REVIEW ARTICLE

RNA interference in parasitic helminths: current situation, potential pitfalls and future prospects

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SUMMARY

RNA interference (RNAi) has become an invaluable tool for the functional analysis of genes in a wide variety of organisms including the free-living nematode *Caenorhabditis elegans*. Recently, attempts have been made to apply this technology to parasitic helminths of animals and plants with variable success. Gene knockdown has been reported for *Schistosoma mansoni* by soaking or electroporating different life-stages in dsRNA. Similar approaches have been tested on parasitic nematodes which clearly showed that, under certain conditions, it was possible to interfere with gene expression. However, despite these successes, the current utility of this technology in parasite research is questionable. First, problems have arisen with the specificity of RNAi. Treatment of the parasites with dsRNA resulted, in many cases, in non-specific effects. Second, the current RNAi methods have a limited efficiency and effects are sometimes difficult to reproduce. This was especially the case in strongylid parasites where only a small number of genes were susceptible to RNAi-mediated gene knockdown. The future application of RNAi in parasite functional genomics will greatly depend on how we can overcome these difficulties. Optimization of the dsRNA delivery methods and *in vitro* culture conditions will be the major challenges.

Key words: RNA interference, RNAi, helminth, nematode, trematode, gene knockdown, reproducibility.

INTRODUCTION

RNA interference (RNAi) is a technology to down-regulate the expression of a gene by the addition of gene-specific double-stranded RNA (dsRNA). It was originally characterized in *Caenorhabditis elegans* (Fire *et al*. 1998) and has since then been applied in a wide variety of organisms to study gene function, including protozoa (Ullu *et al*. 2004), amphibians (Oelgeschlager *et al*. 2000), insects (Kennerdell and Carthrew, 1998) and mammals (Hannon, 2002). The molecular processes and components necessary for a functional RNAi pathway have been extensively investigated in *C. elegans* and were summarized by Grishok (2005). A simplified model of the RNAi mechanism is shown in Fig. 1. In short, the dsRNA is recognized by a protein complex containing a double-stranded RNA binding protein (Rde-4), the dsRNA-specific ribonuclease, dicer, which cleaves the dsRNA into small inhibitory RNAs (siRNAs), a DexH box helicase drh-1 and a Rde-1 protein which is thought to transport the siRNAs to the RNA induced silencing complex (RISC) complex. This interaction results in the activation of the RISC complex which targets homologous mRNA transcripts by a base-pairing mechanism. Once bound, the target transcript is degraded by the RISC complex, resulting in a gene knock-down. In *C. elegans* the silencing effect is systemic, spreading to most cells through the activity of the SID and RSD proteins. siRNAs are subsequently recognized by the RISC protein complex. Thus, RNAi is induced by dsRNA, the RNAi effect can be systemic and, at least in *C. elegans*, is heritable (Grishok, 2005). In *C. elegans*, this RNAi mechanism can be triggered by injection of dsRNA into the adult worm body, by soaking any life-cycle in dsRNA or by feeding worms on bacteria expressing dsRNA. For a more detailed description of the

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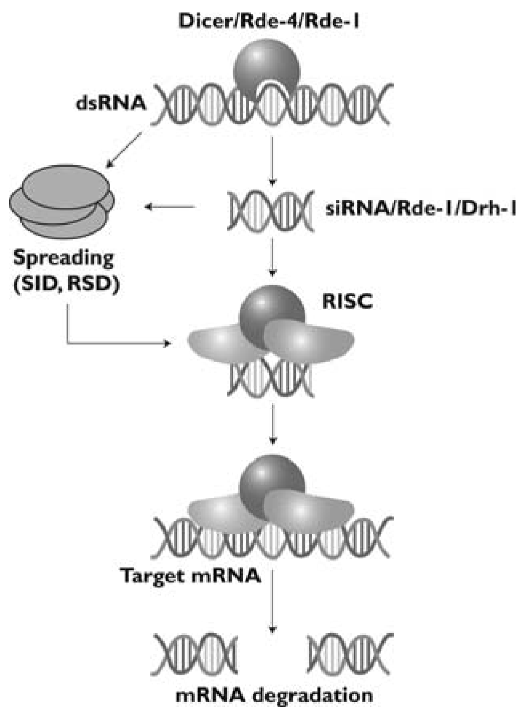

Fig. 1. Simplified schematic model of the RNAi mechanism in *Caenorhabditis elegans*. Double-stranded RNA (dsRNA) is processed into small inhibitory RNAs (siRNAs) by dicer. Processed dsRNA is thought to be transported systemically in the worm by the SID and RSD proteins and siRNAs are subsequently bound by the RISC complex. The activated RISC complex will subsequently bind and degrade the homologous mRNA.

RNAi pathway and all the different components we refer the reader to the excellent review by Grishok (2005).

The ease with which RNAi can be carried out on *C. elegans* has revolutionized functional genomic studies in this organism in recent years. Large-scale screens have been performed on virtually every predicted *C. elegans* gene. The outcome of these studies are increasingly being used in parasite research as well, since it could help in identifying novel drug and vaccine targets (reviewed by Britton and Murray, 2006). However, a large number of parasite genes do not have apparent homologues in *C. elegans*. These genes are probably involved in the parasitic life-style and host-parasite interactions, i.e. the genes that are likely to be the most interesting biologically and important targets for control interventions. It is therefore clear that RNAi applied directly on the parasites would be an extremely useful tool. As currently happens in the *C. elegans* research community, the technology would be used by virtually every molecular parasitology research group to study the function of their genes of interest. The relatively small number of papers describing successful RNAi in parasitic helminths is therefore somewhat surprising considering that RNAi was first reported in parasitic nematodes in 2002 (Hussein *et al*. 2002) and trematodes in 2003 (Skelly *et al*. 2003; overview in Table 1). This could be a sign that the use of RNAi on helminths is not as straightforward as might have been expected. In this review we will focus on the current status of RNAi in parasitic helminths, discuss the problems that have been encountered and consider potential future prospects for this technology.

RNAi IN TREMATODES

The first report of successful RNAi in trematodes was published by Skelly *et al*. (2003). A cathepsin B was targeted in cercariae of *Schistosoma mansoni*. Cercariae were soaked in dsRNA for 6 days and the expression of cathepsin B assayed by reverse transcriptase-PCR (RT-PCR), immunostaining and enzyme activity assays. The outcome of the 3 different methods clearly showed that, compared to the controls, the cathepsin B dsRNA-treated cercariae had lower levels of cathepsin B expression. Shortly after this report, Boyle *et al*. (2003) published the successful down-regulation of a facilitated diffusion glucose transporter (SGTP1) and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in *S. mansoni* sporocysts. Miracidia were allowed to undergo the transition to sporocysts in the presence of dsRNA and were then incubated for 6 days. Targeted transcript levels were reduced by 70–80% as determined by quantitative RT-PCR and in the case of SGTP1, the larval glucose-uptake capacity was reduced by 40%. An identical approach was recently used by Dinguirard and Yoshino (2006), who targeted a CD36-like class B scavenger receptor (SRB) in *S. mansoni*. The SRB protein is a multi-ligand-binding protein which is thought to be important for the binding of low-density lipoproteins (LDL) to the surface of schistosomula and adult *S. mansoni*. Miracidia were soaked in gene specific dsRNA and, after 6 days, the treatment resulted in a significant knockdown (60–70%) of SRB transcript levels in sporocysts. Eight and 10 days post-dsRNA incubation, the sporocysts showed a significant reduction in acetylated LDL binding.

An alternative method to introduce dsRNA in schistosomes is electroporation. The method was originally described by Correnti and Pearce (2004) as an approach for transgene expression in *S. mansoni* schistosomula. A 20 ms pulse at 125 V using a square-wave electroporation generator was optimal to electroporate the parasites with a luciferase-encoding RNA, used to test for efficient introduction of nucleic acid. The majority of the schistosomes expressed the introduced RNA and acute mortality was negligible. The same approach was subsequently used to introduce cathepsin B (SmCB1)-specific dsRNA in schistosomula (Correnti *et al*. 2005). This resulted in a greater than 10-fold

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Table 1. Summary of RNAi studies in helminths

	Life stage	Target	Method	Reference
Schistosoma mansoni	Cercaria	Cathepsin B	Soaking	Skelly *et al.* (2003)
	Miracidium-sporocyst	Facilitated-diffusion glucose transporter glyceraldehydes-3-phosphate dehydrogenase	Soaking	Boyle *et al.* (2003)
		CD36-like class B scavenger receptor	Soaking	Dinguirard *et al.* (2006)
	Schistosomulum	Cathepsin B	Electroporation	Correnti *et al.* (2005)
		Gynecophoral canal protein	Soaking	Cheng *et al.* (2005)
Nippostrongylus brasiliensis	Adult	Acetylcholinesterases	Soaking	Hussein *et al.* (2002)
Brugia malayi	Adult	Beta-tubulin, RNA polymerase II large subunit, microfilarial sheath protein	Soaking	Aboobaker and Blaxter (2003)
Onchocerca volvulus	L3	Cathepsin L-like protease, Cathepsin Z-like protease	Soaking	Lustigman *et al.* (2004)
		Serine protease inhibitor	Soaking	Ford *et al.* (2005)
Trichostrongylus colubriformis	L1	Ubiquitin, tropomyosin	Feeding-soaking-electroporation	Issa *et al.* (2005)
Ascaris suum	L3	Inorganic phyrophosphatase	Soaking	Islam *et al.* (2005)
Haemonchus contortus	L3–L4-adult	Beta-tubuline genes	Soaking	Kotze *et al.* (2005)
	L1–L3	Beta-tubulin, COPII component, Ca2+ binding protein, heat shock protein HSP70, vacuolar ATPase, cathepsinL, paramyosin, Cu-Zn superoxide dismutase, intermediate filament, type IV collagen, GATA transcription factor	Electroporation-soaking	Geldhof *et al.* (2006)
Ostertagia ostertagi	L3	Tropomyosin, beta-tubulin, ATP-synthetase, Superoxide dismutase, polyprotein allergen, ubiquitin, transthyritine-like protein, 17 kDa ES protein	Electroporation-soaking	Visser *et al.* (2006)
Litosomoides sigmodontis	Adult	Actin	Soaking	Pfarr *et al.* (2006)
Meloidogyne species	J2	Cathepsin L	Soaking	Shingles *et al.* (2006)
		Dual oxidase	Soaking	Bakhetia *et al.* (2005)
		Splicing factor, integrase	Plant delivery	Yadav *et al.* (2006)
		Calreticulin, polygalacturonase	Soaking	Rosso *et al.* (2005)
		Parasitism gene *16D10*	Soaking, plant delivery	Huang *et al.* (2006)
	Eggs	Chitin synthase gene	Soaking	Fanelli *et al.* (2005)
Heterodera glycines	J2	Aminopeptidase	Soaking	Lilley *et al.* (2005)
		Cysteine proteinases, C type lectin, major sperm protein	Soaking	Urwin *et al.* (2002)
Globodera pallida	J2	Cysteine proteinases	Soaking	Urwin *et al.* (2002)
Globodera rostichiensis	J2	Beta-1,4 endoglucanase, *gr-ams-1*	Soaking	Chen *et al.* (2005)

reduction in SmCB1 transcript levels that persisted for >20 days. The treated schistosomes also showed a reduced SmCB1 enzyme activity and significant growth retardation when compared to control parasites. The effect on growth was apparent when parasites were maintained both in culture or introduced into mammalian hosts.

At the time of writing this review, no reports have been published on the use of RNAi in other trematode species.

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RNAi IN PARASITIC NEMATODES OF ANIMALS

RNAi has been tested extensively in a variety of parasitic nematodes from different phylogenetic clades. *Nippostrongylus brasiliensis* was the first parasitic nematode on which RNAi was successfully performed (Hussein *et al*. 2002). Soaking adult worms in dsRNA for the acetylcholinesterase (AChE) A isoform resulted in a 80–90% reduction of the secretion of 3 AChE isoforms (A, B and C) throughout a 6-day culture period, as measured by an enzyme activity assay. A smaller dsRNA construct (240 bp) proved to be more potent than dsRNA targeting the full length mRNA (1·8 kb) although the transcript levels were not monitored in this study. Despite this significant suppression, no obvious effects on worm morphology or behaviour were observed.

RNA interference effects have also been reported in filarial nematodes. Aboobaker and Blaxter (2003) described an optimized microvolume culture system to soak the human parasite *Brugia malayi* in dsRNA. They demonstrated that fluorescently labelled dsRNA molecules of 300 bp were able to enter the adult females. The system was subsequently used to target beta-tubulin, RNA polymerase II large subunit and a microfilarial sheath protein. RT-PCRs indicated that all target transcript levels started to drop between 14 and 17 h of soaking. After 24 h, worms soaked in dsRNA for beta-tubulin and the RNA polymerase II large subunit were dead. Targeting the microfilarial sheath protein was not lethal to adult females in culture, an observation which suggests that the lethality phenotype noted above was not due to non-specific toxic effects of the dsRNA soaking, but RNAi induced a marked reduction in microfilariae release and 50% of the released microfilariae did not have fully elongated sheaths.

RNA interference was also used on the L3 life-stage of the human filarial nematode *Onchocerca volvulus* (Lustigman *et al*. 2004; Ford *et al*. 2005). Filarial serine protease inhibitors, a cathepsin L- and a cathepsin Z-like cysteine protease were targeted by soaking the L3 larvae in dsRNA for 18–20 h. Targeting the serine protease inhibitors resulted in the specific knock-down of the target transcript levels and a loss of native proteins. Seven days post-dsRNA incubation, the larvae showed a significant reduction in both moulting and viability (Ford *et al*. 2005). Similar phenotypic effects were obtained for the cathepsin L- and cathepsin Z-like cysteine proteases (Lustigman *et al*. 2004). However, specific transcript levels for the latter were not examined.

RNA interference in filarial parasites was further optimized by Pfarr *et al*. (2006). In this report, the actin gene from the rodent parasite *Litosomoides sigmodontis* was targeted by soaking adult worms in different concentrations of dsRNA, ranging from 0·035 to 35 μM. Actin transcription was suppressed by all tested concentrations. However, 3·5 μM dsRNA, which is a 10-fold lower concentration than that used on adult *B. malayi* worms (Aboobaker and Blaxter, 2003), consistently reduced the actin transcript levels to <10% of the controls. This effect persisted for at least 72 h. Treated worms showed paralysis between 48 and 72 h after the start of the experiment and also released significantly less microfilariae. In *Ascaris suum*, RNA interference was used to study the function of an inorganic pyrophosphatase (Islam *et al*. 2005). Lung-stage larvae were incubated in dsRNA (2 mg/ml final concentration) for 24 h after which they were transferred to dsRNA-free medium and incubated for up to 10 days. Target mRNA and protein levels were significantly suppressed 5 days after the dsRNA treatment, as measured by RT-PCR, enzyme assay, immunofluorescence and immunoblot (Islam *et al*. 2005). After 10 days, the treated larvae showed a reduced moulting from L3 to L4 by 31% compared to control larvae. However, control larvae were not cultured in the presence of nonsense dsRNA to eliminate the possibility of non-specific treatment effects.

Recently, several RNAi studies have been published in trichostrongyloid nematodes of veterinary importance. Issa *et al*. (2005) investigated the efficiency of 3 different RNAi techniques, namely soaking, electroporation and feeding on bacteria producing dsRNA, in the L1 life-stage of the sheep parasitic nematode *Trichostrongylus colubriformis*. Ubiquitin was used as a target since it is well conserved within the nematodes and it induces a strong phenotype when knocked down in *C. elegans*. Electroporation in ubiquitin dsRNA was the only method which significantly inhibited the development of the L1 larvae compared to control larvae electroporated in buffer only. However, transcript knockdown was not assessed by semi- or quantitative RT-PCR. No effects were observed after feeding or soaking. As an alternative to the dsRNA produced by *in vitro* transcription, they also tested the effect of 22 bp synthetic dsRNA (siRNA) derived from ubiquitin coding sequence. Both soaking and electroporation of *T. colubriformis* L1 larvae in this siRNA resulted in significant reductions in the number of L3 stage larvae observed in cultures 6 days later. It is also interesting to note that although the feeding method was unsuccessful for ubiquitin, it did cause a strong phenotype when tested with tropomyosin as a target gene.

Successful RNAi in the blood-feeding nematode *Haemonchus contortus* was first described by Kotze and Bagnall (2005). Two beta-tubulin genes were targeted in exsheathed L3, L4 and adult parasites by culturing the worms in medium containing dsRNA. Quantitative PCRs indicated that the transcript

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levels of both genes dropped significantly in all 3 parasitic life-stages (greater than 1000-fold in some cases) after 24 h of incubation. After 6 days of treatment, L3 worms showed a decreased motility compared to control worms and less worms developed to the L4 stage. Although the adult worms showed marked gene suppression, they did not show any reduced motility compared to controls. Since gene suppression effects occurred in L3 larvae which do not have a functional oral orifice, the authors suggested that the uptake of dsRNA in *H. contortus* might not depend on ingestion. This hypothesis was also supported by the finding that adult worms which were treated with ivermectin to paralyse their pharynx were still susceptible to gene suppression by RNAi.

We have recently carried out an extensive study to further investigate the efficiency of RNAi in *H. contortus* (Geldhof *et al.* 2006). Eleven different genes, including a beta-tubulin gene tested by Kotze and Bagnal (2005), were targeted in exsheathed L3 larvae by soaking in dsRNA. The effect of the treatment was analysed by RT-PCR and, where possible, protein assays. In agreement with the results of Kotze and Bagnal (2005), we also observed a specific decrease in transcript levels of the beta-tubulin gene after 24 h of soaking. Similar results were obtained for *sec-23*, a gene involved in vesicle transport. However, no signs of specific decreases in expression levels were observed for the other 9 genes tested. As soaking did not result in robust RNAi knockdown, we examined electroporation of L1 larvae as a way of introducing dsRNA. Four different genes were targeted: beta-tubulin, superoxide dismutase, cathepsin L and an ATPase. Clear decreases in transcript levels were only found for beta-tubulin and superoxide dismutase 24 h after electroporation at 50 and 100 V. In addition, we observed a high level of larval death following electroporation even in the presence of control dsRNA, indicating that using current protocols this method could not be used for functional analysis.

Similar results were recently published by Visser *et al.* (2006) for the cattle parasite *Ostertagia ostertagi*. Eight different genes were targeted in L1 and L3 larvae by electroporation and soaking in dsRNA. In L3 larvae, variable decreases in target transcript levels were observed for 5 genes (tropomyosin, beta-tubulin, ATPase, superoxide dismutase and a polyprotein allergen). RNA interference for a transthyretin-like protein, a 17 kDa ES protein and ubiquitin never resulted in a decrease of transcript levels. Electroporation of L1 larvae was less effective compared to soaking. Reductions in target transcript levels were only observed for 2 genes and were difficult to reproduce. The authors also investigated the specificity of observed RNAi effects. This was done by checking changes in transcription levels of a large set of non-target genes in worms treated with tropomyosin dsRNA. No differences were observed between control and treated larvae, suggesting that the observed reductions are specific for the target gene.

RNAi IN PLANT-PARASITIC NEMATODES

RNAi has also been demonstrated in plant-parasitic nematodes that cost world agriculture in the region of $125 billion annually (Chitwood, 2003). Like parasitic nematodes of animals, the infective stages are very small making microinjection with dsRNA a major challenge (Bakhetia *et al.* 2005*a*). Moreover, an additional problem is that they do not ingest fluid until they infect the host plant. Oral ingestion can be stimulated by octopamine (Urwin *et al.* 2002) and this approach has been used with success in pre-parasitic second-stage juveniles of *Heterodera glycines, Globodera pallida* (Urwin *et al.* 2002) and *Meloidogyne spp.* (Bakhetia *et al.* 2005*b*; Fanelli *et al.* 2005). Moreover, the inclusion of 1% resorcinol in the incubation solution can induce dsRNA uptake through the alimentary tract (Rosso *et al.* 2005). This reagent acts as a neurostimulant and increases stylet thrusting and accumulation of secretions in *Meloidogyne incognita* (Jaubert *et al.* 2002).

RNAi targeting cysteine proteinases and a C-type lectin homologue in the cyst nematode *Heterodera glycines* resulted in reduced transcript abundance and phenotypic effects (Urwin *et al.* 2002). In the case of the cysteine proteinases, RNAi had no effect on establishment on the plant but did alter the male : female ratio in favour of males 14 days post-infection. With the C-type lectin homologues, sexual fate was unaltered but the number of worms on infected plants was reduced by 41% (Urwin *et al.* 2002). Also in *H. glycines*, RNAi-induced suppression, conducted on J2 juveniles, of an aminopeptidase associated with the reproductive tract of adult females and the genital primordium of pre-parasitic juveniles resulted in a 61% reduction in the number of female nematodes parasitizing soybean roots 21 days after infection with infective juveniles (Lilley *et al.* 2005). Targeting the major sperm protein (MSP) had no effect on development but transcript abundance was reduced when males reached sexual maturity and sperm are produced (Urwin *et al.* 2002). Moreover, Chen *et al.* (2005) used RNAi to define the function of putative pathogenicity proteins in *Globodera rostochiensis* showing that knock-down of a beta-1,4, endoglucanase reduced the ability of the nematodes to invade the roots while gr-ams-1, a protein secreted from the amphids, is essential for host location.

Bakhetia *et al.* (2005*b*) reported a reduction in the number and size of established females as well as a 70% reduction in egg output compared to controls when a dual oxidase expressed in *M. incognita* was targeted by RNAi. Further, chitin synthetase,

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expressed in egg-shells of the root-knot nematode *M. artellia*, was reduced after soaking developing eggs in dsRNA (Fanelli *et al.* 2005). In addition, RNAi of a cathepsin L-like cysteine proteinase expressed in the intestine of young and mature female *M. incognita* reduced transcript abundance, enzyme activity, reduced the number of nematodes infecting plants as well as the number of established females producing eggs (Shingles *et al.* 2006).

Bioengineering crop plants which express dsRNA targeting genes essential for parasitism is now being developed. Yadav *et al.* (2006) cloned 2 genes from *M. incognita* into a plant expression vector that expresses both sense and anti-sense strands in a way that the resultant transcript forms hair-pin dsRNA. When introduced into tobacco plants, root-knot formation was dramatically reduced compared to controls. The authors suggested that the RNAi response was amplified by the host plant RNAi machinery generating siRNAs which may more readily pass through the feeding tube. Using a similar approach, Huang *et al.* (2006) demonstrated that the introduction of a dsRNA construct into *Arabidopsis*, which encoded a conserved root-knot nematode secretory peptide required for parasitism, resulted in effective resistance to infection with 4 major root-knot nematode species.

POTENTIAL PITFALLS

Based on the successful RNAi experiments, some papers have concluded that the current RNAi methods in helminths are useful tools to analyse gene function and could be developed for high throughput analysis to screen for potential drug and vaccine targets. However, we believe that this is an over-optimistic view. It is clear that RNAi is effective in some helminths against some genes but it is becoming apparent that the inconsistencies associated with the technique preclude its use as a reliable screen for potential drug and vaccine targets at the present time. Many questions remain unanswered and, when analysed in more detail, the current RNAi methods show severe problems and inconsistencies when applied to parasitic nematodes in particular.

An essential step in claiming successful RNAi is the evaluation of the experiment. So far, there has been no consistency in the methods used to do this. The first effect of RNAi is the specific decrease of target transcript levels. Surprisingly, some studies have not investigated this and have only provided indirect evidence for RNAi. In *T. colubriformis*, RNAi for ubiquitin was regarded as successful since L1 larvae failed to develop through to the L3 stage (Issa *et al.* 2005). This conclusion is based on the assumption that the function of ubiquitin is conserved between *T. colubriformis* and *C. elegans*. Although it is probably valid to make this assumption for highly conserved genes such as ubiquitin, care is still needed when extrapolating results from *C. elegans* to parasitic nematodes and RT-PCR analysis is needed to confirm specific gene silencing effects. A second evaluation tool is immunolocalization, used in the RNAi experiments targeting cathepsin L and Z-like cysteine proteases in *O. volvulus* (Lustigman *et al.* 2004). This indicated a reduced presence of both proteins in various regions of the moulting larvae. Unfortunately, this type of analysis makes it very difficult to quantify the effect and more importantly, it is unclear in how many worms this effect was visible. Analysis of the transcript levels in both cases would strengthen the claim of successful RNAi.

When RNAi is used to analyse gene function, it is of vital importance that it specifically targets the chosen gene and causes no other effects in the parasites. However, some of the current RNAi methods clearly induce off-target effects. This is especially the case for the soaking method. In *S. mansoni*, Skelly *et al.* (2003) reported a change in cathepsin B expression and distribution in cercariae incubated in control dsRNA. They also observed a substantial decrease in cathepsin activity in different control groups treated with dsRNA in the presence of lipofectamine, which was added to the culture medium to improve the uptake of the dsRNA. It clearly suggests that lipofectamine may have a general detrimental impact on this parasite (Skelly *et al.* 2003). The data published by Dinguirard and Yoshina (2006) indicated a 25% reduction in mRNA levels of a non-target control gene by culturing miracidia in dsRNA. After 10 days in culture, sporocysts treated with control dsRNA also showed a significant decrease in length. Off target effects were also observed in filarial nematodes. Treatment of L3 *O. volvulus* larvae with control dsRNA resulted in a 24·7–49·8% reduction in moulting (Lustigman *et al.* 2004; Ford *et al.* 2005) and adult *B. malayi* parasites soaked in control dsRNA showed a reduced motility (Aboobaker and Blaxter, 2003). Pfarr *et al.* (2006) recently showed that high concentrations of dsRNA were also stressful to the adult stage of the filarial parasite *L. sigmodontis*, as measured by an upregulated expression of a heat shock protein. Finally, some non-target effects were also evident in *H. contortus* L3 larvae after 72 h incubation in dsRNA targeting a beta-tubulin gene (Kotze and Bagnall, 2005). In addition, Bakhetia *et al.* (2005 *a*) also noted that mis-targeting can also arise for genes that have full sequence similarity for just 21 bp. The potential danger is that these non-target effects are mistakenly regarded as specific phenotypes resulting from a gene knock-out or, alternatively, that they mask the specific phenotype. The observation that dsRNA itself can have an effect on the parasites also emphasizes the importance of appropriate controls.

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As well as being specific, a potential RNAi screening method should also be efficient and reliable. In our study on *H. contortus* we showed that although it was possible to suppress gene expression by soaking in dsRNA, it only worked on a limited number of genes (2 out of 11 tested) and in some cases the effect was difficult to reproduce. Similar results were recently published by Visser *et al.* (2006) in *O. ostertagi*. Their data indicated that the problem with reproducibility was caused, in part, by differences between dsRNA preparations. For unknown reasons, some samples induced complete silencing whereas others had only a minor effect. An adequate number of replicates is required to confirm any effects. For example, our initial experiments focussed on superoxide dismutase (SOD) expression in L3 *H. contortus*. This gene was selected because enzyme activity was readily measured, antibody probes were available to us to conduct immunoassays of protein expression and SOD is constitutively expressed throughout the life-cycle. After 5 separate RNAi experiments, where exsheathed L3 were incubated with SOD dsRNA and lipofectin, specific enzyme activity in L3 extracts was reduced by 30% compared to controls although no effect on transcript abundance could be demonstrated. We repeated the experiment on a further 5 occasions and, statistically, the effect on enzyme activity was lost.

It is also unclear why some of the genes tested are more susceptible to RNAi than others. Differences in location, stability and/or levels of target transcript in the parasite could be important. With the exception of some neuronal genes, most *C. elegans* genes are susceptible to RNAi. The significant variation in sensitivity we have observed is therefore unusual and may be a reflection of the inefficiency of the technique in strongylid parasites. However, only a limited number of genes have been tested in the other parasite species. A similar pattern might appear as more genes are tested. Alternatively, more genes may have been tested, but only the ones on which RNAi was successful were reported. For example, Issa *et al.* (2005) quantified L3 development in *T. colubriformis* larvae that were fed HT115 expressing dsRNA from a number of *T. colubriformis* genes available in their laboratory and they reported that one gene, tropomyosin *Tc tmy-1*, did induce a strong RNAi phenotype when fed to L1 larvae under the same conditions as previously used unsuccessfully with *Tc ubq-1*. This statement infers that the RNAi protocols used had variable efficacy depending on the genes targeted. In evaluating the reliability and specificity of the technique, it would be helpful to report the percentage success rate in terms of total number of genes targeted and success should be confirmed by demonstrating reduction in the target RNA transcript in treated worms compared to control worms exposed to nonsense dsRNA and worms not exposed to dsRNA but cultured for the same period of time.

FUTURE PROSPECTS

The first and crucial step in RNAi is the delivery of dsRNA to the parasite. In order to use RNAi for large-scale screening, the delivery method should preferably be simple, quick, cheap and reliable. Despite some successes, all of the methods tested on helminths (soaking, feeding and electroporation) have shown severe limitations. Non-specific effects, variabilty of the outcome and a low efficiency are the major obstacles. The ideal treatment should combine a maximum uptake of dsRNA with a minimum of side-effects. Unfortunately, manipulating parasites under *in vitro* conditions without inducing non-specific effects is extremely difficult. Uptake may also differ in the different stages of the same parasite. Fluorescently-labelled dsRNA was detected in the pharynx and gut of L2 and L4 but not L3 *H. contortus* (Geldhof *et al.* 2006), suggesting this may not be the best stage to target by RNAi using current protocols.

A possible alternative to improve uptake is the use of small interfering RNAs (siRNA). Issa *et al.* (2005) reported that these molecules were more effective in *T. colubriformis* than dsRNA. In addition, the use of siRNAs, which are commercially available, would also circumvent the problem with dsRNA batch differences. Unfortunately, producing the siRNAs is still very expensive. This approach is, therefore, more suitable to analyse selected target genes rather than for large-scale screening. It is also important to note that off target effects can be induced by siRNAs (e.g. Scacheri *et al.* 2004) and by precursor microRNAs (e.g. Bartel, 2004), the latter resulting in cleavage of the target RNA if complementarity is almost perfect or can trigger translational repression following imperfect annealing in the 3'UTR.

Transgenic technology expressing dsRNA from hairpin constructs within cells has been shown to result in inducible and heritable gene silencing in *C. elegans* (Tavernarakis *et al.* 2000). This delivery approach could potentially be used in parasitic nematodes if successful transformation can be achieved.

Based on the published data, RNAi in schistosomes seems to be more robust and reproducible compared to the parasitic nematodes. It is important to note that the consistent RNAi effects in *S. mansoni* only became apparent after 6 days of *in vitro* culture (Correnti *et al.* 2005). This was the case for both the soaking and electroporation method with miracidia, cercariae and schistosomula. Analyses of parasites at earlier time-points revealed inconsistent effects (Correnti *et al.* 2005). The appearance of the RNAi effects in both miracidia and cercariae coincided with the onset of growth and development

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Table 2. Analysis of the *Haemonchus contortus* genome (currently 6-fold coverage) for genes essential in the RNAi pathway

(The preliminary *H. contortus* genome database was searched with 17 *C. elegans* protein sequences by tBLASTn. Putative homologous sequences were analysed for overlapping reads and subsequently used to calculate the percentage identity, similarity and coverage to its *Caenorhabditis elegans* homologue.)

C. elegans gene name	Size (aa)	Protein function	Number of *H. contortus* sequence reads in TBLASTN	% Coverage of *C. elegans* protein	% Identity	% Similarity
Dicer	1845	dsRNase	40	53	59	72
Drh-1	1037	Dicer related helicase	12	47	39	54
Rde-1	1020	Takes siRNAs from initiator complex to rest of pathway	13	19	52	72
Rde-3	441	Possibly engaged in the target	7	38	62	75
Sid-1	776	Systemic RNAi	1	7	48	67
Eri-1	448	RNAi antagonist (exonuclease)	9	47	45	59
Rrf-3	1780	RNA antagonist	8	27	49	68
Tsn-1	914	RNA binding (RISC)	9	48	55	79
Vig-1	378	RNA binding (RISC)	2	13	49	51
Rrf-1	1601	RNA dependent RNA polymerase (RdRP)	10	63	55	70
Ego-1	1632	RNA dependent RNA polymerase (RdRP)	13	47	58	77
Mut-7	910	Predicted RNA-binding protein	9	19	47	67
Rsd-6	689	Systemic RNAi	3 (highly repetitive sequence)			
Sid-2	311	Systemic RNAi	No hit			
Rde-2	578	siRNA accumulation	No hit			
Rde-4	385	RNA binding in dicer complex	No hit			
Rsd-2	1265	Systemic RNAi	No hit			

(Boyle *et al.* 2003; Correnti *et al.* 2005). Therefore, the ability of the parasite to grow and develop under *in vitro* culture conditions could be an important factor in the susceptibility to RNAi. Culturing parasitic nematodes is, unfortunately, more problematic compared to the situation with schistosomes. It has proved to be very difficult to maintain parasitic nematodes *in vitro* for longer periods. For example, Britton and Murray (2006) reported that the early larval stages of *H. contortus* can be maintained in a healthy condition for only around 5 days and development to healthy L4 and adult stages cannot be achieved with the current standard culture systems. The development of new and improved culture methods that enable development of the nematode through successive stages would be a huge step forward. It could potentially improve the efficiency of RNAi and it would also enable us to analyse longer term effects of a gene knock-down. Unfortunately, this is easier said than done. Parasitic nematodes have a complex life-cycle with their own specific hosts and niches. Simulating this under *in vitro* conditions will be extremely difficult. Even if we would succeed, it is still questionable how these worms would compare to worms that are resident in their natural hosts. It is possible that they will transcribe a different panel of genes. Therefore, *in vivo* studies of RNAi effects may still be needed.

The studies on *H. contortus* and *O. ostertagi* have indicated that most of the genes targeted could not be knocked down by RNAi (Geldhof *et al.* 2006; Visser *et al.* in press), putative homologues of which are particularly amenable to RNAi in the larval life-stages of *C. elegans*. Although this might be caused by problems with the dsRNA delivery or the *in vitro* culture conditions, it is also possible that there are fundamental differences between the RNAi pathways of these nematodes. There are multiple examples of closely related species which differ in their susceptibility to RNAi, even within the *Caenorhabditis* clade (Descotte and Montgomery, personal communication). Whereas RNAi in *C. elegans* works by soaking, feeding and injection, it only works in *C. briggsae* by injection. This suggests that *C. briggsae* has the basic RNAi machinery, but lacks the machinery necessary for dsRNA uptake and transport.

A potential way to analyse the RNAi pathway in parasites is by genome database mining. Whole genome shotgun sequencing of *B. malayi* has recently been completed (Ghedin *et al.* 2004) and the *H. contortus* genome is currently being sequenced by the Pathogen Sequencing Unit of the Wellcome Trust Sanger Institute (Cambridge, UK). The genome has been sequenced to approximately 6-fold coverage (based on an estimated haploid genome size

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of 60 Mb), by whole genome shotgun sequencing, which should equate to well over 98% of the genome sequence, using the Lander-Waterman equation (Lander and Waterman, 1988). In a preliminary analysis we have screened this *H. contortus* genome database for 17 genes that have a known function in the RNAi pathway in *C. elegans* (Grishok, 2005). The results of this analysis are shown in Table 2. In short, the 17 *C. elegans* protein sequences were taken from Wormbase (www.wormbase.org) and used to carry out tBLASTn searches against the *H. contortus* genome database using the online Sanger Institute BLAST server (www.sanger.ac.uk/cgi-bin/blast/submitblast/h_contortus). The *H. contortus* sequences identified in each BLAST analysis were used in the ContigExpress programme of Vector NTI to identify overlapping reads (>98% identity over >50 bp). The individual and/or consensus sequences were subsequently used to calculate the percentage identity/similarity to its putative *C. elegans* homologue and percentage of the *C. elegans* protein covered (Table 2). This analysis identified putative homologues of RNAi genes in the *H. contortus* genome, namely *dcr-1, drh-1, rde-1, rde-3, sid-1, eri-1, rrf-1, tsn-1, vig-1, rrf-1, ego-1, mut-7* and *rsd-6*. However, other genes which are thought to be essential for a functional and systemic RNAi pathway have not yet been found; these include *rde-2, rde-4, sid-2* and *rsd-2*.

The absence so far of the *rde-4* gene is intriguing. The RDE-4 protein binds double-stranded RNA and is critical for complex formation with dicer and generation of short interfering RNAs (Parrish and Fire, 2001). Hence, it is critical for an active RNAi pathway in *C. elegans*. RNAi is inoperative in *C. elegans* mutants lacking *rde-4* (Tabara *et al.* 1999) and we have shown that *rde-4* is required for silencing *C. elegans* homologues of genes of interest to us in *H. contortus* (Clark, Geldhof and Knox, unpublished observations). *Rde-4* is also not obviously apparent in the *Brugia malayi* genome, nor is a homologue apparent in the *S. mansoni* gene datasets. Given that successful RNAi has been described in both the latter, are other mechanisms for RNAi operative in helminths?

If *rde-2, sid-2* and *rsd-2* are indeed absent from the *H. contortus* genome, it would infer that an RNAi effect would not spread through the worm and to the germline. If this is the case, effects would only be observed in regions of the worm directly accessible to the dsRNA such as the outer surface, the gut and the reproductive tract. This could explain the variation observed in susceptibility of different genes to RNAi. However, if *rde-4* is not present, how can the dsRNA be processed? Of course, despite the 6-fold coverage, the *H. contortus* genome sequence is not complete and it is still possible that homologues for these genes have yet to be sequenced. However, if their absence holds up, it could mean that RNAi might be impossible in *H. contortus* or, that an alternative pathway is operating. Further analysis of the genome information, combined with gene expression studies, will be extremely valuable in the study of RNAi in parasites.

CONCLUSIONS

It has been clearly established that, under certain conditions, it is possible to interfere with gene expression in helminths by RNAi. However, problems have arisen with the efficiency, specificity and reproducibility of the current techniques. Unless we can further improve the different methods, the application of RNAi for functional analysis will be restricted, the major difficulty being the interpretation of a negative experiment. Is the target gene non-essential to the parasite or is it inaccessible to the RNAi treatment? In order to render the technique more robust, we should use the current knowledge on RNAi and combine it with future research on delivery methods, culturing systems and helminth genomics.

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