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Research Article

CRISPR-Cas9 Mediated Paraflagellar rod 1 Gene Editing in *Trypanosoma Evansi*

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Abstract

Trypanosoma evansi, a blood protozoan parasite of high economic importance, has a wide prevalence in the vertebrates in many tropical countries including India. The parasite is transmitted by the hematophagous flies and causes a chronic debilitating disease 'Surra' in domestic livestock. Development of a protective vaccine has not yet been possible due to the ability of the parasite to evade the host's immune response by periodically changing its surface glycoproteins. The Paraflagellar Rod 1 (PFR1) gene codes for a major flagellar constituent PFR1 protein of *T. evansi*. We report here a specifically targeted editing of the *T. evansi* PFR1 gene by the CRISPR Cas9 tool. We combined the single guide RNA (sgRNA) and recombinant Cas9 protein *in-vitro* to form a ribonucleoprotein complex (RNP) for transfecting *T. evansi* and the induced mutation in the coding sequence of PFR1 was assessed by T7E1 endonuclease assay. The CRISPR-Cas9-mediated PFR1 gene-edited parasites had a significantly shorter lifespan *in vitro*.

Keywords: *Trypanosoma Evansi*; PFR1; CRISPR; Cas9; Gene Editing

Abbreviations

PFR: Paraflagellar Rod; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; Cas: CRISPR Associated; sgRNA: Single Guide RNA; DSB: Double-Stranded Break; RNP: Ribonucleoprotein Complex

Introduction

Trypanosoma evansi belongs to the Phylum Sarcomastigophora and are plasma and tissue fluid-dwelling parasite of mammals. *T. evansi* has an extensive prevalence in Africa, South Asia, parts of Europe, and South America, however, there is no record of the infection from Oceania, Central and North America [1,2]. Among the vector-borne haemoparasitic infections of livestock, the occurrence of surra is significantly high in India [3,4]. The blood-sucking Dipteran flies, such as *Tabanus*, and *Stomoxys,* transmit the infection while feeding on the host. The vampire bats (*Desmodus rotundus)* have been associated with transmission of the haemoparasite in South America [2]. In some sporadic cases, infection may be transmitted per os in the carnivores while feeding on an infected fresh carcass [5-7]. The bloodstream trypomastigote

form is a slender leaf-like unicellular organism and carries a filamentous flagellum, which originates from the blepharoplast posterior to the nucleus. The flagellum remains attached to the cell surface by an undulating membrane before emerging from the anterior tip of the organism.

Unlike other African animal trypanosomes, *T. evansi* is least host-specific and is capable of infecting both wild and domestic ruminants and carnivores [8,9]. The pathogenic outcome of the infection largely depends on the species of host infected. The infection is acute and highly pathogenic for camels, equines, and canines, but usually occurs in a chronic form in ruminants including cattle, buffaloes, sheep, and goats. Although a few infections with *T. evansi* have been reported in humans from India and elsewhere, such incidences are rare [10]. The clinical signs of the infection are non-specific. Intermittent fever, anorexia, anemia, cachexia, nasal and ocular bleeding, and stiffness of the legs are associated with the infection; abortion, neuropathy, and immunosuppression are also reported [11]. World Organization for Animal Health declared surra as a notifiable multispecies animal disease in 2009 [12]. The extensive morbidity and mortality associated with surra inflict an economic loss, to the tune of INR 44740 million per annum in India [13].

The development of a protective vaccine is challenging due to the evolutionary advantage of the trypanosomes to survive in the immunologically hostile environment in the host by changing its surface glycoprotein coat [13-15]. The major Paraflagellar Rod (PFR) proteins PFR1 and PFR2 (70-80 KDa and 68-72 KDa, respectively) are unique in kinetoplastid parasites [16-21] and have been targeted for the development of a protective vaccine and drugs [22-24]. One or more rounds of the selection process for the production of null mutants or transgenics made gene-editing cumbersome in trypanosomes during the pre-CRISPR era. CRISPR has made gene editing a routine laboratory experiment and showed promising results in *T. cruzi* [25,26] and *T. brucei* [27]. The simplicity of the technique has made it an easy-to-handle tool for understanding the biology and function of many genes and thereby facilitated the identification of better diagnostic or prophylactic targets. Lander *et al.* (2015) successfully knocked out the *T. cruzi* PFR1 and PFR2 by CRISPR-Cas9 to study the function of the proteins [28]. Recently the CRISPR-Cas tool has been used for the endogenous gene tagging in *T. cruzi* and for the localization of PFR2 and PFR5 proteins and their visualization by immunofluorescence [29]. Sollellis., *et al*. (2015) first reported the disruption of the PFR2 locus of *Leishmania major* using CRISPR-Cas technology [30]. However, given the absence of any study on the disruption of the PFR gene in *T. evansi,* we undertook the present experiment. We report here the CRISPR-Cas mediated genetic manipulation of *T. evansi* for a better understanding of the biology of the gene-depleted parasite.

Materials and Methods

Parasites

A horse isolate of *T. evansi*, cryopreserved in liquid nitrogen, was revived and propagated *in vivo* in the inbred strain of Swiss albino mice by serial passage [31,32].

Selection of Guide RNA sequence and Designing of Forward primer for *in vitro* transcription

The coding sequence of the PFR1 gene of *T. evansi*, Izatnagar isolate was retrieved from the database (GenBank Accession # FJ968743.1) to determine the nucleotide sequence of the guide RNA using CRISPOR online guide RNA designing tool (http://crispor.tefor.net/). Three guide sequences were selected after evaluation of the on-target and off-target scores and were used for designing the primers for template preparation for the *in vitro* transcription reaction (Table 1).

Sl No.	Primer name	Size (bp)	Nucleotide Sequence
1	Primer 1	58	CCTCTAATACGACTCACTATAGGC-GCGAAGGTTGAAAAGGTTG GTTTA-AGAGCTATGC
2	Primer 2	56	CCTCTAATACGACTCACTATAGGATG-CAACACAGTTGGCGC GTTTAAGAGC-TATGC
3	Primer 3	58	CCTCTAATACGACTCACTATAGGA-CAGACATTGAAGCAAGTGGGTTTA-AGAGCTATGC

Table 1: Primers used for template preparation for *in vitro* transcription. The target guide sequence of the PFR1 gene is represented in bold letters.

PCR amplification of sgRNA template

The DNA template that encodes the guide sequence and T7 promoter was generated by PCR. The reaction was performed in 25µL volume in a 0.2mL PCR tube containing 12.5µL PrimeStar Mx Premix (2X) (Takara Bio), 1µL Guide-it Scaffold Template and 0.5µL self-designed primer (10µM). The volume was made up to 25 µL with Nuclease Free Water (NFW). The thermal cycles of the PCR were set to 98^0C for 10 sec and 68^0C for 10 sec followed by steps 1 and 2 for 33 cycles and 4^0C forever.

In vitro transcription reaction

The reaction was performed in a 20µL reaction volume in a 0.2mL PCR tube. The reaction mixture contained 5µL sgRNA PCR template, 7µL Guide-it *in vitro* transcription buffer, and 3µL Guide-it T7 polymerase mix. The volume was made up to 20µL by NFW. The reaction mixture was incubated at 37°C for 4 hrs and left at 4°C. Two microliters of recombinant DNase I (RNase free) were added to the 20µL reaction mixture and mixed by brief vortexing and spinning. The reaction was performed on a thermal cycler with a preheated lid at 37°C for 15min and left at 4°C. The transcribed single guide RNA was purified using the TAKARA Guide-it IVT RNA Clean-Up Kit and its efficiency was analysed using the TAKARA Guide-it sgRNA screening kit.

Transfection of Trypanosoma evansi

Purified live *T. evansi* were transfected by electroporation. Cas9 (10µg) and sgRNA (6µg) were incubated at 25°C for 10min and transferred to 100µL cytomix buffer pH 7.6. A total of 2×10^6 trypanosomes were suspended in 700µL chilled cytomix buffer. The ribonucleotide protein complex and *T. evansi* suspension were transferred to a 4mm prechilled electroporation cuvette. The electroporation was done at 2.0 kV, 50 Ohm resistance, and 25µF capacitance for 0.4ms in an electroporator (BTX Gemini, USA).

In vitro culture of transfected Trypanosoma evansi

The *in vitro* culture of the transfected parasites was set up in a 24-well tissue culture plate (SPL, South Korea) filled with HMI-9M medium at seeding densities of 1×10^4-10^6 cells mL^{-1} medium. The plates were incubated for 72 hours in a 5% CO_2 atmosphere at 37°C (Eppendorf, New Brunswick, Germany). The growth and viability of the parasites were monitored at 24-hour intervals under an inverted microscope (Nikon, Japan). The medium was replenished and the parasites were sub-cultured every 24h [33].

T7E1 assay

The T7E1 assay was performed for the detection of genomic cleavage (Figure 1). The genomic DNA was isolated from the transfected *T. evansi* cultured *in vitro* using a commercial DNA extraction kit (Qiagen DNeasy Blood and Tissue Kit). The concentration of the dsDNA was determined using a nanodrop spectrophotometer (Nabi MicroDigital, South Korea).

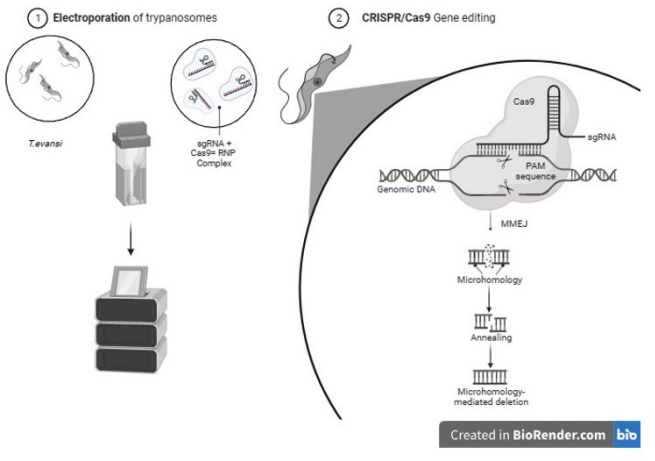

Figure 1: Protocol showing the transfection of RNP complex into *Trypanosoma evansi*. The image was created using Biorender.com.

The reaction was performed in 50µL volume. The reaction mix was composed of Cell lysate 2µL, 10uM forward primer 1µL, 10uM reverse primer 1µL (Table 2), and Dream Taq Master mix 25µL. The volume was made up to 50µL using NFW. The thermal conditions were set to 98°C for 30sec followed by 40 cycles of 55°C for 30sec, 72°C for 30sec, and 72°C for 7min. The product was left at 4°C. The PCR product was checked by electrophoresis on a 2% agarose gel at low voltage alongside a DNA ladder before storing at -20°C until further use. The PCR product was purified using a PCR purification kit (QIAquick, Qiagen).

Two microliters of the PCR product were mixed with 10X NEB buffer in a PCR tube and the volume was adjusted to 9µL with NFW and flash spun for a few seconds. The reaction mix was placed in a thermal cycler with a preheated lid and incubated at 95°C for

Primer name	Size (bp)	Sequence
T7E1 SET 1 FORWARD	22	TGCATGTATCTGACTGGAGCGA
T7E1 SET 1 REVERSE	20	ATGCATCGCAACGTTCTGGA
T7E1 SET 2 FORWARD	24	GACGCGTGTTGTGAGCTTCACGCA
T7E1 SET 2 REVERSE	24	TGCTTCAGGCGACGCT1TCGCATCC

Table 2: The nucleotide sequence and length of the primers used for the T7E1 assay.

5 minutes. Subsequently, 1µL of detection enzyme was added to the test sample, while NFW was used in the negative control. The tube was incubated at 37°C for 1h. The entire 10µL sample was electrophoresed on 2% agarose gel for 30 minutes at low voltage and visualized on a gel documentation system.

Results and Discussion

In vitro culture of Trypanosoma evansi

The wet blood films, prepared from the tail bleeds of the mice, showed increasing parasitemia post-inoculation. At the height of the parasitemia between days 4 to 5 post-inoculation, about 1.5 ml of blood was collected aseptically from the heart of the mice anesthetized using chloroform. The purified host blood cell-free trypanosomes, obtained by DEAE cellulose chromatography, were propagated *in vitro* in an HMI-9M medium supplemented with 3% methylcellulose [34-36]. The yield of the parasite varied between 3 × 10^7 and 1.6 × 10^8 parasites per ml.

The 56-58 nt forward primer along with the Guide-itTM scaffold template containing an inbuilt reverse primer amplified a 130 bp product from the sgRNA template. The sgRNA template was used to transcribe the sgRNA and was purified using the Guide-itTM RNA Clean-Up Kit following the manufacturer's protocol. The efficacy of the sgRNA was screened using a Guide-it sgRNA screening kit. Out of the three guide RNAs tested, only the sgRNA 1 (5'-CGCGAAGGTTGAAAAGGTTG-3') yielded the cleaved band suggesting its suitability for *in vivo* experimentation.

Targeted modification of PFR 1 gene using RNP complex

The number of the transfected parasites increased in the HMI-9M medium and their motility was maintained during the first 72 hours. From 72 h onwards the parasites showed reduced motility that eventually ceased. The parasites were isolated from the medium after 72 h and the genomic DNA was extracted to assess the mutation by T7E1 assay.

T7E1 Cleavage assay

The T7E1 assay showed the cleaved bands indicating a positive manipulation (Figure 2). A specific primer-directed PCR amplified a 509 bp product from the 1770 bp target sequence. This amplified product was used in the T7E1 cleavage assay. The cleaved band fragments were located at positions approx. 390bp and 119bp suggesting the successful endonuclease-mediated fragmentation at the desired position inducing mutation.

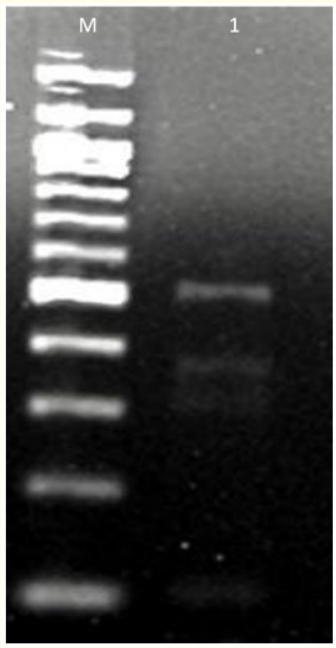

Figure 2: Gel image showing the T7E1 product. Lane M: 100 bp ladder, Lane 1: T7E1 cleaved product.

Conclusion

Several approaches for genetic manipulation have been undertaken since the beginning of the present century for a better understanding of the biology and evolution of *Trypanosoma* species. Stable and efficient inhibition of the expression or translation of some *T. brucei* genes was achieved by RNA interference (RNAi), however, the laborious protocol rendered the technology less appealing [37,38]. Targeting a gene precisely by single-guide RNA (sgRNA) following an efficient Cas9 nuclease-mediated cleavage made the CRISPR-Cas technology an impressive tool for genome editing. The error-prone repair mechanism associated with the double-stranded break (DSB) provided opportunities for manipulation and disruption of the target locus. There are reports of CRISPR-Cas9-mediated gene disruption in *T. cruzi* [39,40], *Leishmania major* [30], and *Leishmania donovani* [41].

We have successfully edited the *T. evansi* PFR1 gene using the CRISPR-Cas9 technique. As the constitutive expression of Cas9 causes toxicity to the cells, we used the ribonucleoprotein complex (RNP) strategy. The guide RNA was *in vitro* transcribed and mixed with Cas9 to form an RNP and the complex was delivered into the *T. evansi* cells by electroporation to avoid the transient expression of Cas9-related toxicity. The approach ensured the functional manipulation of the PFR1 gene using MMEJ-mediated deletion. DNA repair by a non-homologous end-joining (NHEJ) mechanism is absent in the trypanosomatids [40]. In the absence of a donor DNA strand the repair occurs through an alternative pathway known as micro-homology mediated end joining (MMEJ) [39,42], while in the presence of a donor DNA, homology-directed repair (HDR) pathway is active [41]. Compared to the conventional gene-editing mechanism, CRISPR technology has enabled targeted knockdown, knock-in, gene complementation, and endogenous tagging of multicopy gene families [43,44]. The CRISPR technology is easy to perform and helps achieve a precise, and efficient gene mutation [45]. For the detection of mismatches in the sequences, we used the T7E1 assay as it is widely used as a preferred evaluation tool for site-specific nuclease activity in experiments involving the CRISPR-Cas9 system. The presence of the cleaved bands in T7E1 indicated specific editing using the SpCas9-RNP complex approach. However, we do not claim an editing efficiency of 100%. In the absence of a positive marker, we were not able to isolate the positive mutants. However, the positive mutants, generated by the experiments, were not viable beyond 96h in the *in vitro* culture medium. We presume that the manipulation of the PFR1 gene might have a role in the reduced viability of the mutated parasites *in vitro.*

The CRISPR-Cas9 technique allowed manipulation of the PFR1 gene in a short period avoiding molecular cloning and drug selection procedures. The selection of guide RNA was the most important step in the protocol as 100% editing of the target gene depended on the appropriate selection of guide RNA. This approach further saved the time involved in the construction of plasmids and the isolation of mutants by drug selection. The technique had the added advantage of simultaneous multiple gene editing. The guide RNA sequences were identified *in silico* using the online guide RNA design tools (CRISPOR, EuPaGDT, etc) and the best sequence was selected after analyzing the off-target effects and other required parameters [46]. The 20 bp guide sequence was constructed upstream of the Protospacer adjacent motif (PAM), a short 2-6 bp base pair DNA sequence immediately following the targeted DNA sequence at 3′end. Recombinant purified ready-to-use SpCas9 protein was used for the present study along with the 20 bp gRNA sequence upstream of the PAM sequence (5'-NGG-3') [47]. Identification and isolation of the transformants soon after electroporation may be achieved using the marker-tagged Cas9 protein. The rapid editing using the spCas9-RNP complex is useful for studying the gene function and may further help in the discovery of novel diagnostic or prophylactic tools.

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Conflict of Interest

The authors declare no conflict of interest.

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