Molecular & Biochemical Parasitology 180 (2011) 99–105

Contents lists available at SciVerse ScienceDirect

Molecular & Biochemical Parasitology

Candidate anthelmintic resistance-associated gene expression and sequence polymorphisms in a triple-resistant field isolate of *Haemonchus contortus*

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ARTICLE INFO

Article history:
Received 29 July 2011
Received in revised form 1 September 2011
Accepted 6 September 2011
Available online 16 September 2011

Keywords:
Levamisole
Macrocyclic lactone
Nicotinic acetylcholine receptor
Glutamate-gated chloride channel
P-glycoprotein
Quantitative real-time PCR

ABSTRACT

An isolate of *Haemonchus contortus*, UGA/2004, highly resistant to benzimidazoles, levamisole, and ivermectin was isolated from sheep at the University of Georgia, and passaged through experimentally infected goats. We measured the expression of twenty-nine mRNAs encoding drug targets and P-glycoproteins (P-gps), comparing the results to a fully susceptible laboratory passaged isolate. Expression levels of some nicotinic acetylcholine receptor mRNAs were markedly different in UGA/2004. Levels of the *Hco-acr-8b* mRNA, encoding a truncated subunit, were very high in resistant L3, but undetectable in susceptible larvae, with expression of the full-length *Hco-acr-8a* mRNA also significant increased. Expression of *Hco-unc-63* and *Hco-unc-29.3* mRNAs was significantly reduced in the resistant larvae. Expression of the *Hco-glc-3* and *Hco-glc-5* mRNAs, encoding glutamate-gated chloride channel subunits, were slightly reduced in resistant larvae. We observed significant increases in the expression of the *Hco-pgp-2* and *Hco-pgp-9* mRNAs in the UGA/2004 larvae, consistent with previous reports; we also saw a decrease in the levels of *Hco-pgp-1* mRNA. Treatment of the larvae with ivermectin and moxidectin *in vitro* produced variable and inconsistent changes in P-gp mRNA levels. The sequences of the β-tubulin isotype 1 mRNAs showed that the resistant larvae had a resistance-associated allele frequency of >95% at codon 200 and ~40% and codon 167. No changes at codon 198 were present. The presence of the truncated *acr-8b* mRNA may be a reliable indicator of levamisole resistance, but complex changes in gene expression associated with macrocyclic lactone resistance make the identification of a single genetic marker for this resistance difficult.

1. Introduction

Haemonchus contortus is a hematophagous gastrointestinal parasite of small ruminants which is extremely detrimental to animal health and productivity on a global scale [1]. Three classes of widely available broad-spectrum anthelmintics are routinely administered to livestock for the control of parasites such as *H. contortus*. These drugs are the benzimidazoles (BZs), which prevent the polymerization of nematode β-tubulin; the cholinergic agonists, such as levamisole and pyrantel; and the macrocyclic lactones (MLs), such as ivermectin and moxidectin, which modulate ligand-gated chloride channels (LGCCs) in the parasite. However, routine over-use of these compounds has led to the emergence of drug resistance, with widespread reports of parasites, including *H. contortus*, developing resistance to multiple drug classes [2,3].

Despite the high prevalence of drug-resistant parasites, the molecular basis of drug resistance is still not well understood; both changes in the drug target sites, and up-regulation of detoxification mechanisms, have been implicated. In the case of BZ resistance, single nucleotide polymorphisms (SNPs) causing codon changes at positions 167, 198 and 200 of the β-tubulin isotype 1 sequence have been found in resistant parasite populations [4–6]. P-glycoproteins (P-gps), efflux pumps known to expel hydrophobic xenobiotics from cells, have been linked to both BZ and ML resistance [7–9]. Changes in expression levels of levamisole receptor subunits have been observed in conjunction with pyrantel resistance in hookworms, and, recently, truncated nicotinic acetylcholine receptor (nAChR) subunit transcripts have been linked with levamisole

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doi:10.1016/j.molbiopara.2011.09.003

resistance in trichostrongylid parasites [10,11]. In the case of target site changes associated with ML resistance, the situation is much less clear; many different LGCCs may be affected by ivermectin and moxidectin, so identifying resistance mechanisms is more problematic [12–14].

Here we report the investigation of 29 candidate resistance genes in a triple-resistant isolate of *H. contortus* obtained from a clinical treatment failure case and subsequently maintained *in vivo*. cDNAs encoding β-tubulin isotype 1 and various LGCCs were sequenced to identify any SNPs, and the expression levels of 12 LGCC subunits, 8 nAChR transcripts, and 8 P-gps were assessed using quantitative real-time PCR. This approach was designed to both investigate resistance mechanisms reported from previous studies, and to identify any novel resistance-associated changes specific to the triple-resistant isolate.

2. Methods

2.1. Parasites

The UGA/2004 isolate of *H. contortus* was originally obtained from sheep at the University of Georgia (UGA) Sheep Unit in 2004. Just prior to this, clinical evidence suggested *H. contortus* were resistant to multiple anthelmintic classes, and a DrenchRite® Larval Development Assay (LDA) performed in August 2003 confirmed resistance to BZ, levamisole and avermectin anthelmintics. In June 2004, feces were collected from sheep at the UGA Sheep Unit, L3 were harvested from fecal cultures, and these were used to infect two worm-free goats. A pure infection of *H. contortus* was confirmed and L3 collected from these goats were cryopreserved in June 2005. In October 2007, L3 were thawed and used to infect a new set of worm-free goats. Since then the isolate has been passaged in additional laboratory-infected goats housed at the UGA College of Veterinary Medicine. The US/HcS isolate is highly susceptible to all anthelmintics, but the precise history of this isolate is unknown. The only history available is that this isolate was cryopreserved in 1986, then in 2006 was placed back into sheep, and was maintained in sheep until transferred to UGA in 2010. In 2010 this isolate was used to infect a worm-free goat and has been passaged in goats continuously since being acquired. All goats infected with *H. contortus* that were used in this study are housed in AAALAC accredited laboratory animal facilities, all protocols and procedures regarding animal use have been reviewed and approved by the UGA Animal Use and Care Committee, and all infected animals are closely monitored to ensure that they remain clinically healthy.

To obtain the L3 used in this study, fresh feces were collected from the infected goats using canvas fecal collection bags attached to the goats with a harness. Feces were then crushed, and an approximately equal volume of vermiculite added along with a small amount of water to moisten the feces/vermiculite mixture. Fecal cultures were then incubated at room temperature for 10–12 days with daily mixing. The cultured feces were then placed in a Baermann apparatus containing warm water and left overnight at room temperature. L3 larvae were harvested the next morning, rinsed thoroughly with deionized water and stored at 10 °C until used.

2.2. Larval development assay

A commercial LDA (DrenchRite®) was used as described previously [2,15]. Anthelmintics used in the DrenchRite® LDA are thiabendazole, levamisole, and avermectin aglycone, which serve as *in vitro* surrogates for drugs in the BZ, imidizothiazole, and avermectin classes of anthelmintics, respectively. Thus, susceptibility/resistance results are not specific to the particular drug used *in vitro*, but rather apply to members of the anthelmintic class [15]. Data for individual anthelmintics were analyzed by a logistic regression model (Fit Logit algorithm [16,17]) to determine the EC_{50} and EC_{95}, which are defined as the anthelmintic concentration required to block development to the L3 stage in 50% and 95% of the larvae, respectively.

2.3. RNA extraction and reverse transcription

RNA was extracted from L3 stage *H. contortus* from both the US/HcS and UGA/2004 isolates. Approximately 500,000 larvae from each culture were used per RNA extraction. Larvae were concentrated by centrifugation at 3000 × *g* for 15 min. RNA was extracted using Trizol® reagent (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed using qScript™ cDNA SuperMix (Quanta Biosciences) in a scaled-up reaction to generate 200 μl cDNA. Three biological replicates were performed, using L3 from 3 independently prepared cultures of both the US/HcS and UGA/2004 worms.

2.4. PCR and sequencing to identify SNPs

PCR was performed on cDNA samples produced from mRNA extracted from pools of L3 larvae, and amplified the region of β-tubulin isotype 1 covering codons 167, 198 and 200, and also the full-length LGCC sequences, *Hco-avr-14B*, *Hco-glc-5*, *Hco-lgc-37*, and *Hco-glc-6*. Accession numbers for these sequences and primer sequences used for amplification are shown in Table 1. PCR was performed using GoTaq® Green Mastermix (Promega). PCR products were separated by gel electrophoresis, and the bands corresponding to the expected product size were excised from the gel and purified using a gel extraction kit (Qiagen). The gel-purified PCR products were then sequenced directly in both a forward and reverse direction using gene specific primers; for the LGCC sequences, the qPCR primers were used for sequencing. DNA sequencing was performed by Genewiz (Germantown, MD, USA).

2.5. Quantitative real-time PCR

Quantitative real-time PCR was carried out using a Stratagene® Mx3000P instrument and associated software. 20 μl PCR reactions were prepared using PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences) according to the manufacturer's instructions, 2 μl cDNA, and 1 μl each of both a forward and reverse gene-specific primer (concentration 10 μM). Gene specific real-time PCR primers used in the reactions are shown in the tables below. Technical replicates of each reaction were performed in triplicate; this was repeated for the 3 separate biological samples from both *H. contortus* isolates. Efficiency values were calculated for each primer pair/PCR reaction from linear regression of a plot of dilution of template DNA against C_t value (data not shown). These values were used in conjunction with the real-time PCR data to use the Pfaffl method for data analysis [18], using actin as a control gene. Data were analyzed using the REST software freely available from Qiagen, and GraphPad Prism® was used to display the analyzed data in a graphical format.

3. Results

3.1. Resistance status of the two Haemonchus contortus isolates

Results of the DrenchRite® LDA (Table 2) indicate that UGA/2004 is highly resistant to BZs, levamisole and avermectins. In contrast, the US/HcS isolate is highly sensitive to all the drugs tested. Resistance ratios for both EC_{50} and EC_{95} values were very high,

Table 1
Accession numbers of the GluCl, nAChR and tubulin sequences studied, together with the primers used for the real-time reactions. The details of the primers used for the *Hco-pgp* genes have been published previously [25].

Gene	Accession number	Primer sequences
β-Tubulin isotype 1	FJ981633	PCR fw GAAGCTGAAGGTTGTGATTGC
		PCR rv GCTAACTTGCGAAGATCAGCA
		Seq fw ATTGACGCATTCACTTGGAGG
		Seq rv CAGGGAATCGGAGGCAGGTC
Hco-avr-14a	Y14233	qPCR fw GTCTCGTGGGTCTCGTTCTGG
		qPCR rv CATTGTGAGTAGGGTAGTGAC
Hco-avr-14c	JF309448	qPCR fw GTAAAACCAATACCGAGCTCCG
		qPCR rv CGTAGTTGACCAACGCGAAT
Hco-avr-14b	Y14234	qPCR fw CTTATCGATCTCGCATCATATG
		qPCR rv CTGTATTCTCCGGTATTGGT
		PCR fw ATGCGCAATTCCGTCCCTCTG
		PCR rv TCAGTCGAGGTTGCTTTG
Hco-glc-2	Y09796	qPCR fw ACTACGATATGAGGGTTCGA
		qPCR rv TGAGGAATTTTGGTGGGTTGTG
Hco-glc-3		qPCR fw GTGAACATGGAGTACAGCGTGC
		qPCR rv GAAGGTGCATTGGACAGGAGAG
Hco-glc-4	EU006788	qPCR fw GATCACTACTTCCCATGCGA
		qPCR rv TGCTCGTCCAATATCCCCAT
Hco-glc-5	AF076682	qPCR fw AGTCCCTATAGCGATTAGCA
		qPCR rv CTCTCGTCAATCCATTCTTCT
		PCR fw ATGTTCGCCTTGATTCTGCC
		PCR rv GCCTGGACTGCCATGTACGG
Hco-glc-6	EU006789	qPCR fw TGTGGATGTATGGATCGGAG
		qPCR rv CGATCATCTTTGGTGGATCA
		PCR fw ATGAGAATTACATTGATGGAGC
		PCR rv TCAGAGAAACGAATACAATG
Hco-ggr-3	EF202570	qPCR fw TCGCGATTCCCCATAGACGCACA
		qPCR rv CCTTCAGCTGATCCCACATTCC
Hco-lgc-55	FJ817373	qPCR fw GCACGACAATCTTTGAGAGTTAC
		qPCR rv AGAACCATGCAGTAAGTAGGGAG
Hco-lgc-37	X73584	qPCR fw CTCTCGATTGCTACTTCCGAC
		qPCR rv CTGGCACCCATATATCTTTG
		PCR fw CTTCGTGTTCGTCATTCTTCGG
		PCR rv ATGGCTTGATGTCTGTGATG
Hco-LGCC1	EF619540	qPCR fw TACGATGCACTCTAACCGTAGC
		qPCR rv CCGATTTCCGGATGGAACG
Hco-unc-38	GU060984	qPCR fw AGGCTGTACGACGATCTCATGG
		qPCR rv AAGGCACGTAAAGCACGCTC
Hco-unc-63a	GU060985	qPCR fw CGCTCTTCTATACTGTGAATC
		qPCR rv ACTAAGAGTGACCAGCACCATG
Hco-unc-29.1	GU060980	qPCR fw GAATGGGTAGATCTGTCAGAA
		qPCR rv AGCCATTAGCACAGTAGGGAT
Hco-unc-29.2	GU060981	qPCR fw GAATTGGTCGATCTTTCTGAA
		qPCR rv TCCCATCAGAAAGGTAGGAAT
Hco-unc-29.3	GU060982	qPCR fw ACGTTGACAACGTACGTAC
		qPCR rv ACTGTGACTAAGATGGTGATC
Hco-unc-29.4	GU060983	qPCR fw CGAAATAAAACTGGAGTTCGA
		qPCR rv GAGCACGACAGTATAGAAGAGTG
Hco-acr-8a	EU006785	qPCR fw CGTCGTATACATAGTGGAATC
		qPCR rv ATCCGAACGGTATGTGCAAC
Hco-acr-8b	GU168769	qPCR fw CGTCGTATACATAGTGGAATC
		qPCR rv AGACACGACTCGAATATGGA

demonstrating extremely large differences in anthelmintic susceptibility between the two isolates (Table 2). In UGA/2004, large shifts to the right in the sigmoidal dose–response were seen for both thiabendazole and ivermectin–aglycone, and development of L3 did not occur at the highest drug concentrations tested allowing for relative accurate calculation of the EC_{95}. However, with levamisole, larvae developed to the L3 stage even at the highest concentration tested (25 μM). Thus an accurate EC_{95} could not be calculated. This type of *in vitro* result is not uncommon with levamisole; the *in vitro* phenotype of resistance seen with levamisole often differs from the other drug classes. Whereas an overall population shift in susceptibility is typically seen for the BZs and avermectins, with levamisole it is common to see a smaller overall population shift, together with a highly resistant subpopulation ([17,19]; RMK, unpublished observations). In the case of UGA/2004, this highly resistant subpopulation is represented by the 20% of the larvae developing to L3 at 25 μM, which is the highest levamisole concentration tested in the assay. Consequently, an alternative means to evaluate the relative difference in levamisole susceptibility between the two isolates is to compare the highest drug concentration that permitted development to the L3 stage, as well as the percentage of larvae developing to the L3 stage at that concentration. In US/HcS, 0.89% of the larvae developed to the L3 stage at 3.1 μM, whereas 56% of UGA/2004 developed to the L3 stage at this concentration, and 20% of UGA/2004 developed to the L3 stage at 25 μM.

3.2. SNPS in β-tubulin isotype 1

Sequencing β-tubulin isotype 1cDNA from the UGA/2004 isolate of *H. contortus* revealed the presence of two SNPs which have previously been linked to BZ resistance. The sequencing chromatograms showing these regions of the β-tubulin isotype 1 sequence from both the susceptible and triple-resistant isolate are shown in Fig. 1. In the resistant population, analysis of the chromatogram indicated codon 167 is TAC rather than TTC in approximately 40% of the alleles present in the sample (based on relative peak height), encoding a phenylalanine to a tyrosine amino acid change. At codon 200, the UGA/2004 parasites also show the TTC to TAC, phenylalanine to tyrosine, sequence change, but at a higher frequency in the population; we estimate from the chromatogram shown in Fig. 1 that the allele frequency for this SNP is ∼95%. There was no evidence of any sequence difference at codon 198 between the US/HcS and UGA/2004 parasite isolates. The allele frequencies observed in the UGA/2004 population would appear to suggest that most individuals are homozygous for the F200Y allele, and, possibly, heterozygous for the F167Y allele, though it should be noted that this analysis was based on pools of nematodes, and that individual worms were not genotyped. Homozygosity for F200Y or heterozygosity for F167Y has each alone been reported to confer BZ

Table 2
Anthelmintic sensitivity of the UGA/2004 and US/HcS isolates of *Haemonchus contortus* based on the DrenchRite® larval development assay (LDA). DrenchRite® LDA [38] were performed on two separate egg isolations of the US/HcS isolate during 2010, and five separate egg isolations of the UGA/2004 isolate between 2007 and 2010. In all five assays, UGA/2004 was highly resistant to benzimidazole, levamisole and avermectin anthelmintics. Mean and (range) values for both EC_{50} and EC_{95} are given.

Drug tested	US/HcS		UGA/2004		RR[a] EC_{50}	RR EC_{95}
	EC_{50}	EC_{95}	EC_{50}	EC_{95}		
Thiabendazole[b] (μM)	0.03 (0.03–0.04)	0.04 (0.04)	26.09 (26.77–30.68)	145.39 (89.11–216.36)	870	3776
Levamisole (μM)	1.32 (1.32)	2.89 (2.39–3.39)	6.98 (2.38–11.6)	1962.71[c] (101.61–6345)	5.28	679
Ivermectin–aglycone[d] (nM)	0.51 (0.11–0.91)	2.25 (1.88–2.61)	30.43 (12.96–48.06)	305.88 (170.53–492.83)	60	136

[a] RR, resistance ratio. This value is calculated as the ratio of drug concentrations required to achieve the EC_{50} or EC_{95} in the resistant parasite isolate as compared to the susceptible isolate.
[b] EC_{50} and EC_{95} values for UGA/2004 suggest very high resistance to benzimidazole anthelmintics
[c] Value for EC_{95} for levamisole in the UGA/2004 isolate is only an estimate, and this value is prone to a high degree of error since the highest concentration tested (25 μM) was far below the calculated value for EC_{95}.
[d] EC_{50} and EC_{95} values for UGA/2004 suggest high level resistance to avermectins. Based on criteria previously established for defining resistance to moxidectin in the DrenchRite® LDA [15], these values suggest low emerging resistance to moxidectin.

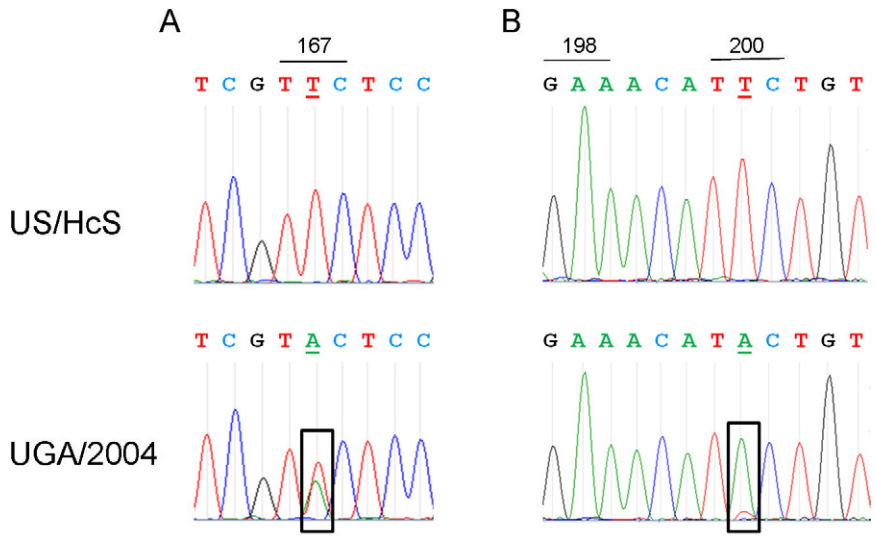

Fig. 1. Chromatograms showing the sequence of the β-tubulin isotype 1 PCR products from the susceptible (top row) and resistant (bottom row) *H. contortus* isolates. (A) Sequence around codon 167, showing a significant proportion of A residues in the resistant compared to susceptible amplicons. (B) Sequence around codons 198 and 200. No differences between the isolates were observed at codon 198, but at codon 200 TTC has been almost completely replaced by TAC in the resistant isolate.

resistance on trichostrongylid nematodes [5,6]. Finding both these SNPs in the same isolate of *H. contortus*, at such high frequencies, seems to be an unusual finding, and may correlate with the very high level of BZ resistance exhibited by UGA/2004 (Table 2).

3.3. Single nucleotide polymorphisms in ligand-gated chloride channel mRNAs

Sequencing of the LGCC cDNAs encoding the AVR-14B, GLC-5, LGC-37 and GLC-6 subunits did not reveal any significant coding differences between the US/HcS and UGA/2004 *H. contortus* isolates. All of the LGCC cDNAs sequenced showed a high degree of genetic polymorphism, and at several nucleotide positions there were different SNPs present within, and between, the different parasite populations. However, none of the SNPs occurring at high frequencies in the UGA/2004 isolate resulted in amino acid substitutions, and were probably incidental variations arising simply from the different genetic backgrounds of the parasites.

3.4. Expression levels of ligand-gated chloride channels

Constitutive expression levels of a range of LGCC mRNAs were compared between the triple-resistant and drug-sensitive parasites using real-time PCR. The levels of the LGCC mRNAs were very similar in the two isolates for the majority of the genes examined. During the course of this work we identified two new transcripts, which were then included in this study. *Hco-glc-3* is a putative GluCl gene with very high sequence identity to the previously described *glc-3* from *Caenorhabditis elegans* [20], and was identified by BLAST searches of the *H. contortus* database (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/h_contortus). *Hco-avr-14c* is a novel splice variant of *Hco-avr-14*, missing the sequences encoding the first and part of the second membrane-spanning regions of Hco-AVR-14A, revealed during RT-PCR experiments. Two LGCC transcripts did show a small but statistically significant ($p = <0.05$) reduction in expression in the UGA/2004 isolate: *Hco-glc-3* mRNA expression was 0.41 (0.11–0.85) and *Hco-glc-5* mRNA expression was 0.59 (0.26–1.26) relative to the US/HcS isolate. The relative expression levels of LGCC mRNAs is shown in Fig. 2A. The finding that *Hco-glc-3* mRNA expression is lower in the drug-resistant parasites may be related to the sensitivity of GLC-3 channels to ivermectin; this has been clearly demonstrated for the *C. elegans* GLC-3 receptor, but the *H. contortus* ortholog has yet to be fully characterized [20]. The GLC-5 channel from *H. contortus* is sensitive to ivermectin [21–23], and has also previously been associated with ML resistance [23,24].

3.5. Expression levels of P-glycoproteins

Constitutive expression levels of P-gp mRNA transcripts were assessed by real-time PCR as previously described [25]. A small but significant decrease in *Hco-pgp-1* mRNA was observed in the triple-resistant isolate; expression was 0.315 (0.097–1.68) relative to the drug sensitive isolate (Fig. 2B). Significant increases were seen in the levels of *Hco-pgp-2* mRNA, which were 5.96 (1.01–50.4) times higher in the resistant isolate, and *Hco-pgp-9* mRNA, where expression was 2.65 (0.82–6.49) times higher in the resistant parasites (Fig. 2). These increases in P-gp mRNA levels is an interesting finding, as these genes have previously been reported to be associated with ML resistance of parasitic nematodes [26,27].

Attempts were also made to measure inducible changes in P-gp mRNA levels after exposure to various concentrations of ivermectin and moxidectin; however, no consistent patterns of up-regulation or down-regulation could be discerned between different biological replicates, so the data have not been included. It seems clear from this result, and from the large variation in constitutive expression levels observed between different samples from the same parasite isolate, as indicated by the large error bars in Fig. 2, that P-gp mRNA levels can vary considerably in *H. contortus*.

3.6. Expression levels of nAChRs

Constitutive expression levels of nAChR subunit mRNAs thought to contribute to levamisole sensitive receptors were quantified by real-time PCR. These results are shown in Fig. 2C. The most notable finding was a dramatic increase in a truncated transcript of *acr-8*, *Hco-acr-8b*, in the resistant parasites. There was at least a 481-fold increase (97.5–3130) in this transcript in the UGA/2004 worms; in some experiments expression levels of this truncated form were too low to be detected by the assay in the US/HcS isolate, so even this increase is perhaps a conservative estimate. This clearly supports the previous findings of Neveu and colleagues that the truncated *Hco-acr-8b* transcript is a marker for levamisole resistance [11]. However, a truncated *Hco-unc-63* transcript also

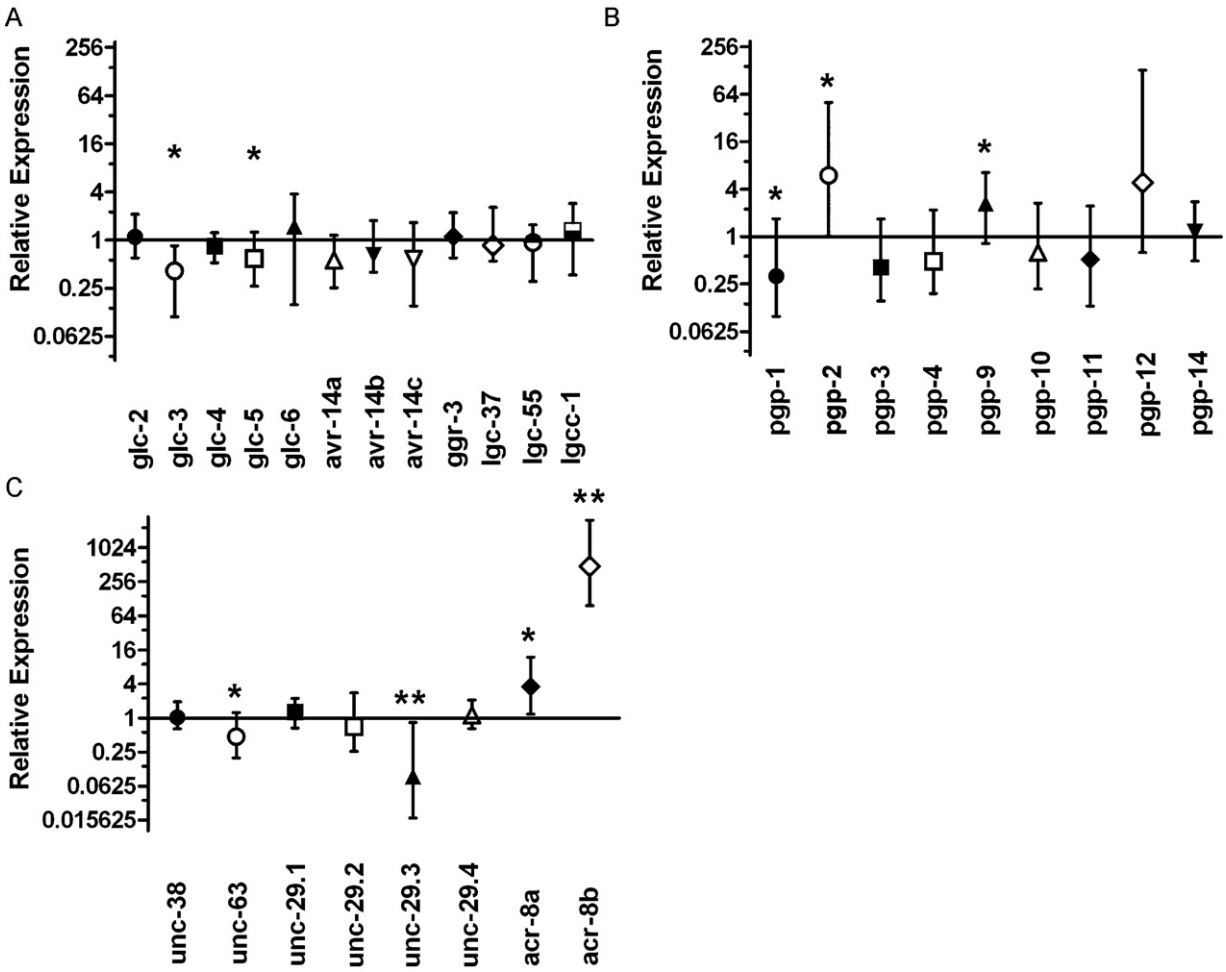

Fig. 2. Changes in mRNA transcript levels between L3 larvae from resistant and susceptible worms. Data are shown ±SEM and significant changes are indicated by $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$. (A) Ligand-gated chloride channels transcripts. (B) P-glycoprotein transcripts. (C) Nicotinic acetylcholine receptor transcripts.

identified in levamisole-resistant *H. contortus* [28] could not be detected at all in the UGA/2004 isolate.

Other statistically significant changes in nAChR subunit mRNA levels in the resistant parasites included 2.14 (0.8–5.0)-fold lower expression of the full-length *Hco-unc-63* transcript, and a 3.53 (1.16–11.89) fold increase in the full-length *Hco-acr-8* transcript. There was also a large reduction in the expression of one of the duplicated *Hco-unc-29* genes, *Hco-unc-29.3*; the expression level in the UGA/2004 parasites was only 0.091 (0.017–0.83) relative to the US/HcS isolate. No difference in mRNA expression levels were detected for *Hco-unc-38*, or for the other three *Hco-unc-29* genes.

4. Discussion

Anthelmintic resistant isolates of *H. contortus* have been reported from almost all parts of the world, and previous studies have shown that parasites resistant to all the currently available anthelmintics are common in the southeast United States [2]. Despite this, our understanding of the molecular basis of this resistance is patchy; several polymorphisms in β-tubulin have been associated with BZ resistance and molecular assays developed to measure the frequency of these alleles within populations [29] but for levamisole and ML resistance no such molecular markers are available. UGA/2004 represents a resistant *H. contortus* isolate that was isolated relatively recently from sheep in Georgia, USA. The resistance status of this isolate has been well characterized by larval development assays, and demonstrates high levels of resistance to BZs, levamisole, and avermectins (Table 2). We are studying the differences between this isolate and a fully susceptible isolate at the molecular and genetic level. As a first step, we report here studies on the expression and sequence of several genes that have been implicated in anthelmintic resistance; this may not represent a complete list of such genes, but the data we present have allowed us to confirm a potential molecular marker for levamisole resistance and to identify other genes that warrant more detailed study for their potential role in ML resistance, as well as eliminating others as serious candidates.

The finding of ~95% allele frequency for F200Y and ~40% allele frequency for F167Y is probably in itself sufficient to explain the high level of BZ resistance observed for UGA/2004 in the DrenchRite® LDA. Finding both these SNPs at such high levels in the same population is unusual, as previous studies of field populations of *H. contortus* have found F167Y mainly in parasites lacking the F200Y SNP [6,30].

In the case of resistance to the MLs however, a single genetic marker remains elusive. There were no convincing new candidate SNPs found in the resistant isolate, and there was no evidence of the mutations such as L256F previously identified by Njue et al. in an ivermectin-resistant isolate of the cattle parasite *Cooperia oncophora* [31]. The up-regulation of *Hco-pgp-2* and *Hco-pgp-9* mRNAs may be a contributing factor to resistance, and these and other P-gps have been implicated in the resistance of several parasitic nematodes to the MLs [12,26,32,33]. However, the

increased expression levels that we observed were relatively modest, rather less than reported for *Tci-pgp-9* by [26], and these changes alone may not be sufficient to confer the high level of resistance to the MLs measured for the UGA/2004 isolate. Examination of multiple isolates, correlating changes in the expression of these genes with the level of resistance, is required to clarify this. There is good and increasing evidence that ML resistance is associated with changes in the expression of P-gps, and our data suggest that this either involves polymorphisms in multiple genes, or the differences that we report here are due to changes in an upstream regulator of P-gp expression that affects multiple genes simultaneously. In addition, it became very clear during these experiments that the levels of P-gp mRNAs in *H. contortus* were quite variable and could apparently change in a rapid and unpredictable manner, at least in L3 maintained in culture. This variability confounded our attempts to measure any biologically relevant changes in expression of these mRNAs following exposure to ivermectin or moxidectin *in vitro*. Similarly, the observed changes in the expression levels of the LGCC mRNAs were fairly small; it seems rather unsatisfactory to claim that these small decreases in two ivermectin sensitive chloride channels, with no accompanying increase in ivermectin insensitive channels, could completely explain the observed resistance phenotype. Though some of the differences reached statistical significance, the real biological significance was not clear – in this study we examined a total of 29 different mRNAs so it would be expected that differences at a probability level of $p \leq 0.05$ would be found for at least one of these by chance. No changes in the expression of any of the *avr-14* transcripts, as observed for the cattle parasites *C. oncophora* and *Ostertagia ostertagi* [34], were detected in our experiments. It has also been observed that selection for ML resistance also selects for the BZ-resistance associated SNP at codon 200 of β-tubulin [30], which is present at very high levels in UGA/2004. In the case of this isolate, it is difficult to dissect the contribution of BZ and ML treatments to the selection of this SNP, and further studies of its importance to ML resistance are required.

For levamisole resistance, we have confirmed that the truncated transcript, *Hco-acr-8b*, originally reported by Neveu et al. [11] is present at high levels in the UGA/2004 isolate, but is almost undetectable in the US/HcS parasites. We have also developed a real-time PCR assay which can distinguish between the different splice variants of the *Hco-acr-8* mRNA, and we intend to further develop this assay into a form that allows rapid detection of this potential resistance marker in populations of parasites. The changes in expression levels of the full-length *Hco-acr-8a*, *Hco-unc-63* and *Hco-unc-29.3* subunit mRNAs may also hint at a potential mechanism of levamisole resistance; it is possible that changes in the subunit composition and stoichiometry of the neuromuscular nicotinic acetylcholine receptor lead to differences in sensitivity to the cholinergic anthelmintics. For example, the nAChR may contain an extra Hco-ACR-8 subunit, which may be severely truncated in the case of Hco-ACR-8B, in place of Hco-UNC-63. This would be consistent with our previous findings from the parasite *Ascaris suum*, where we demonstrated that changing the stoichiometry of a recombinant nicotinic receptor from *A. suum* dramatically changes its *in vitro* sensitivity to levamisole and pyrantel, without markedly affecting the efficacy of acetylcholine [35]. Very recently, Boulin et al. [36] have reported that omitting Hco-ACR-8 from a reconstituted *H. contortus* nAChR expressed in *Xenopus* oocytes altered the response of the receptor to levamisole, changing the drug from a super-agonist to a partial agonist. Changes in the size of the response to a single concentration (100 μM) of pyrantel were also reported, though given the complex pharmacology of this drug, which is both an agonist and a channel blocker (at higher concentrations) [35,37] such data are hard to interpret. Similar changes in the expression of nicotinic receptor subunit mRNAs were reported in pyrantel-resistant isolates of canine hookworm, *Ancylostoma caninum* [10], where higher levels of resistance were correlated with reduced expression of the *Aca-unc-29*, *Aca-unc-38* and *Aca-unc-63* mRNAs. These observations emphasize the complexity of the pharmacology of the nicotinic acetylcholine receptors present in parasitic nematodes and illustrate the way in which the worms may be able to alter the composition of the receptors in order to overcome the challenges of drug treatment. They also confirm the possibility that the parasites may not be able to easily become resistant to multiple nicotinic drugs, though this will probably turn out to vary between species.

All of the mRNA measurements in this study were carried out on L3 larvae, and it is certainly possible that somewhat different results might be obtained from adult worms. However, the level of drug resistance for UGA/2004 and the US/HcS isolates were determined by *in vitro* larval development assay. Thus, the gene sequences and expression levels determined in this study are directly comparable to the drug resistance phenotype of the L3 stage. Additionally, previous work in our laboratory has demonstrated that the DrenchRite® LDA correlates well with *in vivo* drug resistance status for *H. contortus* [15]. Thus the use of the free-living L3 stage seems justified for these experiments, and doing so eliminates the great additional cost and use of experimental animals that is necessary to obtain adult worms.

In summary, the triple-resistant isolate studied here shows the well-established markers of BZ resistance, albeit in an unusual combination, and confirms that expression of the alternatively spliced *Hco-acr-8b* mRNA is an attractive candidate for development as a marker for levamisole resistance. Changes in P-gp mRNA expression are associated with ML resistance, but the complexity of those changes may hamper their development as a marker for resistance.

Acknowledgements

This work was supported partially by a grant from the University of Georgia College of Veterinary Medicine Veterinary Medical Experiment Station. The funders had no input into the experimental design or the writing of the manuscript. We also would like to thank Robert Dobson for sharing the Fit Logit analysis algorithm.

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