides were stored at room temperature in a desktop dessicator until used.

Following completion of the hybridizations, 157 ESTs were re-sequenced using standard Big Dye II reactions on an ABI 3700 automated DNA sequencer. All of the clone identities reported below were obtained by re-sequencing of 500–700 bp of the 3′-end of the clones, and matching Blast output to the clone sequences available at http://www.nematode.net [23].

2.2. Parasites

Infective L3 *A. caninum* were collected from charcoal cultures of egg-infested feces of laboratory Beagle dogs as previously described [24]. These dogs were infected with two different *A. caninum* strains initially derived from natural populations of parasitized dogs: the Maryland strain (kindly provided by Dr. Thomas Nolan, University of Pennsylvania) has been propagated since the early 1970s in laboratory dogs treated with immuno-suppressive doses of steroids and the North Carolina strain has been propagated in laboratory Beagles since 2000. For these studies, both strains were maintained in non-steroid treated dogs. The approximate iL3 infective doses were 250 and 120, respectively to achieve a comparable fecal output of 2000–3000 egg g^{-1} at 4 weeks post-infection. Harvested larvae were washed several times in medicated (20 mg ml^{-1}) gentamycin and lincomycin) phosphate-buffered saline (PBS). To obtain serum-stimulated feeding ssL3 larvae, iL3 were incubated in PBS (5000 L3/500 μl) at 37 °C/5% CO_2 for 20–24 h in the presence of 5% normal dog serum in 24-well plates. To check for feeding and reactivation status, about 250 larvae in 100 μl PBS were incubated at 37 °C/5% CO_2 for 2 h in the presence of an equal volume of a 5 mg ml^{-1} fluorescein isothiocyanate-conjugated bovine serum albumin (FITC-BSA; Sigma) solution. The larvae were repeatedly washed in PBS before examination by fluorescent microscopy. At least 50 larvae from each well were scored; positive feeding larvae display fluorescent intestinal tracts, and a batch of worms was considered 'serum-stimulated' if greater than 85% of the larvae were positive. In the serum stimulation