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Quantitative analysis of gender-regulated transcripts in filarial nematode *Brugia malayi* by real-time RT-PCR

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Abstract

Improved understanding of the biology of reproduction in filarial worms may lead to identification of new targets for drugs or vaccines. Real-time RT-PCR is increasingly being adopted for RNA quantification and genetic analysis. Candidate gender-regulated genes were selected from genes identified in prior studies by differential display RT-PCR and by electronic selection of the *Brugia malayi* expression sequence tag (EST) database for clusters with possible gender-specific expression (four or more transcripts in male cDNA library ESTs but none in female ESTs or vice versa). Expression of candidate genes in male and female worms was compared by real-time reverse transcription PCR with sequence-specific primers. Double stranded DNA product was measured by SYBR Green I fluorescence; melting curves and agarose gel electrophoresis were used to verify the specificity of results. Relative gene expression results were normalized by parallel studies with internal control genes that were shown to be equally expressed in male and female worms (beta actin 2B, histone H3, NADH dehydroxygenase subunit 1) and calculated by the comparative C_t method. Nineteen of 31 candidate genes were verified to have reproducible, gender-biased expression with fold differences between 5 and >30,000. These included several well-known genes (for example, gene encoding major sperm protein and a microfilaria sheath protein) and many novel genes. This paper reports the first large scale use of real time RT-PCR to quantitate and study gene expression in a nematode parasite. Our results represent an important step toward improved understanding of the molecular biology of reproduction in filarial nematodes.

Keywords: Nematode; *Brugia*; Real-time RT-PCR; Sex-regulated genes; Reproduction; Gene expression; SYBR Green I

1. Introduction

Recent years have witnessed an explosion in understanding the biology of the free-living nematode *Caenorhabditis elegans* with the complete sequencing of its genome and development of techniques for functional genetic analysis including transgenesis, targeted mutagenesis, and gene silencing by RNA inactivation [1,2]. Recently, RNAi has been used to identify a number of genes that are essential for reproduction in *C. elegans* [2]. In contrast to this situation, relatively little is known about molecular aspects of reproduction in parasitic nematodes. Consider the filarial nematodes that cause the important diseases of onchocerciasis ("river blindness" caused by *Onchocerca volvulus*) and lymphatic filariasis ("elephantiasis" caused by *Wuchereria bancrofti* and *Brugia malayi*). Existing treatments for these infections (albendazole, diethylcarbamazine, and ivermectin) [3] work in part by killing parasite larvae (microfilariae) and sterilizing adult worms. However, the mechanisms involved and biology of these drug effects are poorly understood. Increased understanding of the basic biology of filarial reproduction could lead to new therapies for filarial and other parasitic nematode infections or to vaccines that interfere with parasite reproduction.

We have previously used a variety of methods to identify genes that appeared to be differentially expressed in

Abbreviations: RT-PC; Rreverse transcription polymerase chain reaction; RNA; ribonucleic acid; DDPCR; differential display PCR; ES; electronic selection; EST; expression sequence tag; cDNA; complementary DNA; C_t; threshold cycle number

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male and female *B. malayi* in hopes of gaining new insight into reproductive mechanisms in filarial parasites [4]. Candidate genes with possible gender-biased expression were identified by differential display polymerase chain reaction (DDPCR) and by analysis of a database of expressed sequence tags (ESTs) generated for this parasite by the Filarial Genome Network [5]. The present study utilized a new technology (real-time RT-PCR) to expand upon that work.

RT-PCR is a time-tested method for studying gene expression and comparing levels of gene expression in different specimens [6]. RT-PCR is especially useful in situations where amounts of RNA are insufficient for Northern blot analysis. However, this qualitative, end-point analysis method lacks precision for quantitative comparisons of gene expression [7,8]. The recent development of kinetic or "real-time" RT-PCR represents a technical breakthrough for research on gene expression. Real-time RT-PCR can be used to compare and quantitate expression of selected genes in different biological specimens. It is especially useful for confirming differential expression of candidate genes identified by other means [9,10].

Real-time RT-PCR quantifies PCR product as it accumulates during the exponential phase of the amplification reaction without the need for a separate assay to detect this product. It has a broad dynamic range and high throughput capacity [11]. Detection of amplified product by real-time RT-PCR can be done with sequence-specific fluorescent probes [12,13] or with a DNA binding dye such as SYBR Green I [8]. Real-time RT-PCR has been used to validate candidate differentially expressed genes identified by DDPCR and DNA array analysis [8,9,14,15]. We now report our use of a rapid and highly sensitive SYBR Green I two-step real-time RT-PCR assay to confirm and quantitate differentially expressed transcripts in *B. malayi* male and female worms. Our results demonstrate that this method is a powerful tool for assessing gene expression in nematode parasites.

2. Materials and methods

2.1. Parasite material

Adult *B. malayi* worms were isolated from the peritoneal cavity of infected jirds (*Meriones unguiculatus*) obtained from the NIAID filariasis Repository (University of Georgia, Athens, GA). Male and female worms were separated carefully by size; broken worms were discarded. The worms were then washed and immediately frozen at −80 °C.

2.2. Selection of housekeeping genes for testing as internal controls

The Filarial Genome Project has produced one of the largest public parasite cDNA databases. This database (NEMBASE, posted at http://nema.cap.ed.ac.uk/nematodeESTs/nembase) contains some 23,000 *B. malayi* EST sequences from randomly selected clones present in cDNA libraries that represent different life cycle stages of the parasite [5,16]. The database contains 4290 ESTs in 2642 clusters from an adult male library and 3333 ESTs in 2021 clusters from an adult female library. A database search engine available at NEMBASE permits data retrieval by text string searches (including gene or cluster names). Three control gene candidates (actin 2B (ACTB), histone H3 (HIST), and NADH dehydrogenase subunit 1 (NADH)) (Table 1) were selected based on their approximate equal representation among ESTs posted from cDNA libraries from adult male and female worms.

2.3. Selection of candidate differentially-expressed genes

Two groups of candidate genes were selected for evaluation. Group 1 transcripts were identified and confirmed (by RT-PCR) to have gender-specific gene expression in a previous study by our group [4]. Group 2 transcripts were newly selected by electronic selection (ES) based on the *B. malayi* EST database cited above (accessed January 2003). We selected candidate genes that had been identified more than three times in the female cDNA library and not in the male cDNA library, and vice versa. The database contained 28 candidate female-specific sequence clusters and 71 candidate male-specific sequence clusters that met these criteria. We selected 25 transcripts with sequences >300 bp (excluding transcripts in group one) as group 2 candidates for further study.

2.4. RNA isolation and reverse transcription reaction

Worms (30 female and 60 male worms for each batch, respectively) were crushed under liquid nitrogen with a ceramic mortar and pestle and re-suspended in TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was prepared according to the manufacturer's instructions with minor modifications. The quantity of RNA was measured with a GeneQuant Spectrophotometer (Pharmacia Biotech, Piscataway, NJ). RNA samples were treated with amplification grade DNase I (Gibco BRL, Gaithersburg, MD) to eliminate any genomic DNA contamination just prior to proceeding with cDNA synthesis (leaving the stock untreated). First-strand complementary DNA was synthesized from male and female total RNA using SuperScript™ II RNase H⁻ Reverse Transcriptase (Invitrogen, Carlsbad, CA) with oligo $(dT)_{12–18}$ primer (Promega, Madison, WI). Briefly, oligo $(dT)_{12–18}$ primed first-strand cDNA was synthesized in a total volume of 20 ul containing 5 ug total RNA either with reverse transcriptase (+RT cDNA) or without the enzyme (−RT control). The quality of single-stranded cDNA was monitored by the actin RT-PCR method described by Michalski and Weil [4]. BmAct primers (different from those used

Table 1
Candidate genes for internal control

Symbol	Protein ID	Accession number	Primer F	Primer R	Amplicon (bp)
ACTB	Actin 2B	BMC00540	CTCCCGAGGAACATCCAGTACT	CTCTGTTTGCCTTTGGGTTCA	59
NADH	NADH subunit 1	BMC02280	GGGTGGCACTCAGTGTCGTA	ACAACGCCTGAAAAATACCAGAGTA	58
HIST	Histone H3	BMC00764	GCTAACGAAAGCACCATCAAGA	TCTTGCAAAGCACGCATACC	64

for real-time RT PCR quantitation of actin mRNA) produce a ∼260 bp PCR product with *B. malayi* cDNA template and a ∼400 bp product with genomic DNA template (due to an intron). The accuracy of worm gender separation was assessed by PCR with *B. malayi* major sperm protein primers (BmMSP) and *B. malayi* embryo-associated fatty acid-binding protein primers (Bm-FAB-1) [4]. Again, primers used for RT PCR are different from those used in real-time RT PCR.

2.5. SYBR Green I quantitative real-time RT-PCR

The PCR reactions were carried out in 96 well microtiter plate wells in a 25 ul reaction volume with SYBR Green Master Mix (PE Biosystems, Forster City, CA) with optimized concentrations of specific primers. An ABI Prism 7000 Sequence Detector (Applied Biosystems, Foster City, CA) was programmed for an initial step of 2 min at 50 °C and 10 min at 95 °C, followed by 40 thermal cycles of 15 s at 95 °C and 1 min at 60 °C. Every assay included duplicate, 10-fold serial dilutions of the calibrator cDNA, test cDNA samples, and controls (no template, internal control gene standards, and template produced with no RT enzyme). Specificity of PCR amplification of each primer pair was confirmed by analyzing PCR products by agarose gel electrophoresis and by melting curve analysis [17].

2.6. Primer design and optimization

Complementary DNA primers were designed from EST sequences obtained from GenBank with Primer Express software (Version 1.0, PE Applied Biosystems, Foster City, CA). We also required that the last five -3′ nucleotides have a maximum GC content of 40%. All primer sets had a calculated annealing temperature of ≥58° C (nearest neighbor method). The primer sequences for internal controls were shown in Table 1 and other primer sequences are available upon request. Primers were ordered from Integrated DNA Technology Inc. (Coralville, IA).

Primer concentrations for each primer set were optimized by checkerboard titration. Briefly, 900, 300 and 50 mM concentrations of each -5′primer were tested with 900, 300 and 50 mM concentrations of the corresponding -3′ primer, with and without template (18 total reactions for each primer set). This optimization step identified primer concentrations that provided the highest sensitivity and specificity for each target sequence.

2.7. Data analysis: standard curves and calculations

Standard curves were generated from calibrator cDNAs made from decreasing amounts of total RNA (10-fold dilutions) to monitor the efficiency of real-time RT-PCR for each assay. Prior to acceptance of data for quantitative work, we required at least four of the five standard curve dilutions in an assay to yield specific product (based on dissociation curve analysis) and that no product was seen in the no-template control. The quality of standard curves can be judged from their slopes and correlation coefficients (r). The PCR efficiency (E_x) was determined using the equation: $E_x = (10^{-1/\text{slope}} - 1) \times 100\%$. The threshold cycle ($C_t$) value was defined as the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value (background noise) [18].

Fold differences for gene expression were calculated by the comparative C_t method [18]. This method compares test samples to a calibrator sample and uses results obtained with a uniformly expressed control gene to correct for differences in the amount of RNA present in the two samples being compared to generate a ΔC_t value. Female cDNA was used as the calibrator for genes believed to be up-regulated in female worms (and vice versa). The formula used for fold difference calculation was $2^{-\Delta\Delta C_t}$, where the value of $\Delta\Delta C_t$ was the difference in ΔC_t values obtained with calibrator and test samples. We considered fold differences of >3 to be significant. C_t values obtained with 1 ng of male and female RNA starting material were used for these calculations. Results shown are mean ± S.D. except as otherwise noted.

3. Results

3.1. Quality assessment of B. malayi RNA

The quality of RNA preparations and cDNA synthesis was assessed by actin PCR amplification. As shown in Fig. 1A, only the expected ∼260 bp product was amplified with female and male cDNA templates, while a 400 bp product was observed with genomic DNA template. Also, control PCR reactions with cDNA templates processed in the ab-

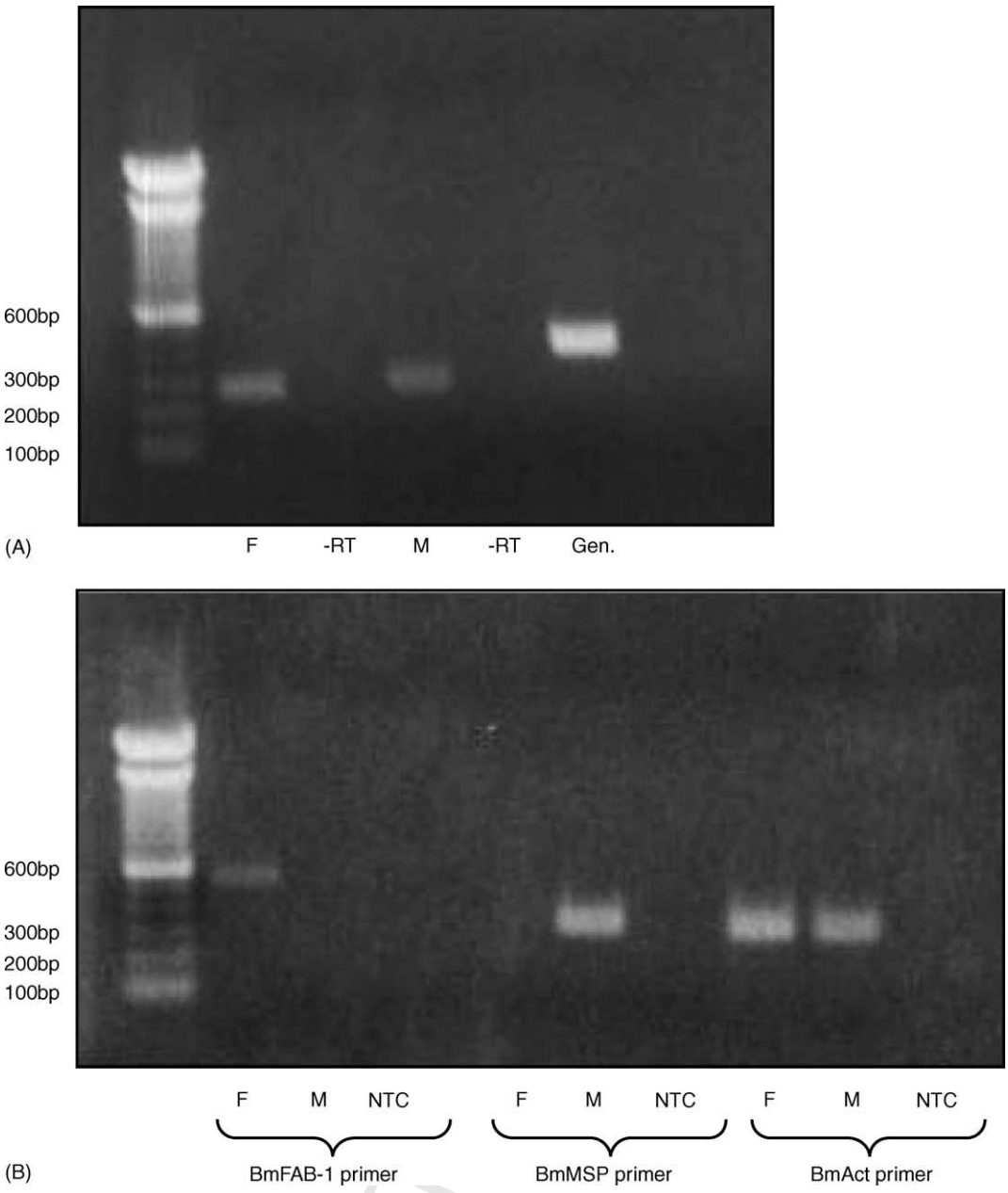

Fig. 1. Quality assessment of *B.malayi* cDNA by RT-PCR. Products shown were electrophoresed on a 4% agarose gel. F, female cDNA template; M, male cDNA template; Gen, genomic template; -RT, PCR with RNA template without reverse transcriptase; NTC, PCR without template. Panel A: BmAct primers were used to amplify actin products from cDNA template (260 bp) and from genomic templates (400 bp). Panel B: male cDNA yielded no Bm-FAB-1 product, while the predicted size product of 550 bp was amplified from female worm cDNA. In contrast, female cDNA yielded no MSP product, while a product of 280 bp was amplified from male worm cDNA.

sence of reverse transcriptase yielded no product. These results show that the DNase I treatment was efficient and that cDNA preparations were substantially free of genomic DNA.

BmMSP and Bm-FAB-1 RT-PCR reactions were used to assess the accuracy of worm gender separation (Fig. 1B). Male cDNA yielded no Bm-FAB-1 product, while the expected 550 bp product was amplified from female worm cDNA. In contrast, female cDNA yielded no BmMSP product, while the expected 280 bp product was amplified from male worm cDNA. These results show that worm gender separation was accurate.

3.2. Evaluation of housekeeping genes as internal controls

We assessed expression levels for the three housekeeping genes in two independent male and female worm RNA sam-

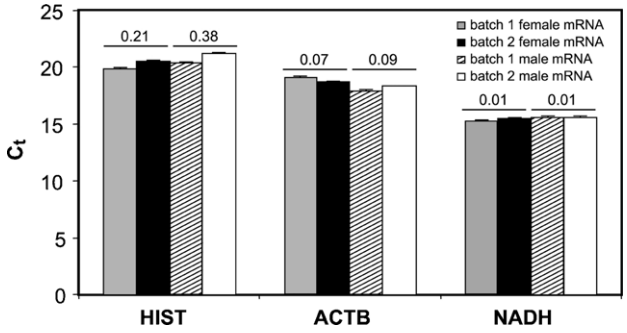

Fig. 2. Quantitation of gene expression for HIST, ACTB and NADH genes in *B. malayi* male and female worms. Numbers above the bars represent differences in mean cycle threshold numbers observed with different batches of female and male worm RNA samples. The C_t values were obtained with 10 ng RNA samples.

ples. All of these genes were equally expressed in male and female worms. NADH was a relatively high abundance transcript with C_t values of 15.6 ± 0.13. HIST and ACTB were moderately abundant genes with C_t values of 20.7 ± 0.57 and 18.9 ± 0.56, respectively. These C_t values were obtained with 10 ng of RNA with two independent female and male RNA samples (Fig. 2).

We considered NADH to be a slightly better internal control gene than HIST or ACTB, because its C_t values were less variable. However, the good overall reproducibility obtained with these control genes showed that potential sources of variability in our real-time RT-PCR assays (variable efficiency of RT reactions, quantitation of mRNA, and differences in handing RNA samples) were under control. Correlation coefficients for standard curves and efficiency values for detection of these genes were excellent. The mean correlation coefficient for these response curves obtained for these three genes (each assessed with four different RNA samples) was 0.992 (S.D. = 0.003, range 0.990–0.998). The efficiency of amplification of the three genes was very similar. The mean slope value for all three genes was −3.512 (S.D. = 0.096, range −3.42 to −3.64) and the mean efficiency of detection for the three genes in four samples was 94.54%.

3.3. Specificity and linearity of the real-time PCR reaction

The specificity of real time RT-PCR results was assessed by agarose gel electrophoresis and by dissociation curve analysis. Fig. 3 shows a typical example obtained with primer set BMC01644 amplifying high morbility group protein. Fig. 3A shows a single dissociation peak for the product of primer pair BMC01644 with a melting temperature (T_m) of 77.3 °C. The agarose gel shown in Fig. 3B shows a single band with the predicted size of 61 bp. These results mean that the BMC01644 real-time RT-PCR assay was gene-specific and that the results were not confounded by non-specific amplification or primer-dimer.

In general, melting curve and gel analyses agreed well. However, in a few cases the dissociation curve had a single melting peak but two bands were observed in the agarose gel. Interestingly, a single band of the expected size was observed with these primer sets when smaller amounts of male and female cDNA templates were used (data not shown).

3.4. Evaluation of gene expression in male and female worms by SYBR Green real-time RT-PCR

Of the initial 35 gender-specific transcript candidates chosen for study, 10 were transcripts that had been previously reported as gender-specific by RT-PCR (group 1) [4], and 25 were new candidate transcripts selected by ES (group 2). Group 1 results are shown in Table 2. Nine of 10 candidates from group 1 were confirmed by SYBR Green real-time RT-PCR to have significant gender-regulated expression with fold differences that ranged from 5 to 2621. Candidate AF118551 was not confirmed as gender-regulated expression by real time RT-PCR. This was not surprising as a faint band had been previously observed with male cDNA by RT-PCR [4]. Ten of 21 transcripts successfully tested by real-time RT-PCR in-group 2 (47.60%) were confirmed as sex-regulated genes with fold differences between 19 and 32768 (Table 3). Four of these transcripts had homology to known proteins, and six of these transcripts were novel.

Candidate genes were classified into three groups (female up-regulated, male up-regulated, or non-biased expression) based on real-time RT-PCR results. We used conservative >3-fold difference criteria for gender-biased gene expression. Female up-regulated genes included some genes previously reported to have female-specific expression (fatty acid-binding protein, high mobility group protein, glutamate-gated ion channel protein, and glutamine-rich protein) and some genes not previously identified as female-associated (MF sheath protein, caveolin-1 and several novel genes). Male up-regulated genes included MSP-1 (previously known to be up-regulated in males) and many novel genes.

Real-time RT-PCR results were highly reproducible. All genes were tested with second batch of RNA and all classifications (female, male or non-biased) were confirmed. The mean coefficient of variation for $\Delta\Delta C_t$ 19 obtained for genes with gender-biased expression was 13.1%.

4. Discussion

4.1. Validation of real-time RT-PCR results

We have successfully adapted the two-step SYBR Green I real-time RT-PCR to assess gender-biased gene expression in the nematode parasite *B. malayi*. The first step involves RNA isolation and cDNA synthesis. The second step is real-time PCR with gene specific primers. This represents the first use of this relatively new technology to study gene-expression

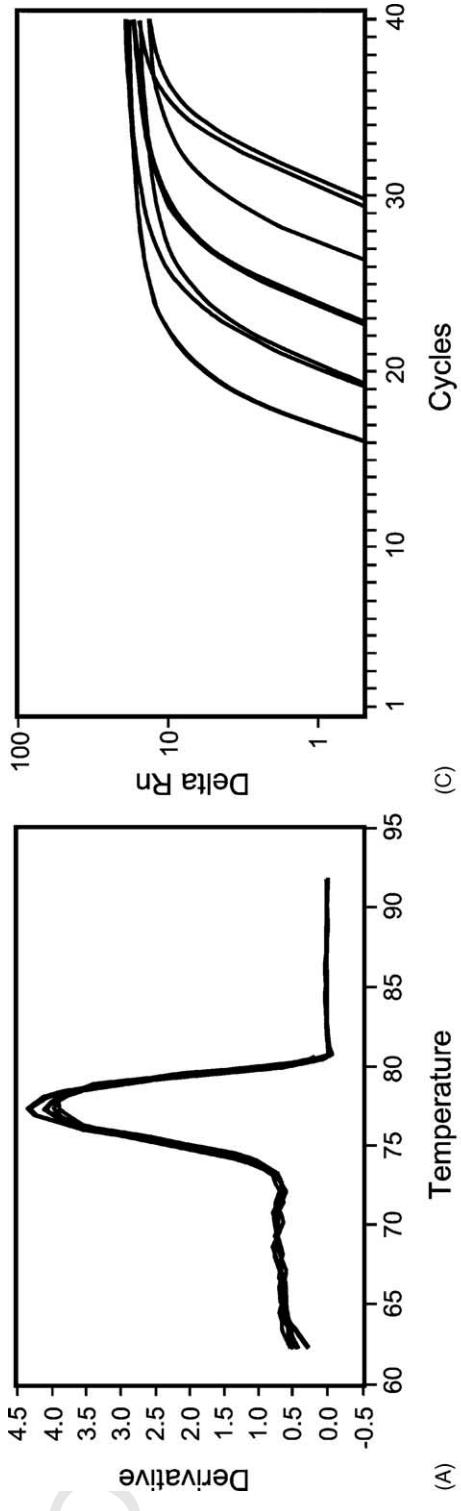

Fig. 3. This figure illustrates a typical quality control assessment of real-time RT-PCR with primer set for BMC01644. Panel A, melting curves; panel B, agarose gel electrophoresis of PCR product(s); panel C, amplification plots produced with varying amounts of a calibrator (female) RNA template. Panel A showed a single peak for the product of primer BMC01644 with a melting temperature value of 77.3 °C. Panel B shows a single 61 bp PCR product of primer BMC01644 with 1 ng female cDNA (F) and male cDNA (M) templates with the predicted size of 61 bp. The negative control (-RT) yielded no products.

Table 2
Relative mRNA expression level of group 1 candidates in male and female worms

Seq ID	BLASTX-similarity	Female ΔC_t [a]	Male ΔC_t [b]	$\Delta\Delta C_t$ [c]	X-fold mRNA level[d]
BMC01644	High mobility group protein	−1.5 ± 0.1	4.3 ± 0.7	−5.8 ± 0.1	55.7 (52–59.7) F
AF118554	Glutamate-gated ion channels	4.5 ± 0.55	9.1 ± .85	−4.6 ± 0.55	24.3 (16.6–35.5) F
AF118551	No hit	6.8 ± .01	8 ± 0.1	−1.2 ± 01	2.3 (2.28–2.31) N
BMC01764	No hit	−1.1 ± 0.04	5.1 ± 0.13	−6.2 ± 0.04	73.5 (71.5–75.6) F
BMC00903	Fatty acid-binding protein	0.3 ± 0.06	2.7 ± 0.08	−2.4 ± 0.06	5.3 (5.1–5.5) F
BMC02037	Glutamine rich protein	8.7 ± .06	13.1± 1.1	−4.4 ± 0.06	21.1 (20.3–22) F
BMC01751	No hit	17.3 ± 0.12	7.8 ± 0.12	−9.5 ± 0.12	724 (666–787) M
BMC03514	No hit	9.9 ± 0	−1.4 ± 0	−11.3 ± 0	2521 (2521) M
BMC03373	No hit	6.7 ± 0.12	12.9± 0.13	−6.2 ± 0.12	73.5 (67.6–79.9) F
BMC03552	No hit	8.4 ± 0.05	0.6 ± 0.05	−7.8 ± 0.05	222 (215–230.7) M

[a] Female ΔC_t is the C_t for the target gene normalized to an internal control gene (NADH) ($C_{tfemale} - C_{tcontrol}$) ± S.D.
[b] Male ΔC_t is the C_t for the target gene normalized to an internal control (NADH) ($C_{tmale} - C_{tcontrol}$) ± S.D.
[c] $\Delta\Delta C_t$ values are net differences in normalized C_t values obtained with male and female RNA ($\Delta C_{tcalibrator} - \Delta C_{ttest}$) ± S.D.
[d] Gene expression fold difference ($2^{-\Delta\Delta C_t}$, means and range). Genes were classified as having female (F), male (M), or non-biased (N) gene expression.

on a large scale in nematode parasites. Real-time RT-PCR has advantages over Northern blot or conventional RT-PCR, because it requires less RNA and provides quantitative results. We chose the two-step real-time RT-PCR method over the one-step method, because it has been reported to be more sensitive than the one-step method and less prone to problems related to production of primer-dimer artifacts and contamination with genomic DNA [19,20]. Others have reported that pseudogene sequences in genomic DNA are sometimes co-amplified by primers that were expected to be specific for cDNA templates [21]. We routinely treat RNA samples with DNase I and control its success by performing PCR without RT. This minimizes the problems of pseudogenes and non-specific DNA amplification.

Real-time RT-PCR results were obtained for all 10 transcripts in group 1 and 21 of 25 transcripts in group 2. No specific PCR products were obtained with four of the primer sets for genes in group 2. The mean slope of C_t versus RNA template concentration was -3.4 ± 0.05 for the genes tested. This was very close to the slope obtained with the control gene NADH (-3.41 ± 0.037). This indicated that relative differences in target genes could be calculated by the comparative C_t method [18]. For all 31 transcripts tested, the mean correlation (r^2) of detection was 0.993 (range, 0.971–0.999).

SYBR Green I detection of PCR products has a similar sensitivity, reproducibility, and dynamic range as methods that employ fluorescent probes [22,23]. It is also much less

Table 3
Relative mRNA expression level of group 2 candidates in male and female

Seq ID	BLASTX-similarity	Female ΔC_t [a]	Male ΔC_t [b]	$\Delta\Delta C_t$ [c]	X-fold mRNA expression [d]
BMC01695	Microfilaria sheath protein 1	1.2 ± 0.03	5.5 ± 0.08	−4.3 ± 0.3	19.7 (19.3–20.1) F
BMC00485	NADH dehydrogenase subunit 1	1.2 ± 0.06	1 ± 0.08	−0.2 ± 0.08	1.1 (1.09–1.2) N
BMC01735	Hypothetical protein	1.2 ± 0.01	2.6 ± 0.01	1.4 ± 0.01	0.6 (0.6) N
BMC01695	Microfilaria sheath protein 1	4.8 ± 0.17	9.2 ± 0.08	−4.4 ± .17	21.1 (18.8–23.8) F
BMC02383	Caveolin 1	0.8 ± 0.04	5.8 ± 0.14	−5 ± 0.04	32 (31.1–32.9) F
BMC01750	Hypothetical protein	5.6 ± 0.14	7.1 ± 0.07	−1.5 ± .14	2.8 (2.6–3.1) N
BMC01672	No hit	3.3 ± 0.14	7.6 ± 0.21	−4.3 ± 0.14	19.7 (17.9–21.7) F
BMC01737	Hypothetical protein	3.6 ± 0.06	5.2 ± 0.01	−1.6 ± 0.06	3 (2.9–3.21) N
BMC01863	No hit	7.1 ± 0.18	12.5 ± 0.17	−4.8 ± .18	27.9 (24.6–31.6) F
BMC11914	No hit	13.2 ± 0.18	0.8 ± 0.04	−12.4 ± 0.04	5404 (5257–5557) M
BMC03274	No hit	12.7 ± 0.81	2.8 ± 0.06	−9.9 ± 0.06	955 (917–996) M
BMC01685	Major sperm protein 1	6.7 ± 0.05	−2.7 ± 1.8	−8.9 ± 1.8	478 (137–1663) M
BMC10335	rRNA promotor binding protein	−9.5 ± 0.6	−9.8 ± 0.01	−0.3 ± 0.01	1.2 (1.2) N
BMC07890	No hit	−8.5 ± 0.1	−8.4 ± 0.07	0.1 ± 0.07	1.1 (1.0–1.13) N
BMC03414	24 kD secreted protein	4.6 ± 0.05	3.2 ± 0.07	−1.4 ± 0.07	2.6 (2.5–2.8) N
BMC12255	Macrophage migration inhibitory factor	−0.3 ± 0.11	1.2 ± 0.01	1.5 ± 0.01	0.5 (0.5) N
BMC06508	Putative senescence associated protein	−3.8 ± 0.04	−3 ± 0.08	0.8 ± 0.08	1.2 (1.12–1.28) N
BMC04232	No hit	10 ± 0.13	1.1 ± 0.02	−8.9 ± 0.02	477.7 (471–484) M
BMC03272	No hit	15.3 ± 1.4	0.3 ± 0.08	−15 ± 0.08	32,768 (31,000–34,636) M
BMC03310	Hypothetical protein	3.4 ± 0.45	3.7 ± 0.17	0.3 ± 0.17	1.7 (1.68–1.32) N
BMC03472	NADH dehydrogenase subunit 2	2.1 ± 0.06	1.3 ± 0.52	−0.8 ± 0.52	1.7 (1.2–2.5) N

[a] Female ΔC_t is the C_t for the target gene normalized to an internal control gene (NADH) ($C_{tfemale} - C_{tcontrol}$) ± S.D.
[b] Male ΔC_t is the C_t for the target gene normalized to an internal control (NADH) ($C_{tmale} - C_{tcontrol}$) ± S.D.
[c] $\Delta\Delta C_t$ values are net differences in normalized C_t values obtained with male and female RNA ($\Delta C_{tcalibrator} - \Delta C_{ttest}$) ± S.D.
[d] Gene expression fold differences ($2^{-\Delta\Delta C_t}$, means and range). Genes were classified as having female (F), male (M), or non-biased (N) gene expression.

expensive than probe-based detection methods, which require a different probe for each gene being tested.

Great care was taken to ensure the validity of our results. This included careful isolation and handling of total RNA and analyses to verify both the accuracy of gender separation of parasite material and the absence of genomic DNA in cDNA templates. We tested and validated three housekeeping genes as internal control genes. These should be broadly useful in studies of gene expression in filarial parasites.

The quality of real-time RT-PCR data was assessed by measuring the efficiency of PCR and correlation coefficients of standard curves. The specificity of SYBR Green detection of PCR products was verified by melting curve analysis and agarose gel electrophoresis. While our study focused on gender-biased gene expression, the methods we employed should be generally useful for studies of gene expression in nematode parasites.

We obtained real-time RT-PCR results for most of our candidate gene transcripts (31 out of 35). It may be possible to assess the "problem" candidate genes with different primer sets or other methods. Our results confirmed nine of 10 transcripts previously reported to be gender-specific by conventional RT-PCR [4] and 10 of 21 (47%) of new candidates identified by electronic selection. The latter confirmation rate is similar to that obtained in prior work with candidates selected by this method [4]. Thus, our new results confirm that electronic selection is a useful way to identify genes that may have gender-biased or stage-specific gene expression. It is not surprising that not all identified candidate genes were confirmed; some candidates would be expected to be falsely identified by ES by chance, given the relatively low numbers of male and female ESTs in the *B. malayi* database. Considering its sensitivity and specificity, real-time RT-PCR is well-suited for validation of differences in gene expression identified by other means, such as DDPCR and microarray analysis [9].

Expression data obtained by real-time RT-PCR were cross checked and verified several ways. First, genes were considered to be confirmed only if they showed consistent changes in two independent assays. Second, we had positive controls; some of the candidate genes had been previously shown to exhibit higher expression in male or female worms by RT-PCR. Third, expression patterns for several known genes were biologically plausible.

4.2. Gender-regulated gene expression in Brugia malayi

Our real-time RT-PCR studies identified 11 female-enriched messages and eight male-enriched messages based on the conservative criteria of expression ratios >3 (all were >5). We are confident that expression of these genes is gender-biased. It is possible, however, that some of the other genes with lower ratios have minor degrees of gender-biased expression. It is interesting that no transcript was absolutely gender-specific by real-time RT-PCR, although genes with fold differences greater than 100 are functionally gender-specific. This may be explained by very minute amounts of contamination of gender-specific RNA preparations not observed by the conventional RT-PCR methods we employed but detectable by real-time RT-PCR. It is also possible that female worms contain small amounts of male messenger RNA, either in sperm deposited by males or in developing male embryos and microfilariae.

Gender-regulated genes provide clues regarding sexual differences in function and behavior in parasitic nematodes. For *B. malayi*, where the male and female worms live in the same environment, genes that are expressed in a gender-regulated manner are likely to be involved in reproduction. For example, male-specific major sperm protein (MSP), a small cytoskeletal component that comprises ∼15% of total sperm protein, controls sperm motility in filarial worms [24]. In contrast, MF sheath protein is concentrated within the microfilarial sheath, which is a remnant of egg structures [25]. The inactivation of MF sheath protein by RNAi in adult female *B. malayi* worms has been reported to inhibit MF release from female worms [26]. Bm-FAB-1 is a homologue of a major protein found in the perivitelline fluid of first and second stage *Ascaris* larvae [27] that has been localized to the perivitelline fluid of early *B. malayi* embryos and on the surface of unhatched *B. malayi* microfilaria [28]. It is not clear what roles that high mobility group (HMG) proteins and glutamate-gated ion channel proteins play in filarial reproduction. However, it is interesting to note that HMG proteins are involved in mammalian sex determination [29,30]. The female up-regulated glutamate-gated ion channel protein is part of the molecular target of the avermectin family of anthelmintic and insecticidal compounds [31]. Interestingly, ivermectin has been reported to temporarily sterilize female *O. volvulus* worms [32].

Of course, many of the gender-biased genes we identified are novel. Additional studies will be needed to further validate these genes with localization and knockout studies to establish their function and assess their potential as targets for chemotherapy.

Our list of gender-biased genes is notable for the absence of many genes reported to be associated with reproduction in *C. elegans* [33]. This may be partially due to real differences between *C. elegans* (nematode Clade V) and the distantly related *B. malayi* (nematode clade III). However, apparent differences may also be due to insufficient sampling of the *B. malayi* cDNA libraries. We expect that a broader analysis of gene expression by microarray and future annotation of the emerging genome of *B. malayi* [34] will identify homologues for many of the "missing" *C. elegans* genes.

In summary, we have successfully used the SYBR Green I two-step real-time RT-PCR to identify sex-regulated genes in *B. malayi*. We have generated a partial list of internal control genes and sex-regulated genes for future study. And we have shown that real-time RT-PCR is an excellent technique for studying gene expression in parasitic nematodes.

References

[1] The *C. elegans* sequencing consortium. Genome sequence of the nematode *C. elegans*: a platform for investigating biology. Science 1998;282:2012–8.
[2] Maine E. RNAi as a tool for understanding germiline development in *Caenorhabdidtis elegans*: uses and cautions. Dev Biol 2001;239:177–89.
[3] Ottesen EA, Duke BO, Karam M, Behbehani K. Strategies and tools for the control/elimilation of lymphatic filariasis. Bull World Health Organ 1997;75:491–503.
[4] Michalski ML, Weil GJ. Gender-specific gene expression in *Brugia malayi*. Mol Biochem Parasitol 1999;104:247–57.
[5] William S, Lizotte-Waniewske MR, Foster J, Guiliano JD, Daub J, Scott AL, et al. The filaral genome project: analysis of the nuclear, mitochondrial and endosymbiont genomes of *Brugia malayi*. Int J Parasitol 2000;30:411–9.
[6] Orlando C, Pinzani P, Pazzagli M. Developments in quantitative PCR. Clin Chem Lab Med 1998;36:255–69.
[7] Siebert PD. Methods in molecular medicine. In: Reischi U, editor. Molecular diagnosis of infectious diseases. Clifton, NJ: Humana Press; 1997. p. 13.
[8] Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP. Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques 1997;22:130–8.
[9] Rajeevan MS, Ranamukhaarachchi DG, Vernon SD, Unger ER. Use of real-time quantitative PCR to validate the results of cDNA array and differential display PCR technologies. Methods 2001;25:443–51.
[10] Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 2002;29:23–39.
[11] Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 2000;25:169–93.
[12] Lie YS, Petropoulos CJ. Advances in quantitative PCR technology: 5′ nuclease assays. Curr Opin Biotechnol 1998;9:43–8.
[13] Schmittgen TD, Zakrajsel BA, Mills AG, Gorn V, Singer MJ, Reed MG. Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. Anal Biochem 2000;285:194–204.
[14] Weis JH, Tan SS, Martin BK, Wittwer CT. Detection of rare mRNAs via quantitative RT-PCR. Trends Genet 1992;8:263–4.
[15] Dhar AK, Roux MM, Klimpel KR. Quantitative assay for measuring the Taura syndrom virus (TSV) and yellow head virus load in shrimp by real-time RT-PCR using SYBR Green chemistry. J Virol Methods 2002;104:69–82.
[16] Parkinson JC, Witton D, Guiliano JD, Blaxter ML. 200,000 nematode ESTs on the net. Trends Parasitol 2001;17:394–410.
[17] Ririe KM, Rasmussen RP, Wittwer CT. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal Biochem 1997;245:154–60.
[18] Livak K. ABI Prism 7700 Sequence Detection System, User Bulletin 2. PE Applied Biosystem; 1997.
[19] Battaglia M, Pedrazzoli P, Palermo B, Lanza A, Bertolini F, Gibelli N, et al. Epithelial tumor cell detection and the unsolved problems of nested RT-PCR: a new sensitive one step method without false positive results. Bone Marrow Transplant 1998;22:693–8.
[20] Vandesompele J, De Paepa A, Speleman F. Elimination of primer-dimer artifacts and genomic coamplification using a two -step SYBR Green 1 real-time RT-PCR. Anal Biochem 2002;303:95–8.
[21] Kreuzer KA, Lass U, Landt O, Nitsche A, Laser J, Ellerbrok H, et al. Highly sensitive and specific fluorescence reverse transcription-PCR assay for the pseudogene-free detection of beta-actin trascripts as quatification reference. Clin Chem 1999;45:297–300.
[22] Lekanne Deprez RH, Fijnvandraat AC, Ruijter JM, Moorman AF. Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR Green I depends on cDNA synthesis conditions. Anal Biochem 2002;307:63–9.
[23] Steuerwald N, Cohen J, Herrera RJ, Brenner CA. Analysis of gene expression in single oocytes and embryos by real-time rapid cycle fluorescence monitored RT-PCR. Mol Hum Reprod 1999;5:1034–9.
[24] Scott AL, Dinman J, Sussman DJ, Yenbutr P, Ward S. Major sperm protein genes from *Oncocerca volvulus*. Mol Biochem Parasitol 1989;36:119–26.
[25] Zahner H, Hobom G, Stirm S. The microfilarial sheath and its proteins. Parasitol Today 1995;11:116–20.
[26] Aboobaker AA, Blaxter ML. Use of RNA interference to investigate gene function in the human filarial nematode parasite *Brugia malayi*. Mol Biochem Parasitol 2003;129:41–5.
[27] Mei B, Kennedy MW, Beauchamp J, Komuniecki PR, Komuniecki R. Secretion of a novel, developmentally regulated fatty acid-binding protein into the perivitelline fluid of the parasitic nemotode, *Ascaris suum*. J Biol Chem 1997;272:9933–41.
[28] Michalski ML, Monsey JD, Cistola D, Weil GJ. An embryo-associated fatty acid-binding protein in the filarail nematode *Brugia malayi*. Mol Biochem Parasitol 2002;124:1–10.
[29] Fechner PY. The role of SRY in mammalian sex determination. Acta Paediatr Jpn 1996;38:380–9.
[30] Bowles J, Koopman P. New clues to the puzzle of mammalian sex determination. Genome Biol 2001;2:1–4.
[31] Cully DF, Vassilatis DK, Liu KK, Paress PS, Van Der Ploeg LH, Schaeffer JM, et al. Cloning of an ivermerctin-sensitive glutamate-gated chloride channel from *Caenorhabditis elegans*. Nature 1994;371:707–11.
[32] Duke BO, Zea-Flores G, Castro J, Cupp EW, Munoz B. Effects of multiple monthly doses of ivermectin on adult *Oncocerca volvulus*. Am J Trop Med Hyg 1990;43:657–64.
[33] Jiang M, Ryu J, Kiraly M, Duke K, Reike V, Kim SK. Genome-wide analysis of developmental and sex-regulated gene expression profiles in *Caenorhabditis elegans*. Proc Natl Acad Sci USA 2000;98:218–23.
[34] Ghedin E, Wang S, Foster JM, Slatko BE. First sequences genome of a parasitic nematode. Trends Parasitol 2004;20:151–4.