**Review**

**CRISPR/Cas9: a new tool for the study and control of helminth parasites**

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**Summary**

Recent reports of CRISPR/Cas9 genome editing in parasitic helminths open up new avenues for research on these dangerous pathogens. However, the complex morphology and life cycles inherent to these parasitic worms present obstacles for the efficient application of CRISPR/Cas9-targeted mutagenesis. This is especially true with the trematode flukes where only modest levels of gene mutation efficiency have been achieved. Current major challenges in the application of CRISPR/Cas9 methods for the study of parasitic worms thus lie in enhancing gene mutation efficiency and overcoming the issues involved in host passage so that mutated parasites survive. Strategies developed for CRISPR/Cas9 studies on *Caenorhabditis elegans,* protozoa and mammalian cells, including novel delivery methods, the choice of selectable markers, and refining mutation precision represent novel tactics whereby these impediments can be overcome. Furthermore, employing CRISPR/Cas9-mediated gene drive to interfere with vector transmission represents a novel indirect approach for the control of parasitic worms that is worthy of further exploration.

**Keywords:** CRISPR/Cas9, DNA repair, Mutagenesis, Functional genomics, Genome editing, Parasitic helminth

1. **Introduction**

Globally, parasitic helminths afflict 1.5 billion people, and 150,000 die of resulting complications every year throughout Africa, Asia and South America.[1] Helminths comprise two main groups: the phylum Nematoda (roundworms) and the phylum Platyhelminthes which includes the Trematoda (flukes), Cestoda (tapeworms) and Monogenea. Currently, no effective vaccinesare available for human use against any of these parasitic worms.[2] The limited chemotherapy options for treatment of the diseases caused by parasitic helminths increase the risk that drug resistance will develop.[3] It is thus paramount that improved control strategies be developed using advanced techniques that can mitigate this 21st-century threat to global health through the identification of novel drug or vaccine intervention targets. The International Helminth Genomes Consortium recently reported new genome assemblies for 31 nematode and 14 platyhelminth species, providing a solid base for the development and application of advanced technologies, such as genomic editing, to help unravel the functions of unknown helminth genes.[4] Post-transcriptional gene silencing, developed over the past 15 years for loss-of-function research in helminths, resulted in variable levels of efficiency and RNA interference outcomes were either transient or the inheritance of gene silencing was not fully penetrant.[5-9] CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology, however, is a powerful genetic approach for interrogating the genomes and defining the function of key genes in various organisms by triggering specific and heritable genome editing, not only in somatic cells but also in germ-line cells.[10-23][9] Utilizing CRISPR/Cas9 technology for the study of parasitic worms sets the scene for effectively characterizing helminth gene products, thereby providing improved understanding of genomic functions, parasite biology and parasite-host interactions. Successful adaptation of CRISPR/Cas9 in parasitic helminths provides a molecular genetic toolbox for identifying novel drug targets or vaccine candidates, thereby accelerating the pace towards more effective ways to prevent and treat the diseases caused by these worms.

**1.1. CRISPR/Cas9: A New Tool for Genome Editing**

CRISPR was first identified in bacteria as a defence mechanism against viruses, utilizing Cas endonucleases to deactivate the invading viral DNA with the guidance of CRISPR RNA, or ‘crRNA’.[24-27] In CRISPR/Cas9-mediated editing, Cas9 first interacts with the *protospacer-adjacent motif* (PAM) to locate the appropriate substrate and then the Cas9-single guide RNA (sgRNA) complex will open the double strand structure of DNA.[28] SgRNA will recognize the complementary target strand by RNA-DNA base pairing. Subsequently, the HNH domain and RuvC domain of Cas9 cut the target stand and non-target strand, respectively, to introduce site-specific double strand breaks (DSBs).[29] The breaks are then repaired through Non-Homologous End Joining (NHEJ) resulting in random nucleotide insertions and deletions (indels) or Homology-Directed Repair (HDR) utilizing a homologous template for highly accurate repair when template DNA is provided for alternative repair pathways (such as polymerase theta (Pol θ)-mediated end joining (TMEJ)).[30-34]

**1.2. CRISPR/Cas9 Editing in Helminths: What is the Current Picture?**

To date, the CRISPR/Cas9 gene editing system has been successfully established in four free-living nematodes (*Caenorhabditis elegans*,[17, 35-42] *Pristionchus pacificus*,[21, 43] *Auanema rhodensis* and *A. freiburgensis*[44]), three parasitic nematodes (two human parasites, *Strongyloides stercoralis* and *Brugia malayi*, and a parasite of rats, *Strongyloides ratti*[20, 44-47]), and two parasitic trematodes (the human liver fluke *Opisthorchis viverrini* and the human blood fluke *Schistosoma mansoni*[22, 23, 48, 49]). In these studies, Cas9 and target specific sgRNAs were expressed using either an *in vivo* plasmid-based system or by *in vitro* synthesis of the CRISPR components. The delivery of CRISPR components involved microinjection, electroporation or lentiviral-mediated transduction (Table 1).

**Table 1.** **Use of the CRISPR/Cas9 Editing System in Helminths**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Organisms** | **Delivery method** | **Targeted life stage(s)** | **DNA repair pathways** | **Selectable marker(s)** | **Reference** |
| *Caenorhabditis elegans* | Microinjection | Adult worm | HDR,  NHEJ,  TMEJ | Fluorescence donor template and Fluorescence labeled vector,  Drug- resistance,  Co-CRISPR\* | [17, 33, 36, 37, 50, 51] |
| *Pristionchus pacificus* | Microinjection | Adult worm | HDR,  NHEJ | Co-CRISPR | [21, 43] |
| *Auanema rhodensis and A. freiburgensis* | Microinjection combined with Lioposome-based transfection | Adult worm;  Dauer larvae | HDR | Not reported | [44] |
| *Strongyloides stercoralis* | Microinjection | Free-living adult female worm;  Infective third-larval stage (iL3) | HDR,  Large deletions (>500bp) | A reporter, *mRFPmars* was included in the plasmid donor template | [20, 44-46] |
| *Brugia malayi* | Lipofection | Infective larvae (L3) | HDR | Secreted Gaussia luciferase (GLuc) activity | [47] |
| *Opisthorchis viverrini* | Electroporation | Metacercariae;  Newly excysted juvenile worm;  Adult worm | NHEJ  (No DNA template was added) | Not reported | [22] |
| *Schistosoma mansoni* | Electroporation/ lentiviral transduction | Egg;  Mother sporocyst; Adult worm | HDR,  NHEJ | A fluorescence marker attached to ribonucleoprotein (RNP) complexes | [23, 48, 49] |

\*Note: Co-CRISPR uses a highly visible phenotype at one locus to facilitate the identification of custom modifications at the other locus.[51, 52]

1. **CRISPR-mediated Genome Editing is Inheritable in Parasitic Nematodes**

**2.1 CRISPR/Cas9 Toolkit in *Strongyloides***

Soil-transmitted nematodes cause some of the most severe of the neglected tropical diseases and are responsible for the infection of more than 1 billion people worldwide.[53] Genomic sequences are available for six nematodes from the *Strongyloides* clade: four *Strongyloides* species including *S. stercoralis* (a parasite of humans and dogs), *S. venezuelensis* and *S. ratti* (important laboratory models of nematode infection; both parasites of rats) and *S. papillosus* (a parasite of sheep); Parastrongyloides *trichosuri* (a parasite of the brushtail possum *Trichosurus vulpecula*); and the free-living nematode *Rhabditophanes* sp. KR3021.[54] There are 12,451–18,457 predicted genes across the six genomes but very little is known about the function of their expressed products.[54]

CRISPR/Cas9-mediated genome mutagenesis has been achieved in *S. stercoralis* and *S. ratti* (Figure 1A).[20, 44-46] The CRISPR/Cas9-based gene editing efficiencies in both species were relatively consistent among genes and target loci.[20, 44, 45] A CRISPR toolkit has been established for *S. stercoralis* whereby plasmids encoding Cas9 and sgRNA, and a plasmid donor template containing a red fluorescent reporter (*mRFPmars*), were introduced into developing eggs in free-living adult females by gonadal microinjection. The F1 infective third-larval (iL3) progeny of these female worms were screened for the expression of the fluorescent marker. Fluorescent-positive iL3s were then subjected to behavioral assays to analyze phenotype changes followed by PCR genotyping.[20, 45] The CRISPR/Cas9 induced mutation in *S. stercoralis* was shown to be heritable from the F1 iL3s to the F2 and F3 iL3s following host passage.[20, 45] Notably, large deletions (> 500 bp), instead of small indels, were observed during the repair of DSBs in *S. stercoralis.* [20] Further investigation will be required to prevent undesirable outcomes in these DNA repair mechanisms before utilizing CRISPR/Cas9 to induce customized mutations inthis worm. Strategies developed in mammalian cells and unicellular parasites for increasing the precision of CRISPR/Cas9-mediated gene editing include inhibiting NHEJ,[55-61] activating the HDR pathway,[62] and using engineered Cas9[11, 63, 64], might be applicable to this and other parasitic helminths, although the adoption may be challenging due to the complex morphology and life cycles inherent to these worms.

Encouragingly, however, homozygous deletion of a target gene (the twitchin gene *unc-22*) was obtained by CRISPR/Cas9 in the F1 iL3 progeny of *S. stercoralis*,thereby representing an important step for future work in this area.[20, 45] Usually, CRISPR/Cas9-mediated gene editing only affects one of the two alleles of a target gene; therefore several breeding cycles are required to generate offspring with homozygous mutants.[19] However, conducting many breeding cycles with parasitic helminths is difficult due to the challenges in the host passage of mutant worms and this will be discussed further below. Achieving homozygous mutants in the F1 generation would reduce the requirement of host passage and provide an enabling avenue for the study of worm genes with recessive phenotypes including those that have important roles in host infectivity.

**2.2 Integration of Reporter Genes in the *Brugia malayi* Genome**

*B. malayi* is a filarial parasite that causes lymphatic filariasis; it is endemic predominantly in rural areas of Asia and affects 150 million people worldwide. The draft genome sequence of *B. malayi* has been released[65-68] that facilitates the genetic manipulation of this nematode parasite. Very recently, Liu *et al.* developed a CRISPR/Cas9-mediated strategy for specific transgenesis in *B. malayi*.[47] RNP complexes and the plasmid donor DNA encoding a green fluorescent protein (GFP) and Gaussia Luciferase (GLuc) were delivered into infective larvae (L3) of *B. malayi* by lipofection.[47] The transfected L3 were injected intraperitoneally into gerbils and were allowed to develop into adult worms which produced F1 progeny microfilariae. Around 3% of the F1 microfilariae were identified as being transgenic by measuring the secreted GLuc activity and no off-target insertions were detected.[47] Whereas this study provides an enabling avenue for precision editing of the *B. malayi* genome, the CRISPR/Cas9 editing efficiency will need improving if this approach is to be used for developing novel drug targets or vaccine candidates against this filarial parasite.

**2.3 A Novel CRISPR/Cas9** **Delivery Method**

In terms of CRISPR/Cas9 delivery, Adam *et al.* were able to combine microinjection with liposome-based transfection to deliver CRISPR formulations into the free-living adult worms and larvae of *S. stercoralis* and *Auanema* spp.,a procedurethat significantly enhanced the efficiency of CRISPR/Cas9 mutagenesis in these nematodes.[44] Although conventional microinjection has been successfully utilized in delivering CRISPR components into the gonads of *C. elegans* and *S. stercoralis* adults, it failed to introduce mutagenesis in *Auanema* spp. This outcome likely resulted because of the differences in gonad morphology between the genus *Auanema* and *C. elegans*.[44] In the hermaphroditic *C*. *elegans*, the distal germ line is a syncytium. Germ cells have incomplete borders and are connected to one another via a central canal called the rachis.[69] This morphology facilitates the process of microinjection for generating mutagenesis as injected components can be readily incorporated by many oocytes when they mature, finally being incorporated into eggs. However, it is not clear whether there is a similar syncytium of germline nuclei in the genus *Auanema,* and the mitotic zone of the gonad in this nematode group is much smaller than in *C. elegans,* a feature that makes microinjection into this region difficult.[44] However, by adding lipofectamine reagent to the microinjection mixture to enhance the transport of CRISPR components across the cell membranes of *Auanema*, CRISPR/Cas9 mutagenesis was achievable.[44] This liposome-based delivery method expands the application of CRISPR technology to a wider range of free-living and parasitic nematodes, which have very different gonad morphologies compared with *C. elegans*. This is important as in some parasitic nematodes, adult germlines are not readily accessible for CRISPR/Cas9 mutagenesis due to the absence of free-living reproducing stages. This delivery method can thus be used successfully to introduce heritable mutations into the larvae of these nematode species before they develop into adult worms in their hosts, thereby effectively passing on gene mutations from one life cycle stage to the next.

In addition, novel delivery methods promoted for mammalian cells, such as the cell penetrating peptide-mediated method[70] or the biodegradable and well tolerated lipid nanoparticles (LNP)-mediated delivery system,[71] could induce higher gene editing efficiency, minimal damage to cells and less off-target events. Combining these new procedures with the current delivery methods may foster an effective way of delivering CRISPR components into helminth parasites.

1. **Applying the CRISPR/Cas9 System inTrematodes: the First Step in Precision**

**Genome Editing**

**3.1. Successful Programmed Gene Editing in *Opisthorchis viverrini***

*O. viverrini* is the cause of human liver fluke disease or opisthorchiasis, which can result in hepatobiliary morbidity and increased risk of bile duct cancer and cholangiocarcinoma (CCA).[72]The liver fluke is mainly distributed in Thailand, the Lao People's Democratic Republic, Cambodia and Vietnam, and is responsible for infecting around 10 million people.[72] Infection occurs after an individual [ingests](https://www.sciencedirect.com/topics/medicine-and-dentistry/ingestion) undercooked fish or crabs harboring *O. viverrini* metacercariae; these excyst in the small intestine and migrate to the biliary duct of the liver (Figure 1B). The draft genome and transcriptomes of *O. viverrini* have been characterized[73] and provide a valuable resource to identify critically important genes and gene products that can be validated using genetic manipulation tools including CRISPR RNA-guided Cas9 nuclease-based genome editing. Indeed successful CRISPR/Cas9-mediated knockout targeting the *Ov-grn-1* (granulin growth factor) gene was recently achieved in adult worms of *O. viverrini* transfected by electroporation with a reconstructed CRISPR/Cas9 vector encoding specific sgRNA driven by the U6 promoter and Cas9 nuclease driven by the CMV promoter.[22] Deep sequencing of amplicon libraries from genomic DNA of the gene-edited adults showed 1.3% NHEJ indels (0.6% insertions and 0.7% deletions) and 98.7% substitutions.[22] This gene mutation resulted in considerably depleted transcripts and reduced expression of the target genein gene-edited adult flukes. Notably, significantly reduced pathology, as evidenced by decreased biliary hyperplasia and fibrosis, was observed in hamsters infected with gene-editednewly excysted juvenile worms(NEJs).[22] This study illustrates the potential of employing CRISPR/Cas9-mediated editing system to interfere with *O. viverrini* and potentially to control opisthorchiasis. In addition, the work emphasizes the importance of selecting an appropriate life cycle stage for targeting, as direct gene editing of metacercariae achieved only modest downregulation in the level of *Ov-grn-1* transcription.[22] However, when the cyst walls of the metacercariae were removed, thereby allowing the parasites to develop into NEJs before they were subjected to electroporation, the transcription level decreased substantially.[22]

It is highly likely the CRISPR methodology developed for use with *O. viverrini* could readily be extended to the genetic manipulation of the related trematode species *Opisthorchis* felineus,found in eastern Europe, and the Chinese liver fluke, *Clonorchis sinensis*, which also cause CCA,[74] thereby promoting the exploration of alternative therapies and treatments against these parasites as well. Also, akin to the situation with schistosomiasis, which will be discussed later in this review, CRISPR/Cas9 could also be used to manipulate the *Bithynia* spp. intermediate host of *O. viverrini* in order to disturb its transmission and is an approach that should be investigated since proteomic and transcriptomic profiles of this snail are available.[75, 76]

* 1. **CRISPR/Cas9 Editing in Schistosomes: A Good Start but Room for Improvement**

Schistosomiasis, which is first on the scale of devastating parasitic helminth diseases, affects 250 million people in 78 countries.[77] Complete genome sequences for the 3 major schistosome species of humans, [*S. mansoni*](https://www.sciencedirect.com/topics/immunology-and-microbiology/schistosoma-mansoni),[78, 79] *S. japonicum*[80, 81], and *S. haematobium*[82, 83] have been released, providing critical insights into the molecular biology of blood flukes. The schistosome genomes in general comprise ~11,000 protein-encoding genes, although relatively few have been characterized. To date, studies of CRISPR/Cas9-mediated editing in schistosomes have been undertaken exclusively with *S. mansoni* (the life cycle of *S. mansoni* is shown in Figure 1C). However, the considerable genomic information currently available for *S. japonicum* and *S. haematobium* also renders these two schistosome species entirely amenable to the approach.

**3.2.1 Programmed Editing of a Schistosome Gene Resulting in Reduced Host Pathophysiology**

The first study of site-specific genome editing in schistosomes was conducted by Ittiprasert *et al*.[23] and utilized CRISPR/Cas9 on *S. mansoni* eggs targeting the gene encoding omega-1 ribonuclease (ω1). This secreted enzyme is crucial for T helper type 2 (Th2) polarisation and granuloma formation, a process that eventually leads to severe tissue damage and hepatointestinal schistosomiasis.[23] Eggs isolated from liver of mice infected with *S. mansoni* were transduced with lentivirus encoding a sgRNA and Cas9 nuclease combining with/without a ssODN (single stranded oligodeoxynucleotide) donor template.[23] Next generation sequencing (NGS) analysis of reads of amplicon libraries spanning the predicted Cas9-catalyzed ω1 mutations indicated a frequency of NHEJ modifications of ~4.5% and a 0.19% frequency of HDR in the knock-in eggs when donor template was provided.[23] By using a window size as large as the entire amplicon (202 bp) -- which defines the size (in bp) of the quantification window extending from the DSB -- it was revealed that NHEJ substitutions comprised the majority of the NHEJ modified reads (> 98% of NHEJ modified reads were substitutions). As was evident with *O. viverrini,* the transcriptional level of the target gene was markedly downregulated in *S. mansoni* eggs, despite the fact that only a modest level of gene mutation efficiency was achieved.[22, 23] Furthermore, mice, tail-vein injected with the ω1 gene-edited eggs, exhibited substantially reduced pathology compared with those injected with non-edited eggs, confirming an important role for ω1 in the pathophysiology of schistosomiasis mansoni.[23] A key anomaly that remains to be addressed concerns how the low gene modification efficiency generated resulted, nevertheless, in the paradoxical substantial downregulation of the transcriptional level of the target genes and the reduction in host pathophysiological outcomes both with *S. mansoni* and *O. viverrini.* It has been reported that random large gene deletions might be missed by amplicon sequencing during CRISPR/Cas9 editing in *Strongyloides* and *C. elegans*.[20, 84] Accordingly, undetectable off-target mutations generated during gene editing may have led to the pronounced phenotypic changes evident at the protein level in both flatworm species. In addition, the editing of the target genes may also have affected the expression of other key genes in downstream pathways resulting in the phenotypic outcomes observed.

**3.2.2 HDR DNA Repair is Predominant in *S. mansoni* Eggs**

Acetylcholinesterase is a recognized anthelminthic target, playing key roles in the adult schistosome neuromusculature cholinergic system, and in muscular function.[85] Recently, You *et al.*[48] undertook CRISPR/Cas9 editing of *S. mansoni* eggs targeting the gene (*AChE*) encoding acetylcholinesterase. Eggs were transfected by electroporation with reconstructed CRISPR/Cas9-plasmids encoding specific sgRNAs driven by the U6 promoter and Cas9 nuclease driven by the CMV promoter.[48] Given sgRNAs with different target sites might exhibit different endonuclease activities, You *et al.* pre-screened the activity of two sgRNAs (X5 land X7 located on exon 5 and exon 7 of *AChE*, respectively) combined with /without a ssODN template. It was anticipated that the six-stop-codon transgene in the ssODN would be inserted into target sites in *SmAChE* (*AChE* knock-in). The study demonstrated that major modifications induced by CRISPR/Cas9 in *S. mansoni* eggswere generated by HDR and extremely rare NHEJ indels were detectable in *AChE*-edited eggs.[48] In the NGS analysis of amplicons from *AChE* knock-in eggs, using CRISPResso2 software and a 1 bp window size, You *et al.* found that the majority (> 95%) of modified reads with NHEJ were due to substitutions, and not indels; however, the frequency of NHEJ substitutions increased equally across all analyzed samples, including control groups, when the window size was changed from 1 bp to the entire amplicon length.[48] This suggests the NHEJ substitutions detected by CRISPResso2 were likely false-positives introduced from PCR/sequencing errors, but further study is required to confirm these observations.

Nevertheless, a consistent frequency of HDR reads (~0.12%) was detected by NGS analysis in both the X5 and X7 target loci of *AChE*-edited eggs.[48] This was subsequently reflected in a phenotypic difference including decreased AChE activity (by 8.3-10.7%) measurable in the *AChE* knock-in eggs. When the *AChE* knock-in eggs were injected into either the tail veins or the small intestinal subserosa of mice, an enhanced *in vivo* Th2 response (with elevated levels of IL-4, IL-5, IL-10 and IL-13) was detected only in those mice injected with eggs conferring knock-in at the X5 target locus, compared with those identified in mice injected with unmutated eggs.[48] This observation indicates that CRISPR/Cas9-mediated knock-in at X5 site in eggs triggered a superior host immune response than that at X7 site. This further suggests that motifs or domains of AChE that are involved in regulating the Th2 immune response might be located between the X5 and X7 sites in the AChE gene. This study adds support to a previous report by You *et al*. demonstrating the critical role of egg-secreted AChE in inhibiting the host IL-4 response.[86]

**3.2.3 CRISPR/Cas9 Gene Knockout is More Efficient in Adult Worms of *S. mansoni***

In another recent study, Sankaranarayanan *et al.* achieved CRISPR/Cas9-based knockout in *S. mansoni* targeting the *SULT-OR* gene in which mutations confer resistance to the drug oxamniquine.[49] Using electroporation to deliver *in vitro* assembled ribonucleoprotein (RNP) complexes by combining specific sgRNA with the Cas9 nuclease to adult worms, mother sporocysts and eggs, variable gene mutation efficiency was evident across the different life cycle stages.[49] The highest *SULT-OR* modification efficiency was observed in adult worms (0.3%-2% deletions), followed by sporocysts (0.1%-0.2% deletions) but no NHEJ-mediated modifications were detected in eggs,[49] observations supporting those of You *et al.*.[48]

Possible explanations for the observed differences in gene mutation efficiency can be summarized as follows: 1) Adult worms have a larger surface area to volume ratio than sporocysts and eggs, a feature that may more readily facilitate the delivery of CRISPR components by electroporation.[49] 2) There are higher expression levels of some key enzymes in the NHEJ pathway in adult worms than in sporocysts and eggs, an observation that suggests that DNA repair activities in the NHEJ pathway may be more efficient in adults.[49] 3) The egg shell of *S. mansoni* has three layers and is a hardened and tanned structure.[87] There are often serpiginous branching and anastomosing channels[87], which might impede effective transfection. 4) Different protocols employed for different genes (ω1, *AChE* and *SULT-OR* as highlighted here) may result in variable CRISPR/Cas9 editing efficiency. 5) Variation in the pattern of CRISPR/Cas9-induced modifications may depend on the function or distribution of the targeted gene. Further studies are needed to improve the efficiency of CRISPR/Cas9 in schistosomes to facilitate future gene functional studies in these parasites.

The genome editing approach developed for *S. mansoni*[23, 48, 49] is readily amenable to *S. japonicum* and *S. haematobium*, and other clinically relevant platyhelminth species, thereby laying a solid foundation for high-throughput functional analysis of flatworm genes. The pivotal studies described here provide the blueprint for editing other important schistosome genes in the future. Indeed, the application of this powerful but affordable technique may prove of value not only for targeting specific protein-encoding genes implicated in the pathogenesis attributable to schistosomiasis, but also for the identification of novel anti-schistosome vaccine and drug candidates.

**3.2.4 CRISPR/Cas9 in Schistosomes** **and Other Trematodes: Challenges to Overcome and Future Perspectives**

**3.2.4.1 A Selectable Marker for Enriching Gene-edited Worms**

The showcased studies presented here emphasize the potential of utilizing CRISPR/Cas9 to manipulate trematode flukes. However, no appropriate robust markers have been developed in trematodes for the recovery of mutagenesis, and that represents an important step prior to further screening. A fluorescence marker attached to RNP complexes was used in the study by Sankaranarayanan *et al.* to identify successfully transfected schistosomes wherein fluorescence signal was observed under confocal microscopy without any further enrichment required.[49] One disadvantage lies in sorting and selecting positively labelled parasites that possibly possess the desired mutations or traits. Separating worms may be feasible under fluorescent microscopy but sorting through the eggs and sporocysts can be challenging. This is because considerable numbers of labelled eggs and sporocysts (~10,000) are often required to obtain reliable results.[23, 88] Moreover, schistosome eggs and sporocysts are too large and/or fragile to be sorted using conventional flow cytometry. Auto-fluorescence[89] also renders fluorescent sorting difficult. Other selectable markers, such as drug resistance markers, or utilizing a co-CRISPR strategy have been applied in *C. elegans* and in parasitic protozoa,[36, 50, 51, 90-94] and these might be applied to parasitic worms but their feasibility requires further exploration. Co-CRISPR uses a significantly visible phenotype at one locus to facilitate the identification of custom modifications at the other locus.[51, 52] The main issue of adapting co-CRISPR in parasitic helminths is that many robust, easily identifiable phenotypes tend to be detrimental to parasite fitness, making it difficult for host passage and to maintain stable transgenic lines. Thus, only when a visible phenotype that does not affect parasite fitness is obtained will co-CRISPR be feasible. Summarizing, identifying an appropriate marker and relevant sorting method to enrich gene-modified parasites are important hurdles to overcome in order to improve the efficiency of CRISPR/Cas9 in trematode worms.

**3.2.4.2 Establishing Transgenic Lines from Schistosomes**

The heritance of CRISPR/Cas9 mutagenesis in trematodes is also an important angle for future study. Rinaldi *et al.*[95] found eggs provided access to *S. mansoni* germ-line cells when using pseudotyped murine leukemia virus (MLV) to introduce random insertional mutagenesis in chromosomes of this parasite. Furthermore, the study, employing high throughput sequencing approaches, provided the first report of vertical or germline transmission of retroviral transgenes in schistosomes in that the transgenes introduced into eggs were passed down from the first to the second (F1) generation, [95] reinforcing the feasibility of establishing transgenic lines of schistosomes.

However, developing a stable transgenic line of trematodes and other parasitic helminth requires the passage of mutant parasites through hosts. Passaging mutant parasitic worms across their hosts for several life cycle generations is very challenging as their life cycles are complex and it usually takes several months to complete a cycle; for example, it takes around 3 months to complete one *S. mansoni* life cycle round in the laboratory. Consequently, undertaking many breeding cycles is very time-consuming. Since, as discussed, modest gene editing efficiency has been observed in trematodes, it would be very labor intensive to obtain sufficient mutant parasites to support propagation in hosts. In addition, gene editing may negatively affect the fitness of parasitic worms during their passage through hosts. Importantly as well, targeting genes involved in host infectivity may result in parasites failing to propagate in their hosts. One interesting future direction in this respect might be in making conditional knockouts, thereby enabling the passage of worms with mutated genes that would otherwise prevent host infection. The CRISPR-based conditional knockout strategy has been successfully utilized in *C. elegans* for the study of essential genes [96-98]. For example, Shen *et al.*[96] established a somatic CRISPR-Cas9 platform whereby Cas9 was driven by a heat-shock-inducible promotor or a tissue specific promotor, thereby enabling the triggering of gene mutations in various cell types from different developmental stages of *C. elegans*. Shen *et al.*[96] successfully utilized this efficient and robust method to study the function of an essential embryonic gene (Coronin) in *C. elegans*, and the technique provides the basis for developing a conditional knockout platform for future genomic studies of parasitic helminths.

**3.2.5 CRISPR/Cas9 Can be Utilized in *Biomphalaria* for Population Replacement**

With the World Health Organization's goal of eliminating schistosomiasis as a global health problem by 2025, there has been renewed interest in improved snail control. The recent reporting of the complete genomic sequence of the *S. mansoni*-transmitting freshwater snail, *Biomphalaria glabrata*[99] provided critical insight on snail biology in terms of stress responses, phero-perception, immune function and regulation of gene expression; it also identified a wealth of potential targets not only for developing novel snail control measure but also timely and valuable information that could lead to the sustainable genetic intervention of schistosomiasis transmission.[99]

**3.2.5.1 CRISPR/Cas9-based Gene Drive: A New Approach for Combatting Schistosomiasis**

“Gene drive” is an emerging technology that can be used to potentially spread particular genomic alterations through targeted wild populations over many generations. CRISPR/Cas9-based gene drive has considerable advantages over other gene drive systems, such as engineering zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENS), which have been shown to achieve only limited efficiency and are much more labor-intensive.[100] Therefore, the potential of using CRISPR/Cas9-mediated gene drive to spread anti-*Schistosoma* effector genes into the genomes of natural snail host strains for field control of schistosomiasis, although in its infancy, has generated considerable recent interest.[101] Significant advances have been achieved in the development of CRISPR/Cas9 gene drives in *Anopheles* aimed at population replacement and suppression which might, in future, render mosquitoes unable to transmit *Plasmodium* spp., thus preventing the spread of malaria.[102-104] However, whereas only females can transmit malaria,[103] *Biomphalaria* and *Bulinus* (the intermediate host of *S. haematobium*) are hermaphrodites, capable of self- and cross-fertilization.[105] This reproductive plasticity represents a major challenge in applying gene drive inheritance for the population reductionof snails. Thus, the current focus is to explore the importance of particular snail genes that are implicated in resistance to schistosome infection, resulting in the breeding and release of transgenic schistosome-resistant vectors to limit schistosomiasis spread.[101]

The immune system of molluscs comprises immune cells (hemocytes) and humoral immune factors of the hemolymph, and these are the major physiological determinants of pathogen resistance.[106] *B*. *glabrata* genes including *Sod1* and *RADres* (a restricted-site associated DNA-determined resistance locus) and Guadeloupe Resistance Complex (GRC, < 1 Mb gene region) can affect the susceptibility of snails to *S. mansoni*.[107-109] Mutagenesis by CRISPR/Cas9-based methods may provide an approach to precisely investigating the function of these genes, a development that would lead to more in-depth understanding of this complicated snail-pathogen defense system.

In a recent advance, Coelho *et al.*[110] applied CRISPR/Cas9 into the *B*. *glabrata* embryonic (*Bge*) cell line, targeting the gene encoding the allograft inflammatory factor of *B. glabrata* (*Bg*AIF). A plasmid encoding Cas9 and sgRNA was delivered into *Bge* cells by electroporation. This resulted in the adherence between the gene mutated *Bge* cells and *S. mansoni* sporocysts being significantly reduced,[110] illustrating the value of using CRISPR/Cas9 technology to study *B*. *glabrata*-*S. mansoni* interactions and snail-parasite compatibility and the possibility of conducting CRISPR/Cas9 mutagenesis in schistosome snail vectors.

**3.2.5.2 The Challenges in Developing CRISPR/Cas9-basedGene Drive for *Biomphalaria***

A major barrier to applying gene drive for schistosome snail vector control is that there are many kinds of snail species that can harbor schistosomes and snail-schistosome interactions are highly variable.[111, 112] Thus, developing a single transgenic target effective for all snail-schistosome interactions based on a single snail species presents a challenge. However, this could be achieved with genes having wide-spectrum schistosomal-resistance activities, and which are conserved across various snail-schistosome combinations. On the other hand, conducting gene manipulation experiments on snails may affect their fitness, leading to the biased killing of transgenic individuals. Releasing gene-edited snails into areas with fewer predators or competitors might improve the survival rate of transgenic snails. A major public health concern about open field release of transgenic schistosome-resistant snails is that gene drive failure may occur with potentially adverse effects on human health.[113] It is possible that gene-edited vectors might evolve to transmit more virulent and pathogenic strains. Moreover, the spread of gene drive schistosome-resistant snails might generate additional concerns if they spread beyond the target areas or into areas where the gene drive concept is not acceptable.[113] This highlights the importance of restricting transgenic snails to limited areas to decrease the risk of these potential issues arising. Nonetheless, employing CRISPR/Cas9-mediated gene drive to efficiently spread schistosome resistance traits in snail populations to reduce disease transmission in the long term is worth exploring.[114]

1. **Conclusions and Outlook**

CRISPR/Cas9 has been recently applied to several helminth parasite species raising the possibility that this powerful genetic tool can be used for in-depth biological studies on these worms and to explore new effective strategies for the control and prevention of diseases caused by these and other parasites. A CRISPR/Cas9 work pipeline has been established in *S. stercoralis* and it is plausible that homozygous deletions in F1 iL3 were obtained and that CRISPR/Cas9 mutagenesis is heritable in this parasite. Indeed, CRISPR/Cas9-based methods developed in nematodes can pave the way for improving programmed genome manipulation in trematodes and other helminths. Moreover, the newly developed liposome-based transfection/microinjection delivery method in free-living *Auanema* broadens the potential application of CRISPR/Cas9 for future studies on helminth parasites.

The efficiency of CRISPR/Cas9 mutagenesis in trematodes obtained thus far is modest but appears to result in substantial phenotype change, the reasons for which are not entirely clear and require further study. Importantly, identification of a robust marker for recovering gene mutated worms will be important for future CRISPR/Cas9 studies in trematodes. It is noteworthy that HDR was predominant in CRISPR/Cas9 edited *S. mansoni* eggs and this finding may provide a unique opportunity in future to generate loss-of-function insertions and to introduce drug selection genes precisely into the schistosome genome. Employing CRISPR/Cas9-mediated gene drive to spread anti-schistosome resistance genes into the genome of wild-type strains of snail intermediate hosts for field control of schistosomiasis is also worthy of future investigation and can serve as a template for the control of other important vector-borne medical and veterinary parasitic helminths.

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**Conflict of interest**

The authors declare no conflict of interest.

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Figure Legend

**Figure 1**. Life cycle stages used in CRISPR/Cas9-mediated genome editing in *S. stercoralis*, *O. viverrini*, and *S. mansoni*. (A) Life cycle of *S. stercoralis*. Genomic editing using CRISPR/Cas9 technologies has targeted the free-living adult worm. (B) Life cycle of *O. viverrini*. CRISPR/Cas9-mediated gene editing has been undertaken in metacercariae (MC), newly excysted-juvenile flukes (NEJ) and adult worms. (C) Life cycle of *S. mansoni*. CRISPR/Cas9-mediated gene editing has been conducted using eggs, sporocysts and adult worms.