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REVIEW ARTICLE

RNA Interference in Veterinary Parasitology- An Overview

S R Tramboo[1], Z A Wani*[2], R A Shahardar[3], I M Allaie[2] and K H Bulbul[2]
1 Ex-Ph. D Scholar (shahana_qx@yahoo.com)
2Assistant Professor (zahoorwani_103@yahoo.co.in; drkhbulbul@gmail.com; dr.idreesallaie@gmail.com)
3 Prof. & Head (rafiqshahardaar@gmail.com)
* Corresponding author
Division of Vety. Parasitology, F.V.Sc. & A.H, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shuhama, Jammu and Kashmir 190 006, India

ABSTRACT
The phenomenon of RNA interference is biological response to double-stranded RNA, which regulates the expression of protein coding genes. Therefore, this technology has the ability to identify and assess the function of thousands of genes within the genome that are actually involved in disease phenotypes. In case of parasites, miRNAs are being evaluated for their role in parasite development as well as host parasite interactions. The availability of genome sequence data as well as small RNA sequencing has enabled to profile miRNAs which are expressed at particular developmental stage. Though this process has proved to be inconsistent in parasitic nematodes, but recent success of this technique in schistosome and liver fluke encourage further efforts to enhance delivery of RNA and improve in vitro culture systems and assays to monitor phenotypic effects in nematodes. RNAi technology for sequence-specific gene silencing promises to revolutionize experimental biology and thus may have important applications in functional genomics, therapeutic intervention, diagnosis, disease control agriculture and other areas.
Key words: RNA interference, Parasite, Applications

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INTRODUCTION

RNA interference (RNAi) is a molecular mechanism in which fragments of double-stranded ribonucleic acid (dsRNA) interfere with the expression of a particular gene that shares a homologous sequence with the dsRNA. It can also be defined as a sequence specific RNA degradation process characterized by the formation of double stranded RNA and can be introduced by virus or transgenes. Duplexes 21- nucleotide (nt) RNAs with symmetric 2-nt 3'overhangs are introduced into the cell mediating the degradation of mRNA [64]. Before RNA interference was well characterized, the phenomenon was known by several names like post transcriptional gene silencing, transgene silencing and quelling. The technique was first discovered in petunias plant [57]. A phenomenon called co suppression occurred due to high level of transgenic chalcone enzyme expression. The revolutionary finding of RNAi resulted from the work of Fire and co-workers, who demonstrated that injection of double-stranded RNA (dsRNA) into the free-living nematode *Caenorhabditis elegans* leads to efficient sequence-specific gene silencing [24]. Since then, it has been applied in a wide variety of organisms to study gene function, including protozoa [80], amphibians [60], insects [40] and mammals [30]. Advances in RNAi technology have also been used to establish functional links between genes and phenotypes in parasites over the past few years. However, it has been shown that the RNAi approach does not result in gene knockdown in some parasite species, either due to biological or technical reasons. RNAi can be used both in cell culture and living organisms as the synthetic dsRNA introduced into cells can selectively induce suppression of specific genes of interest. RNAi has been best studied in parasites like *Trypanasoma brucei*, *Leishmania braziliensis* [8], *Giardia lamblia*, *Trichinella spiralis* [18], *Entamoeba histolytica* [84], *Brugia malayi* [73, 43] and *Toxoplasma gondii* [11]. Besides being

helpful in gene function analysis, it has potential in disease therapy, drug development and control of parasite transmission and development also [80].

COMPONENTS OF RNAI

Though being an RNA-dependent gene silencing process, it is controlled by the RNA-induced silencing complex (RISC) that is initiated by dsRNA molecules [20]. The various components of RNAi include; Double stranded RNA (dsRNA) which may be of exogenous, viral or transposans dsRNA; Dicer, a ribonuclease III enzyme which degrades dsRNA into 22 bp small interfering RNAs (siRNAs); siRNA that induces the formation of RNA induced silencing complex; Argonaute (AGO), a group of proteins having three main domains viz., PAZ, MID and Piwi which helps in target identification and cleavage of the transcripts; RNA dependent RNA polymerase (RdRp) which triggers the synthesis of RNA from an RNA template and Proteins like Systemic RNA interference deficient (SID-1,SID-2) and RNAi spreading defective (RSD-4) which help in intake and spread of dsRNA in the cells.

MECHANISM OF RNAI

Introduction of double stranded RNA (dsRNA) molecules into the cell cytoplasm trigger this process. Initially, the "trigger" dsRNA molecule is cleaved by dicer i.e. ribonuclease III enzyme to form 21-23 bp double stranded fragments known as short interfering RNAs (siRNAs) which have high binding affinity for the target mRNA template. In the effector step, the duplex siRNA are then unwound by a helicase activity associated with a distinct multiprotein complex known as the RNA- induced silencing complex (RISC). Binding of siRNA, activates the RNA induced silencing complex (RISC complex), leading to degradation of the homologous RNA, thus affecting the phenotypic gene expression. This is also termed as post transcriptional gene silencing (PTGS) and it is said that other forms operate at the genomic or transcriptional level in some organisms. This phenomenon reduces the transcripts of specific mRNA, rather deleting or inserting a gene unlike genetic modifications.

METHODS OF DELIVERY

Delivery of dsRNA remains the biggest challenge among non-model organisms, as it is necessary that dsRNAs should reach the cytoplasm of target cell to become effective and induce silencing. An ideal dsRNA delivery approach will tend to optimize all the steps, from the need for efficient uptake and trafficking into the cytoplasm to the need to maintain its integrity and stability of the RNAs inside the target cells. Artificial dsRNA delivery strategies for RNAi include soaking, injection, feeding, transfection and electroporation [83]. Nanotechnology is nowadays giving new solutions to the problem of gene silencing delivery methods.

DSRNA DELIVERY IN PARASITES

Delivery of dsRNA in translating RNAi to parasitology research as a conventional option still remains a challenge. Many attempts using several delivery and carrier strategies have been applied in parasites, although strategies and outcomes vary depending on the type of parasite to manipulate. Four methods which are mainly used in parasites include: i) feeding of *Escherichia coli* expressing double stranded RNA (dsRNA); (ii) soaking of short interfering (synthetic) RNA oligonucleotides (siRNA) or in vitro transcribed dsRNA molecules; (iii) electroporation of siRNA or in vitro transcribed dsRNA molecules and iv) Micro–injection dsRNA in to the organism.

RNAI IN HELMINTHES

Since the discovery of the RNAi mechanism in the free living nematode *C. elegans*, the RNAi has been applied as a tool for the study of gene function in variety of animals, including parasitic worms [49]. Besides the RNAi-related methodological particularities for each group of helminths, two main reasons behind the slow and disappointing development of RNAi in parasitic helminths include: (i) the apparent lack of homology between some *C. elegans* genes and the parasite genes, which are involved in parasitic lifestyle and parasite-host relationship [28] and (ii) the complexity in the parasitic lifecycle, along with the difficulties in the invitro culturing of their developmental stages and the lack of immortal cell lines. Till now, only 10 species of parasitic helminthes have been studied for RNAi effects after its successful application in *C. elegans* in 1998 [28].

TREMATODES

***Schistosoma* spp.**

The first successful RNAi in trematodes carried out by Skelly et al [74] was used to access the function of cathepsin B in cercariae of *Schistosoma mansoni.* Soaking of cercariae in dsRNA for 6 days was used to study the expression of cathepsin B by reverse transcriptase-PCR (RT-PCR), immunostaining and enzyme activity assays. The study proved that RNAi treated cercariae had lower levels of cathepsin B expression. Boyle and coworkers targeted two *schistosoma manmsoni* genes i.e. glucose transporter gene SGTP1, a facilitated

diffusion glucose transporter and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene [16]. The study indicated that RNAi leads to the reduced level of expression of both genes, which was then transferable from miracidium to sporocyst stage in the life cycle of schistosomes. Another experiment was used by Dinguirard and Yoshino [21] who targeted a CD36-like class B scavenger receptor (SRB) in *S. mansoni*. Miracidia soaked in gene specific dsRNA for 6 days, resulted in a significant knockdown (60–70%) of SRB transcript levels in sporocysts. After about 8-10 days post-dsRNA incubation, the sporocysts showed a significant reduction in acetylated LDL binding. Silencing of peroxiredoxins (Prx) 1 and 2 in *S. japonicum* schistosomula showed that Prx1 may act as a scavenger against reactive oxygen species, but are not essential for parasite survival [42].

***Fasciola* spp.**

Subsequently RNAi was applied to *Fasciola hepatica* to know the function of cathepsin L and B in parasitic invasion mechanism [66]. The studies showed that RNAi by dsRNA in newly excysted juveniles resulted in knocking down of the leucin aminopeptidase [66] and the proteases cathepsin B and L [52]. Significant reduction in penetration of the rat intestinal wall by newly excysted juveniles was observed in an exvivo model experiment due to silencing of either of these two enzymes. McCammick et al [51] focused on the role of Calmodulins (CaM) in *Fasciola hepatica* and concluded that RNAi process helps in deduction of underlying fundamental processes of CaM like phosphorylation of protein kinases, transcription process of gene, calcium transport and smooth muscle contraction. Anandanarayanan et al [6] studied the RNAi protocols in *F. gigantica* where long dsRNA was delivered to the NEJs by simple soaking method and reported an efficient and persistent gene silencing up to 15 days of observation, thus opening new pathways for functional validation of putative vaccine and therapeutic targets. Long dsRNA and siRNA delivered to the newly excysted juveniles by soaking method was found to be efficient in the above experiment.

Opisthorchis viverrini

Functional genomics have not been reported for *O. viverrini* or *C. sinensis*. However, RNA interference and other methods used for genetic manipulation will enable characterization of the role and importance of genes for these flukes [65, 66]. Sripa et al [78] targeted cathepsin B of *O. viverrini* as a model gene for development of RNAi and other fish-borne flukes and showed that cathepsin B is susceptible to RNAi knockdown, as evidenced by both reductions in levels of transcription and enzyme activity of cathepsin B.

CESTODES

The first work report on the successful application of RNAi to a cestode, *M. expansa,* demonstrates that cestodes also possess a functional RNAi [62]. RNAi targeting with dsRNA in cultured *E. multilocularis* primary cells for three different genes: glyceraldehyde-3-phosphate dehydrogenase, 14-3-3 and ezrin–radixin–moesin-like protein has been demonstrated [76]. Although obvious phenotype was detected, but significant decrease in mRNA and protein levels was observed after dsRNA electroporation. The scarcity of RNAi assays for monogenea as well as cestodes, maybe due to the difficulties inherent in the invitro and in vivo maintenance of their life cycles rather than to other factors.

NEMATODES

Nematode parasites are a major cause of disease in animals and the worldwide economic impact of nematode parasites to the livestock industry is estimated to be more than 10 billion per annum [68]. In nematodes, the RNAi technology has been applied with variable results. Problem encountered in the application of such technology in nematodes may be due to the lack of invivo and invitro maintenance and propagation alternatives, difficulties in dsRNA delivery that is related to outer structures of nematode and also potentially lack of the SID-1 molecule present in *C. elegans*, schistosomes and insects.

The first RNAi study was performed on *Nippostrongylus brasiliensis* [32] in which the adult worms were soaked in dsRNA to knock down the acetylcholinesterase A isoform gene and this process resulted in 80–90% reduction of the secretion of ACh A, B and C isoforms. Besides this, RNA interference effects have also been reported in filarial nematodes. In adult females, beta-tubulin, RNA polymerase II large subunit and a microfilarial sheath protein was targeted [1] by soaking the human parasite *Brugia malayi* in dsRNA and it was observed that fluorescently labelled dsRNA molecules of 300 bp were able to enter the adult females. All target transcript levels started to drop between 14 and 17 h of soaking by RT-PCR. After 24 hrs of soaking in dsRNA for beta-tubulin and the RNA polymerase II large subunit, worms were dead. Microfilarial sheath protein targeting did not have lethal effect on adult females. The experiment resulted in marked reduction of microfilariae release and about 50% of them did not have fully elongated sheaths. The introduction of dsRNA and siRNA targeting the cathepsin L-like cysteine protease gene of *B. malayi* in parasitized mosquitoes resulted in reduction of gene transcription and thereby consequent reduction in

worm motility which prevented the migration of larvae to the mosquito proboscis[76]. RNA interference in filarial parasites was further optimized [61] wherein 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 mM. Actin transcription was suppressed by all tested concentrations. Cathepsins play important roles in filarial biological processes within the host, like moulting, cuticle remodelling, embryogenesis, feeding and immune evasion. Two cathepsins (L and Z) as well as serine protease inhibitor (OV-SP1) genes were targeted by soaking of L_3 stage larvae in dsRNA and it significantly reduced larval moulting in *Onchocerca volvulus* [25,46]. Decreased numbers of released microfilariae and the disruption of embryogenesis was noticed when cathepsins L and Z genes were knocked down in *B. malayi* [26]. The RNAi assays done in *A. suum* have also been effective. Soaking the L_3 larval stage in dsRNA for targeting the pyrophosphatase (PPi) gene resulted in reduction of transcript levels, native protein expression and inhibition of the moulting process [35]. When these results were compared with previous results, they were found consistent with the same group that showed moulting inhibition in L_3 treated with PPi-specific inhibitors [34]. As these PPi homologs have also been seen in *Trichinella spiralis*, *A. lumbricoides*, *Toxocara canis*, *B. malayi* and *Loa loa*, it increases the chance to characterize a common factor for L3 moulting, to manipulate it and thus block development in nematodes. Ablation of the *A. suum* enolase gene expression and the gene corresponding to a specific L_3 transcript of unknown ontology, reducing the in vivo survival rate of treated nematodes was also noticed [18, 81]. In case of blood-feeding nematode *Haemonchus contortus* first successful RNAi was described by Kotze and Bagnall [41]. When two beta-tubulin genes were targeted in exsheathed L_3, L_4 larvae and adult parasites by culturing the worms in medium containing dsRNA, it resulted in the decrased transcript levels of both genes in all 3 parasitic life-stages (greater than 1000-fold in some cases) after 24 h of incubation which was accessed by Quantitative PCR. L_3 worms showed a decreased motility compared to control worms and less worms developed to the L_4 stage after 6 days of treatment.SiRNA-mediated silencing of Hc-MMP-12 gene in *H. contortus* significantly reduced the egg counts, larval hatchability and adult worm counts and sizes [58, 69] silenced *H. contortus* aminopeptidase H_{11} gene in L_3 larvae which resulted in decreased worm number and eggs recovered from lambs upon infection with the silenced parasites. They further evaluated the effect of transcript levels and gene expression site due to soaking *H. contortus* L_3 in dsRNA, when several genes were targeting and found that genes represented by high ESTs numbers or not expressed in the intestine, excretory cells or amphids were inconsistently silenced. Dim-1 is a member of the disorganized muscle family of *H. contortus*. Effective silencing of Dim-1 in third stage larvae (L_3) led to reduced L_3 migration and slowed larval development from L_3 to early L_4. Thus, potentiating the therapeutic effect against *H. contortus* [7]. In *T. colubriformis* the efficacy of RNAi was investigated [36] by using three different delivery methods, i.e., soaking, electroporation and feeding on dsRNA-carrying bacteria, in which ubiquitin and tropomyosin were taken as target genes. Ubiquitin siRNA or dsRNA delivery by soaking or electroporation method inhibited the development of *T. colubriformis*, but tropomyosin gene was successfully silenced by feeding method.

PROTOZOA

The mechanisms which regulate gene expression in protozoa are poorly understood, which may be due to the fact that protozoan parasites are with highly divergent genetic backgrounds, and with different regulatory mechanisms. The RNAi technology was first applied in *T. brucei* and appeared to be the technique of choice for down-regulating gene products in African trypanosomes [8, 13]. In *T. brucei* blocking the actively expressed VSG by RNAi resulted in the arrest of cell cycle, but it was reversible when a second VSG was expressed [75]. Repeated application of RNAi in bloodstream and promastigote *T. brucei* stages has resulted in the genetic validation of numerous new potential drug targets [5]. Some other *T. brucei* molecules have been found to be essential for parasite survival by RNAi technology include ornithine decarboxylase and the spermidine synthase, which are important for growth arrest and cell death in trypanosomes [63, 79]. Besides this, a small conserved mitochondrial protein namely frataxin has been linked through RNAi studies with parasite protection against reactive oxygen species [44]. The procyclic forms found in tsetse fly vector have been manipulated by RNAi, which indicated that procyclic vacuolar proteins plays an important role in the intracellular iron utilization system, in parasite 'defences' and maintenance of normal cellular morphology in *T. brucei* [45]. Unfortunately, every RNAi trial attempted in *T. cruzi* to date has failed due to the presence of alternative epigenetic control mechanisms. In *Leishmania braziliensi*, a successful silencing experiment was carried out by Lye et al [47]. Gene replacement techniques are the only resource actually developed for epigenetic approaches in *Leishmania* [9]. Rather disappointingly, direct experimentation showed that neither *T. cruzi* nor *Leishmania major* and *L. donovani* were able to respond to dsRNA, indicating lack of functional RNAi pathway [19, 67]. This was confirmed at the genome sequence level, which revealed the absence of RNAi genes [22, 37]. RNAi attempts have been

done mainly in *P. falciparum* and *T. gondii* in Apicomplexan parasites, which initially confirmed that in *Plasmodium falciparum* asexual forms, dsRNA could trigger downregulation of gene expression [48, 53, 56] but latter on could not be reproduced by several laboratories. Al-Anouti and Ananvoranich [2] demonstrated that RNAi could be a very useful tool for the study of gene expression in *T. gondii* interrupting expression of the enzyme uracil phosphoribosyl transferase. The expression of *T. gondii* enolase, adenosine kinase and hypoxanthine–xanthine–guanine phosphoribosyl transferase genes have been successfully silenced as well [82, 31]. Database mining has enabled that *G. lamblia* has an RNAi pathway, which has a role in control of variant-specific surface protein (VSP) [8]. Externally delivering dsRNA in *E. histolytica* RNAi methodologies, which down-regulate gene expression has been shown promise and has been applied very successfully [8, 82]. E.g. transcriptional silencing of the amoebapore (Ehapa) and the cysteine protease 5 genes, result in development of trophozoites which exhibit attenuated virulence [10, 54]. TRNA gene-mediated silencing mechanism, which is known to function in *Saccharomyces cerevisiae*, is also used by *E. histolytica* for epigenetic transcriptional gene silencing also [33]. Regarding *T. vaginalis*, both miRNAs and siRNAs have been characterized [19].

ARTHOPODS

Alphey [4] stated that among the parasitic arthropods, RNAi has been applied to mosquitoes and ticks affecting man and animals worldwide.

TICKS

The first RNAi assays in tick i.e. *Amblyomma americanum* was performed [3]. An aberrant tick feeding patern was observed when the salivary histamine binding protein from female ticks was silenced by dsRNA injection. Similarly silencing of synaptobrevin and nSec-1 genes in *A. americanum* resulted in inhibition of secretion of anticoagulant proteins and aberrant tick feeding, thus highlighting their role in salivary glands vesicle exocytosis [39]. Besides this, an impaired salivation and decreased the amount of ingested blood was noticed by RNAi of the *Haemaphysalis longicornis* valosin-containing protein and *I. scapularis* Na+/K+ -ATPase [14, 38]. The role of the *Ixodes ricinus* aquaporin-1 (IrAQp1) in blood meal water handling was studied by silencing with RNAi [17]. Role of the ferritin-1, ferritin-2 and iron regulatory protein-1 of *I. ricinus* in the non-heme iron storage in digestive gut cells, its transport through hemolymph and its utilization by peripheral tissue cells was demonstrated by Hajdusek et al [29] through RNAi. Similarly, RNAi has allowed the disruption of VgR expression in *Dermacentor variabilis* and *H. longicornis* [55, 15]. When the gene coding for immunophilin in *Rhipicephalus microplus* gene was silenced [12], it resulted in increased infection by *B. bovis* in larval progeny, and reflected that immunophilin controls the transovarial transmission of these protozoa to eggs and larvae. Furthermore, up to 90% reduction in gene expression of subolesin has been successfully knocked down in the soft tick species *Ornithodoros erraticus* and *Ornithodoros moubata* by injecting dsRNA into adult ticks. Silenced females fed normally, but their oviposition was completely inhibited [50], showing that subolesin is also essential in this tick group and also this is the first report of RNAi silencing in soft ticks. It is expected that with the increase in tick genome sequence information the application of RNAi for functional genome-wide studies to address important issues on tick physiology, development and gene regulation will be carried out.

MOSQUITOES

For characterizing gene function in diverse fields of mosquito biology and mosquito–pathogen interactions, RNAi has now rapidly become the tool of choice [27]. In temperate climates, adult female mosquito undergoes diapauses in winter and manipulation of this regulation could result in the induction of dormant, non-host-seeking mosquitoes. Dr. Denlinger's team have used RNAi to investigate the diapause mechanism in *C. pipiens*, to ellucidate the participation of the insulin/FOXO signalling pathway in the regulation of diapause [70, 71]. They have evidence for the involvement of some fatty acid synthases in the accumulation of fat reserves in overwintering females [72]. With the help of RNAi, vitellogenesis and reproduction have also been characterized.

Sarcoptes scabiei

Scabies, which has a considerable medical relevance with regards to complications is due to mite associated secondary infections. Non-invasive immersion of *S. scabiei* in dsRNA encoding an *S. Scabiei* glutathione S-transferase mu-class1 enzyme (SsGST-mu1) resulted in a 35% reduction in the transcription of the target gene compared to controls [23].This is first the first experimental gene silencing by RNA interference (RNAi) in *S. Scabiei*.

LIMITATIONS OF RNAI

Besides the numerous advantages of RNA interference, the main drawback is that similar sequence to the targeted gene is also repressed. Off-site effects are more frequent when dsRNA is delivered by the soaking

method [59]. Multiple barriers are faced by artificially delivered dsRNA molecules to reach their specific targets inside the parasites' cells, some of which are solely attributable to the parasite itself, external parasite's structures and components: the extracellular matrix at the surface of unicellular parasites and additionally, the tegument in flatworms and cuticle in roundworms. Host cell internalization, entrapment in endosomes and lysosomal degradation could result in losing of dsRNA activity.

CONCLUSION

The RNAi phenomenon is recently described process and major advances have been accomplished in model organisms such as *C. elegans* and *Drosophila*. But, many of these advances cannot be directly translated to parasites. The information about RNAi and gene silencing in parasites is evolving very fast, and shows its enormous potential in the field of functional annotation of genes and genomes. But this is possible for parasites in which the RNAi pathway is present and from which the genome is already known. For parasites lacking RNAi pathway, an alternate mechanism similar to that of RNAi should be looked down. Application of high-throughput-based RNAi assays has lead to the understanding of parasite physiology and further definition of new targets to fight against them.

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DISCLOSURE

The authors declare that they have no conflict of interest.

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