Int.J.Curr.Microbiol.App.Sci (2020) *9*(4): 1175-1184

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 9 Number 4 (2020)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2020.904.139

RNA Interference of the *Hemonchus contortus* Astacin-like MTP Gene by the Soaking Method

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Keywords

RNA interference, *H. contortus*, Astacin-like MTP gene, Soaking method

Article Info

Accepted:
10 March 2020
Available Online:
10 April 2020

A B S T R A C T

RNA interference (RNAi) is a powerful molecular tool for gene discovery and functional validation for candidate vaccine antigens from the vast amount of information available within EST datasets. In this paper we report the knock down in the phenotypic expression of *Hemonchus contortus* astacin-like MTP gene associated with reproduction and blood-feeding of this parasite which is predominantly expressed in late larval and adult stages by RNAi interference. The RNAi experiments were conducted utilizing the soaking protocol. Results showed that there was interference in expression of each *H. contortus* astacin 3' or 5'ends transcripts individually as judged by RT-PCR's. The resultant dsRNA products were also found to be gene-specific. However no visible phenotypes were detected after simultaneous RNAi treatments for both *H. contortus* astacin 3'and 5'end gene transcripts.

Introduction

Gut expressed antigens of the parasitic nematode *Haemonchus contortus* are effective targets for vaccine development (Redmond *et al.,* 2004; Loukas *et al.,* 2004). These proteins play an important role in food digestion within the worm (Williamson *et al.,* 2003) and antibody is thought to mediate the protective immunity after vaccination by blocking the activity of the enzymes. This would interfere with food digestion and may ultimately lead to worm starvation. However, the assumption that intestinal secreted enzymes are important for food digestion is only based on circumstantial evidence. The enzymes are localised in the intestinal villi and are able to degrade proteins involved in nutrition of the worms, such as haemoglobin (Williamson *et al.,* 2003). Nevertheless no

conclusive experimental evidence to support a specific role in feeding is available. Subjecting specific gut expressed enzymes to RNAi is the way forward to verification of their functions.

A family of astacin-like MTPs associated with the transition to parasitism which are developmentally regulated exist in parasitic nematodes (Zhan *et al.,* 2002; Gomez Gallego *et al.,* 2005). Suggested roles for the MTPs include moulting, feeding, tissue penetration and immunomodulation, thus making them prime targets for intervention by vaccination to control infection.

Astacin-like MTP activity was reported in *H. contortus* L3 EF and in ExL3 ES (Gamble *et al.,* 1989; Geldolf *et al.,* 2005). As with many of the hookworm vaccine candidates, levels of protection using a bacterially-expressed AcMTP have been highly variable.

Assessing astacin-like MTPs functions in RNAi experimental models would provide an insight to their true role in nematodes. This paper describes RNA i of astacin-like MTP in *H. contortus* using the soaking method.

Materials and Methods

Extraction of RNA from *H.contortus* parasites

Total RNA was extracted from exsheathed L3, L4 and adult male and female parasites as described (Hashmi *et al.,* 2002). In brief, parasites were re-suspended in 200µl of lysis buffer (0.5% SDS, 5% β-mercaptoethanol, 10 mM EDTA, 10 mM Tris–HCl, pH 7.5, and 0.5 mg/ml proteinase K), quick frozen at -80 ^{0}C for 10 min, followed by incubation at 55 ^{0}C for 1 h. The RNA was extracted using Total RNA Isolation Reagent, (Advanced Biotechnologies Ltd.) and stored at -80^{0}C until use.

Generation of *H. contortus* astacin cDNA

Primers

Four gene specific primers designated HCF1Nco-Ⅰ (5'-ccatgga ATGAGGCTCAC TATACTACTG-3'); HCR1Xho-Ⅰ (5'-ctcgaga GGCAGCAAGTTAA

CCAGAT -3'); HCF2-Nco-Ⅰ (5'-ccatggaAA CTCGGCCACGCTCTTGG-3'); HCR2Xho-Ⅰ (5'-ctcgag GGTGCTTCTCATA ACAA GAAA -3') respectively, based on *H.contortus* astacin-like MTPs gene (Acc. No.AM159505) (Fig. 1), were synthesized (EurofinS MWG Operon, Germany).

Single step RT-PCR

Reverse transcriptase coupled PCR (RT-PCR) was used to determine the specificity of *H. contortus* astacin cDNA mRNA transcription using SuperScript One-Step RT-PCR System (Invitrogen) according to the manufacturer's instructions. The thermo cycle conditions were cDNA synthesis and pre-denaturation phase of 55^{o}C (30min) (1-cycle) and 94^{o}C (5min) (1-cycle), followed by PCR amplification of 30cycles at 94^{o}C (30s), 56^{o}C (1min), 72^{o}C (2min), and a final extension for 7min at 72^{o}C in the last cycle.

H. contortus astacin 3'or 5'end*s* amplified products were analyzed by gel electrophoresis on 1% (w/v) agarose gels (Sigma*), stained with red gel (BIOTIUM*) and submerged in 1xTAE electrophoresis buffer. The gels were visualized under ultraviolet illumination and recorded on a Polaroid 667-film. Amplified 3'and 5'ends of *H. contortus* astacin DNA fragments were excised from agarose gels and purified with QIAqiuck Gel extraction Kit(QIAGEN*), as per manufacturer instructions, and cloned into pGEM*-T cloning vector.

Cloning of *H. contortus* astacin 3'and 5'ends

The pGEM*-T cloning vector (Promega*), and each of the 3' or 5'end fragments of purified *H. contortus* astacin DNA obtained from step 1.4.2 above for sub-cloning were combined in approximately 3:1 molar ratio (fragment: vector). The cloning reactions were assembled as follows:

Reagents	3'ends	5'ends	+Ve	BG
2 × rapid ligation buffer T_4 DNA ligase	5µl	5µl	5µl	5µl
pGEM – T (50ng)	1 µl	1 µl	1 µl	1 µl
Purified PCR products	3 µl	3	-	-
Control DNA insert	-	-	2 µl	-
T_4 DNA ligase (3Weiss units/µl)	1 µl	1 µl	1 µl	1 µl
dH_20	-	-	1 µl	3 µl
Total volume	10 µl	10 µl	10 µl	10 µl

The ligation reactions were incubated overnight at 4°C. *E. coli* JM 109 competent cells (STRATAGENE*) were transformed with recombinant *C.* pGEM* -T/ *H. contortus* astacin 3'or 5'end DNA products, respectively.

Transformation of *E. coli* JM 109 with recombinant 3'or 5'ends

The *E. coli* JM 109 bacteria strain was transformed with recombinant *C.* pGEM* –T/ *H. contortus* astacin 3' or 5'end*s* DNA as per manufacturer instructions (STRATAGENE*). Briefly; 100µl of thawed JM 109 competent cells were mixed with 0.8µl β-mercaptoethanol (1.42M) and placed on ice for 10min. A 2µl of ligation mix (recombinant *C.* pGEM* –T/ *H. contortus* astacin 3'or 5'end*s* DNA) was added and the mixture put on ice for 30minutes.

This was heated at 42°C for exactly 45 seconds, and rapidly placed on ice bath for another 2min. The cells were then cultured in 900µl of SOC media on a slow rotating shaker set at 150rpm for 90min at 37°C. Transformed cells (200µl) were plated on 1% (w/v) agarose LB-agar/X-gal (50mg/ml)/1M-IPTG plates supplemented with 25mg/ml of ampicillin antibiotic and incubated at 37^0C for 16hrs. Largest single colonies were randomly selected and cultured in LB (Luria Bertani medium), (Tryptone (10g), Yeast extract (5g), and NaCl (10g) growth media pre-mixed with ampicillin (25mg/ml) and incubated overnight at 37°C on a slow rotating shaker. The 3'or 5'end*s* plasmid DNA were extracted utilizing Wizard* Plus SV Minipreps DNA purification kit as per manufacturer instructions (PROMEGA*) and stored at -20°C until use.

Identification of recombinant 3'or 5'end*s*

Recombinant *C.* pGEM* –T/ *H. contortus* astacin 3'or 5'end*s* plasmid DNA were digested with *Xho* I and *Nco* I restriction enzymes followed by gel electrophoresis for identification purposes and verified by sequencing (EurofinS MWG Operon, Germany).

Construction of recombinant L4440 RNA_i expression vector

Purified recombinant 3'or 5'end*s* DNA products obtained from step 1.5.1 above were

subsequently excised from pGEM*-T using *Nco* Ⅰ and *Xho* Ⅰ restriction enzymes and ligated in the L4440 RNAi vector (Promega*), previously linearized with similar enzymes for construction of RNAi recombinant expression vector (Fig 2). *E. coli* cells were transformed with recombinant L4440 /*H. contortus* astacin 3'or 5'end*s* DNA product.

Transformation of *E. coli* with L4440/*H. contortus* astacin 3'or 5'end*s*

The *E. coli* XL-10 GOLD* bacteria strain (STRATAGENE*), were transformed with recombinant L4440/*H. contortus* astacin 3'or 5'end*s* DNA essentially as described in step 1.5.1 above and stored at -20^{0}C until use.

Identification of recombinant positive clones

The recombinant L4440/*H. contortus* astacin 3'or 5'end*s* plasmid DNA were digested with *Xho* I and *Nco* I restriction enzymes, separated by agar gel electrophoresis for identification and verified by sequencing (EurofinS MWG Operon, Germany).

RNA interference of *H. contortus* astacin gene

Synthesis of dsRNA

The L4440 constructs were linearised with *Nco* I and *Xho* I restriction enzymes separately and dsRNA prepared from these templates using the T7 Ribomax Express RNAi System (Promega*) according to manufacturer instructions.

Soaking of *H. contortus* worms with dsRNA

RNAi using the soaking protocol was performed on ~100 L_3 and L_4 worms. The worms were washed with PBS and soaked in 2% sodium hypochlorite for 15min. and washed again.

The parasites in a 30µl of PBS volume containing 1 mg/ml dsRNA pre-mixed with 3 µl of lipofectin reagent (Invitrogen) were stood at room temperature for 48 h. Larvae soaked in either PBS alone or lipofectin reagent without dsRNA served as the respective controls.

Detection of dsRNA treated worms

The loss of 3'or 5'end transcripts following RNAi was detected by RT-PCR with pairs of *H. contortus* astacin 3' or 5'end*s* specific primers viz: HCF1Nco- Ⅰ (5'- ATGAGGCT CACTATACTACTG-3'); HCR1Xho- Ⅰ (5'-GGCAGCAAGTTAA CCAGAT-3'); HCF2-Nco-(5'-AACTCGGCCACGCTCTTGG-3'); HCR2Xho- Ⅰ (5'-GGTGCTTCTCATAACA AGAAA -3'), respectively.

Extraction of t-RNA and the RT-PCR's were performed as previously described. After 35 cycles of amplification, the RT-PCR products were separated on 1% agarose gels.

Results and Discussion

H. contortus astacin 3'or 5'end PCR products

H. contortus astacin 3'or 5'end*s* PCR products migrated to the expected size of ~200bp (Fig. 2.0), following gel electrophoresis on 1% (w/v) agarose (Sigma) gels, stained with 0.5mg/ml red gel.

Recombinant pGEM –T/ *H. contortus* astacin 3'or 5'end*s*

The 3'or 5'end*s* DNA fragments of *H. contortus* astacin migrated to an expected size of ~200bp (Fig. 3.), after digesting recombinant pGEM –T/ *H. contortus* astacin

3'or 5'end*s* with *Xho* I and *Nco* I restriction enzymes followed by gel electrophoresis.

L4440 RNA$_i$ vector DNA

Digested L4440 RNAi vector DNA of ~2790 (Fig. 4), was excised from agarose gels, purified and ligated to purified *H. contortus* astacin 3'or 5'end*s* fragments of ~200bp for construction of RNA interference recombinant expression vector of *H. contortus* astacin.

Recombinant L4440/*H. contortus* astacin 3'or 5'end*s*

The recombinant L4440/*H. contortus* astacin 3'or 5'end*s* plasmid DNA digested with Nco I and Xho I restriction enzymes followed by electrophoresis on 1% agarose gels migrated to the expected size of ~200bp (Fig. 5).

RNAi of *H. contortus* astacin 3'or 5'end transcripts

To confirm the absence or presence of transcripts following RNAi treatment, RT-PCRs using gene specific primers was carried out in dsRNA treated as well as control worms.

The loss of *H. contortus* astacin 3'or 5'end*s* cDNA from RNAi-treated worms is shown (Fig. 6), Lane 1: worms soaked with *H. contortus* astacin 3'end*s* dsRNA; Lane 2: worms soaked with *H. contortus* astacin 5'end*s* dsRNA. The transcripts are noticeably visible in the negative control worms, using appropriate primers to check uniformity of the RNA purifications, see lanes 3 ~ 6, Fig. 6.

In this paper we describe interference in the expression of astacin-like MTP gene of *H. contortus in vitro*. The RNAi experiments were set up to knock down each *H. contortus* astacin 3'or 5'end*s* fragment individually. The effects on transcripts and specificity of the silencing following RNAi treatment was demonstrated by RT-PCR's using gene-specific primers.

The expression for both gene fragments were non-existent compared to the negative control samples. The RT-PCR results also showed the new dsRNA samples were gene-specific.

However no unusual phenotypes were associated with the F1 and F2 generations, feeding and movement appearing normal in both cases after RNAi. Lack of abnormal phenotypes observed in this study are in agreement with findings elsewhere (Geldhof *et al.*, 2006), who reported that worms treated with individual dsRNA or mixed dsRNA samples had no atypical phenotypes as judged by egg production and viability. Majority of astacin-like MTP genes belong to multicopy of gene families which are highly prevalent in nematode parasites.

Genes within such large families probably show high levels of functional redundancy (Kamath *et al.*, 2003; Geldolf *et al.*, 2005; Geldhof *et al.*, 2006). Compensation for lost function between and within *H. contortus* astacin gene family or its homologues in the parasite could explain why we did not detect abnormal phenotypes.

RNAi offers a powerful tool for gene discovery and functional validation for the vast amount of information available within EST datasets. Traditionally, identification of novel candidate antigens from this big pool of genes is complex, time-consuming and expensive. Consider search for possible vaccine candidates, one of the important questions in the screening process is gene identification, sequence analysis and whether an antigen is accessible by an immune response.

Fig.1 *H. contortus* astacin-like MTPs gene (Acc. no. AM159505)

The forward and reverse primers designated HCF1Nco-Ⅰ (5'-ccatggatgaggctcactatactactg-3'); HCR1Xho-Ⅰ (5'-ctcgagatctggttcaacttgctgcc-3'); HCF2Nco-Ⅰ (5'-ccatggaactcggccacgctcttgg-3'); HCR2Xho-Ⅰ (5'-ctcgagtttcttgttatgagaagcacc -3') respectively, are highlighted in the open reading frame.

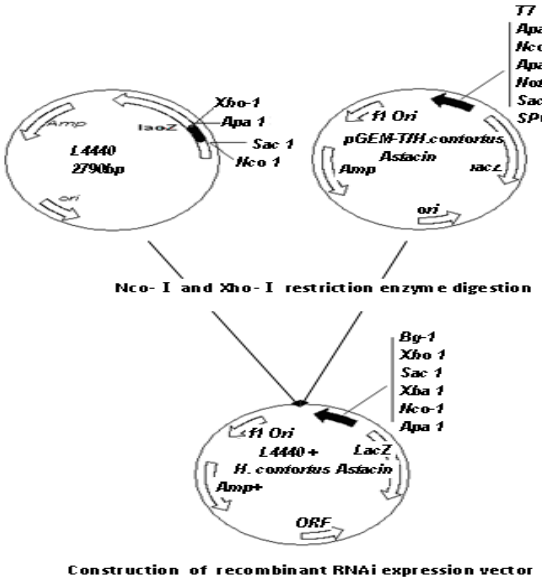

Fig.2 Construction of recombinant L4440/*H. contortus* astacin expression plasmid

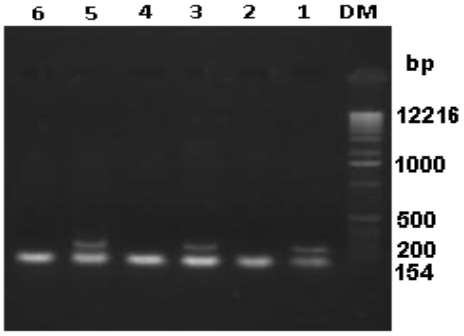

Fig.2 *H. contortus* astacin 3'or 5'ends PCR products

Lane DM: DNA molecular weight marker (0.075 – 12.21 kbp). *H. contortus* astacin amplified in Lane 1: *H. contortus* astacin 3'end*s* with HCF1=HCR1 primers Lane 3: *H. contortus* astacin 3'ends with HCF1Nco-Ⅰ= HCR1Xho-Ⅰ primers Lane 5: *H. contortus* astacin 5'end*s* with HCF2Nco-Ⅰ= HCR2Xho-Ⅰ primers. The negative controls in lanes 2, 4 and 6 with no template. Lanes 1, 3 and 5 are PCR products of ~200bp.

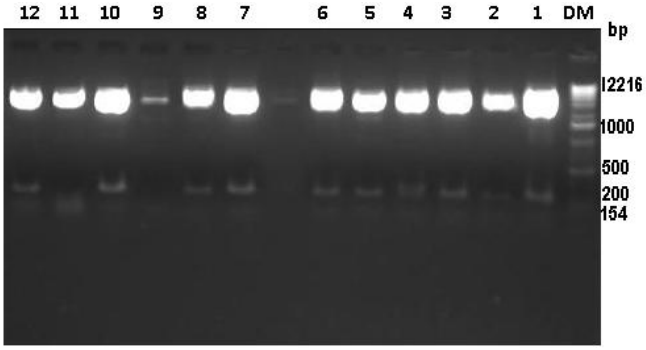

Fig. 3 Plasmids containing inserts of recombinant pGEM –T/ *H. contortus* astacin 3'or 5'end*s*

Key: DM: DNA molecular weight marker X (0075 – 12 21kbp). Lanes 1~6: recombinant pGEM –T/ *H. contortus* astacin 3'ends plasmids digested with Nco-Ⅰand *Xho* Ⅰenzymes. Lanes 7-12: recombinant pGEM –T/ *H. contortus* astacin 5'end*s* plasmids digested with Nco-Ⅰand *Xho* Ⅰenzymes. Digested plasmid DNA products were ~200bp

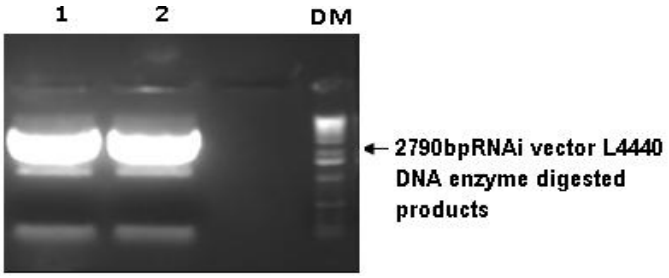

Fig.4 The plasmid of L4440 RNAi vector digested with Nco- Ⅰ and *Xho* Ⅰ restriction enzymes

Lane DM: DNA molecular weight marker X (0075 – 12 21kbp); Lanes 1 and 2: Digested L4440 RNAi vector DNA of ~2790bp

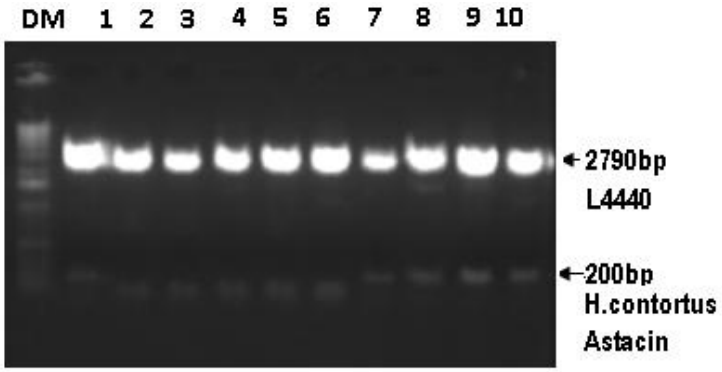

Fig.5 Identification of recombinant plasmids containing inserts of recombinant L4440/ *H. contortus* astacin 3'or 5'end*s* DNA plasmids digested with Nco- Ⅰ and *Xho* Ⅰ enzymes

Key: DM: DNA molecular weight marker X (0075 – 12 21kbp). Lanes 1~5: recombinant L4440/ *H. contortus* astacin 3'end*s* DNA plasmid digests. Lanes 6-10: recombinant L4440/ *H. contortus* astacin 5'end*s* DNA plasmid digests. Digested products were ~200bp.

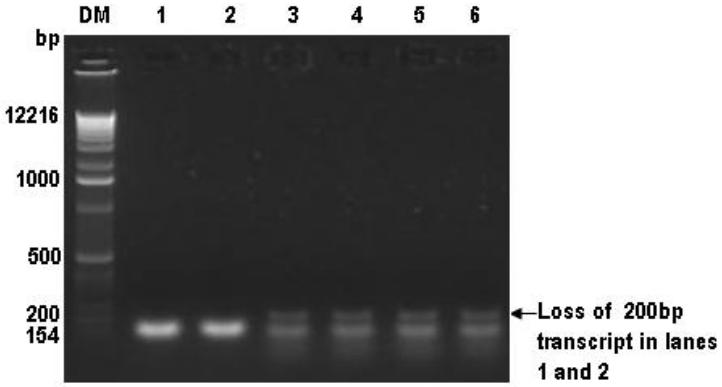

Fig.6 Loss of *H. contortus* astacin 3'or 5'ends transcripts following RNAi. RT-PCRs were carried out using total RNA isolated from RNAi-treated and untreated worms

Key: DM: DNA molecular weight marker X (0075 – 12 21kbp); Lane 1: worms soaked with *H. contortus* astacin 3'ends dsRNA; Lane 2: worms soaked with *H. contortus* astacin 5'ends dsRNA , lanes 3 ~ 6: negative control worms using appropriate primers to check uniformity of the RNA purifications. The major band at 200 bp is lacking from dsRNA treated worms in lanes 1 and 2 for both *H. contortus* astacin 3'or 5'ends transcripts

The later can only be addressed through immune-localisation studies. It is apparent starting from an expressed sequence tag (EST) it takes months to make a recombinant antigen, raise antiserum, and localise the native protein on worm sections, perform *in-vitro* functional tests and run animal vaccination trials. RNAi steps are shorter, specific and could provide the much needed option to the afore-going drawbacks.

Acknowledgements

This work was supported under the commonwealth scholarship commission academic fellowship in the United Kingdom, ACU, 20-24 Woburn House, Tavistock Square, London, WC1H 9HF. The authors acknowledge generous support given by the entire parasitology lab team at Moredun Research Institute, U.K. (David Smith; George Newlands; Philip Skuce; Alasdair Nisbet; Aileen Halliday; Alison Dicker; Jacqui Matthews; Mairi Mitchell; Rachael Smith; Samantha Ellis; Stephen Smith; Stuart Smith; Frank Turnbull; Keith Ballingall John Parkinson, Alasdair Anthony, James Wasmuth and Ralf Schmid)

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How to cite this article:

Charles Muleke Inyagwa and Dave Knox. 2020. RNA Interference of the *Hemonchus Contortus* Astacin- like MTP Gene by the Soaking Method. *Int.J.Curr.Microbiol.App.Sci*. 9(04): 1175-1184. doi: https://doi.org/10.20546/ijcmas.2020.904.139