Investigative study on the role of the Toxo 5699 gene in the *Toxoplasma gondii* lytic cycle using the CRISPR/Cas9 system

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Abstract. The focus of the current study was to disrupt the Toxo 5699 gene via CRISPR/Cas9 to evaluate the effects of gene disruption on the parasite lytic cycle. In the present work, a single plasmid expressing both the guide RNA and Cas9 nuclease together with a selectable marker of human dihydrofolate reductase (DHFR) was introduced into *Toxoplasma gondii*. Targeted disruption of the Toxo 5699 gene was carried out via the CRISPR/Cas9 system and confirmed by PCR, sequencing, and immunofluorescence microscopy. Disrupted and non-disrupted control parasites were allowed to invade HS27 cell monolayers and plaques were counted. The average number of plaques from three replicates per group was obtained between the disrupted and non-disrupted *T. gondii* RH strain and was compared using a one-tailed t-test. It was observed that there was a significant decrease in number and size of plaque formation in the Toxo 5699 gene disrupted parasite line. This is an indication that the Toxo 5699 gene may play a role in the lytic cycle of the parasite, particularly during the replication phase and thus would be a novel target for disruption or silencing. The Toxo 5699 gene presented in the current work is an important part of the *T. gondii* lytic cycle, therefore meriting further inquiry into its potential as a target for further genetic-silencing or disruption studies.

INTRODUCTION

Toxoplasma gondii (*T. gondii*) is a ubiquitous and obligate intracellular protozoan parasite having a broad range of warm-blooded hosts that causes toxoplasmosis (Dubey, 2010). Toxoplasmosis is a common infection globally distributed affecting up to one-third of the world's human population (Jackson and Hutchinson, 1989). The infection poses a danger to Acquired Immune Deficiency Syndrome (AIDS) patients and pregnant women which may result in fatality and abortions can result. Toxoplasmosis also triggers abortions in livestock especially sheep and goats, leading to great economic losses within the food industry (Buxton, 1998).

An initial study of six independent loci from Toxoplasma isolates through PCR-RFLP categorized these isolates into three major clonal lineages, namely type I, II and III which were mainly from Europe and North America. However, there are several isolates resulting from natural combinations of type II with III and type I with III (Howe and Sibley, 1995). Type I strains are highly virulent while type II and III isolates are avirulent (Robert-Gangneux and Darde, 2012).

Our previous study was conducted via whole-genome sequencing and *de novo* assembly of the *T. gondii* type I RH strain (Lau *et al.*, 2016). It was discovered that there was a total of 111 unique genes in this strain. However, most of the genes were annotated to proteins with unknown functions. Only 17

unique genes were annotated to proteins with known functions and appeared to be accountable for the virulence differences among the parasites from the three major clonal lineages; type 1 (GT1), II (ME49) and III (VEG). Among the 17 genes, TOXaeaD_GLEAN_10005699 (Toxo 5699), was selected to determine its contribution to the *in vitro* infectivity of the *T. gondii* RH strain through a clustered regularly interspaced short palindromic repeats-associated 9 (CRISPR/Cas9) system. Annotation of Toxo 5699 indicated that this unique gene encodes the 60S ribosomal protein L24 (RPL24) and is involved in both gene expression and replication regulation, specifically translation "patrolling" and mitotic cell cycle regulation, respectively (Lau *et al.*, 2016). We are thus keen to investigate the effects of Toxo5699 gene disruption on both the replication and invasion capabilities of the *T. gondii* parasite in this study.

The feasibility and high specificity of CRISPR/Cas9 genome editing technology makes it our top alternative to perform the gene silencing research (Ran *et al.*, 2013). CRISPR is a defensive adaptive immunity mechanism naturally found in bacteria and archaea against bacteriophages. Following viral attack, they developed into phage-resistant mutants by integrating foreign genetic elements; in this case, the phage genomic sequence-derived spacers through a mechanism based on RNA interference using the CRISPR system together with the associated cas gene (Barrangou *et al.*, 2007)(Jinek *et al.*, 2012). CRISPR/Cas9 genome-editing technology has thus been widely investigated and incorporated into genome-modification studies with the first two reports on *T. gondii* in 2014 (Shen *et al.*, 2014)(Sidik *et al.*, 2014).The type II CRISPR system originated from *Streptococcus pyogenes* and is commonly chosen despite having three different types (type I, II and III) available owning to its simplicity and ease of manipulation. This system involves guiding of the Cas9 endonuclease by a single guide RNA (sgRNA) to the targeted site of a genome with sequence homologous to the sgRNA in order to introduce a site-specific double-stranded breaks (DSB) followed by activation of a DNA repair mechanism. Two types of repair mechanisms are error prone non-homologous end joining (NHEJ) which occurs naturally at higher frequency in *T. gondii*, and homologous recombination (Jinek *et al.*, 2012)(Sidik *et al.*, 2014).

In the present study, we have introduced a single plasmid CRISPR/Cas9 system encoding both the sgRNA (targeting Toxo 5699 gene) and Cas9 endonuclease together with a selectable marker of human dihydrofolate reductase (DHFR) into *T. gondii*. The selectable marker serves as a tool for disrupting the target locus and for the selection of positively transfected parasites.

MATERIAL AND METHODS

T. gondii tachyzoites of the virulent wild-type RH strain were provided by the Department of Parasitology, University of Malaya, Kuala Lumpur, Malaysia. They were maintained according to the procedures previously described (Ching *et al.*, 2016). The pU6 universal plasmid carrying the Cas9 insert and encoding *Bsa*I site in place of a protospacer/gRNA was purchased from Addgene (ID #52694). The construct targeting the *RPL24* locus was generated by cloning the annealed Toxo5699gRNA duplex into BsaI-digested pU6 universal plasmid using In-Fusion® HD Cloning Kit (Clontech, Japan). The annealing oligos are: Toxo5699gRNAF (5' ATGGGGATGTCAAGTGCACATGTCCGT CTTGATGGGTTTTAGAGCTAGAAA-3') and Toxo5699gRNAR (5'-TTTCTAGCTCTAAAA CCCATCAAGACGGACATGTGCACTTGA CATCCCCAT-3'). Annealing and cloning procedures were performed in accordance with the instruction's manuals. (Supplementary 1) Pyrimethamine resistance cassette was amplified through conventional PCR directed against 1.2x DNA plasmid carrying the human DHFR gene using primers: dhfrF: 5'-ATGTCGGCGGTCAAAA CCAATGCATGGTTCGCTAAACTG-3' and dhfrR: 5'-GGTAAATACGGTACTCCGAGA AGGATTAATCATTCTTCTCATATA-3'. PCR cycling conditions for DHFR amplification are as follows: an initial denaturation step at

94°C for 5 min followed by 30 cycles of 94°C for 45 seconds, 62°C for 30 seconds, and 72°C for 1 min. Finally, an elongation step at 72°C for 10 min was added to the cycle.

Forty micrograms of Toxo5699-pU6 plasmid and 10 µg of purified DHFR amplicons were co-transfected into RH *T. gondii* parasites by electroporation (Lonza, Switzerland). Briefly, precipitated plasmid DNA and approximately 9 x 105 parasites were resuspended in 20 µl of Buffer P3 (P3 Primary Cell 4D Nucleofector X kit, Lonza). Parasites were transfected in a cuvette format using a program FI-158, and were subsequently transferred to HS27 monolayer. Transgenic parasites were left to grow for 72 h after transfection and positively transgenic parasites were obtained via antibiotic selection using 3 µM pyrimethamine (Sigma-Aldrich, St. Louis, USA).

Toxo 5699 gene disruption was confirmed by PCR, sequencing, and immunofluorescence microscopy. PCR amplification for the DHFR gene was carried out as detailed above to detect the presence of the DHFR gene in transfected RH *T. gondii* parasites and the control parasites. The Toxo 5699 gene from both transfected RH *T. gondii* and control *T. gondii* were subsequently sent for sequencing to identify disruption in the gene sequence of transfected parasites compared to control parasites.

HS27 monolayers containing transfected and control *T. gondii* were fixed with 4% formaldehyde and permeabilized with 0.1% Triton-X. The cells were then incubated with anti-Anti-FLAG MAb (Sigma, MI, U.S.A.) as the primary antibody and FITC-labelled anti-mouse IgG (K.P.L Inc., U.S.A.) as the secondary antibody. Cells were then incubated with DAPI/Antifade (Merck, U.S.A.) and mounted on glass slides using Calbiochem Floursave reagent (Merck, U.S.A.). Images were acquired using an Olympus BX51-FL-CCD microscope (Olympus, Japan).

Parasites were allowed to go through one infectious cycle prior to infection with $1x10^3$ freshly lysed parasites seeded into confluent HS27 cells and incubated at 37°C in 5% CO_2 for seven days before the cells were fixed with 100% methanol. The cells were then subsequently stained for 10 minutes with crystal violet. The plaques were visualized via microscopy using an Olympus CKX41 inverted microscope (Olympus, Japan). The plaque assay performed with three replicates per group and 10 fields were counted per replicate. The average number of plaques from three replicates per group was obtained between the transfected and non-transfected *T. gondii* RH strain and was compared using a one-tailed t-test using GraphPad prism. (GraphPad Software, Inc, U.S.A).

RESULTS

To investigate the function of Toxo 5699 within the *T. gondii* lytic cycle, we used the CRISPR/Cas9 system to disrupt the gene via the insertion of DHFR at the guide-targeted region through NHEJ. The Toxo5699-pU6 plasmid was transfected concurrently with DHFR amplicons into the RH strain of *T. gondii*. PCR and sequencing of transgenic and control parasites was carried out confirming the disruption of the Toxo 5699 gene and insertion of the DHFR amplicon in the transgenic parasite line (Figure 1 and 2). The insertion of the large DHFR amplicons
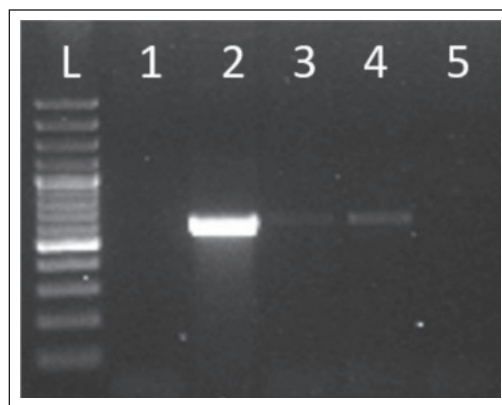

Figure 1. PCR results in (A) indicate the presence of DHFR in the transgenic *T. gondii* line with a band at ~650bp. This band is absent in Lane 5 which is the control *T. gondii* group. Lane L: 100bp plus ladder, Lane 1: No template control, Lane 2: Positive control using 1.2x DNA plasmid carrying the human DHFR gene, Lane 3&4: Transgenic *T. gondii* line, Lane 5: Control *T. gondii* line.
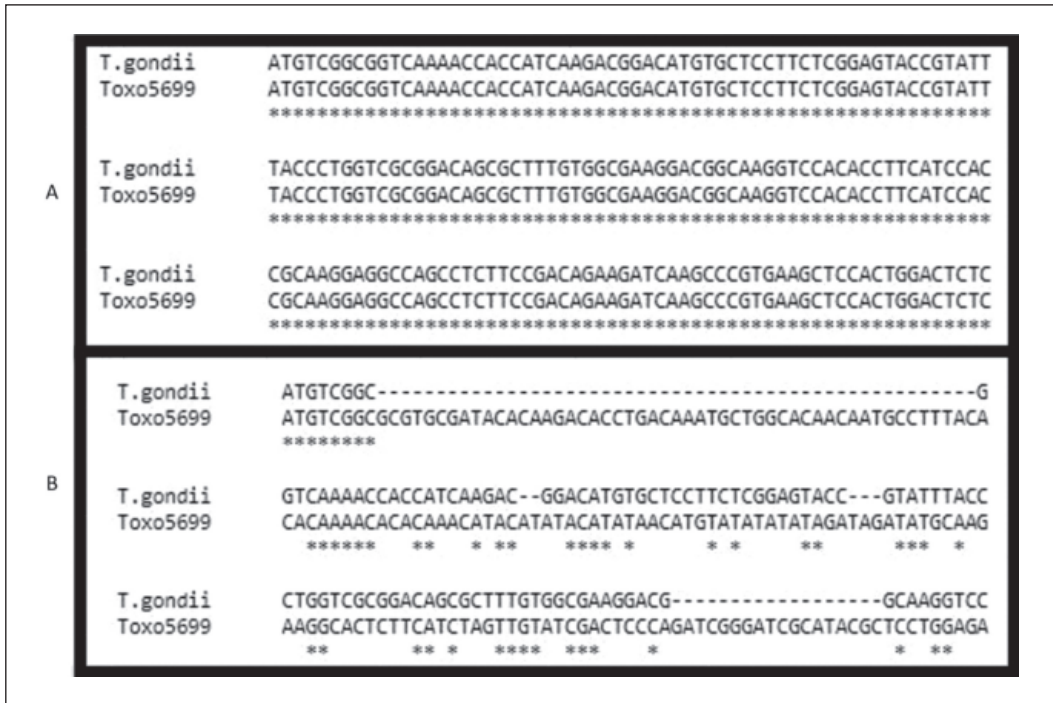
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     T.gondii    ATGTCGGCGGTCAAAACCACCATCAAGACGGACATGTGCTCCTTCTCGGAGTACCGTATT
     Toxo5699    ATGTCGGCGGTCAAAACCACCATCAAGACGGACATGTGCTCCTTCTCGGAGTACCGTATT
                 ************************************************************

A
     T.gondii    TACCCTGGTCGCGGACAGCGCTTTGTGGCGAAGGACGGCAAGGTCCACACCTTCATCCAC
     Toxo5699    TACCCTGGTCGCGGACAGCGCTTTGTGGCGAAGGACGGCAAGGTCCACACCTTCATCCAC
                 ************************************************************

     T.gondii    CGCAAGGAGGCCAGCCTCTTCCGACAGAAGATCAAGCCCGTGAAGCTCCACTGGACTCTC
     Toxo5699    CGCAAGGAGGCCAGCCTCTTCCGACAGAAGATCAAGCCCGTGAAGCTCCACTGGACTCTC
                 ************************************************************
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     T.gondii    ATGTCGGC--------------------------------------------------G
     Toxo5699    ATGTCGGCGCGTGCGATACACAAGACACCTGACAAATGCTGGCACAACAATGCCTTTACA
                 ********

B
     T.gondii    GTCAAAACCACCATCAAGAC--GGACATGTGCTCCTTCTCGGAGTACC---GTATTTACC
     Toxo5699    CACAAAACACACAAACATACATATACATATAACATGTATATATATAGATAGATATGCAAG
                 ******    **   * **    ****  *      * *      **      *** *

     T.gondii    CTGGTCGCGGACAGCGCTTTGTGGCGAAGGACG-----------------GCAAGGTCC
     Toxo5699    AAGGCACTCTTCATCTAGTTGTATCGACTCCCAGATCGGGATCGCATACGCTCCTGGAGA
                 **          ** *   ****  ***       *                 *  **
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Figure 2. Sequencing results in (A) show an intact Toxo 5699 gene and a disrupted Toxo 5699 gene in (B). Sequence labelled *T. gondii* is the PCR amplified and sequenced product from the transgenic parasites and the sequence labelled Toxo5699 is the intact Toxo 5699 gene.

caused frame-shift mutations in the Toxo 5699 gene, thereby producing transfected *T. gondii* parasites without a functioning Toxo 5699 gene.

Subsequent IFA detection (Figure 3) of the FLAG tag was carried out to determine transfection efficiency and whether transfected parasites had taken up plasmid. In Figure 3 it is observed that transfected parasites in (A) fluoresced under FITC indicating successful insertion of the Toxo5699-pU6 plasmid compared to control parasites in (B). To further confirm the disruption of the gene, PCR amplification and sequencing of the Toxo5699 gene was carried out on transfected and non-transfected *T. gondii*. We also carried out PCR amplification for the DHFR gene from both transfected and non-transfected *T. gondii* to confirm the integration of the DHFR gene into the *T. gondii* genome indicating disruption at the target site. The sequencing results confirmed the disruption of the Toxo 5699

gene when aligned with the Toxo 5699 sequence from control RH *T. gondii* and positive amplification of the DHFR gene in transfected *T. gondii* indicated integration of the DHFR gene into the genome.

To investigate the importance of the Toxo 5699 gene to the lytic cycle of the parasite, plaque assays were performed to compare plaque formation between Toxo 5699-disrupted and control RH *T. gondii* (Figure 4). Parasites were plated, harvested and stained with crystal violet for both groups. It was observed that there was a significant increase in plaque formation in control Toxo 5699 parasites compared to the disrupted parasites, suggesting that a disruption of the Toxo 5699 gene adversely affected the lytic cycle of the parasite (Figure 5). Furthermore, the plaques formed in control Toxo 5699 parasites were observed to be larger in comparison to the disrupted parasites, further reinforcing this observation (Figure 6).
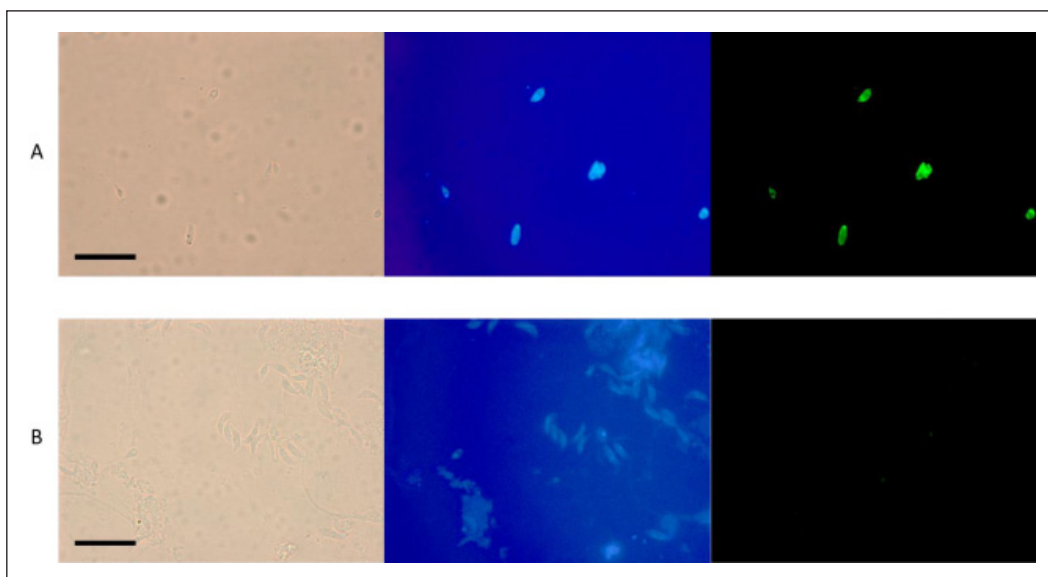
Figure 3. IFA of *T. gondii* with disrupted (A) and intact (B) Toxo 5699 gene using anti-Anti-FLAG MAb as the primary antibody and FITC-labelled anti-mouse IgG as the secondary antibody. The first panel is under phase-contrast view, the second panel with the DAPI filter applied and the third with the FITC filter applies. Scale bar is 10µM.
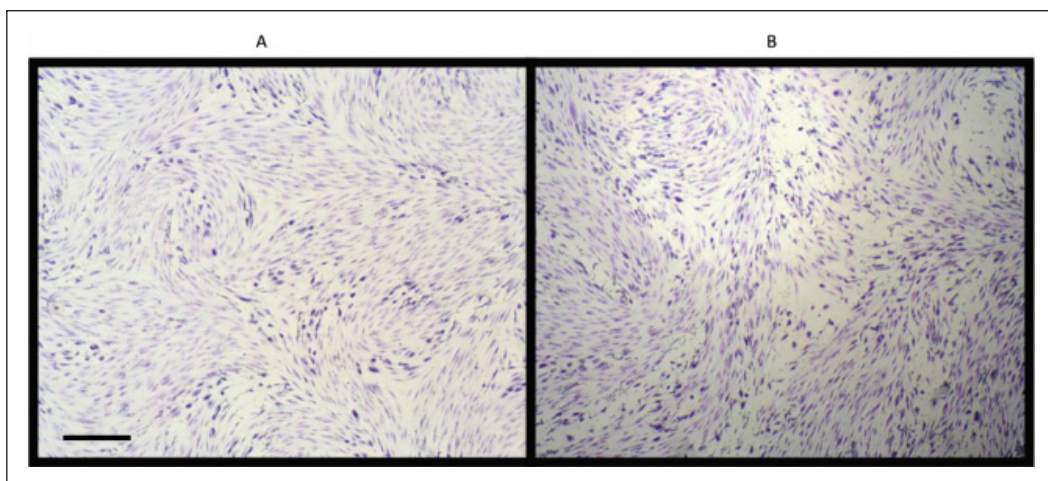

Figure 4. Plaque formation following transfection with parasites containing the Toxo 5699-pU6 plasmid (A) and control RH *T. gondii* (B). Parasites were allowed to go through one infectious cycle prior to infection with 1×10^3 freshly lysed parasites seeded into confluent HS27 cells. Scale bar is 40 µM.

DISCUSSION

In this study, we utilized the RNA-guided Cas9 endonuclease type II CRISPR system. This system facilitates the development of an easily programmable platform for genome editing (Mali *et al.*, 2013). With an emphasis on crucial construction and design of the knockout vector, there is the allowance for rapid, targeted disruption or removal of targets genes compared to other genome-editing methods. With respect to *T. gondii* genetic manipulation specifically, this system enables the knockout of other *T.*
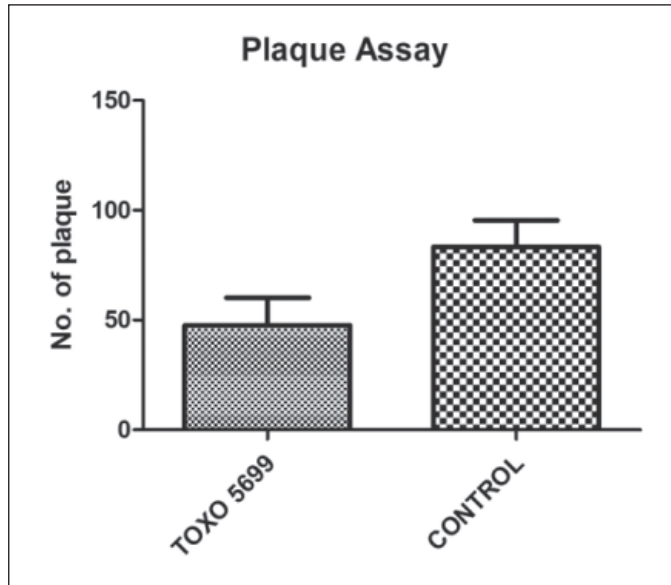
Figure 5. The average number of plaque from three replicates between the transfected and non-transfected RH *T. gondii* were compared using a one-tailed t-test. The average number of plaque formed in the control group of *T. gondii* parasites that were transfected without the Toxo 5699-pU6 plasmid was significantly higher compared to the group transfected with Toxo 5699-pU6 plasmid. (p-value=0.05).
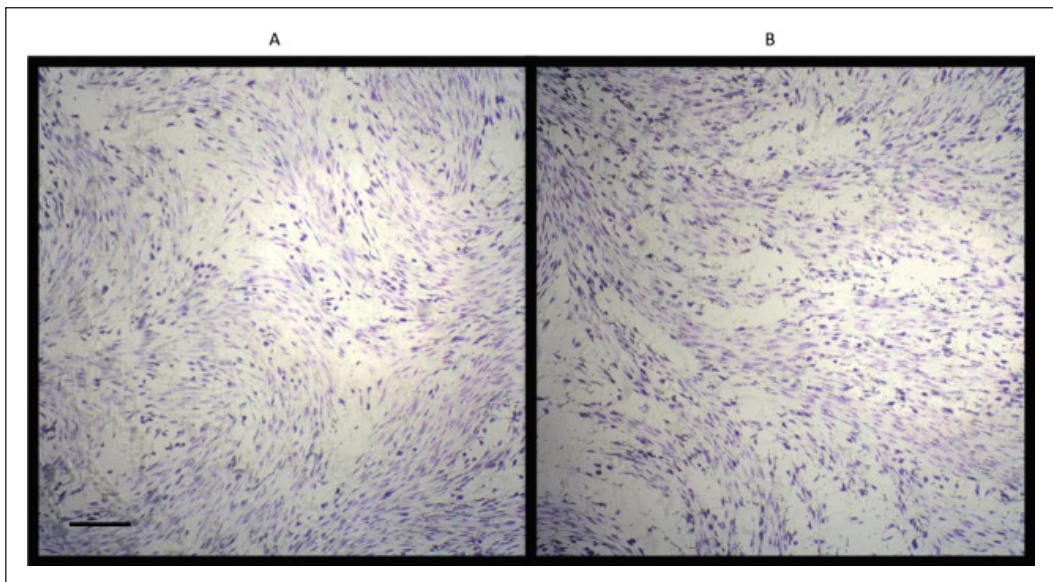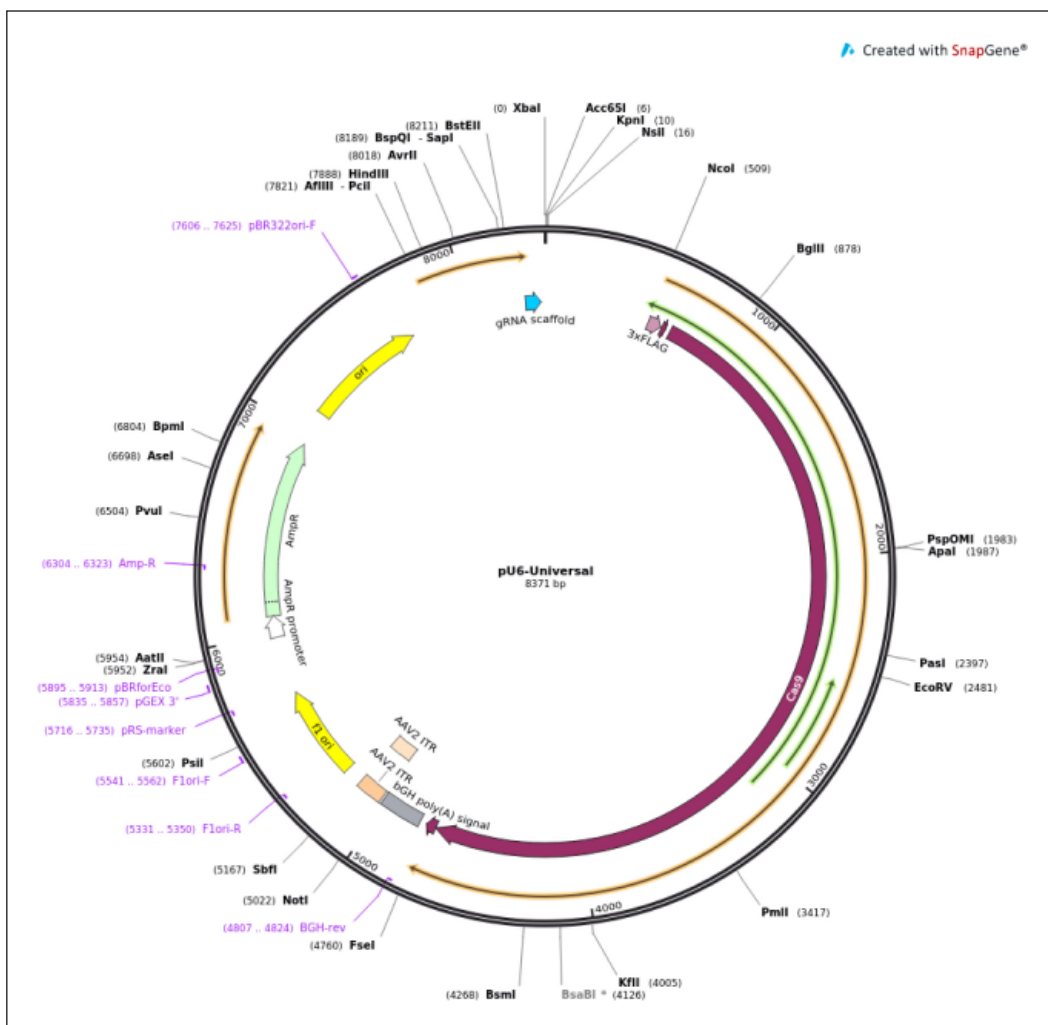

Figure 6. Plaque size following transfection with parasites containing the Toxo 5699-pU6 plasmid (A) and control RH *T. gondii* (B). Parasites were allowed to go through one infectious cycle prior to infection with 1×10^3 freshly lysed parasites seeded into confluent HS27 cells. Scale bar is 40 µM.

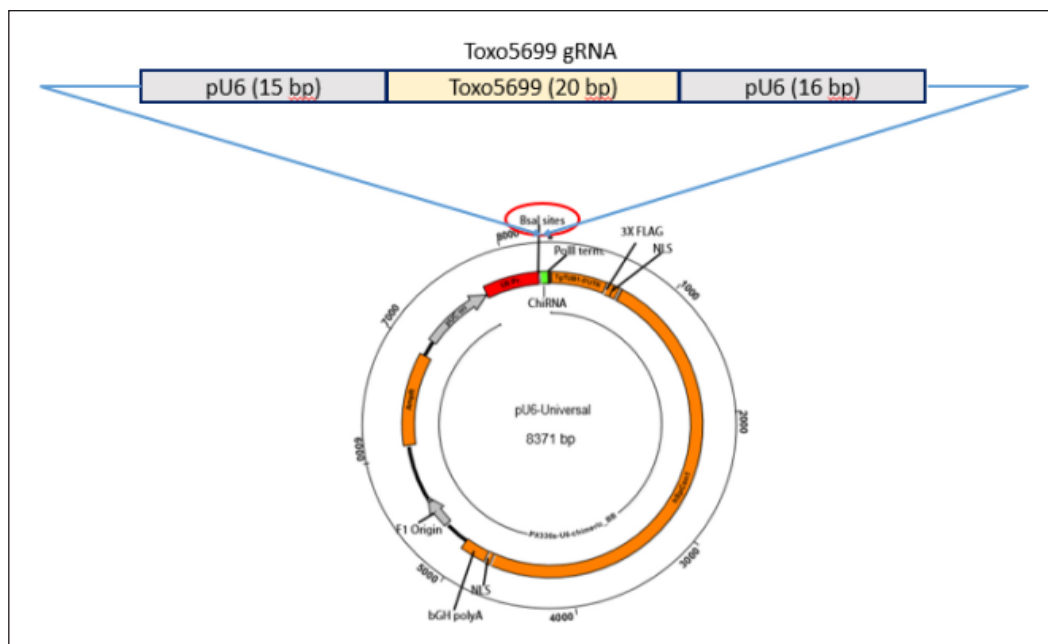**Full sequence map for pU6-Universal** (https://www.addgene.org/52694/)

Purpose: This plasmid can be used to mutate genes in *Toxoplasma gondii* using the CRISPR/Cas9 system after having an appropriate protospacer cloned into it.

gondii genes simply by replacing the guide-targeted RNA sequence with one corresponding to another gene of interest. The CRISPR/Cas9 tool has been used in multiple organisms for successful genome editing. With regards to *T. gondii*, CRISPR/Cas9 has been employed to disrupt the SAG1 locus in the *T. gondii* genome (Sidik *et al.*, 2014) and six different calcium-dependent protein kinases (Wang *et al.*, 2016).

The results of this study lead us to believe that the Toxo 5699 gene, which expresses the RPL24 protein and was previously ascribed to acting during mitotic cell division (Lau *et al.*, 2016), is critical for the lytic cycle of the parasite, particularly in the replication phase. Although previous whole genome sequencing of *T. gondii* by Lau *et al.*, hypothesized that Toxo 5699 plays an important role in the *T. gondii* mitosis cycle this was yet to be confirmed *in vitro* thus the purpose of this study. In this study we chose to disrupt the Toxo 5699 gene to then observe how the disruption of this gene affects the

Supplementary Figure 1: Schematic diagram of Toxo5699-pU6 plasmid construction

replication and invasion capabilities of the *T. gondii* parasite. With its disruption in our study, we observed that the RH *T. gondii* parasite replication is adversely affected with the lower number of plaques formed and the smaller size of the plaques seen. The lower number of plaques formed indicates that the replication of the parasite was hampered when the Toxo 5699 gene was disrupted hence the lower number of parasites able to re-invade the HS27 cells and form plaques in the HS27 cell monolayer. In addition to that, the smaller plaque size also indicates that again parasite replication was hampered resulting in lower re-infection of the surrounding HS27 monolayers thus the smaller plaque size. As such, this gene can represent a novel target for disruption or silencing to reduce the infectivity of the parasite within a host.

This study is however a preliminary study and future studies that can further elucidate the function of the Toxo 5699 in *T. gondii* must be carried out to provide confirmation of the role of this gene. These future studies may include *in vivo* studies on replication of Toxo 5699 disrupted and non-disrupted parasites in mice and the effects of Toxo 5699 gene silencing and complete gene knockout compared to gene disruption on parasite invasion and replication both *in vitro* and *in vivo*.

CONCLUSION

Therefore, CRISPR/Cas9 is a welcomed addition to the toolkit for genetic manipulation in *T. gondii* and the Toxo 5699 gene presented in the current work is an important part of the *T. gondii* lytic cycle, therefore meriting further inquiry into its potential as a target for further genetic-silencing or disruption studies.

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