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Application of CRISPR-Cas9 Technology in Visceral Leishmaniasis Research: Current Understanding and Future Perspective

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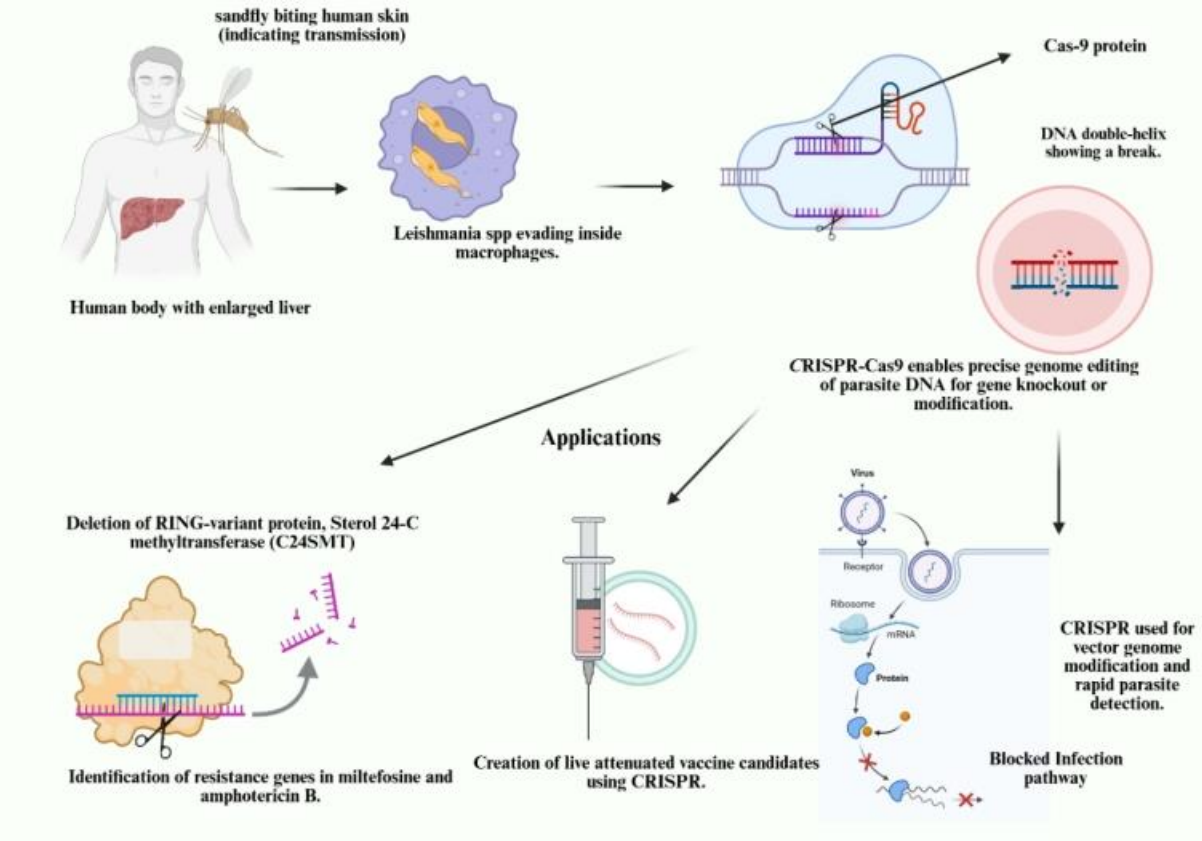
Abstract

Visceral leishmaniasis (VL) is a severe disease that affects millions of people in the endemic areas due to *Leishmania donovani* parasites. There are no licensed vaccines, and chemotherapy is the mainstay to combat the disease. The clinical value of commonly used chemotherapeutic agents for the treatment of VL is now threatened due to the emergence of acquired drug resistance and toxicity. The CRISPR-Cas9 genome editing system have significantly advanced *Leishmania* research in terms of drug target validation and vaccine development. The CRISPR-Cas9 system is an adaptive immune system in bacteria, and it uses Cas9, an endonuclease guided by single-guide RNA (sgRNA) to introduce double-strand breaks at specific genomic loci. It is therefore more efficient for gene knockout, knock-in or base editing via HDR pathways, which makes it a better tool of gene editing than other currently available tools such as ZFNs and TALENs because of its flexibility, simplicity and scalability. To date, in *Leishmania*, CRISPR-Cas9 has helped in dissecting the drug resistance mechanisms, for example, RING-variant and transmembrane proteins in miltefosine resistance and the sterol 24 C methyltransferase (C24SMT) in amphotericin B resistance. It has also exposed new genes involving parasitic qualities that help it survive, multiply, and be virulent, and helped create new shapes of weakened strains for use in vaccines. Therefore, CRISPR-Cas9 proves to be an indispensable weapon in the assault on *Leishmania* biology and the development of VL treatment.

Key Words: Visceral Leishmaniasis, CRISPR-Cas9, *Leishmania donovani*, Gene Editing, Drug Resistance

Graphical Abstract



1. Introduction

Visceral Leishmaniasis is an acute and chronic parasitic disease caused by two kinds of *Leishmania* protozoa, *Leishmania donovani* and *Leishmania infantum*. These species are geographically distinct: L. donovani is found in East Africa and the Indian subcontinent and is transmitted between sand fly vectors to humans, while *L. infantum* is found in the Mediterranean, parts of Europe, North Africa, and the Americas, and is transmitted from dogs to man as a zoonosis [1]. The parasites multiply intracellularly in macrophages in the important organs, including the liver, spleen, and bone marrow. The dynamics between the parasite determine clinical manifestations of VL and host immune system. Th1 activity is necessary to stimulate macrophages to kill the parasite, and, at the same time, an overproduction of Th2 cytokines has been linked to increased parasite load in the animal host. Disease susceptibility and severity are strongly determined by the genetics of the host and interference of the parasite, as well as the effect of the sand fly saliva. PKDL refers to the cutaneous skin lesions after successful treatment of VL that contain parasites and sustain transmission [2].

CRISPR-Cas9 has been an emerging genetic tool in the biological sciences during the past few years due to its versatility, precision, and reduced cost. CRISPR-Cas9 employs Cas9 nuclease and synthetic single guide RNA (sgRNA) to cleave target double-stranded (ds) DNA. These are corrected by different cellular processes, such as homology-directed repair HDR to allow for gene editing. Compared to the other similar platforms such as ZFNs and TALENs, CRISPR-cas9 technology is much more flexible, requires less

design, can target more than one locus at once and its enhancements are regularly being made to make it more specific and safe [3].

Leishmania is one area where CRISPR-Cas9 has been used; one of them is investigating the mechanisms of drug resistance. For instance, screens adopting CRISPR discovered the role played by RING-variant proteins and transmembrane proteins within miltefosine, an antileishmanial drug of first choice. These proteins affect the membranes' contents and transporters, making it difficult for the parasite to withstand drug pressure [4] [5]. Likewise, genes coding Sterol 24 C methyltransferase (C24SMT), which contribute to ergosterol synthesis in the parasite membrane, have been implicated in resistance to the action of amphotericin B when ambiguous or excised. They change the sterol patterns, thus having less medicament available to bind to and be effective [6]. Also, CRISPR functional genomics showed novel hypothetical genes involved in drug resistance that were never considered important before, and they also outlined the flexibility of the parasite's genome and ability to adapt [7][8].

Apart from the drug resistance mechanism, the ergosome functions of the CRISPR-Cas9 strategy have been used to knock out genes that encode for multicopy virulence factors. For instance, these works give us hypotheses on the pathogenesis and have suggested potential gaps for developing recombinant vaccine strains [9]. Furthermore, CRISPR has been applied for exploring crucial metabolic genes for P. falciparum, including calmodulin and serum palmitoyltransferase subunits, for targeting drugs because these are the genes that are essential for its survival [6].

In conclusion, the disease caused by *Leishmania* parasites has various factors, such as parasite species, host immunological reactions, and the environment. The CRISPR-Cas9 system has become a valuable tool in studying the *Leishmania* parasite due to the capacity of the method to modify the genome precisely, thus contributing to the understanding of various aspects of the parasite, such as drug resistance and pathogenicity. Such progress opens up new horizons in treating those diseases and enhancing disease control methods in the endemic zones globally [10]. In this review, the current advancement in the application of CRISPR-Cas9 technology in *Leishmania* research has been discussed and also highlighted its future perspective.

2. CRISPR-Cas9 System: Basics and Mechanisms

2.1 Mechanism of CRISPR-Cas9

Processes of bacteria and archaea involve combining CRISPR arrays with Cas proteins to cut foreign genetic elements. In 2012, Doudna and Charpentier [11] revealed that the Cas9 nuclease could use synthetic single-guide RNA (sgRNA) to make double-stranded breaks at desired DNA sequences. The accurate genome-editing capability of this technology has enabled efficient and cost–effective modification of genetic sequences in various organisms, helping scientists to study disease mechanisms and gene functions, though direct therapeutic use against *Leishmania* is under research.

Visceral leishmaniasis represents a complex parasitic disease because specific *Leishmania* species need sand flies for transmission, while the immune interactions of hosts determine disease outcomes. Environmental factors and geographic location shape the way this disease spreads [12]. The molecular

tool CRISPR-Cas9 enables promising research to understand parasites better and create new intervention methods.

The DNA editing system CRISPR-Cas9 provides superiority to traditional gene-editing approaches using zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). CRISPR-Cas9 depends on simple RNA guides (gRNA) to target DNA sequences, thus eliminating the requirement for complex protein engineering in ZFNs and TALENs while providing fast operation, flexibility and reduced cost [13]. CRISPR allows users to edit multiple genomic sites simultaneously by implementing multiple gRNA guides, which existing methods lack. Researchers have developed optimisation techniques for CRISPR-Cas9 to minimise unintended DNA targeting and enhance its accuracy and safety [14]. CRISPR-Cas9 leads to safe editing because it does not insert foreign DNA permanently into the genome, avoiding insertional mutagenesis risks. CRISPR-Cas9 operates autonomously without auxiliary nucleases, thus making the editing process simple. The large gRNA libraries enable fast design work that extends their application throughout diverse organisms and cell types [15]. The mechanism of action of CRISPR-Cas9 has been shown in Figure 1.

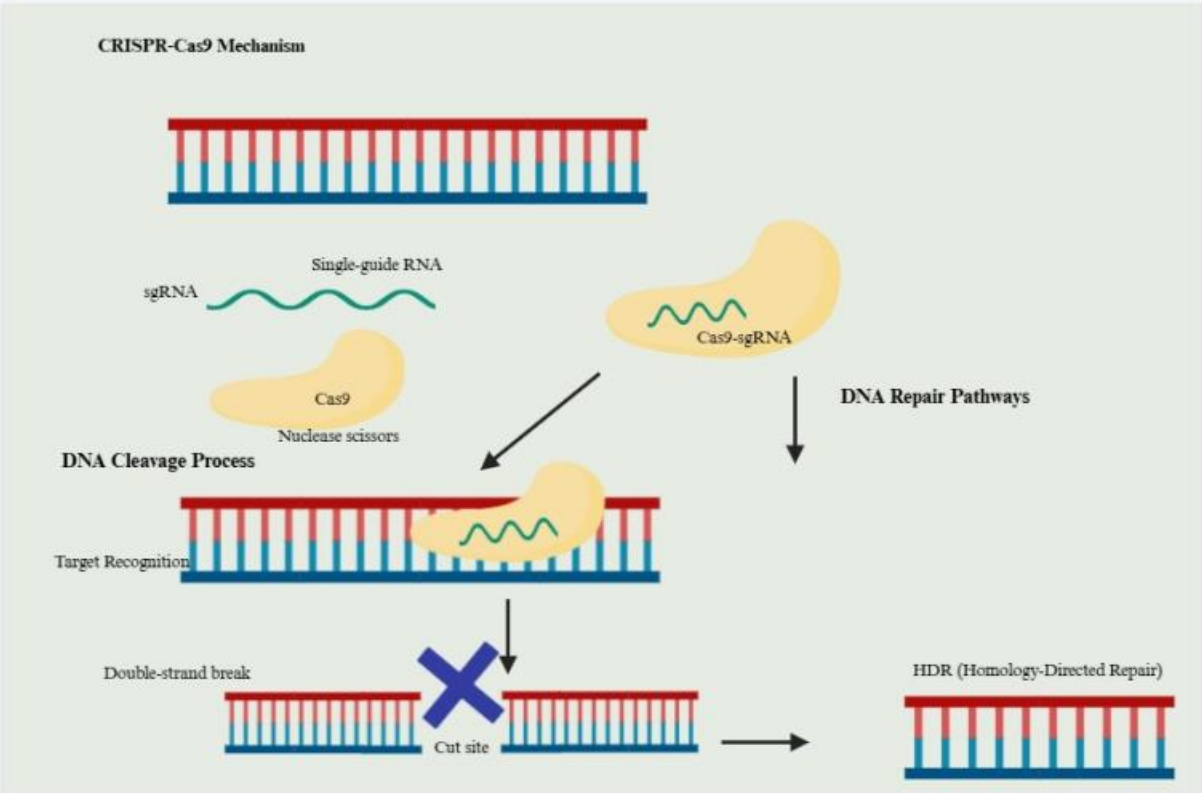


Figure 1: Mechanism of action of CRISPR-Cas9, shows Cas9 nuclease is been guided by single guided RNA (sgRNA) to the targeted DNA sequence, where it introduces a site-specific break. The break is further repaired by cell through insertions or deletions or homology-directed repair (HDR)

2.2 Comparative Positioning of CRISPR (CRISPR Vs ZFN/TALEN, newer Cas systems)

Genome-editing technologies have developed at a fast pace over the last decade, and three main platforms, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and

CRISPR-associated (CRISPR-Cas) systems, have established specific standards of targeted genome control. Both of them mark the next step in the technological development of the programmable endonuclease, with CRISPR-Cas assuming the centre stage due to its unprecedented versatility and massive dissemination [13].

2.2.1 ZFNs and TALENs: Pioneering but Limited

One of the earliest programmable nucleases was ZFNs, which took advantage of customizable DNA-binding zinc finger motifs attached to a FokI endonuclease domain. ZFNs provide high specificity of gene editing, though are challenging and expensive to design because of the sequence-context selectivity of zinc finger domains. TALENs, however, are based on arrays of DNA-binding repeats which are based on Xanthomonas TALE proteins, with increased modularity and specificity, particularly in repetitive or mitochondrial DNA targets [16][17]. Both systems involve intensive protein engineering of every new target and are both expensive and difficult to scale. The difficulties are listed below:

a. Specificity On both platforms, high specificity is by custom protein-DNA recognition, but off-target activity of TALENs is usually lower than that of ZFNs [18].

b. Efficiency and Scalability ZFNs and TALENs are not as user-friendly and scalable as they require complex protein construction in case of every new target [19].

c. Multiplexing Multiplex genome editing is terribly scarce in ZFNs and TALENs as each site needs a specific pair of engineered proteins [19].

d. Clinical Applications TALENs are, in particular, still clinically useful in ultra-high-fidelity editing where off-target effects are to be avoided at all costs [16].

e. Delivery Both systems have problems with delivery of larger constructs, particularly in the case of large constructs, and are commonly introduced by viral vectors or electroporation [16].

Genome engineering has been transformed by the introduction of the CRISPR-Cas system, and especially by CRISPR-Cas9. The RNA-guided properties of Cas9 allow easy retargeting of the nuclease to new loci, whereby just the short guide RNA is changed which eliminates the requirement of custom protein design. This not only provides low-cost and rapid design, but also allows multiplex editing and increased applicability to other types of cells and organisms [20] [21].

The advantages are listed below

a. **Specificity** CRISPR-Cas9 is an effective method, but specification must be considered in terms of guide RNA fidelity and PAM-site limits; low off-target activity has been observed, yet recent advances (e.g. high-fidelity Cas9, enhanced computational design) provide better specificity than previous generations [21].

b. **Efficiency & Scalability** CRISPR-Cas can be scaled and is compatible with high-throughput editing to enable large-scale or genome-wide screens [20].

c. **Multiplexing** Due to the simplicity of guide RNA synthesis, CRISPR-Cas9 can be used to perform multiple loci of CRISPR-mediated editing, or multiplexing, simultaneously, which is not possible with ZFNs/TALENs [20].
d. **Delivery & Cost** CRISPR uses a smaller set of RNA instructions (compared to protein-based editors) and can be delivered through multiple different delivery strategies, such as ribonucleoprotein complexes, viral, and non-viral vectors. CRISPR reagents are far less expensive than ZFNs/TALENs [20].
e. **Clinical and Research Applications** CRISPR is dominating in gene therapy and functional genomics but ZFNs and TALENs are needed in special situations, such as mitochondria or repetitive DNA regions [20]. Table 1 provides an overview of the major genome editing techniques used in *Leishmania* research

Table 1: Comparative Analysis between ZFNs, TALENs and CRISPR-Cas9

Characteristics	ZFNs	TALENs	CRISPR-Cas9
Design Complexity	Complex requiring protein engineering for each target [16]	Moderate [17]	Low, since only guide RNA can redesign [13]
Editing efficiency	Moderate [18]	High editing performance [17]	Very high across kinetoplastids [22][24]
Multiplexing Ability	Difficult; each target requires a new protein pair [19]	Limited multiplexing potential [19]	Easy via multiple gRNAs [20]
Specificity	Usually high, but it is dependent on protein-DNA context [18]	High target specificity [17]	Adjustable using high-fidelity Cas9 variants
Delivery options	Delivered by viral vectors and electroporation [16]	Delivered by electroporation or by plasmids [17]	Delivered by RNPs, plasmids, viral and non-viral vectors [35]
Application	Minimal [58]	Mainly conceptual not widely implemented [33]	Extensive due to gene knockouts, multigene family editing, vaccine strain development [32]

3 Genetic manipulation of *Leishmania* species: Targeting genes for attenuation or drug resistance

3.1 Background and Implementation

The implementation of CRISPR-Cas9 in *Leishmania* parasite research took place during 2015, which achieved superior gene targeting through enhanced efficiency compared to conventional homologous recombination approaches. The Cas9 endonuclease receives constitutive promoter activation through DHFR-TS, while single guide RNAS (sgRNAs) use U6snrna RNA polymerase III promoters to generate expression.

The targeted knockout of genes becomes possible because CRISPR-Cas9 makes double-stranded breaks in target areas, and the parasite repairs those breaks through mechanisms, including microhomology-mediated end joining or homologous recombination. CRISPR-Cas9 performs better in disrupting solitary genes, multiple gene duplicates, and genes situated across multiploid chromosomes beyond previous technology capabilities [22]. The research application of CRISPR-Cas9 for *Leishmania* investigations includes genetic attenuation through inactivation of virulence-sustaining and survival-related genes. A single transfection round allowed scientists to remove the paraflagellar rod-2 locus, which consists of essential tandemly repeated genes required for parasite motility. The knockout of genes through this method enables scientists to discover vital genes and pathways that represent potential treatment and vaccine development targets [23].

3.2 Genes involved in drug resistance

Laboratories extensively employ CRISPR-Cas9 technology to manipulate genes responsible for resisting frontline antileishmanial drugs. The miltefosine transporter gene (LdMT) becomes resistant to miltefosine when its structure or function becomes mutated, and alterations of sterol 24 C methyltransferase DNA sequences transform membranes to become resistant to amphotericin B medication. The drug-resistance genome screening approach using CRISPR screens discovered new resistance genes like transmembrane proteins and RING-variant proteins, expanding available genomic targets [24][7].

3.2.1 RING-Variant Protein and Transmembrane Protein Genes

During COVID-19 pandemic, researchers applied CRISPR-Cas9 screen technology to *Leishmania infantum* and found sgRNAs targeting two gene, including the RING-variant protein and the transmembrane protein, which became enriched under miltefosine selection. The studied gene modifications demonstrated higher drug resistance properties, thus confirming their role in miltefosine susceptibility mechanisms outside the classical transporter gene [23]. Recent genetic and molecular studies of miltefosine resistance in *Leishmania infantum* have extended the research conducted with CRISPR-Cas9 screens that pointed to RING-variant protein and transmembrane protein genes as elements in resistance development. The research of Saboia-Vahia et al., 2022 [4] utilised quantitative proteomics to identify how *L. infantum* cells, which developed resistance to miltefosine, showed substantial changes in membrane components alongside elevated levels of ABC transporters and phospholipid transport ATPases that are linked with drug resistance and membrane function [4]. Based on this research, membrane-

associated proteins and transmembrane proteins play a pivotal role in developing the miltefosine resistance phenotype.

Using genomic and phenotypic analyses, Hendrickx et al., 2014 [10] studied experimental miltefosine resistance development in intracellular amastigotes of *L. infantum* to recognise gene function changes and mutant phenotypes connected with resistance. This research demonstrated that miltefosine resistance extends beyond transporter genes to include altering genome functions involved in membrane maintenance, stress response functions that could involve RING-variant protein genes, and transmembrane protein genes. A comprehensive review by [5] et al., 2022 examined *Leishmania* drug resistance mechanisms by explaining how they consist of multiple factors that modify membrane structure and control transporter functions and protein regulation activities. RING-variant proteins and membrane proteins have been identified as key drug susceptibility components that help *Leishmania* adapt to medication changes.

These findings reveal that miltefosine resistance in *Leishmania* consists of intricate changes across genetic materials and proteomic information. Recent CRISPR-Cas9 screening techniques validate the central function of transmembrane and RING-variant proteins throughout resistance development.

3.2.2 Sterol 24 C Methyltransferase Genes

The ergosterol biosynthesis pathway in *Leishmania* species depends on Sterol 24 C methyltransferase (C24SMT) genes for proper functioning, and their deletion generates resistance against amphotericin B [25]. Several studies throughout the previous five years have revealed comprehensive molecular evidence concerning this resistance development.

Modifying the C24SMT genes in *Leishmania* through CRISPR-Cas9 leads to significant alterations of parasite sterol composition. The leishmanicidal mechanism of amphotericin B depends on the sterol substances resembling ergosterol, which exist in parasite cell membranes [6]. When C24SMT dysfunction occurs, the sterol biosynthetic pathway experiences modifications that cause ergosterol destruction and alternative sterol intermediates to build up. The parasite membrane becomes less receptive to amphotericin B, thus granting resistance [6]. The discovery of sterol profiles through whole-genome sequencing confirms that drug-resistant strains of *L. mexicana* and *L. infantum* demonstrate alternative sterol intermediates instead of wild-type ergosta-5,7,24-trienol molecules. The resistance-developing changes in the sterols stemmed from mutations together with structural changes found at the C24SMT gene locus, which in some cases also required mutations in the sterol C5 desaturase gene [6].

Additional research through molecular dynamics simulations and docking studies thoroughly explained how resistance occurs at the structural level. The SMT enzyme binding affinity for amphotericin B becomes weaker through mutations like V131I, V321I and F72C because these modifications cause higher binding energies and reduced molecular interactions compared to wild-type proteins. The study shows that gene knockout and point mutations act as resistance-driving agents through sterol profile modification of parasite membranes that stem from changes in enzyme shape [26].

3.2.3 Hypothetical Genes Identified in Screens

Research teams using CRISPR-Cas9 functional genomic methods have found hypothetical *Leishmania* genes that become enriched when exposed to drug pressure in situations where these genes had no previously identified purpose [27]. These recent discoveries expanded the exploration of new drug resistance mechanisms in this parasite. Antileishmanial drugs such as pentavalent antimonials, miltefosine, or amphotericin B produce resistant *Leishmania* parasites with increased activity of hypothetical genes with unknown or unpredicted functions. Lab experiments that eliminate hypothetical genes directly demonstrate that their absence provides survival advantages against drug pressure, thus validating their link to drug resistance phenotypes. The latest reviews and systematic studies confirm that *Leishmania* achieves drug resistance by using classical mechanisms alongside adaptive plasticity in its genome. This enables rapid development of new resistance factors, including hypothetical genes [7].

Hypothetical genes likely help drug resistance occur through cellular modifications of drug uptake or efflux, stress response, or metabolic adaptation. However, institutional research remains essential to determine their specific molecular activities. These resistance factor genes are important because they create new possibilities for medical research to develop therapeutic targets and biomarker applications in drug resistance monitoring at clinical sites. Such genes demonstrate the sophisticated nature of resistance mechanisms in *Leishmania*, requiring additional genome-wide explorations to discover all drug resistance genetic components [8]. Table 2 depicts an overview of the application CRISPR-Cas9 in *Leishmania* Research i.e Research in targeting genes for attenuation or drug resistance.

3.3 Virulence and Survival Genes

Leishmania relies on multicopy gene families such as the A2 virulence cluster to sustain infection and survive in host cells. Researchers applied CRISPR-Cas9 to delete the entire A2 gene family, which showed the A2 family's essential function for parasite infectivity and host survival. The proof of concept to target multiple gene copies in *Leishmania* pathogenesis serves two purposes: understanding pathogenic processes and developing less virulent parasite strains for vaccine development. Several research groups applied the same methods to knock out LPG2 virulence factor genes and other genes, including those within the A2 cluster. Past attempts at knocking out LPG2 failed due to gene duplications until CRISPR-based approaches successfully removed the genes [9].

CRISPR gene editing in *Leishmania* research usually entails the stable expression of Cas9 and guide RNA (gRNA) in the parasite via *Leismania*-specific plasmid vectors, including those with a *Leismania* ribosomal RNA promoter to transcriptionally regulate the gene in the cell. The system has a stable expression which enables easy production of the gene knockouts, such as multiple-copy virulence genes, A2, LPG2, and GP63 [28]. It is done by causing the break of two strands of DNA at loci of interest, and then repairing the DNA with homology-directed repair (HDR) or microhomology-mediated end joining (MMEJ) [29]. To increase specificity of targeting and simplify the process of selecting mutant parasites, repair templates with antibiotic resistance markers are frequently inserted at the locations of cleavages allowing the targeting cells to be selected using the markers. Such plasmid vectors are designed such that they express all the required components: Cas9, gRNA expression cassettes and selection markers in one construct, simplifying the process of transfection and selection [30]. Cases of successful application of

such all-in-one CRISPR plasmid systems include insertion of genes, knock-out of target sequences or knock-out of virulence factor genes in Leishmania to functional ends. Thus, introduction of the CRISPR constructs is done through transfection of Leishmania promastigotes or amastigotes with the plasmids and thereafter the antibiotic selection and molecular screening of the mutations in the desired genes is done. This method is used in different studies which break down virulence and immune evasion genes such as those which affect macrophage infection and cytokine modulation [31].

3.4 Essential Genes and Metabolic Pathways

Researchers apply CRISPR-Cas9 technology to knockout genes, which helps discover essential metabolic and cellular mechanisms. The calmodulin gene family (LmxM.09.0910, LmxM.09.0920, LmxM.09.0930) serves as an illustration.09.0910, LmxM.09.0920, LmxM.09.The targeted essential genes LmxM.09.0910, LmxM.09.0920, LmxM.09.0930 at 0930 hours solely required a single guide RNA to cause parasite death, demonstrating the calmodulin requirement for survival and cell movement. Similarly, the catalytic subunit of serine palmitoyltransferase (LCB2), critical for sphingolipid biosynthesis, was found to be essential in L. mexicana; Through CRISPR researchers gain access to essential tools for studying cellular metabolism alongside new understandings of possible drug targets. Genomic instability *in Leishmania* provides the organism with considerable adaptability when facing environmental changes and medicine applications [6].

3.5 Genome Plasticity and Chromosomal Rearrangements

Through the CRISPR-Cas9 system, scientists can recreate chromosomal translocations and deletions to observe evolutionary changes in gene expression and phenotypic outcomes. Experimental chromosomal translocation studies have examined polycistronic transcript controls and extensive genomic adaptation mechanisms. Scientists used engineered chromosomal translocations as investigative tools to study *Leishmania's* management of multiple gene clusters with its capacity for extensive genetic amendments. Scientists recognize research about *Leishmania* adaptation and stress-evolutionary processes as fundamental for genetic investigation [6].

3.6 Technical Advancements (Base Editing, Prime Editing, CRISPRi/a, delivery systems)

Recent developments of CRISPR platforms have opened a vast range of new genetic tools to study *Leishmania*, beyond simple knockout methods, to more specific and flexible alterations. Base editing technologies, including cytosine base editors, allow the efficient insertion of a stop codon or single-nucleotide mutation, with no formation of a double-strand break, enabling the efficient and rapid generation of loss-of-function mutations in multi-copy gene families [32]. Prime editing can also go beyond this specificity, enabling an extensive repertoire of targeted sequence alterations, such as insertions, deletions, and targeted conversion of individual bases, without the need of donor DNA templates or induction of genome-wide instability. Along with DNA modifications, CRISPR interference (CRISPRi) and activation (CRISPRa) systems also use catalytically dead Cas9 (dCas9) to block or activate the expression of genes in a reversible manner, allowing essential functions of genes and phenotype correlations to be dissected without permanently modifying the parasite genome [33]. The optimization of efficient delivery of these tools into *Leishmania* cells by episomal expression, ribosomal

and T7 RNA polymerase promoters and better constructs to allow stable integration of the tools at safe genomic locations has overcome former obstacles to high transfection efficiency and species-specific expression issues. This combination of technical advances is facilitating advanced functional genomic screens, high-throughput drug target screening and eventually hastening the application of genome editing to new therapeutics and diagnostics in *Leishmania* [34].

Table 2: Application of *Leishmania* Research in targeting genes for attenuation or drug resistance

Serial No	Application area	Key points
1.	Genetic Manipulatiom	Plays a major role in gene knockout using CRISPR-Cas9 [57].
2.	RING and Transmembrane Proteins	It is linked to miltefosine resistance, alters membrane functions [4]
3.	Sterol 24C Methyltransferase (C24SMT) Genes	Its deletion leads to amphotericin B resistance, hence affects ergosterol biosynthesis [6]
4.	Hypothetical Genes	Newly identified genes contribute to drug resistance [7][8].
5.	Drug Resistance Targets	Includes classical (LdMT) and novel genes, extends the resistance gene knowledge [24].
6.	Virulence and Survival genes (A2, LPG2)	Knockouts reduces infectivity [9].
7.	Essential Metabolic Genes (e.g: Calmodulin)	CRISPR reveals vital survival pathway, useful for identifying druggable targets [6].
8.	Genome Plasticity	CRISPR helps model chromosomal changes, shows *Leishmania*'s adaptive genome [6].
9.	CRISPRi/a Systems	Allows reversible control of genes without DNA breaks [24].

4 Functional Genomics and Essential Gene Mapping in *Leishmania*

4.1 Functional Genomics and Essential Mapping of key genes

Functional genomics and critical mapping of key genes in *Leishmania* studies have been transformed by CRISPR-Cas9 and base-editing techniques to allow precise identification of the important genes that are essential in the survival of the parasite, including kinases, phosphatases, proteases, and metabolic enzymes (Zhang & Matlashewski, 2024)

Extending these functional insights, bioinformatic approaches have complemented experimental tools to refine essential gene identification and regulatory mapping as discussed below:

4.1.1 Bioinformatic Essential Gene Mapping and Genomic Regulation

CRISPR-Cas9 genome editing has enabled an easy and efficient method of gene knockout and base-editing screens in several kinetoplastids, including different species of *Leishmania.* These technologies have made possible the interrogation of the entire kinome and gene families, where key genes necessary in maintaining the viability, growth as well as drug resistance pathways of the parasites are identified [35].

4.1.2 Identification of Essential Genes around the whole genome

Genome-wide CRISPR screens of *Leishmania*, particularly of *L. mexicana* and *L. infantum* have shown that a number of genes are essential, including protein kinases (e.g., AGC essential kinase 1 and CDPK4) and core metabolic genes needed by the parasite to grow and differentiate. The survival of promastigotes and amastigotes has been confirmed to depend on the presence of calmodulin and sphingolipid biosynthesis enzymes, the central kinase, KKT2 and CDK related kinase, CRK9. The deep mapping of these elements is essential in prioritization of drug targets [36].

4.1.3 Conditional Essentiality in the Lifecycle Stages

In recent years, the essentiality of the genes has been observed to be conditional, depending on the life stages (promastigote vs. amastigote), the cell cycle phases, and in various conditions of environmental stress. For example, transporter genes and protein kinases were discovered using high-throughput CRISPR screens, which are only required during the mammalian amastigote phase. This observation guides the choice of therapy, where the essential genes at each stage are ideal targets of a drug or vaccine [36].

4.1.4 Mapping of gene Networks: Cell cycle, Stress Response and Virulence

Systematic mapping of cell cycle, signal transmission, stress response, and virulence-determinant gene networks has been enabled by functional genomic approaches. To illustrate, kinase-wide CRISPR knockout and tagging revealed kinase functions in differentiation and host adaptation. Also, the mechanisms behind parasite persistence and pathogenicity have been elucidated through the use of CRISPR-based network mapping of the oxidative stress response network and virulence factors (e.g., gene cluster, LPG2 [37].Table 3 summarizes essenbtial genes and their biological functions identified using CRISPR

Table 3: Essential genes Identified in *Leishmania* via CRISPR Functional Genomics

Gene/Protein	**Function**	***Leishmania* Species responsible**	**Observed Phenotype**	**Reference**
Calmodulin (LmxM.09.0910)	Ca signalling and motility	*L. mexicana*	Lethal, loss of motility	[6]

Serine Palmitoyltransferase (LCB2)	Sphingolipid biosynthesis	*L. mexicana*	Growth defect; non-viable	[6]
AGC Kinase 1`	Cell cycle control	*L.infactum*	Impaired cell division	[36]
CRK9 (CDK-like kinase)	mRNA processing	*L. donovani*	Reduced viability	[35]
KKT2 (Kinetochore kinase	Chromosome segregation	*L.mexicana*	Cell cycle arrest	[36]

5 CRISPR-Cas9 in Vector Control: Targeting Sandflies

Through CRISPR-Cas9 technology, scientists can control *Leishmania* transmission through vector modification, engineered vector immunity, and transmission reduction research. Recent research and review literature from the past five years form this detailed overview of the methods.

5.1 Genetic Modification of Sandfly Populations

Researchers explore using CRISPR-Cas9 technology to control how sandflies transmit *Leishmania* as their natural vector. A detailed procedure for sandfly embryo microinjection emerged from Martín-Martin et al. 2018 [37] as a vital process to introduce CRISPR-Cas9 components for accomplishing gene editing in non-model insects. The core aspects of the CRISPR-Cas9 transmission involve targeting specific genes, generating sgRNAs, and producing Cas9 protein before microinjection of the Cas9-sgRNA mix into sandfly embryos. Developing genetic manipulation methods for sandflies depends on solving issues with embryo survival and microinjection techniques. Kumari et al., 2025 [38] achieved the first CRISPR-Cas9 mutagenesis success with *Phlebotomus papatasi* and *Lutzomyia longipalpis*. Research teams proved the feasibility of CRISPR editing for sandflies by producing *Leishmania* major infection response mutants. The technique proved successful in producing many genetic mutations among surviving individuals, even though few bugs survived, thus demonstrating potential in vector control research.

5.2 Scientific researchers have developed Methods to Engineer Sandflies with Resistance Against Leishmania Infection

The CRISPR-Cas9 targeting of sandfly immune pathways provides an approach to limit their ability to spread *Leishmania*. Using CRISPR on *P. papatasi* sandflies, scientists manipulated the immune deficiency (IMD) pathway transcription factor Relish, which caused infection-susceptible mutants [39]. Research showed that the immune systems of sandflies play a substantial role in how competent vectors become. The engineering of sandfly genes that control immune processes or influence parasite growth in vectors can create vectors that show reduced receptivity to *Leishmania* infection. Genetically modified vectors engineered to stop parasite life cycle progression would enable lower transmission rates between humans and carriers [38].

5.3 The development of CRISPR-based vector control strategies shows promise in diminishing transmission spread

Research shows that CRISPR-Cas9 gene drives can potentially transmit engineered features throughout natural sandfly populations. Gene-driven intervention directs inheritance patterns toward the spread of essential alleles, demonstrating resistance to *Leishmania* and reproductive sterility traits to decrease vector numbers or change their transmission efficiency [40]. Nateghi Rostami , 2020 [41] studied the applications of CRISPR/Cas9 gene drive technologies toward blocking vector parasite development while obstructing vector fertility. Research on gene drives mainly concentrates on mosquitoes, but scientists can apply these principles to sandflies despite their obstacles.

6 Challenges in CRISPR-Cas 9 Application for Visceral Leishmaniasis

6.1 Delivery methods of CRISPR-Cas9 in Leishmania and Sandflies

Solving the problem of CRISPR-Cas9 delivery to *Leishmania* parasites and sandfly vectors represents a crucial yet unresolved scientific hurdle. Scientists conduct delivery of CRISPR-Cas9 components into *Leishmania* parasites and sandfly vectors by electroporation or nucleofection of plasmids carrying Cas9 and guide RNAs (gRNAs) or ribonucleoprotein (RNP) complexes. Zhang et al., 2017 [24] built a stable episomal expression system that used *L. donovani* rRNA promoter sequences to express Cas9 and gRNA genes, which allowed effective gene editing of multiple *Leishmania* species. Current transfection methods are ineffective for all species or strain types, and some clinical isolates follow a pattern that is not amenable to traditional techniques.

6.2 Off-taget risks and Cas9 fidelity cariants, Parasite genome plasticity

There are a number of technical and biological constraints to CRISPR-Cas9 gene editing of *Leishmania* limiting its potential. Delivery also is one of the key obstacles: although episomal vectors can be used to express Cas9 and sgRNA, continued drug pressure is needed to maintain it, which poses a risk to experimental fidelity and is challenging to do in *Leishmania*, whereas ribonucleoprotein (RNP) complexes can be expressed transiently and avoid off-target effects, but are technically challenging to deliver into Leishmania, and often yield lower editing rates than plasmid-based methods [35]. Off-target effects; the unwanted changes in DNA unintended at the locus of interest; continue to be a significant constraint particularly with the complex and repetitive *Leishmania* genomes [42]. The promiscuity of Cas9 has the potential to introduce spurious edits, although variants of Cas9 with high fidelity and careful design of guide RNA have minimized this risk, extensive off-target interrogation of *Leishmania* has not been done yet, and is needed in both research and clinical settings. Moreover, *Leishmania* shows a remarkable genomic plasticity, such as mosaic aneuploidy, large-scale gene duplication and rapid genomic rearrangements under environmental pressure [23]. This plasticity does not only complicate gene knockout and phenotypic analysis because compensatory mutations or gene amplifications can obscure the result of edits, but has also been found to enable escape of CRISPR-induced damage and may hamper making stable and reproducible mutants, particularly when essential or multi-copy families of genes are targeted [33]. To overcome these weaknesses, more insightful and efficient delivery platforms, uptake of second-

generation CRISPR nucleases to enable greater specificity, and integrative genomics methods to consider the incredible versatility of *Leishmania* would be beneficial [43].

6.3 Parasite Genomic Plasticity

The genomics of l*eishmania* and other protozoan parasites have shown a great deal of plasticity such as aneuploidy, frequent gene amplification, and rapid adaptation. This plasticity makes the gene knockout experiments and drug resistance studies more difficult: rearrangements in the genome and copy number fluctuations both have the potential to instigate or compensate individual gene deletions, making the production of stable mutants more difficult. The magnitude of this plasticity is now exposed by new CRISPR-based screens and long-read genomic technologies and recent studies from 2022-2025 studies suggest combined approaches, combining CRISPR knockout libraries with whole-genome sequencing or whole-genome screen profiling to decouple actual gene action and adaptive genomic change [43][44]

6.4 Episomes vs RNPs

The effectiveness of gene altering in *Leishmania* are closely related with the CRISPR-Cas9 delivery technique. Episomal plasmids make it possible to maintain stable Cas9 expression but rely on drug selection and may randomly integrate especially in genomes with active recombination mechanisms. Conversely, the ribonucleoprotein (RNP) complexes reduce sustained expression, reduce off-target risks, reduce cytotoxicity, but tend to be technically difficult to transfect parasites. Recent comparative analysis of fungal and protozoan pathogens highlights that plasmid-based solutions are more effective in editing but pose a higher risk on genomic collateral consequences, whereas the RNP-delivery (through sophisticated electroporation or nucleofection techniques) technology holds prospective in transient, specific editing of a broad selection of microbes [45][23].

6.5 Off-Target Risks and Cas9 Fidelity Variants

To address the long-established issue of off target effects, several recent studies have designed and tested high-fidelity variants of Cas9, including Cas9-HF1, HypaCas9, eSpCas9 and evoCas9 which have significantly increased cleavage specificity without cleavage activity. New Cas12a RNP strategies as well as truncated guide RNAs have demonstrated increased targeting specificity in protozoa and microalgae systems. The use of high-fidelity and orthogonal Cas proteins in therapeutic and functional genomic studies is now widely advised in order to minimize undesirable genome perturbations [46].

7. Current Advances and Case Studies in CRISPR-Cas9 Applications for Leishmaniasis

7.1 Recent Studies Applying CRISPR-Cas9 in *Leishmania*

Since *Leishmania donovani* adapted to CRISPR-Cas9 in 2015, scientists have transformed this parasite's genetic engineering capabilities, which causes fatal visceral leishmaniasis VL. Zhang and Matlashewski (2015) [57], developed DNA editing vectors containing single-gRNA or dual-gRNA expression elements that use an RNA polymerase I promoter to produce robust gene editing of *L. donovani*. The newly developed system offered advanced genome editing capabilities that enabled practical high-speed analysis

of parasite genes. The research showed *Leishmania's* double-strand breaks resulting from Cas9 activity primarily underwent repair through homology-directed repair (HDR) and microhomology-mediated end joining (MMEJ) pathways [57]. Research activities now present tailored CRISPR cytosine base editors (CBES) that work on *Leishmania* species L. mexicana, *L. major*, *L. donovani* and *L. infantum*. Implementing CBEs allows for efficient stop codon introduction through cytosine-to-thymine conversions that avoid double-strand break production and eliminate donor DNA requirements (May et al., 2025). This methodology reaches 100% editing effectiveness in non-clonal populations through its ability to perform scalable functional genomic screens, which overcome DNA repair pathways and gene copy number variations constraints [24].

CRISPR-Cas9 editing has also played a role in the production of *Leishmania* major centrin knockout strains (LmCen–/–) that are nonpathogenic, induce strong immunity and lacks antibiotic resistance markers- solving a major regulatory obstacle to human application. In contrast to earlier lines of gene-Disrupted created using homologous recombination, CRISPR-Cas9 also enables markerless editing to facilitate candidates of vaccines that are regulatory compliant. In preclinical models, it has been demonstrated that LmCen–/– is safe and immunogenic and provides protection against needle and sand fly challenge without disease symptoms. The Phase I clinical trials are currently under consideration by scientists, which indicates its translational potential [22][47].Other virulence genes, including universal minicircle sequence binding protein (UMSBP), that is a critical mitochondrial regulator, have been targeted using CRISPR/Cas9 instead of centrin. The UMSBP knockouts (LmUMSBP- /-) parasites have attenuated growth, higher levels of apoptosis and in the case of UMSBP-/- parasites, protection as Th1-type immunity in mice using Th1-immune responses. This potential to make such knockouts without having to insert foreign DNA or sets of antibiotic resistance is essential in clinical translation [48].CRISPR-related systems can be used to specifically eliminate marking genes of choice, which is a requirement of live attenuated vaccines in humans. This move is a great improvement to the previous technologies since strains with antibiotic resistance markers are unlikely to be approved by regulatory agencies. Also, LmCen and other marker-free attenuated strains have proven genomic stability and reproducibility in animal models further enhancing their use in clinical development [9] [49].

7.2 Integration with Omics and Systems Biology

The future applications of CRISPR-Cas9 in *Leishmania* are more dependent on the combination with omics and systems biology methods to have a more profound understanding of the biology of parasites and their resistance to drugs [24]. Transcriptomics, proteomics and metabolomics allows researchers to comprehensively describe genome edits on a variety of scales, and identify downstream consequences on gene expression, protein activities, and metabolic processes that may not be available to classical genetic screen [50]. More recent CRISPR-based reverse genetic screens are coupled to transcriptomic profiling, which assists in the connection of engineered mutations to global changes in parasite transcriptomes and the discovery of regulatory networks involved in virulence and drug response. Proteomic studies complemented these studies by correlating genetic disturbances with variability in protein levels, localization, and post-translational modifications which give both therapeutic and diagnostic search points [51]. Further joint treatment with single-cell sequencing technologies maximises the resolution of these analyses and enables dissection of cellular heterogeneity and functional diversity in the *Leishmania*

populations following genome editing. Altogether, these synergies of CRISPR tools and multi-omics systems provide an effective systems design, defining the core sets of genes, anticipating phenotypic responses, and directing the rationale in designing combination therapies and diagnostics to treat leishmaniasis [52].

8. Future Perspectives and Potential Applications

8.1 CRISPR-Cas12 and CRISPR-Cas13 Systems

Research studies on the topic of *Leishmania* infections have been revolutionised by the use of novel CRISPR technology by scientists. Currently, the development of the CRISPR technology has progressed forward through CRISPR-Cas9 to CRISPR-Cas12 and CRISPR-Cas13 platforms, each having specific benefits to the research of Leishmaniasis diseases.

8.1.1 CRISPR-Cas12: A group applied the technology to identify the *Leishmania* DNA in clinical samples and showed high diagnostic specificity in cutaneous leishmaniasis diagnosis. The process of point-of-care molecular diagnostics testing can continue to the next level because Cas12 has the capacity to perform collateral cleavage, thereby enhancing the outcomes of the field detection in endemic areas [54][55].

8.1.2 CRISPR-Cas13: Cas13 is different when compared to Cas9 and Cas12 since the former targets RNA as its nucleic acid. RNA detection assays are applicable in this feature. In *Leishmania*, laboratory scientists are discussing the use of the CRISPR-Cas13-based diagnostic system to detect protozoan parasites on the RNA level. Gene knockdown studies and ways of exploring the potential of using Cas13 as an antiviral therapeutic are possible because of its capacity to target RNA [56].

8.2 Gene editing for Drug Target Validation: CRISPR-based biomechanisms still exploit potential beside-the-barrel applications with existing cures to enhance medical outcomes. Researchers also identify drug targets through gene editing by ensuring the CRISPR technology is used. *Leishmania* gene editing with the CRISPR-Cas9 technology has benefited by the sufficient characterisation of the knockout and other functional studies involved in the characterisation of vital genes to be used in treatment development. The data derived in the research contributes to the drug therapy development designs based on the parasite survival system and the immune management system of the host [57].

8.3 Synergy with Antileishmanial Drugs: The disruption of genes associated with drug resistance or virulence through CRISPR technology enables researchers to create drug-sensitive parasites, thereby enhancing the effectiveness of existing antileishmanial treatments [46].

8.4 The CRISPR-Cas9 system can enhance combination treatments alongside vaccine development possibilities

8.4.1 Live Attenuated Vaccines CRISPR-Cas9 technology has developed live attenuated versions of *Leishmania* parasites through virulence gene depletion, generating protective vaccine candidates without actual disease manifestation. CRISPR-generated vaccines continue their preclinical success while Phase I human trials are currently in progress because they lack markers (Singh et al., 20022).

8.4.2 Combination Therapies The use of CRISPR-based gene editing in conjunction with conventional medication and immunotherapy strategies have proved to be very promising in creating enhanced therapeutic effect. This approach involves the implementation of gene-edit parasites as a method of providing vaccination and augmentation with the addition of immunomodulators that assist in enhancing the immune system response to parasites [57].

8.4.3 Next-Generation Vaccine Strategies The CRISPR tool helps the medical professionals to design rational multi-antigen or multi-species vaccines against many *Leishmania* parasites. A vaccine plan of this kind holds the prospect of settling the unavailability of one human vaccine that will be effective against leishmaniasis [57].

9. Case Study

One groundbreaking case study is the creation of the live attenuated *Leishmania* major centrin gene deletion mutant strain (LmCen–/–), which has been designed by CRISPR-Cas9 and in which the centrin gene is deleted without the addition of antibiotic resistance markers, thereby fulfilling regulatory safety concerns. This candidate has shown great immunogenicity and protection against sand fly-borne cutaneous leishmaniasis in preclinical models and is under Phase I human clinical trials which is a major breakthrough in developing a vaccine against leishmaniasis. In addition to single-strain vaccines, CRISPR has made it possible to design multi-antigen and multi-species vaccine candidates and has been used to simultaneously edit multiple virulence genes of different *Leishmania* species, which have a complex antigenic diversity that is important in broad protection. Also, the concept of combination therapy is becoming increasingly popular, in which CRISPR is applied to sensitize parasites via knockout or knockdown of drug resistance or virulence genes, increasing the efficacy of already available antileishmanial compounds and preventing therapeutic failures. This interplay of gene editing and pharmacological intervention will lead to a new opportunity of finding more effective and customized treatment of leishmaniasis patients. Combined, these developments demonstrate the future of CRISPR technology in developing the next generation of leishmaniasis interventions, not only in vaccine innovation, but also combination-based therapeutic approaches.

10. Discussion

Leishmania donovani and *L. infantum* are the parasites causing visceral leishmaniasis (VL), an important public health burden, primarily in South Asia, East Africa, and Brazil. Drug resistance and toxicity are also increasing and hence, traditional treatment methods are becoming less efficient. *Leishmania* research

changes were revolutionised by the improvement of the CRISPR-Cas9 technology, which allows strict genome editing. This has gained the identification of major genes involved in drug resistance, e.g. RING-variant proteins, sterol 24 -C methyltransferase, and hypothetical genes with hypothesized functions in the adaptation of the parasite. CRISPR has also helped in deleting genes that contain niches of virulence and this has produced genetically attenuated parasite strains that may be utilized in the development of vaccines.

Besides the use in those applications that target the parasite, CRISPR has also been implemented in sandfly vectors to investigate the prospect of controlling transmission by editing the genome. Moreover, there are also improved methods of functional genomics based on CRISPR interference (CRISPRi), activation (CRISPRa), and base editing. New Cas12 and Cas13-based CRISPR-based diagnostics improve on-site applications due to the increased speed of detection of *Leishmania* DNA and RNA, respectively. In sum, the CRISPR-Cas-like technologies offer good prospects to learn *Leishmania* biology, vaccines, therapeutics, and diagnostics, as well as to devise novel tools against the VL.

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12. Author's Biography

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