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# Identification of gender-regulated genes in *Ancylostoma braziliense* by real-time RT-PCR

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**Abstract**

*Ancylostoma braziliense* belongs to the family Ancylostomatidae and infects cats and dogs in various parts of the tropical world. It is also a zoonotic parasite causing cutaneous larva migrants in humans. There are very few, either biological or molecular, studies of this species. In this study, differential display was used to identify differentially expressed genes in male and female *A. braziliense*. Nineteen new sequences were identified and examined by real-time RT-PCR to confirm male–female specificity. Ten were more expressed in males, while two were more expressed in females. Molecules shown to be important in other host–parasite relationships were also found in this study.


*Keywords:* *Ancylostoma braziliense*; Sex-enriched expression; Differential display; RT-PCR; Expressed sequence tag

## 1. Introduction

Infection by hookworms continues to be a serious problem in the developing world (Crompton, 2000; De Silva et al., 2003). Members of the family Ancylostomatidae that infect humans cause anemia and arrest growth in infected people (Hawdon and Hotez, 1996). Other members of the family, such as *Ancylostoma caninum* and *Ancylostoma braziliense*, are zoonotic and, besides infecting cats and dogs, also infect humans, causing cutaneous larva migrants (Kalkofen, 1987; Morrison, 2001).

Parasitic nematodes have major socio-economic importance as pathogens of humans, animals, and plants, and investigating their developmental and reproductive processes is of fundamental importance for understanding their epidemiology and control (Nisbet et al., 2004). Knowledge of gene characterization for parasitic nematodes is advancing, and sex-specific genes have been identified, coding for enzymes such as serine/threonine phosphatases, that are known to be involved in spermatogenesis and/or regulation of sperm motility in other organisms (Smith et al., 1996; Varmuza et al., 1999). For example, a serine-threonine protein phosphatase gene is expressed specifically by male *Oesophagostomum dentatum* (Boag et al., 2003) and male *Trichostrongylus vitrinus* (Hu et al., 2007).

Most work addressing sex-related gene expression in nematodes has been done with the model-free living nematode *Caenorhabditis elegans* (reviewed by Stothard and Pilgrim, 2003). However, parasitic nematode species may exhibit different reproductive mechanisms. For example, a novel gene has been found

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in a parasitic nematode of plants (Llado et al., 1998). This suggests that studies on reproductive genes of parasitic nematodes should be extended. Furthermore, there is no record of an expressed gene of *A. braziliense* in the gene databases, reinforcing the need to expand studies of this parasite. In the present study, gene expression in male and female worms was compared.

## 2. Materials and methods

### 2.1. Parasites

Adult worms were collected at necropsy from the small intestines of 10 dogs of undefined breeds 6–24 months old which were routinely killed by procedures approved by the Municipality Health Center from Belo Horizonte-MG-Brazil by the Zoonoses Control Center (CCZ-BH).

### 2.2. RNA purification and cDNA synthesis

Total RNA was extracted from a pool of 22 male and 18 female adult *A. braziliense* using Trizol (Life Technologies Inc., Rockville, MD) according to manufacturer's instructions. The RNA pellet was washed with 75% ethanol, air-dried for 10 min, and diluted in 50 μl of diethyl pyrocarbonate (DEPC) treated water. Total RNA aliquots were DNase I (Gibco BRL, Gaithersburg, MD) treated to eliminate possible traces of contaminant genomic DNA. First-strand cDNA was synthesized in a final volume of 20 μl by incubating approximately 1 μg of total RNA with 25 ng/μl oligo $(dT)_{12–18}$ and 500 μM each of dNTP (dATP, dGTP, dCTP, and dTTP) (Life Technologies Inc., Rockville, MD) at 65 °C for 5 min followed by quick chilling on ice. Subsequently, the following were added to the tube: 10 mM of dithiothreitol, 4 μl of 5× first-strand reaction buffer (final concentration 50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$, pH 8.3), 40 units of RNase OUT™ (Recombinant Ribonuclease Inhibitor), and 200 units of Reverse Transcriptase (SuperScript™ II) (Life Technologies Inc., Rockville, MD). The tube contents were mixed and incubated at 42 °C for 50 min. For each sample, a parallel reaction was prepared without reverse transcriptase to be used as a negative control for the DD RT-PCR and semi-quantitative RT-PCR.

### 2.3. Differential display

Male and female pattern expression differences were analyzed by a modification of the differential display approach of Liang and Pardee (1992). This comprised the use of oligo dT with no anchored nucleotides (A, C and G) at the 3′ terminus and a single arbitrary primer (17–24 nucleotides) to the PCR reaction. The reaction was performed by combining 1 μl of each cDNA first-strand sample, 10 mM Tris–HCl (pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.1% Triton®X-100, 200 μM of each dNTP (Life Technologies Inc., Rockville, MD), 2.6 μM of one of the primers [αPR (5′-AAGTGGATATTTG-GAGCGTT-3′), DimpYU (5′-ATGGCTAGTGTTTTG-TTGC-3′), RG1 (5′-GCAGGTGTGTGAGCATTGGC-3′), or RB1.1 (5′-CAGGTGTGTGAGCATGGGC-3′)] and 3 units of *Taq* DNA polymerase (Phoneutria, MG, Brazil) in a final volume of 25 μl, repeated for each primer. The protocol for the PCR program was: 2 cycles of 2 min at 95 °C, 1 min at 37 °C, and 2 min at 72 °C followed by 29 cycles of 1 min at 95 °C, 1 min at each primer-specific temperature, and 2 min at 72 °C with a final extension for 8 min at 72 °C.

Ten microlitres of each PCR product were resolved on 6% polyacrylamide gels (16 mm × 13 mm × 1 mm) and run for 3.5 h at 100–120 mV. The gels were subsequently silver-stained following Bassam et al. (1991) and air-dried onto cellophane. Bands that displayed differentially between sexes were excised from the dried gel and eluted into 50–100 μl of buffer [10 mM Tris–HCl (pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.1% Triton®X-100] by heating for 20 min at 95 °C. The samples were re-amplified as triplicates in a PCR reaction using 2.5 μl of the eluted product, 10 mM Tris–HCl (pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 1% Triton®X-100, 200 μM of dNTP (Life Technologies Inc., Rockville, MD), 0.6 μM of the specific primer, and 1.25 units of *Taq* DNA polymerase (Phoneutria, MG, Brazil) in a final volume of 25 μl, employing a similar protocol, but excluding the initial cycle with annealing at 37 °C. PCR products were purified using the GFX kit (Amersham Pharmacia Biotech inc.) following the manufacturer's instructions, ligated to pGEM®-T (Promega, Madison, WI), transformed into *Escherichia coli* strain DH5α, and selected using the system ampicillin/IPTG/X-GAL after incubation for 16 h at 37 °C (Sambrook et al., 1989).

The accuracy of cloned fragments sizes was confirmed by PCR. A small colony fragment was used as a template in a PCR reaction in a 10-μl final volume using the same re-amplification PCR parameters.

### 2.4. DNA sequencing and analyses

Recombinant plasmid DNA was isolated using the Wizard® Plus SV Minipreps (Promega, Madison, WI).