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Genotyping of Athelmintic Resistant *Haemonchus contortus* Reveals New β-Tubulin Allelic Variants

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Abstract: Anthelmintic resistance detection of ruminant gastroenteric parasitic nematode *Heamonchus contortus*, can be achieved by molecular probes and DNA amplification assays, this advanced methodologies are faster and cheaper to apply than conventional bioassays, however for better accuracy of a DNA-based test, it is necessary first to clarify the details of the molecular mechanisms underlying anthelmintic resistance. β-tubulin is Benzimidazoles (BZ) anthelmintic target, several anthelmintic detection methods rely on the identification of mutations within the coding gene leading to BZ tolerance or resistance. *H. contortus*, β-tubulin genes are highly variable and new alleles are frequently detected in small ruminant livestock infected with this parasite. This research was done with the objective to identify geographical allelic variants of β-tubulin genes in Mexican *H. contortus* field isolates. Specific DNA was obtained from the nematodes by RT-PCR starting from mRNA, amplicons were cloned, submitted to automate DNA sequencing and translated to aminoacid (aa) sequences that were analyzed by multiple sequence alignment software and compared against similar nematodes proteins reported at the GenBank. Six allelic variations were identified in the analyzed sequences one at aa position 200 conferring a change from Phe to Tyr regarded as responsible for BZ resistance in *H. contortus*. Five more allelic variations not previously identified were found at position: 64, Val to Ala., 170 Val to Ala., 190 His to Gln,, 198 Asp to Glu and 343 Asp to Glu, this findings corroborate the allelic variability of *H. contortus* β-tubulin and highlight the necessity to identify new geographic alleles useful for DNA-based anthelmintic resistance assays.

Key words: Anthelmintic resistance, β-tubulin, *Haemonchus contortus*, DNA sequencing, allelic variance

INTRODUCTION

Anthelmintic resistance is widely spread among grastroenteric parasitic nematodes including *H. contortus*, this phenomenon neutralizes the control efforts of significant helminth parasites originating an adverse economic impact on the affected livestock industries (Jabbar *et al*., 2006). Small ruminants are particularly susceptible with a relevant incidence on wool, meat and milk production (Coles *et al*., 1992). The scientific search for the molecular basis underlying this problem lead to the finding that Benzimidazole (BZ) resistance mechanisms in gastroenteric helminth parasites are caused by mutations in β-tubulin genes, this protein is the intracellular target for BZ compounds, BZ interfere with β-tubulin polymerization into microtubules by binding to the protein altering the mitotic phase during cell replication (Hollomon *et al*., 1998). β-tubulin gene expression shows different isotypes in nematodes each with different affinities to BZ binding, these genes have demonstrated to be highly variable from one geographical region to another leading to a constant identification of new alleles conferring resistance against BZ (Beech *et al*., 1994). Mutations on β-tubulin DNA sequence originate changes in the aminoacid sequence altering the structural properties of the protein observed in resistant isolates of *H. contortus* (Ghisi *et al*., 2007). Some studies have demonstrated that BZ resistance in trichostrongylid nematodes are related to a particular mutation in the β-tubulin gene at aminoacid (aa) 200, this mutation produces an aa substitution of a Phe in the BZ susceptible nematodes to Tyr in BZ resistant (Beech *et al*., 1994; Humbert *et al*., 2001; Jabbar *et al*., 2006). Resistance against BZ in a number of nematode,

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fungi and some protozoa species has been shown to be caused by aa substitutions at either position 167 or 200 of β-tubulin (Hollomon *et al*., 1998; Silvestre and Humbert, 2002). In general eukaryotes that normally code for tyrosine instead of Phe at codon 167 and 200 appear to be uniformly tolerant for BZ in a form that otherwise, would affect mitotic cell replication (Hollomon *et al*., 1998). The knowledge of the molecular basis of the BZ resistance, allowed the development of DNA-based methods for the diagnosis of BZ resistance in worm populations since DNA point mutations and allelic variations are straight fordward and easy to identify with a well designed protocol (Geary *et al*., 1992; Silvestre and Humbert, 2000, 2002; Walsh *et al*., 2007). In addition to its diagnostic value, resistance detection by DNA-based methods, makes it possible to study resistance traits with implications for the management of resistance in relation to avoidance of selection of resistant worms and a rational administration of different anthelmintic compounds based on a scientific judgment (Jabbar *et al*., 2006).

MATERIALS AND METHODS

Two *Haemonchus contortus* field isolates previously reported were used (Campos *et al*., 1990; Miranda-Miranda *et al*., 2006), one resistant to benzimidazoles named BzRHc; the other one is resistant to ivermectin named IvRHc. Total RNA was isolated from 50,000 L3 larvae using RNA aqueous according to manufacturer's instructions (Ambion). Reverse Transcriptase PCR (RT-PCR) cDNA was derived from total RNA using first strand cDNA Amplification Kit (Ambion), the cDNA was used in a PCR reaction with a forward oligonucleotide primer 5'-ATGCGTGAAATC GTTCATGTG-3' and the reverse oligonucleotide primer 5'-CTCCTCGGGATATGCCTCTTC-3', oligonucleotides were designed to amplify the *H. contortus* β-tubulin DNA sequence reported at the Genbank database (Accession No. HAEBTUBC; Geary *et al*., 1992).

The cycling conditions were 94°C for 4 min followed by 25 cycles of 94°C for 45 sec, 61°C for 45 sec, 72°C for 1 min and a 4°C hold in a Sprint thermal cycler (Thermo Scientific USA). The PCR amplicon was verified by 1% agarose electrophoresis in 1X TBE buffer (Shambrook and Russel, 2001). Amplicons were cloned directly into plasmid pCRII using the TA/TOPO cloning system (Invitrogen USA), plasmids were used to transform *E. coli* HC101 bacteria and plate them on Luria agar plates (Sambrook and Russell, 2001). Individual colonies were used to seed 5 mL Luria broth, where bacteria were allowed to grow at 37°C with agitation for 18 h, bacteria cell pellets were collected by centrifugation and subjected to a plasmid isolation protocol using a commercial kit (Wizard Promega USA). Plasmids were screened for cloned amplicons by Eco R1 restriction enzyme digestion of the plasmid, DNA insertions were verified by 1% agarose electrophoresis (Sambrook and Russell, 2001).

Plasmids were submitted to nucleotide sequencing reactions, carried out by Dye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems) and automated sequencing (ABI PRISM Model 373A or ABA Model 377 automated sequencer with Version 1.2.2 or Version 2.1.1 software, respectively) were carried out by the Institute of Biotechnology UNAM. The obtained sequences were analyzed using the online DNA sequences software analysis at the National Center for Biotechnology Information of the National Institute of Health NCBI/NIH (www.ncbi.nih.gov), in search for an Open Reading Frame (ORF), identified ORF's were translated to aa sequences and subjected to BLAST similarity searches (Altschul *et al*., 1997) and search for phylogenetic matches compared to the GenBank sequences database. Translated aa sequences were aligned against β-tubulin gene sequences of *H. contortus* (GenBank accession number HAEBTUBC) using the Geneworks® 2.45 multiple sequence alignment program (Intelligenetics California USA).

RESULTS

Two 1.34 Kbp were obtained from the RT-PCR reaction on both samples of nematodes (Fig. 1). Cloned products in the PCRII plasmids were identified also as 1.34 Kbp from the Eco R1 restriction enzyme analysis (Fig. 1). Nucleotide sequencing and subsequent sequence

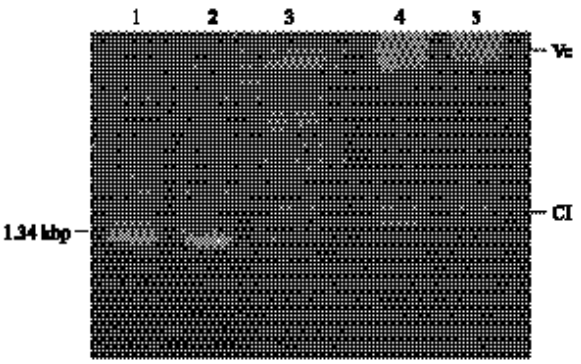

Fig. 1: Agarose DNA electrophoresis. RT-PCR 1.34 Kbp amplicons from nematodes BzReHc and IvReHc were identified at lane 1 and 2, respectively. Sequencing plasmid Cloned RT-PCR products were subjected to Eco R1 digestion analysis lanes 3 and 4. Ve stands for PCR II Vector, Cl stands for Cloned RT-PCR products. Lane 3 contains Molecular weight references

A

Sequences producing significant alignments:		Score (Bits)
gb|AAB28077.1|	beta-tubulin [Haemonchus contortus, Peptide, 448	912
emb|CAA56353.1|	unnamed protein product [Haemonchus contortus]	911
gb|ABM92348.1|	beta tubulin isotype 1 [Haemonchus contortus]	910
gb|AAP20434.1|	beta-tubulin isotype 1 [Cooperia oncophora]	907
gb|ABM92349.1|	beta tubulin isotype 1 [Cooperia pectinata]	904
emb|CAA93249.1|	beta-tubulin [Teladorsagia circumcincta]	904
gb|ABM92349.1|	beta tubulin isotype 1 [Cooperia pectinata]	904
emb|CAA93249.1|	beta-tubulin [Teladorsagia circumcincta]	904

B

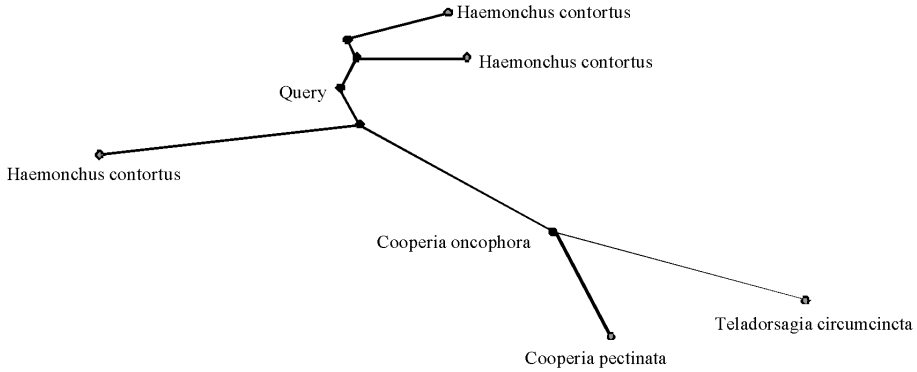

Fig. 2: Blast query and phylogenetic tree. A. ORF found in the raw DNA data from the automated sequence reaction was submitted to a BLAST query, the best matches were found at the GenBank database for -tubulin from the nematodes: *Haemonchus contortus* (AAB28077, CAA56353, ABM92348), *Cooperia oncophora* (AAP20434), *Cooperia pectinata* (ABM92349), *Teladorsagia circumcicta* (CAA93249). B. Best matches from the BLAST query were depicted in a phylogenetic tree, relative distances between sequences within the tree, represent similarities

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                                                                64
IvReHc      MREIVHVQAG QCGNQIGSKF WEVISDEHGI QPDGTVKGES DLQLERINVY YNEAHGGKYV PRAVLVDLEP GTMDS    75
HAEBTBC     MREIVHVQAG QCGNQIGSKF WEVISDEHGI QPDGTVKGES DLQLERINVY YNEAHGGKYV PRAVLVDLEP GTMDS    75
BzReHc      MREIVHVQAG QCGNQIGSKF WEVISDEHGI QPDGTVKGES DLQLERINVY YNEAHGGKYV PRAALVDLEP GTMDS    75
Consensus   MREIVHVQAG QCGNQIGSKF WEVISDEHGI QPDGTVKGES DLQLERINVY YNEAHGGKYV PRAVLVDLEP GTMDS    75

IvReHc      FRSGPYGQLF RPDNYVFGQS GAGNNWAKGH YTEGAELVDN VLDVVRKEAE GCDCLQGFQL THSLGGGTGS GMGTL   150
HAEBTBC     FRSGPYGQLF RPDNYVFGQS GAGNNWAKGH YTEGAELVDN VLDVVRKEAE GCDCLQGFQL THSLGGGTGS GMGTL   150
BzReHc      FRSGPYGQLF RPDNYVFGQS GAGNNWAKGH YTEGAELVDN VLDVVRKEAE GCDCLQGFQL THSLGGGTGS GMGTL   150
Consensus   FRSGPYGQLF RPDNYVFGQS GAGNNWAKGH YTEGAELVDN VLDVVRKEAE GCDCLQGFQL THSLGGGTGS GMGTL   150

                      170                 190     198 200
IvReHc      LISKIREEVP DRIMASFSVA PSPKVSDTVV EPYNATLSVQ QLVENTDETF CIDNEALYDI CFRTLKLTNP TYGDL   225
HAEBTBC     LISKIREEVP DRIMASFSVV PSPKVSDTVV EPYNATLSVH QLVENTDETF CIDNEALYDI CFRTLKLTNP TYGDL   225
BzReHc      LISKIREEVP DRIMASFSVV PSPKVSDTVV EPYNATLSVH QLVENTDDTV CIDNEALYDI CFRTLKLTNP TYGDL   225
Consensus   LISKIREEVP DRIMASFSVV PSPKVSDTVV EPYNATLSVH QLVENTDETF CIDNEALYDI CFRTLKLTNP TYGDL   225

IvReHc      NHLVSVTMSG VTTCLRFPGQ LNADLRKLAV NMVPFPRLHF FMPGFAPLSA KGAQAVRAST VAELTQQMFD AKNMM   300
HAEBTBC     NHLVSVTMSG VTTCLRFPGQ LNADLRKLAV NMVPFPRLHF FMPGFAPLSA KGAQAVRAST VAELTQQMFD AKNMM   300
BzReHc      NHLVSVTMSG VTTCLRFPGQ LNADLRKLAV NMVPFPRLHF FMPGFAPLSA KGAQAVRAST VAELTQQMFD AKNMM   300
Consensus   NHLVSVTMSG VTTCLRFPGQ LNADLRKLAV NMVPFPRLHF FMPGFAPLSA KGAQAVRAST VAELTQQMFD AKNMM   300

                                                343
IvReHc      AACDPRHGRV LTVAAMFRGR MSMREVDDQM MSVQNKNSSV FVDWIPNNVK TAVCDIPPRG LKMAATFIGN STAIQ   375
HAEBTBC     AACDPRHGRV LTVAAMFRGR MSMREVDDQM MSVQNKNSSV FVEWIPNNVK TAVCDIPPRG LKMAATFIGN STAIQ   375
BzReHc      AACDPRHGRV LTVAAMFRGR MSMREVDDQM MSVQNKNSSV FVEWIPNNVK TAVCDIPPRG LKMAATFIGN STAIQ   375
Consensus   AACDPRHGRV LTVAAMFRGR MSMREVDDQM MSVQNKNSSV FVEWIPNNVK TAVCDIPPRG LKMAATFIGN STAIQ   375

IvReHc      ELFKRISEQF TAMFRRKAFL HWYTGEGMDE MEFTEAESNM NDLISEYQQY QEATADDMGD LDAEGGEEAY PEE     448
HAEBTBC     ELFKRISEQF TAMFRRKAFL HWYTGEGMDE MEFTEAESNM NDLISEVQQV QEATADDMGD LDAEGGEEAV PEE     448
BzReHc      ELFKRISEQF TAMFRRKAFL HWYTGEGMDE MEFTEAESNM NDLISEVQQV QEATADDMGD LDAEGGEEAV PEE     448
Consensus   ELFKRISEQF TAMFRRKAFL HWYTGEGMDE MEFTEAESNM NDLISEVQQV QEATADDMGD LDAEGGEEAV PEE     448
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Fig. 3: Multiple aminoacid sequence alignment. Sequences from IvReHc and BzReHc nematodes obtained from the ORF finder software, were comparatively aligned against GenBank database sequence HAEBTBC Isotype I BZ susceptible -tubulin. Software GeneWorks® 2.45

analysis and aminoacid (aa) translation produced a 1.34 Kbp nucleotide sequence with a complete ORF translated to a 448 aa sequence. Blast analysis showed high homology with reported *H. contortus* β-tubulin isotype I (GenBank accession numbers: AAB28077, CAA56353, ABM92348) as well as with analogous proteins of the nematodes: *Cooperia oncophora* (AAP20434), *Cooperia pectinata* (ABM92349) and *Teladorsagia circumcicta* (CAA93249) (Fig. 2). Sequence analysis demonstrated 3 nucleotide substitution for IvReHc nematodes originating aa changes at, 170, Val to Ala., 190 His to Gln and 343 Glu to Asp, as well as three nucleotide substitutions in BzReHc nematodes with aa changes at 64 Val to Ala, position 198 Glu to Asp and 200 Phe to Tyr (Fig. 3).

DISCUSSION

The mutation involved in BZ resistance which causes the replacement of a phenylalanine by a tyrosine at aminoacid (aa) residue 200 of β-tubulin gene, occurs in about 60% of resistant nematodes (Ghisi *et al*., 2007) this mutation is an easy target for a DNA-based BZ resistance detection and allele-specific PCR makes it possible to determine the BZ resistance genotype of ruminant gastroenteric worms (Silvestre and Humbert, 2000, 2002; Walsh *et al*., 2007). This DNA based assays has been applied for anthelmintic resistance diagnostic purposes on the three most prevalent trichostrongylid species: *Teladorsagia circumcincta*, *Trichostrongilus colubriformis* and *Haemonchus contortus*, in order to establish their BZ resistance or susceptibility (Silvestre and Humbert, 2000; Walsh *et al*., 2007).

However, the accuracy of a DNA based diagnostics for BZ resistance is compromised for the fact that β-tubulin is highly variable and polymorphic, for that reason continuously new alleles are found including β-tubulin allelic variants not previously reported that are likely candidates to confer BZ resistance (Beech *et al*., 1994) one mutation have been recently reported in *H. contortus* β-tubulin gene producing an aa change from Glu to Ala in position 198 in South African *H. contortus* (Ghisi *et al*., 2007). Our BzReHc nematodes showed an aa change at aa position 200 of Phe to Tyr which is consistent wit BZ resistance in our BzReHc nematodes. Another nearby as substitution was spotted at position 198 this position is similar to allelic variant reported for BZ resistant South African *H. contortus* (Ghisi *et al*., 2007) but Mexican nematodes showed a substitution from Gln to Asp instead of Ala, this change in Mexican nematodes implicates the substitution of an acid aa for another with similar properties, since BzReHc nematodes are already BZ resistant it is difficult to ascertain this aa 198 mutation roll on the anthelmintic phenomenon without an extensive bioassay or phenotyping, however it does demonstrates the necessity to do more research on geographically new allelic variants.

Another aa that may confer BZ resistance was spotted on the BzReHc at position 64 from Val to Ala; this implicates a neutral aa for another one with similar properties, substitution for similar aa may not represent a significant change for conferring anthelmintic properties, yet indicates the high tendency for aa substitution in the β-tubulin gene of gastroenteric parasites. IvReHc nematodes are not resistant to BZ compounds, nevertheless they are Ivermectine resistant a type of anthelmintic resistance not related to β-tubulin. Our results showed no aa substitution at Phe 200 which is consistent with the absence of Bz resistance, still three aa substitution were found at position 170 were a Val was changed for Ala, at aa position 190 were His was changed to Gln and at position 343 were Glu was change to Asp, only 170 and 343 substitution are not relevant since changes were made for similar aa, however substitution 190 is relevant since histidine is a sulfur bridge generating aa and a change for an acid aa should confer an important structural difference, only further biochemical assay could demonstrate the relevance of this aa change in β-tubulin function or protein structure.

DNA-based anthelmintic resistance detection in *H. contortus* depends on an accurate previous knowledge of β-tubulin allele distribution on the geographic location were the survey will take place. A highly variable gene like β-tubulin requires a constant genotyping to maintain an up to date catalog of BZ resistance allelic geographical distribution (Walsh *et al*., 2007), only 60% of the gastroenteric parasites BZ resistance can be predicted by the amino acid 200 of the β-tubulin gene (Silvestre and Humbert, 2000, 2002) our work may help to determine new allelic variants in order to increase the diagnostics probabilities for a DNA-based assay dedicated to BZ resistance identification in *H. contortus*.

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REFERENCES

Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman, 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Research, 25: 3389-3402.

Beech, R.N., R.K. Prichard and M.E. Scott, 1994. Genetic variability of the beta-tubulin genes in benzimidazole-susceptible and -resistant strains of Haemonchus contortus. Genetics, 138: 103-110.

Campos, R.R., D.R. Herrera, R.H. Quiroz and J.S. Olzaran, 1990. Resistencia de *Haemonchus contortus* a bencimidazoles en ovinos en México. Tec: Pe. Mex. 28: 30-34.

Coles, G.C., C. Bauer, F.H. Borgsteede, S. Geerts, T.R. Klei, M.A. Taylor and P.J. Waller, 1992. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol., 44: 35-44.

Geary, T.G., S.C. Nulf, M.A. Favreau, L. Tang, R.K. Prichard, N.T. Hatzenbuhler, M.H. Shea, S.J. Alexander and R.D. Klein, 1992. Three beta-tubulin cDNAs from the parasitic nematode Haemonchus contortus. Mol. Biochem. Parasitol., 50: 295-306.

Ghisi, M., R. Kaminsky and P. Maser, 2007. Phenotyping and genotyping of Haemonchus contortus isolates reveals a new putative candidate mutation for benzimidazole resistance in nematodes. Vet. Parasitol., 144: 313-320.

Hollomon, D.W., J.A. Butters, H. Barker and L. Hall, 1998. Fungal beta-tubulin, expressed as a fusion protein, binds benzimidazole and phenylcarbamate fungicides. Antimicrob. Agents Chemother., 42: 2171-2173.

Humbert, J.F., J. Cabaret, L. Elard, V. Leignel and A. Silvestre, 2001. Molecular approaches to studying benzimidazole resistance in trichostrongylid nematode parasites small ruminants. Vet. Parasitol., 101: 405-414.

Jabbar, A., Z. Iqbal, D. Kerboeuf, G. Muhammad, M.N. Khan and M. Afaq, 2006. Anthelmintic resistance: The state of play revisited. Life Sci., 79: 2413-2431.

Miranda-Miranda, E., E. Liebano-Hernandez, M.E. Lopez-Arellano, P. Mendoza-de-Gives and R. Cossio-Bayugar, 2006. Marcadores enzimáticos como indicadores de resistencia a los antihelmínticos en el nematodo parásito de rumiantes *Haemonchus contortus*. Bioquimia, 31: 6-12.

Sambrook, J. and D.V. Russell, 2001. Molecular Cloning: A Laboratory Manual. 3rd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, Vol. 1.

Silvestre, A. and J.F. Humbert, 2000. A molecular tool for species identification and benzimidazole resistance diagnosis in larval communities of small ruminant parasites. Exp. Parasitol., 95: 271-276.

Silvestre, A. and J.F. Humbert, 2002. Diversity of benzimidazole-resistance alleles in populations of small ruminant parasites. Int. J. Parasitol., 32: 921-928.

Walsh, T.K., A.A. Donnan, F. Jackson, P. Skuce and A.J. Wolstenholme, 2007. Detection and measurement of benzimidazole resistance alleles in Haemonchus contortus using real-time PCR with locked nucleic acid Taqman probes. Vet. Parasitol., 144: 304-312.