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Published in:
International Journal for Parasitology

Document Version:
Publisher's PDF, also known as Version of record

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

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RNA interference in adult Ascaris suum – an opportunity for the development of a functional genomics platform that supports organism-, tissue- and cell-based biology in a nematode parasite


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ARTICLE INFO

Article history:
Received 3 April 2015
Received in revised form 19 May 2015
Accepted 22 May 2015
Available online 4 July 2015

Keywords:
Ascaris suum
RNA interference
Nematode parasite
Reverse genetics

ABSTRACT

The sustainable control of animal parasitic nematodes requires the development of efficient functional genomics platforms to facilitate target validation and enhance anthelmintic discovery. Unfortunately, the utility of RNA interference (RNAi) for the validation of novel drug targets in nematode parasites remains problematic. Ascaris suum is an important veterinary parasite and a zoonotic pathogen. Here we show that adult A. suum is RNAi competent, and highlight the induction, spread and consistency of RNAi across multiple tissue types. This platform provides a new opportunity to undertake whole organism-, tissue- and cell-level gene function studies to enhance target validation processes for nematode parasites of veterinary/medical significance.

Nematode parasites are a major cause of disease in humans and animals where they undermine health and global food security. Over one billion people living in the developing areas of sub-Saharan Africa, Asia and the Americas are infected with at least one nematode parasite (see Lustigman et al., 2012), and the worldwide economic impact of nematode parasites to the livestock industry is estimated to be >GBP 10 billion per annum (Roebet et al., 2013). Ascaris suum is a gastrointestinal parasite of pigs which impacts significantly on pig health through reductions in finishing weights, feed conversion efficiencies and carcass quality. Recent publications have highlighted the zoonotic potential of A. suum and there is now evidence to suggest that A. suum is more than a close relative of the human pathogen, Ascaris lumbricoides (see Peng and Criscione, 2012). This is significant given that more than one billion people worldwide suffer from ascariasis and its resultant chronic morbidity (see Lustigman et al., 2012).

In the absence of viable, commercially available vaccines, the control of nematode parasites relies heavily on a small collection of chemotherapeutic drugs in both human and veterinary medicine (see Prichard et al., 2012). Significantly, multi-drug resistance has been reported in many of the key parasitic nematodes of livestock (see Kaplan, 2004) and drug resistance in human therapy is a rising concern (see Prichard et al., 2012).

Our future defence against nematode parasites in both humans and livestock is dependent on the identification and validation of novel chemotherapeutic targets. The availability of genomic and transcriptomic sequence data for nematodes is rapidly progressing; 25 published genomes and >100 significant transcriptomic datasets are now available for mining (Blaxter and Koutsovoulos, 2014; Cotton et al., 2014; Foth et al., 2014; Jex et al., 2014; Rödelsperger et al., 2014; Tang et al., 2014). Genome-directed drug discovery has made significant inroads towards the identification of putative novel drug targets for nematode pathogens but validation will require the incorporation of functional tools in therapeutically relevant parasite species.

RNA interference (RNAi) is an appealing reverse genetics tool and, whilst its utility for the validation of drug targets in veterinary parasites is widely accepted, issues with their variable sensitivity to standard RNAi approaches have undermined the development of robust gene silencing platforms (see Maule et al., 2011). Although successful RNAi has been reported in ~11 animal parasitic nematode (APN) species (see Maule et al., 2011; Tzelos et al., 2013), the utility of these data is hampered by a variety of species/tissue/target-specific inconsistencies in RNAi responsiveness. A key hurdle to the exploitation of putative targets identified
in silico is the absence of validation tools that allow the elucidation of target function in relevant parasites.

Importantly, the adult stage of A. suum is well established as a parasite model system and more is known about the basic biology of A. suum than any other veterinary parasite. Amongst nematode parasites, A. suum has the most genomic/transcriptomic resources and it offers unrivalled utility for cell biology/physiology/biochemistry studies (Jex et al., 2011), supported by an extensive array of physiology- and cell-based assays for functional studies (Stretton and Maule, 2013). The tractability of A. suum as an experimental model makes the application of RNAi to the adult stage very appealing. To date, only larval stage A. suum have been shown to be RNAi susceptible (Islam et al., 2005; Xu et al., 2010; Chen et al., 2011).

Here we describe the development of an RNAi platform in adult A. suum that has the potential to significantly advance drug target validation in nematode parasites. Our data demonstrate that: (i) RNAi is readily achievable in adult A. suum. We have developed a method for the induction of RNAi in adult A. suum through the injection of double-stranded RNA (dsRNA) (100 µl; 200 ng/µl) into the pseudocoelomic cavity of female worms (see Fig. 1A). Adult A. suum were collected from the abattoir and transferred to the laboratory in mammal saline (0.9% NaCl). Worms were maintained in Ascaris Ringers Solution (ARS: 13.14 mM NaCl, 9.47 mM CaCl₂, 7.83 mM MgCl₂, 12.09 mM C₄H₁₁NO₃/Tris, 99.96 mM NaC₂H₃O₂, 19.64 mM KCl, pH 7.8) for a maximum of 24 h prior to use, and for the duration of the RNAi experiments (for specific methodology see Fig. 1).

We have achieved target transcript knockdown that: (a) is robust (e.g. > 60.0% (P < 0.05) knockdown across multiple targets (A. suum elongation factor 1a (As-eft-1); Elongation factor 1b (As-eft-2); GMP reductase (As-gmpr), troponin C (As-tnc-1), Ras-related protein (As-rab-3), haemoglobin (As-hb-1), and two nicotinic acetylcholine receptor subunits (As-unc-29 and As-unc-38)) at ≥ 3 days post-RNAi trigger delivery; (b) occurs relatively quickly (significant transcript knockdown recorded as early as 24 h post-RNAi trigger delivery (e.g. 67.7 ± 4.4% knockdown of As-eft-1 in the tail region at 24 h, P < 0.05)); (c) is consistent and reproducible (100% success rate across eight targets with differential expression (see below)); and (d) persists (for up to 8 days; e.g. 98.5 ± 0.4% knockdown of As-eft-1 in the tail region at day 8) (see Figs. 1 and 2). Our findings are in contrast to the early literature describing RNAi susceptibility for some of the veterinary APNs including Haemonchus contortus, Ostertagia ostertagi and Teladorsagia circumcincta where hypervariability in the induction of gene silencing, that appeared to be largely target- and RNAi trigger delivery-dependent, was common (see Maule et al., 2011). One study has described the development of an RNAi platform for
**H. contortus** through the investigation of different RNAi trigger delivery approaches across developmental stages and target types (Zawadzki et al., 2012), providing confidence in the application of RNAi in this species. In other APNs where inconsistencies were not highlighted (e.g. for *Nippostrongylus brasiliensis*, Brugia malayi, Onchocerca volvulus, *Trichinella colubriformis*, *A. suum*, Litomosoides sigmodontus, *Heterorhabditis bacteriophora*, Acanthocheilonema vitea) the majority of studies only targeted one or two genes (see Maule et al., 2011; Tzolos et al., 2013), making it more difficult to assess the reliability of RNAi as a reverse genetics tool in these species.

(ii) RNAi is capable of spreading in adult *A. suum*. Our RNAi trigger delivery approach involves the injection of dsRNA into the pseudocoelomic cavity at a position approximately 1 cm anterior to the gonopore on the ventral side, where worms were injected at an angle (approximately 20°) to avoid piercing the gut (see Fig. 1A). We have recorded robust transcription knockdown for five targets in tissues distant from the site of injection (head and tail regions; see Figs. 1 and 2; As-eft-1, As-eft-2, As-rab-3, As-ncmr-1, As-gmp; As-unc-29, As-unc-38), and also in muscle bag cells (As-unc-29, As-unc-38; see Fig. 3) highlighting the spread of gene silencing triggers in adult *A. suum*. Note that the RNAi response in tissues adjacent to the injection site consistently lags behind that observed at the worm extremities across all time points (see Fig. 1B). The significance of the apparent lag is unclear.

The fact that the temporal dynamics of the RNAi responses in the head, gonopore and somatic muscle is similar enhances the interpretation of phenotypic readouts, and means that distinct tissues from the same worm can be used for a range of analyses such as transcript and protein quantitation and physiology/biochemical assays. This enhances the appeal of *A. suum* as a model nematode for reverse genetics.

The observed efficiency in adult *A. suum* RNAi is in contrast to that described for other APNs (see Maule et al., 2011). It seems likely that our injection of dsRNA directly into the pseudocoelom would enhance RNAi trigger delivery and could reduce variability in RNAi induction compared with that achieved in other APNs using alternative dsRNA delivery approaches (see Maule et al., 2011). Large adult worms contain ~1 ml of pseudocoelomic fluid that is continuously circulated within the pseudocoelomic cavity by the activity of the somatic body wall muscle. This likely enables the efficient spread of the dsRNA within the pseudocoelomic fluid, bringing it into close contact with the various organs/tissues of the worm. This is likely to enhance the efficiency of RNAi in terms of both spread and direct access to cells. It is interesting to note the absence of *sid-1* and *sid-2* homologues from *A. suum*, known to be important for the uptake and spread of environmental RNAi in nematodes, and the presence of...
an \textit{rsd-3} homologue, which is the most highly conserved of the proteins associated with the intercellular spread of RNAi (see Dalzell et al., 2011). The presence and/or absence of these proteins clearly does not limit RNAi capability in \textit{A. suum} as is the case with other RNAi-competent nematode species which also lack these proteins (e.g. \textit{Meloidogyne incognita} and \textit{Globodera pallida}) (Dalzell et al., 2011; Cotton et al., 2014). As suggested by Dalzell et al. (2011), there may be uncharacterised RNAi pathway proteins that fulfil these roles.

(iii) Multiple \textit{A. suum} tissue-specific targets are susceptible to RNAi including ‘neuronal’ genes. We have shown that a range of targets are susceptible to RNAi in adult \textit{A. suum} including those expressed in: multiple tissues (\textit{As-unc-1}; \textit{As-unc-2}; \textit{As-gmpr}); muscle (\textit{As-tnc-1}); nerve (\textit{As-rab-3}); gut/body wall (\textit{As-hb-1}); and the neuromuscular system (\textit{As-unc-29}, \textit{As-unc-38}) (based on spatial expression of homologous genes in \textit{Caenorhabditis elegans}) (see Figs. 1–3). Samaranasinghe et al. (2011) suggest a correlation between target accessibility and RNAi susceptibility in that targets genes that are expressed in tissues accessible to an external source of dsRNA are more likely to be susceptible to RNAi. Here we do not see any tissue-specific differences in our ability to induce RNAi in adult \textit{A. suum}. We believe that dsRNA delivery to the pseudocoelomic circuits any potential RNAi trigger accessibility issues. The physiology of \textit{A. suum} is such that the pseudocoelomic fluid perfuses multiple target tissues including the intestine, muscle (body wall, pharynx, ovijector, anus), nerve, hypodermis, and systems associated with reproduction and excretion, and it is therefore an ideal vehicle to carry dsRNA cargo to its target, offering a systemic delivery system.

Significantly, we have not noted any neuronal RNAi refractoriness that has been observed in other nematode species e.g. \textit{C. elegans} (Timmons et al., 2001). Here we report significant silencing for three neuronal targets (\textit{As-rab-3}, \textit{As-unc-29} and \textit{As-unc-38} (see Figs. 2 and 3)), providing confidence that neuronal targets are as susceptible to RNAi as those expressed in other tissues. Until now, successful RNAi of neuronal targets in parasitic nematodes has been restricted to the plant parasitic nematodes (see Maule et al., 2011).

(iv) A multi-target dsRNA cocktail delivery approach is efficient in inducing RNAi. Here we demonstrate that effective gene silencing is achievable when two dsRNA species, targeting different transcripts (e.g. \textit{As-unc-29} and \textit{As-unc-38}) are delivered as a cocktail (Fig. 3). This is significant with respect to experimental set up as it reduces time, cost and worm/tissue requirements. In addition, from a gene function perspective, this approach enables the co-silencing of multiple related genes where functional redundancy may be a concern, but promiscuous dsRNA design is not possible. It will be interesting to determine the limitations of this approach with respect to RNAi efficiency, and to assess the efficacy of small interfering RNA (siRNA)-mediated RNAi in \textit{A. suum}.

(v) RNAi is consistent across geographical isolates of \textit{A. suum}. Here we present data to show that \textit{A. suum} from different geographical regions were equally susceptible to RNAi (Fig. 3), and that there was no significant difference in the dynamics associated
with the induction of knockdown across geographical isolates. These data are significant given that different strains of *C. elegans* appear to be variably susceptible to somatic RNAi across multiple targets and tissues and present a novel, efficient delivery approach. The appeal of the *A. suum* RNAi platform described here is enhanced by the unique experimental tractability of the adult worm and the availability of a number of post-RNAi functional bioassays at the whole worm (body waveform, egg output, development), tissue (body wall muscle, pharynx, ovipositor) and cell (muscle and neuron electrophysiology) levels (see Pecson et al., 2006; Stretton and Maule, 2013). This provides an appealing platform for the identification and validation of drug targets in parasitic nematodes of veterinary and human importance.

**Acknowledgements**

The authors are grateful to Dunbia Meats, Ballymena, Northern Ireland and the JBS packing plant Marshalltown, Iowa, USA for assistance in the collection of worms. This work was supported by the Biotechnology and Biological Sciences Research Council, UK (BB/H019472/1), the Department of Employment and Learning for Northern Ireland, the Department of Agriculture and Rural Development for Northern Ireland, and the National Institute of Allergy and Infectious Diseases (NIH), USA (R01 AI 047194). The authors would like to acknowledge the British Society for Parasitology for an International Travel Award to CMC.

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