DEVELOPMENT OF HIGH-THROUGHPUT GENE KNOCKOUT STRATEGIES IN *TRYPANOSOMA CRUZI* FOR PRODUCTION OF ATTENUATED LINES

by

DAN XU

(Under the Direction of Rick L. Tarleton)

ABSTRACT

In contrast to the substantial *in silico* studies of the *T. cruzi* genome, only a few genes have been experimentally characterized, mainly due to the lack of convenient methods for gene manipulation for reverse genetics studies. In this study we have compared the conventional multi-step cloning technique with two knockout strategies that have been proven to work in other organisms, One-Step-PCR- and Multisite Gateway-based systems. Our results show that while the One-Step-PCR strategy is faster than the other methods, it does not efficiently target genes of interest. The Multisite Gateway based approach, although not as fast and easy as the One-Step-PCR strategy, is less time-consuming than the conventional method and is able to efficiently delete target genes. Using this strategy, we have successfully generated single- and double- gene knockout parasites with genes that are predicted to be non-essential for epimastigote survival but essential for growth of *T. cruzi* amastigotes.

INDEX WORDS: *Trypanosoma cruzi*, Chagas disease, vaccine, gene knockout, One-Step-PCR, MultiSite Gateway, homologous recombination, *ECH*, *DHFR-ts*

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DEDICATION

To my husband, Jie, without whom I could not have made it to this point.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1. *Trypanosoma cruzi*

1.1 Biological Characteristics

Trypanosoma cruzi (*T. cruzi*) is a hemoflagellate parasite species of protozoan trypanosomes which is transmitted through the feces of triatomine insect-vectors to over 100 species of mammalian hosts, including rodents, sloths, armadillos, marsupials, dogs, and primates.

T. cruzi has four morphologically and physiologically distinct stages. The infective vector-borne metacyclic trypomastigotes of *T. cruzi*, as well as the mammalian host blood-form trypomastigote, is highly motile and able to efficiently infect a wide variety of phagocytic and non-phagocytic cells. Following transmission to the mammalian host, circulating metacyclic trypomastigotes of *T. cruzi* invade an amazing variety of cell types, including macrophages, smooth and striated muscle cells, fibroblasts and even neurons, in which they transform into amastigotes within the cytoplasm. After cell invasion, the trypomastigotes reside for a short time in an endosome-derived parasitophorous vacuole, but then escape into the cytosol and convert to the amastigote form, which is the replicative stage in the mammalian host. Intracellular amastigotes divide by binary fission, and convert back into flagellated trypomastigotes, eventually causing the host cell to rupture, and trypomastigotes are released into the circulation, to continue the cell invasion process. The circulating trypomastigotes can be ingested by triatomine bugs and then convert into the epimastigotes, which replicate in

the gut of the insects. A new cycle of infection starts when the triatomine bug defecates, releasing trypomastigotes near the site of the bite wound [1]. In addition to spreading of contaminated feces by triatomine bugs to mucous membranes of mammalian hosts, alternative transmission modes of *T. cruzi* have also been documented, including oral transmission [2], placental passage [3], blood transfusion [4], and organ transplantations [5-7].

The transition of trypomastigote to amastigote appears to be accompanied by a dramatic shift from carbohydrate- to lipid-dependent energy metabolism, as analysis of the *T. cruzi* proteome demonstrates the virtual absence of glucose transporters and the detection of enzymes that oxidize fatty acids to generate acetyl-coenzyme A in amastigotes [8]. Enzymes of the citric acid cycle, which oxidize acetyl coenzyme A to carbon dioxide and water, are also abundant in amastigotes [8]. The lipid-dependent energy production would also be consistent with the finding that *T. cruzi* shows a preference for growth and persistence in adipose tissues [9].

1.2 Genetic Characteristics

T. cruzi has been partitioned into major groups *T. cruzi* I and *T. cruzi* II that can be further divided into six subgroups, I, IIa, IIb, IIc, IId and IIe [10-12]. The different subgroups not only vary in tissue tropism, replication rate, morphology, isoenzyme patterns, and virulence [13-15], but also have very different sizes of chromosomes [16]. *T. cruzi* strain CL Brener, the strain chosen for genome sequencing, is a member of subgroup IIe, but also a hybrid between subgroup IIb and subgroup IIc, which itself is also apparently a hybrid derived from *T. cruzi* I [17].

Genome analysis of *T. cruzi* CL Brener strain revealed that the diploid genome of *T. cruzi* has a size between 106.4-110.7 Mb, with at least 30 chromosomes ranging from 0.45 to 3.5 Mb [18]. The genome contains a predicted 22,570 protein-encoding genes, of which 12,570 represent allelic pairs, and allelic copies of genes may vary in sequence by about 1.5% [19]. Trisomy also occurs in the case of some chromosomes [17, 20]. Putative function could be assigned to 50.8% of the predicted protein-coding genes on the basis of significant similarity to previously characterized proteins or known functional domains. In addition, the *T. cruzi* genome contains an extensive range of repetitive elements [21], consisting mostly of large gene families of surface proteins, retrotransposons, and subtelomeric repeats [22, 23]. Although lacking several classes of signaling molecules, the genome of *T. cruzi* contains a large and diverse set of protein kinases and phosphatases [19]; their size and diversity imply previously unknown interactions and regulatory processes, which may be targets for intervention.

In common with other trypanosomatids, including *Leishmania* and the African trypanosome *T. brucei*, *T. cruzi* exhibits several unusual genetic traits. They virtually lack mechanisms to control gene expression at the transcriptional level, and rely mostly on post-transcriptional regulation processes [24], like mRNA stabilization and degradation [25] to control protein level. Protein-coding genes are generally arranged in long clusters of tens-to-hundreds of genes on the same DNA strand, with no recognizable RNA polymerase II-specific promoters [26, 27]. Polycistronic primary transcripts are converted into translatable monocistronic mRNA by 5′-end trans splicing and 3′-end polyadenylation [28]. Trans-splicing/polyadenylation site skipping is also suggested to be part of a mechanism to control gene expression through which RNAs in a “translational

latency state" can be stored for further processing into a mature transcript when required by the cell [29].

1.3 *T. cruzi* Invasion of Host Cells and Host Immune Response

1.3.1 *T. cruzi* Invasion of Host Cells

T. cruzi invasion of host cells which begins with the attachment of the parasite to the host cell, immediately followed by the recruitment of host cell lysosomes to the site of attachment, where they transiently fuse with the plasma membrane [30-33]. This fusion results in a vacuolar compartment allowing parasite entry [33-37]. Recent evidence indicates that infective *T. cruzi* trypomastigotes trigger transient increases in the $[Ca^{2+}]_i$ of a variety of mammalian cells [38-41], which is required for lysosome-mediated *T. cruzi* entry [38, 42-44]. Ultimately, the parasites escape from the parasitophorous vacuole and, thus liberated, multiply freely in the cytosol as amastigotes.

Although many parasite proteins are likely important for *T. cruzi* invasion of host cells and successful completion of the life cycle, few have been proven experimentally. One such proteins is TC-TOX, a secreted acid-stable hemolytic protein [45]. It has been postulated that TC-TOX is activated by the acidification of the parasitophorous vacuole, and mediates the escape of *T. cruzi* from the parasitophorous vacuole into the cytosol [46]. Another protein suggested to be involved in *T. cruzi* infection is a trypomastigote-secreted peptidyl-prolyl cis or trans isomerase [47], although its specific target on the host cell remains to be elucidated. A third *T. cruzi* protein, which plays an important role in host cell invasion, is oligopeptidase B. It is demonstrated that this enzyme mediated production of a signaling agonist for mammalian cells that is required for efficient invasion and infectivity [48]. A fourth protein, LYT1, is suggested to be required for

efficient *in vitro* infection by a gene knockout study that shows the deletion of the gene encoding this protein result in attenuation of infection, which is associated with diminished hemolytic activity [49].

1.3.2 Host Immune Response Against *T. cruzi*

Given the complex life cycle and the presence of both extra- and intracellular forms of *T. cruzi* in the mammalian host, a wide spectrum of immune responses is involved in the control of *T. cruzi* infection. Strong and consistent antibody responses to *T. cruzi*, capable of cooperating with the complement system, macrophages, or polymorphonuclear leukocytes, have been well documented in infections in most hosts, including humans [50-55].

T. cruzi amastigotes reside in the cytoplasm of infected mammalian cells. Since $CD8^+$ T-cells survey cytoplasm-derived microbial or self-proteins displayed in the context of MHC-class I molecules, it is no surprise that $CD8^+$ T-cells play an important role in control of *T. cruzi* -infected cells. This conclusion was first suggested by the observation that $CD8^+$ T-cells are the major lymphocyte infiltrate in infected tissues [56]. β2-microglobulin deficient mice, which lack stable expression of class I MHC antigens and therefore lack mature $CD8^+$ T-cells and cytotoxic T-cell activity, were found to be highly susceptible to infection and quickly succumbed to death, demonstrating the importance of $CD8^+$ T-cells during the acute phase [57]. Furthermore, MHC class I gene knockout mice succumb to high parasitemias leading to 100% mortality in the acute phase following infection with the Brazil strain of parasite [58]. $CD4^+$ T-cells, though have a limited role in the initial priming of *T. cruzi* -specific $CD8^+$ T-cells [59], were demonstrated to assist in the control of *T. cruzi* through secretion of T-helper type 1

cytokines, enhancement of the phagocytic activity of macrophages, and stimulation of B-cell proliferation and antibody production [60].

1.4 Epidemiology and Infection of Chagas Disease

T. cruzi is the etiological agent of Chagas disease in humans, also known as American trypanosomiasis. The World Health Organization (WHO) estimates that 28 million people in Latin America are at risk of becoming infected [7]. 16-18 million people are infected with *T. cruzi* in Central and South America where Chagas disease is the leading cause of heart failure [61, 62]. Approximately 12, 500 people die each year as a result of the clinical complications of *T. cruzi* -induced heart disease and the lack of effective treatment [63]. Due to continuous population migration from endemic countries into developed countries and increased business and tourism interests, Chagas disease threatens the human populations outside the traditional geographic boundaries [64]. Based on sero-epidemiologic studies, it is estimated that there are at least 100,000 infected individuals living in the United States [65, 66].

Different strains of *T. cruzi* vary in the site and size of inoculation, as well as genetic susceptibility and general health of infected individuals. As a result, infection of *T. cruzi* exhibits a spectrum of disease expressions [67]. The acute phase of *T. cruzi* infection lasts 2-4 months, and is usually characterized by detectable parasites in the blood and widespread tissue parasitism. Death rarely occurs during the initial acute phase of infection, which is usually asymptomatic or mildly symptomatic [68]. A chronic phase then ensues in which circulating parasites are virtually undetectable, but certain tissues, such as muscle and nerve tissues, remain persistently infected by low numbers of parasites for the life of the subject [69]. Most Chagasic patients do not exhibit any severe

disease symptoms, and are thus referred to as "indeterminate." However, over decades after initial infection, 30-40% of infected individuals develop a range of clinical symptoms of Chagas disease such as cardiomyopathy, heart failure, digestive problems, and gut megasyndrome [70, 71].

1.5 Vaccine Development for Chagas Disease

Despite the high risk of *T. cruzi* infection in endemic areas and severity of Chagas disease, vaccination for Chagas disease is unavailable, and the only two approved drugs, nifurtimox and benznidazole, both have serious side effects and are not available to most subjects in endemic countries [72-75]. For a long period of time, Chagas disease was considered an autoimmune disease resulting from inappropriate immune responses to self-antigens that are induced and perhaps perpetuated by *T. cruzi* infection [76-78]. Thus, vaccination against *T. cruzi* infection was proposed to cause exacerbation of the disease. However, parasite DNA, parasite protein and even intact parasites [79] have been detected in the site of disease. In addition, re-infection or continued exposure is found to increase parasite load in addition to disease severity [69, 80, 81]. Furthermore, Chagas disease progresses in the presence of higher parasite burden and is reduced when parasite burden is reduced. These observations support the hypothesis that Chagas disease fails to meet the most crucial criteria for being considered an autoimmune disease, and that the disease is actually a result of the inability to adequately control *T. cruzi* infection without significant cumulative damage. Thus, vaccines that could heighten immune responses at the right location and time to decrease tissue-parasite load would possibly prevent or arrest development of Chagas disease. A cheap, safe, effective and reliable

vaccine will also help alleviate the costs for vector control and drugs used to dispel the infection in endemic countries.

Early vaccine development for Chagas disease focused on utilizing heat-killed or subcellular fractions of *T. cruzi* [82, 83]. These vaccines turned out to be either ineffective or provided only a degree of protection. *T. cruzi* strains attenuated either by drug treatment, or by serial passage in cultures were also tested as live vaccines [84-88]. However, the vaccinated animals are found to lack sterile immunity and develop delayed cardiomyopathy, possibly due to the unclear genetic profile, and possible reversion of these attenuated strains to a virulent form.

With the increased understanding of the immunity to *T. cruzi*, vaccine development studies have been focusing on identification of effective antigen delivery systems, in the hope to elicit potent immune responses against potential subunit vaccine candidates. Some recombinant or purified parasite proteins were demonstrated to provide a substantial control of acute parasitemia [89-91], however, only 40-60% of immunized mice survived lethal challenge. This result is not surprising because although protein vaccines are more suitable to induce antibody responses, they are not efficient in eliciting cell-mediated immunity that is essential for controlling the intracellular stage of *T. cruzi*.

More recent studies found out that immunization with DNA is a superior vaccination strategy not only because of the stability of DNA, as well as the antibodies, Th1 cytokines and $CD8^+$ T-cell responses it elicits [92], but also the construction and production of the vectors is relatively easy. Most of the current vaccine candidates for *T. cruzi* are dominant epitope-targets of specific CTLs, such as trans-sialidase (TS) family members [93, 94], and have afforded protective immunity in multiple, but not all mouse

strains. Furthermore, it has been demonstrated that epitope-dominance patterns vary depending on the infective strain of *T. cruzi* [95]. Attempts to enhance the protective efficacy of TS family members by a DNA prime-protein boost approach were also unsuccessful [96].

Because an effective 'subunit' vaccine remains elusive, and the complexity of *T. cruzi* might preclude the successful development of such a vaccine, there has been renewed interest in whole-organism vaccine approaches using genetically attenuated parasites (GAP) as vaccines [97-100]. The recent availability of complete genome sequences [19] now permits the development of live-attenuated parasites by more precise and defined genetic manipulations [101]. Genetic attenuation disrupts specific genes, creating homogeneously arrested parasites, and it is possible that this genetic arrest best reflects the repertoire of protective antigens and might be superior for the induction of protection in that it is more immunogenic. GAPs could produce a broader spectrum of parasite antigens, some with high binding affinities to MHC class I molecules. Studies with malaria parasites revealed that a GAP with double gene deletions induced protective immunity that was long-lived and linked to MHC class I–dependent, IFN-γ–producing hepatic memory $CD8^+$ T-cells in a mouse malaria model [97]. *T. cruzi* depleted of the calmodulin-ubiquitin (*cub*) gene displayed attenuated virulence and is able to provide protective immunogenicity [102]. A strong protection against a virulent *T. cruzi* challenge was also observed in mice immunized with *T. cruzi lyt1* null mutant parasites [103]. It is thus conceivable that, genes that are involved in processes that are unique and essential to amastigotes could be mutated in epimastigotes, *in vitro* conversion of these epimastigotes to metacyclic trypomastigotes could result in parasites that are unable to

complete development as amastigotes. The dead and dying GAPs would be expected to stimulate immune responses that may provide protective immunity to the host, possibly through the function of memory $CD8^+$ T-cells generated after the antigen is cleared. Then, these GAPs could be superb vaccines offering complete, long-lasting protection to *T. cruzi* infection.

2. Reverse Genetics and Gene Knockout in *T. cruzi*

Reverse genetics is an approach to discovering the function of a gene in an organism by disruption or deletion of the gene from the genome and examine the organism for phenotype or biochemical activity. The first step – disruption or deletion of a gene - is usually accomplished by RNA interference (RNAi), antisense oligonucleotides, or gene knockout through homologous recombination.

RNAi is a mechanism for RNA-guided regulation of gene expression in which double-stranded RNA inhibits the expression of genes with complementary nucleotide sequences [104-106]. The RNAi pathway is initiated by the enzyme dicer, which cleaves double-stranded RNA to short double-stranded fragments of 20–25 base pairs. One of the two strands of each fragment, known as the guide strand, is then incorporated into the RNA-induced silencing complex and base-pairs with complementary sequences. The selective and robust effect of RNAi on gene expression makes it a valuable research tool, in experimental biology to study the function of genes, by inducing suppression of specific genes of interest, in cell culture and *in vivo* in model organisms. Double-stranded RNA is synthesized with a sequence complementary to a gene of interest and introduced into a cell or organism, where it is recognized as exogenous genetic material and

activates the RNAi pathway. Using this mechanism, researchers can cause a drastic decrease in the expression of a targeted gene, also called gene knockdown. However, although RNAi is operational in *T. brucei*, *T. cruzi* and *L. major* do not seem to have the full RNAi machinery [107-109].

Antisense oligodeoxynucleotides are designed to function as inhibitors of specific mRNAs and can be made resistant to nucleases by the introduction of modifications such as phosphorothioate linkages [110]. Antiparasitic activities of antisense oligonucleotides towards obligatory intracellular protozoans have been reported [111]. Antisense oligonucleotides targeted to *gp90* gene sequences was used in *T. cruzi* to determine the effect of this treatment on metacyclic trypomastigote *gp90* expression and on the parasite's ability to enter host cells [112]. Antisense phosphorothioate-modified oligonucleotides was also used to inhibit the expression of phosphoinositide-specific phospholipase C in *T. cruzi* trypomastigotes [113]. However, attempts to use antisense RNA to inhibit trypanothione reductase production in *T. brucei* failed [114]. In addition, using of antisense oligonucleotides is only a means of knocking down a gene without total depleting the expression of its product. More importantly, it will not generate stable avirulent lines and therefore is not a good approach for generation of a safe vaccine.

Homologous recombination is a process of physical rearrangement occurring between two strands of DNA. It can be utilized to delete a gene from the genome and is a general tool to knockout genes. Recombination requires the alignment of similar sequences, a crossover between the aligned DNA strands, and breaking and repair of the DNA to produce an exchange of material between the strands [115]. The process of homologous recombination naturally takes place during chromosomal crossover, which

occurs in meiosis in eukaryotic organisms and results in a shuffling of genetic material. But it is also utilized as a molecular biology technique for introducing genetic changes into organisms [116]. Homologous recombination has been well documented in *T. cruzi* as a means of experimental gene knockout strategy, although some critical genes required for homologous recombination in *Saccharomyces cerevisiae* [117] are notably absent in the trypanosomatids. The *T. cruzi* genes that have been experimentally characterized by reverse genetics, including *dihydroorotate dehydrogenaseare*, *calmodulin-ubiquitin*, *TcGALE*, *gp72*, *LYT1*, *Met-III*, *oligopeptidase B*, *UDP-Glc: glycoprotein glucosyltransferase*, *Tc52* and *TcOYE*, were all knocked out through homologous recombination [48, 49, 118-124].

In *T. cruzi*, a general gene knockout strategy that takes advantage of the homologous recombination machinery involves three major steps. The first step is to construct a DNA cassette that has a selectable marker gene, normally a drug selectable marker, flanked by the 5' and 3' untranslated regions (UTRs) of the target gene. The second step is to transfect this DNA into *T. cruzi*, normally through electroporation, after which homologous recombination occurs between the identical sequences flanking the marker and target genes, resulting in replacement of the target gene by the marker gene. The third step is a 3- to 4-week-long drug selection, at the end of which all the viable parasites should be single knockout parasites with one copy of the target gene replaced by the drug selectable marker. If the target gene has two or more copies, then the above processes must be repeated until both or all copies are replaced by drug selectable markers.

2.1 Conventional Approach

A rate-limiting step for knocking out a gene through homologous recombination is the construction of a DNA cassette that has a drug selectable marker flanked by the UTRs of the target gene. The conventional way to generate this cassette is through multiple Polymerase Chain Reactions (PCRs), restriction digestions and ligations.

Typically, the process starts with a PCR using primers with restriction sites (RSs) to amplify the 5′ and 3′ flanking sequences of the target gene plus the target gene coding sequence (CDS) from genomic DNA. This PCR product is then digested and cloned in a cloning vector previously digested with compatible restriction enzymes to the digested PCR product. A second PCR amplification using specific primers with different RSs enables deletion of the CDS of the target gene, resulting in a linear DNA with the cloning vector backbone plus the flanking regions of the target gene. A drug selectable marker such as Hyg^R (gene encoding hygromycin phosphotransferase) and Neo^R (gene encoding neomycin phosphotransferase), is also obtained by PCR or enzyme restriction digestions from another vector. In many cases for *T. cruzi*, the drug selectable marker is accompanied by 450 basepair (bp) of upstream and 750 bp of downstream sequence from *glyceraldelyde-3-phosphate dehydrogenase* (*gapdh*) to ensure strong expression [125]. This drug selectable cassette is then inserted into the linearized DNA with the cloning vector backbone plus the flanking regions of the target gene, to become the final plasmid. This final plasmid can be amplified in bacteria and subject to a restriction digestion to liberate the linear targeting cassette from the plasmid backbone.

All gene knockout studies in *T. cruzi* have used this conventional approach to construct gene knockout cassettes [48, 49, 118-123]. This approach provides enough gene

specific regions for homologous recombination, and has multiple checkpoints to ensure sequence fidelity. Through bacteria transformation, a large amount of DNA can be obtained for transfection. However, this approach has several disadvantages. Not only does it require intensive genomic DNA mapping to identify multiple compatible restriction sites for cloning, it also involves multiple restriction digestions, ligations, and cloning steps that make the whole process extremely time consuming [126]. A quick and simplified strategy to knockout genes is needed to facilitate reverse genetic studies of *T. cruzi* genes.

2.2 MultiSite Gateway® System Based approach

Gateway® technology (Invitrogen, Carlsbad, CA, USA) provides increased speed, versatility, and efficiency over conventional restriction enzyme-based cloning methods. This technology is based on the well-characterized λ phage site-specific recombination [127]. Any DNA fragment flanked by a pair of recombination sites (attL, attR, attB, and attP) can be transferred into any vector with a corresponding site using Gateway® BP and LR Clonase® II enzyme mixtures that mediate λ recombination reactions. Orientation and reading frame are maintained with high efficiencies - typically >99% - effectively eliminating secondary sequencing or subcloning after the initial entry clone is made. Once a DNA fragment is cloned into a Gateway® vector, it can be shuttled into as many expression and functional analysis systems as needed.

MultiSite Gateway® based cloning for generating gene-knockout constructs is performed in three steps. First, the 5' and 3' flanking region of a target gene are amplified by PCR using primers with specific attB recombination sites. Through a BP reaction with BP clonase enzyme mix and corresponding Entry vectors, the flanking regions are cloned

into two different Entry vectors, respectively. Similarly, a drug selectable marker is also cloned into a third Entry vector. Next, the above three Entry clones are mixed in vitro with the appropriate Gateway® expression vector (Destination vector) and LR Clonase® II enzyme mix. The resulting expression clone is ready for restriction digestion to release the fragment that bears a drug selectable marker flanked by 5' and 3' UTRs of the target gene. This technology enables efficient and convenient assembly of multiple DNA fragments in the desired order and orientation into a Gateway® Expression vector. Primers with specific att sites are easy to design, and BP and LR reactions are robust. Furthermore, the success rate in cloning is high. This method has enabled rapid generation of knockout constructs for *T. cruzi* within a week. Application of this approach to construct gene-targeting vectors in human cell line has allowed for high-efficiency gene targeting [128].

2.3 One-step PCR Approach

The One-step PCR approach is also called Long-Primer PCR, which is first used for gene knockout in fission yeast [129-131]. In this strategy, a pair of primers is designed to amplify a selectable marker. While the 5' forward primer has 30 to 40 nucleotides of homology to the 5' end of the target gene, the 3' reverse primer has 30 to 40 nucleotides of homology to the 3' end of the target gene. Using any DNA that bears the selectable marker as a template, the PCR generates DNA with the desired drug selectable marker flanked by 30 to 40 nucleotides of homology to the target gene as the gene disruption cassette. Because only very small regions of homology between DNA sequences are required to direct homologous recombination in yeast, those 30 to 40 nucleotides are sufficient for homologous recombination and lead to the replacement of

target gene by the selectable marker after transformation into yeast. The efficiency of this technique depends upon two factors. First, the homology of the PCR product to the target gene should be as long as possible, typically 40 nucleotides. Second, this technique works best if the sequences encoding the selectable marker have been completely deleted from the yeast genome, thus minimizing the chances for homologous recombination elsewhere in the genome.

This strategy has also been examined to be successful in *Leishmania* [132] and *T. brucei* [130]. It was found that as short as 42 nucleotides are sufficient for homologous recombination in *T. brucei*, whereas at least 150 nucleotides are needed in *L.tarentolae* to guide homologous recombination. One concern with this technique is that the short gene-specific region in the primers might not be sufficient for homologous recombination, as compared to gene-specific regions composed of several kilo-base-pair nucleotides provided by other approaches. Furthermore, multiple PCR reactions are required to obtain sufficient DNA for transfection, and the long primers are often costly. Nevertheless, this strategy is significantly faster than both the conventional approach and the MS/GW based approach, and is extremely useful for applications in which only limited sequence information of the target is available.

3. Conclusion

Trypanosoma cruzi, a kinetoplastid protozoan parasite that causes Chagas disease in humans, infects approximately 16-18 million people in Central and South America. *T. cruzi* has four morphologically and physiologically distinct stages. Both humoral and cell-mediated immune responses are necessary for the control of *T. cruzi* infection. No

effective vaccines are currently available for the disease, although there is emerging interest in whole-organism vaccine approaches using genetically attenuated parasite as vaccines. In contrast to the substantial *in silico* studies of the *T. cruzi* genome, transcriptome, and proteome, only a few genes have been experimentally characterized, mainly due to the lack of convenient methods for gene manipulation for reverse genetics studies. Current strategies for gene disruption in *T. cruzi* are tedious and a quick and easy strategy to knockout genes is needed. The development of such a strategy will not only facilitate reverse genetic studies of *T. cruzi* genes, but also will facilitate the generation of live attenuated parasites to be tested as experimental vaccines for Chagas disease.

CHAPTER II

DEVELOPMENT OF HIGH-THROUGHPUT GENE KNOCKOUT STRATEGIES IN *TRYPANOSOMA CRUZI* FOR PRODUCTION OF ATTENUATED LINES[1]

[1] Xu, D. and Tarleton, R.L. To be submitted to *Molecular & Biochemical Parasitology*.

1. ABSTRACT

Trypanosoma cruzi, a kinetoplastid protozoan parasite that causes Chagas disease in humans, infects approximately 16-18 million people in Central and South America. In contrast to the substantial *in silico* studies of the *T. cruzi* genome, transcriptome, and proteome, only a few genes have been experimentally characterized, mainly due to the lack of convenient methods for gene manipulation for reverse genetic studies. Current strategies for gene disruption in *T. cruzi* are tedious and a quick and easy strategy to knockout genes is needed. In this study we have compared the conventional multi-step cloning technique with two knockout strategies that have been proven to work in other organisms, One-Step-PCR- and Multisite Gateway-based systems. Our results show that while the One-Step-PCR strategy is faster than the other methods, it does not efficiently target genes of interest and gene-specific sequences of 78 nucleotides are not sufficient to guarantee homologous recombination. The Multisite Gateway® based approach, although not as simple as the One-Step-PCR strategy, is less time-consuming than the conventional method and is able to efficiently delete target genes. Using the Multisite Gateway® strategy, we have successfully generated single- and double- gene knockout parasites with genes that are predicted to be non-essential for epimastigote survival but essential for growth of *T. cruzi* amastigotes. Those knockout parasites are currently being screened for their ability to persist in mice. The results of this study might also facilitate reverse genetic studies of *T. cruzi* genes, but also will provide valuable contribution to the development of live attenuated parasites as experimental vaccines for Chagas disease.

2. INTRODUCTION

Trypanosoma cruzi is a protozoan parasite and the etiological agent of Chagas disease in humans, also known as American trypanosomiasis. *T. cruzi* is transmitted through the feces of triatomine insect-vectors to over 100 species of mammalian hosts, including rodents, sloths, armadillos, marsupials, dogs, and primates. 16-18 million people are infected with *T. cruzi* in Central and South America where Chagas disease is the leading cause of heart failure [61]. Approximately 12, 500 people die each year as a result of the clinical complications of *T. cruzi* -induced heart disease and the lack of effective treatment [7].

T. cruzi has four morphologically and physiologically distinct stages. In the insect vector, the flagellated epimastigote proliferates in the midgut before differentiating into the non-dividing metacyclic trypomastigote found in the hindgut of the insect. Upon introduction to the blood stream of the mammalian host, the metacyclic trypomastigote infects cells and differentiates into a rapidly dividing amastigote. The amastigote ultimately differentiates into the blood stream trypomastigote which can either infect other cells or be taken up by the insect vector thus completing the parasite's life cycle [1].

T. cruzi strain CL Brener was chosen for genome sequencing. The diploid genome of *T. cruzi* has a size between 106.4-110.7 Mb, with at least 30 chromosomes ranging from 0.45 to 3.5 Mb [18]. The genome contains a predicted 22,570 proteins encoded by genes, of which 12,570 represent allelic pairs, and allelic copies of genes may vary in sequence by about 1.5% [19]. Trisomy also occurs in the case of some chromosomes [17, 20]. Putative function could be assigned to 50.8% of the predicted protein-coding genes

on the basis of significant similarity to previously characterized proteins or known functional domains [19].

In contrast to the substantial *in silico* studies of the *T. cruzi* genome, only 9 of *T. cruzi* genes are experimentally characterized by reverse genetics, which include *dihydroorotate dehydrogenaseare*, *calmodulin-ubiquitin*, *TcGALE*, *gp72*, *LYT1*, *Met-III*, *oligopeptidase B*, *Tc52* and *TcOYE* [48, 49, 118-123]. These genes were all disrupted through homologous recombination, using a DNA cassette that has a drug selectable marker flanked by the untranslated regions (UTRs) of the target gene. The conventional way to generate this cassette is through multiple polymerase chain reactions (PCRs), restriction digestions and ligations. Not only is identification of multiple compatible restriction sites for ligation reactions and for vector linearization required, this conventional approach also involves multiple restriction digestions, ligations, and cloning steps that make the whole process time-consuming and complicated. A quick and simplified strategy to knockout genes is needed.

A wide spectrum of immune responses, including both humoral and cell-mediated immune responses are necessary for the control of *T. cruzi* infection. Disruption of either B-cell or T-cell function during the acute or chronic phase of *T. cruzi* infection can result in eventual failure to control infection. Studies in mice lacking T-cell subpopulations due to antibody treatment, mutations in the class II MHC, or β2-microglobulin genes have established that both $CD4^+$ and $CD8^+$ T-cells are required for control of *T. cruzi* infection [58, 133]. When antigen-specific $CD8^+$ T-cells detect their cognate *T. cruzi* -derived antigen in the context of MHC-I, they are induced to perform their effector function,

which may include the secretion of interferon gamma (IFN-γ) and the killing of target cells [134].

In part because of the wide-spectrum of immune responses involved in controlling the infection and the complexity of *T. cruzi* itself, a vaccine for Chagas disease is currently unavailable. There has been renewed interest in whole-organism vaccine approaches using genetically attenuated parasite (GAP) as vaccines. Through specific gene disruption, GAPs can be generated as homogeneously arrested parasites, while keeping the repertoire of antigens as in the WT parasites. It is thus conceivable that, if genes that are involved in processes that are unique and essential to amastigotes are deleted in *T. cruzi* epimastigotes, the epimastigotes can be converted to metacyclic trypomastigotes *in vitro*, and used to infect animals. Those GAPs will convert to amastigotes but would not able to develop further. These arrested and dying GAPs would be expected to stimulate immune responses that provide protection to the host, possibly through the function of memory $CD8^+$ T-cells generated after the antigen is cleared. These GAPs have the potential to be superb vaccines offering complete, long-lasting protection to *T. cruzi* infection.

In order to facilitate the generation of GAPs for *T. cruzi* vaccine development, and to develop an easy-to-use tool for depletion of *T. cruzi* genes for reverse genetics studies, in this study, we compared the conventional multi-step knockout technique with two knockout strategies that have been proven to work in other organisms, One-Step-PCR- and Multisite Gateway® (MS/GW) -based systems. We attempted to knockout the Dihydrofolate Reductase-Thymidylate Synthase (*DHFR-ts*) and Enoyl-CoA Hydratase (*ECH*) genes using One-Step-PCRbased system, while employing Multisite Gateway-

based system to knockout the *ECH1* and *ECH2* genes. Our results show that while gene-specific sequences of 78 nucleotides used in One-Step-PCR strategy are not sufficient to guarantee homologous recombination, the Multisite Gateway® based approach is able to efficiently disrupt target genes. The results of this study will not only provide a powerful tool for reverse genetic studies of *T. cruzi*, but also will facilitate the development of live attenuated parasites to be tested as experimental vaccines for Chagas disease.

3. MATERIALS AND METHODS

3.1 Culturing, transfection and cloning of *T. cruzi*

The CL and TCC stocks of *T. cruzi* epimastigotes were cultured at 26^{o}C in supplemented liver digest-neutralized tryptose (LDNT) medium as described previously [135]. Early-log cultures containing 1×10^6 to 2×10^6 parasites/ml, were used for electroporation using the Human T Cell Nucleofector Kit (Amaxa AG, Cologne, Germany). Briefly, a total of 1×10^7 early-log epimastigotes were centrifuged at 3,000 rpm for 15 min and resuspended in 100 μl room temperature Nucleofector^{TM} Solution. The resuspended parasites were then mixed with 3-20 μg DNA (3-10 μg of each DNA construct generated with One-Step PCR strategy and 5-15 μg of each KO construct based on MS/GW system, in a total volume of 5-10 μl) and transferred to an AMAXA compatible cuvette. The cuvette was placed into the cuvette holder in the Nucleofector Device and electroporated using program "U-33". The electroporated parasites were then transferred into a 25cm^2 cell culture flask (Corning Incorporated, Lowell, MA, USA) with 10 ml LDNT medium. 250 μg/ml G418 (geneticin, for transfectants with *Neo*^{R}-containing cassette) and/or 600 μg/ml Hyg (Hygromycin B, for transfectants with *Hyg*^{R}-

containing cassette) was added at 24 hrs post-transfection. For single-cell cloning, drug selected lines were deposited into a 96-well plate to a density of 1 cell/well using a MoFlow (Dako-Cytomation, Denmark) cell sorter and cultured in 250 μl LDNT supplemented with G418 and/or Hyg. Each population from an individual well was considered an individual clone.

3.2 Construction of knockout DNA cassettes based on MS/GW strategy

All plasmids were constructed based on MS/GW system using commercially available MultiSite Gateway® Three-Fragment Vector Construction kit (Invitrogen, Carlsbad, CA, USA), which includes all the Entry vectors and Destination vectors used in this study.

3.2.1 pDEST/ECH_Neo-GAPDH and pDEST/ECH_Hyg-GAPDH

To construct the pDEST/ECH_Neo-GAPDH plasmid, 1.0-kb 5' sequence of *ECH2* was amplified with primers attB4_ECH5'UTR_f and attB1_ECH5'UTR_r (Table 1) from the genomic DNA (gDNA) of WT CL using Platinum® PCR SuperMix (Invitrogen), gel purified with QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and cloned into the Entry vector pDONR^{TM}P4-P1R through a BP reaction using the BP Clonase II Enzyme Mix (Invitrogen), resulting in the Entry clone pDONR-ECH5'UTR. Similarly, 1.0-kb 3' sequence of *ECH1* was amplified with primers attB2_ECH3'UTR_f and attB3_ECH3'UTR_r (Table 1) and cloned into pDONR^{TM}P2R-P3 to generate pDONR-ECH3'UTR. Using plasmid pTrex-YFP as a template, the *Neo^{R}* and its downstream *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) 3'UTR was amplified with primers attB1_Neo_f and attB2_Neo_r (Table 1) and cloned into Entry vector pDONR^{TM}221. The three Entry clones were then mixed with a Destination vector

pDEST^{TM}R4-R3 to generate pDEST/ECH_Neo-GAPDH through a LR reaction. The product of the LR reaction was then transformed into DH5α competent cells. Colonies were inoculated into 3 ml LB and grown overnight, followed by a miniprep with the GenElute™ HP Plasmid Miniprep Kit (Sigma-Aldrich Corporation, St. Louis, MO, USA) to extract plasmid DNA. The plasmid DNA was screened through restriction digestions and selected clones were sequenced. Based on the sequencing report, Positive colonies was amplified by liquid culture and plasmids were grown in bacteria and purified with GenElute™ Endotoxin-free Plasmid Midiprep or Megaprep Kit (Sigma-Aldrich Corporation, St. Louis, MO, USA). The KO DNA cassette was liberated from the Gateway® plasmid backbone, purified with QIAquick Gel Extraction Kit, and resuspended in water to a final concentration of 1-2 μg/μl. 10 μg DNA was used for each transfection.

Similarly, to construct pDEST/ECH_Hyg-GAPDH, *Hyg*^{R} and its downstream intergenic region of *GAPDH* (*GAPDH-IR*) was amplified from plasmid pTEX-Hyg.mcs using primers attB1_Hyg_f and attB2_Hyg_r (Table 1) and cloned into Entry vector pDONR^{TM}221. This Entry clone was then mixed with pDONR-ECH5'UTR, pDONR-ECH3'UTR and a Destination vector pDEST^{TM}R4-R3 to generate plasmid pDEST/ECH_Hyg-GAPDH (Figure 1A) through a LR reaction. The final plasmid was digested with restriction enzymes Pvu II and Pci I. The gel-purified 4.0-kb KO DNA cassette was ethanol precipitated and resuspended to 1-2 μg/μl for transfection.

3.2.2 pDEST/ECH2_Hyg and pDEST/ECH2_Neo

With gDNA of WT CL as the PCR template, 316 bp 5'UTR of *ECH2* was amplified using primers attB4_ECH2H5'_f and attB1_ECH2_5'_r, and 378 bp of *ECH2* 3'UTR

amplified with attB2_ECH2_3'_f and attB2_ECH2E3's_r, respectively. *Hyg*^{R} was amplified from plasmid pTEX-Hyg.mcs with primers attB1_KHyg_f and attB2_1F8Hyg_f, whereas *Neo*^{R} was amplified from plasmid pTrex-YFP with primers attB1_KNeo_f and attB2_1F8Neo_r. The PCR products were cloned into corresponding Entry clones to obtain pDONR/ECH2 5'UTR, pDONR/ECH2 3'UTR, pDONR/KHygCDS, and pDONR/KNeoCDS respectively. pDONR/ECH2 5'UTR, pDONR/ECH2 3'UTR and pDONR/KHygCDS were mixed with pDEST^{TM}R4-R3 and LR Clonase^{TM} II Plus to yield pDEST/ECH2_Hyg, whereas pDONR/ECH2 5'UTR, pDONR/ECH2 3'UTR and pDONR/KNeoCDS were cloned into pDEST^{TM}R4-R3 through a LR reaction to yield pDEST/ECH2_Neo. The final plasmids were digested with restriction enzymes Hind III and EcoR V. The gel-purified 1.79-kb knockout DNA cassette from digested pDEST/ECH2_Hyg and1.55-kb knockout DNA cassette from digested pDEST/ECH2_Neo were ethanol precipitated and resuspended to 1-2 μg/μl for transfection.

3.3 Construction of knockout DNA cassettes via One-Step-PCR

Primers LP_ECH_Neo_f and LP_ECH_Neo_r (Table 2) were used to amplify the *Neo*^{R} from plasmid pTrex-YFP, while primers LP_ECH_Hyg_f and LP_ECH_Hyg_r (Table 2) were used to amplify the *Hyg*^{R} from plasmid pTEX-Hyg.mcs.

Neo^R was amplified with primers LP_dhfr_Neo_f and LP_dhfr_Neo_r (Table 2) from plasmid pTrex-YFP.

PCR reactions were carried out as follows: initial denaturation at 94^{o}C for 3 min followed by 30 cycles of: 98^{o}C for 20s; 55^{o}C for 30s; and 72^{o}C for 2 min followed by 72^{o}C for 10 min using Gradient Master Thermocycler (Eppendorf, Westbury, NY, USA).

Products were collected and purified with QIAquick PCR Purification Kit. The eluted DNA was further ethanol precipitated and resuspended to 0.2-1 μg/μl. 3-10 μg DNA was used for each transfection.

3.4 Southern blot

For Southern blot analysis, gDNA was purified using Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA), digested with Ban I or EcoR I, and analyzed by 0.7% agarose gel electrophoresis. Gels were blotted onto nylon membranes (Hybond-N 0.45-mm-pore-size filters; Amersham Life Science) using standard methods [136]. For probe generation, a 1030 bp DNA (*Hyg*^{R}) was amplified using primers Hyg_f and Hyg_r (Table 4) from plasmid pTEX-Hyg.mcs. *ECH1* gene was amplified using primers ECH1_pb_f and ECH1_pb_r (Table 4) from gDNA of WT CL. The PCR products were purified with QIAquick PCR Purification Kit. Labeling of the probe and DNA hybridization were performed according to the protocol supplied with the PCR-DIG DNA-labeling and chemiluminescence detection kit (Roche Applied Science, Indianapolis, IN, USA).

3.5 Parasite growth measurements

All strains were cultured in 5-ml LDNT with antibiotics (G418 and/or Hyg) in 25cm^2 cell culture flasks and quantified daily for 10 days using a hemocytometer cell counting chamber. Each 5-ml culture was inoculated mid-log epimastigotes to give an initial density of approximately 1×10^6 parasites/ml and cultured in a 25cm^2 cell culture flask. A student T-test was used to determine statistical significance.

3.6 Infection of cell cultures

Metacyclic trypomastigotes were obtained from stationary phase LDNT cultures by complement lysis selection [137]. Vero cells (continuous cell line derived from a normal African green monkey kidney) were grown at 37°C in RPMI with 10% (vol/vol) FBS (fetal bovine serum). For infection, 10^5 Vero cells were transferred into each well of a Lab-Tek 8-well chamber slide (Nunc, Rochester, NY, USA) and cultured over night at 37^oC. 8×10^5 metacyclic trypomastigotes were added to the Vero cell culture and cultured for 24 hrs, after which the culture was washed with PBS to remove non-adherent parasites and fresh RPMI supplemented with 10% FBS was added. After 3 days of culture, slides were separated and Dif-quick stained. Numbers of amastigotes and Vero cells were counted using an oil-immersion objective lens of an Olympus BX 40 microscope (Olympus Inc., Center Valley, PA, USA). A student T-test was used to determine statistical significance.

3.7 Real-time Quantitative PCR

Genomic DNA from WT CL and clone 4 of *ECH1*$^{+/-}$*ECH2*$^{-/-}$ were serial diluted to 15, 1.5, 0.15, 0.015 and 0.0015 ng/µl and used as templates for standard curve calculation. 0.15 ng/µl gDNA was also used as an unknown sample. Each reaction mixture consisted of 2 µl of template DNA, 1 µl each of forward (5 µM) and reverse primers (5 µM), 6 µl of nuclease-free water and 10 µl of SYBR Green PCR Master Mix (Bio-rad, Hercules, CA, USA) in a total reaction volume of 20 µl (96-well optical plates). The reactions were performed in duplicate for each sample in an iQ5 real-time PCR detection system (Bio-rad). The thermal cycler program consisted of a denaturation for 10 min at 95°C, followed by 50 cycles of : 95°C for 6 s; 54°C for 6 s; and 72°C for 15 s followed by 95°C

for 1 min. For each sample, the amounts of DNA for *ECH2* were analyzed and calculated from *ECH2* standard curve.

4. RESULTS

4.1 Consecutive *ECH1* and *ECH2* genes are simultaneously replaced by a construct generated based on MS/GW system

Trypanosoma cruzi ECH1 and *ECH2* are tandemly arranged genes (Figure 1A) with a DNA identity of 72%. They both encode for putative enoyl-CoA hydratase/isomerase family proteins, which catalyze the second step in the physiologically important beta-oxidation pathway of fatty acid metabolism. *T. cruzi* proteome studies have detected high expression level of both genes in amastigote stage only [8]. Analysis of the *T. cruzi* proteome also demonstrated the up-regulation of enzymes in the fatty acid oxidation pathway in *T. cruzi* amastigotes, suggesting that amastigotes probably require fatty acid metabolism for energy generation. Thus, we hypothesize that the gene products of *ECH1* and *ECH2* are essential to amastigote stage but not for other stages of *T. cruzi*. Therefore, our initial plan was to knockout both *ECH1* and *ECH2* genes in the epimastigote stage.

Using MS/GW system, 10 colonies from each BP and LR reactions were screened and at least 9 colonies were demonstrated to be positive colonies. The plasmid from one positive colony generated from the final LR reaction was named pDEST/ECH-Hyg-GAPDH (Figure 1B). Transfection of linearized DNA from pDEST/ECH-Hyg-GAPDH resulted in parasite lines that were resistant to Hyg selection. Figure 1A shows the expected genomic loci of *ECH* and Hyg^R-*GAPDH-IR* in the genome of *ECH+/-/*Hyg^R parasites. PCR analysis with the genomic DNA from the drug resistant parasites and WT

CL confirmed the expected gene replacement of *ECH1* and *ECH2* genes by *Hyg*^{R}-*GAPDH-IR* (Figure 1C). No product was obtained when using WT CL gDNA as the template with primer combinations f2 and D, f2 and F, C and r2, and E and r2, whereas products of expected size were observed with gDNA from *ECH*$^{+/-}$*/Hyg*^{R} as the template. There was no contamination of undigested plasmid in the *ECH*$^{+/-}$*/Hyg*^{R} parasites, as PCR with primers that only amplify the circular plasmid did not yield any product (data not shown). Southern blot with EcoR I digested gDNA using *ECH1* gene as a probe (Figure 1 A and D) revealed that a 4.9 Kb band that correspond to the replaced allelic copy of both *ECH*s was undetected in *ECH*$^{+/-}$*/Hyg*^{R}. In addition, a 3.0 Kb band and a 1.5 Kb band corresponding to the inserted *Hyg*^{R}*-GAPDH-IR* were only observed in Ban I digested gDNA of *ECH*$^{+/-}$*/Hyg*^{R}, but not that of WT CL. Taken together, these results confirmed that both *ECH1* and *ECH2* genes are replaced by the *Hyg*^{R}*-GAPDH-IR* knockout cassette.

Similarly, using linearized DNA from pDEST/ECH_Neo-GAPDH (Figure 2A), we also generated knockout parasites *ECH*$^{+/-}$*/Neo*^{R} with one copy of both *ECH1* and *ECH2* gene replaced by *Neo-GAPDH-3'UTR* knockout cassette (Figure 2B). This result is confirmed by both PCR amplification (Figure 2C) and Southern blot hybridization (Figure 2D) of gDNA of the drug resistant parasites. However, the attempts to obtain parasites in which both copies of *ECH1* and *ECH2* were deleted were unsuccessful, when *ECH*$^{+/-}$*/Hyg*^{R} was transfected with linearized DNA from pDEST/ECH_Neo-GAPDH, or when WT CL was co-transfected with both linearized DNA from pDEST/ECH_Hyg-GAPDH and pDEST/ECH_Neo-GAPDH. This result suggests that deletion of both copies of both *ECH* genes is lethal in epimastigotes. Subsequent transcriptome analysis of WT *T. cruzi* (Minning *et al*, data unpublished) revealed that whereas the transcript for

ECH2 was observed to be upregulated in the amastigote stage, *ECH1* was detected to be most abundant in the epimastigote stage (Figure 2E). These data might explain why deletion of both *ECH1* copies is lethal in *T. cruzi* epimastigotes.

4.2 *ECH1*$^{+/-}$*ECH2*$^{-/-}$ parasites are generated based on MS/GW system

Since the attempt to generate *ECH1*$^{-/-}$*ECH2*$^{-/-}$ parasites was not successful, we next attempted to delete both copies of the *ECH2* gene, while leaving at least one copy of *ECH1* gene intact. Therefore, *ECH*$^{+/-}$*/Neo*^{R} parasites were transfected with the knockout cassette liberated from digested pDEST/ECH2_Hyg (Figure 3A). Dual (G418 and Hyg) drug resistant parasites were obtained, cloned and analyzed by PCR based upon the expected map of *ECH1*, *Neo-GAPDH-3'UTR* and *Hyg*^{R} genomic loci in *ECH1*$^{+/-}$*ECH2*$^{-/-}$ parasites as depicted in Figure 3B. PCR using any combination of primers that only amplifies *Hyg*^{R} or *Neo*^{R} yielded the expected products in the drug resistant population (both cloned and uncloned) but not in controls (Figure 3C); no products were generated in neither cloned nor uncloned drug resistant population with primers M13_f and M13_r (Figure 3C), which are only expected to amplify circular plasmids pDEST/ECH_Neo-GAPDH and pDEST/ECH2_Hyg, indicating no contamination of plasmid in the knockout parasites. Therefore, the targeting of *Hyg*^{R} construct to the *ECH2* locus is successful, the second transfection depleted the second allelic copy of *ECH2* gene, and the dual drug resistant line is *ECH1*$^{+/-}$*ECH2*$^{-/-}$.

However, gDNA of parasites from both uncloned *ECH1*$^{+/-}$*ECH2*$^{-/-}$ and a clone of *ECH1*$^{+/-}$*ECH2*$^{-/-}$ (clone 4) was amplified with primers that are specific to *ECH2.* The resulting PCR product is about 1.0 Kb, which is the same size as that of WT parasites (Figure 3D), indicating the existence of another copy of *ECH2* in *ECH1*$^{+/-}$*ECH2*$^{-/-}$. This

is further supported by our preliminary real-time quantitative PCR results. Using primers 170_f and 170_r (Table 3), which are specific for *ECH2* gene amplification, the PCR amplified genomic DNA from both WT CL and clone 4 of *ECH1*$^{+/-}$*ECH2*$^{-/-}$, respectively. The copy number of *ECH2* in WT CL is calculated to be approximately twice of that in clone 4 of *ECH1*$^{+/-}$*ECH2*$^{-/-}$ (data not shown), indicating that an extra copy of *ECH2* gene is present in clone 4 of *ECH1*$^{+/-}$*ECH2*$^{-/-}$ parasites.

4.3 Two allelic copies of ECH2 gene are replaced by the MS/GW approach

A second way to delete both copies of the *ECH2* gene, while leaving at least one copy of *ECH1* gene intact is to knockout both copies of *ECH2* gene to create *ECH2* double knockout parasites (*ECH2*$^{-/-}$). In order to generate *ECH2*$^{-/-}$, plasmids pDEST/ECH2_Neo (Figure 4A) and pDEST/ECH2_Hyg were both linearized and co-transfected into WT CL. The expected genomic loci of *ECH1*, *Neo*^{R} and *Hyg*^{R} in *ECH2*$^{-/-}$ is illustrated in Figure 4B. PCR analysis revealed that the gDNA from dual drug resistant parasites was amplified by primers that only anneal to the *Neo*^{R} or *Hyg*^{R} gene, and that the sizes of products were as expected for *ECH2*$^{-/-}$ (Figure 4C). Thus, two allelic copies of *ECH2* gene are replaced by drug selectable markers constructed based on the MS/GW system. Notably, PCR of the drug resistant parasites using primers that only amplify *ECH2* specifically also generated a product of 1.0 Kb, which is the same size as expected from WT parasites (Figure 3D).

4.4 No knockout parasites are obtained with constructs produced using One-Step-PCR strategy to delete *ECH* genes and *DHFR-ts* gene

Since we demonstrated that at least one allele of *ECH1* and *ECH2* gene can be simultaneously deleted using the MS/GW based system, we next tested the One-Step-

PCR strategy for targeting the deletion of *ECH1* and *ECH2* genes. However, the transfection and drug selection of either LP-ECH-Neo or LP-ECH-Hyg individually or simultaneously into WT CL did not yield any drug resistant parasites (Figure 5A). Numerous repeated attempts of the transfection and selection also failed.

As there are 78 nts of the CDS of *ECH2* gene in both forward long primers used to produce LP-ECH-Neo and LP-ECH-Hyg, the drug selectable markers were to be expressed as a fusion protein, with 26 amino acids of the start of *ECH2* gene fused at the N terminal. It is possible that the knockout parasites were not achieved because the drug selectable marker has reduced enzyme activity when expressed as a fusion protein. To evaluate this possibility, as well as to determine if this long-primer strategy might be effective for use with genes other than *ECH*, we constructed LP-DHFR-ts-UTR-Neo to knockout DHFR-ts gene in *T. cruzi* TCC strain. *DHFR-ts* has been shown to be non-essential to epimastigotes of both Tulahuen and TCC strain of *T. cruzi*, as the knockout of this gene has been obtained by both conventional strategy and MS/GW strategy by Perez et al (data unpublished). The knockout construct LP-DHFR-ts-UTR-Neo has 78 nts of the UTRs of *DHFR-ts* at both ends (Figure 5B). Transfection and selection of parasites with the knockout cassette LP-DHFR-ts-UTR-Neo failed to yield drug resistant parasites, despite numerous attempts. Therefore, the constructs generated with One-Step-PCR strategy that bear 78 nts gene CDS or UTR specific sequence are likely to be insufficient for homologous recombination in *T. cruzi*.

4.5 *ECH2*$^{-/-}$ Epimastigotes are not defective in growth while metacyclic trypomastigotes exhibit an attenuated ability for host cell infection

Since both proteome and transcriptome study agree that *ECH2* gene is undetectable in epimastigote stage, the growth of *ECH2*$^{-/-}$ epimastigotes should not be strongly affected. This is indeed the case, as the growth curve of epimastigotes of *ECH2*$^{-/-}$ and WT CL are comparable (Figure 6A). In contrast, *ECH1*$^{+/-}$*ECH2*$^{-/-}$ epimastigotes exhibit a modest but statistically significant defect in growth (Figure 6A). Thus, these results suggest that the deletion of one copy of *ECH1* gene already has consequences for growth of epimastigote, which is consistent to the fact that attempts to obtain *ECH1*$^{-/-}$ *ECH2*$^{-/-}$ was unsuccessful. We hypothesized that the amastigotes of *ECH2*$^{-/-}$ parasites should display a defect in the ability to infect host cells and to replicate inside host cells. To test this hypothesis, purified metacyclic trypomastigotes obtained from stationary phase cultures of epimastigotes were used to infect Vero cells. A significant difference was observed not only in the invasive capability of *ECH2*$^{-/-}$ compared with WT, but also in the replication ability of *ECH2*$^{-/-}$, as less Vero cells were infected, and there are less amastigotes within each infected Vero cells (Figure 6B-6D).

5. DISCUSSION

In this study, we have compared the conventional multi-step cloning technique with two knockout strategies that have been proven to target gene deletion in other organisms, One-Step-PCR- and MultiSite Gateway-based system. We found that although the One-Step-PCR- strategy is significantly faster than the other methods, and in addition, required little genomic DNA mapping, the attempts to knockout either *ECH* and

DHFR-ts genes was not successful. No drug resistant parasites were obtained after transfection with knockout DNA cassette generated through One-Step-PCR strategy, regardless of whether the drug selectable marker was expressed as a fusion protein or not. Therefore, we conclude that the 78 nucleotides gene-specific regions used in our constructs were insufficient for homologous recombination in *T. cruzi*. This is not surprising as study in *Leishmania* [132] demonstrated that at least 150 nucleotides are needed in *Leishmania tarentolae* to guide homologous recombination. Since nucleotides over than 100 nts are costly, it is not economical to generate longer primers to use in this strategy. It is possible that two primers each with less than 100 nts can be combined to amplify the drug selectable marker gene with two consecutive PCR reactions, so as to yield longer gene-specific region for homologous recombination. However, this approach requires more time to accomplish than the original One-Step-PCR protocol, and has more chance of getting PCR artifacts.

Other studies have used fusion PCR strategy to generate KO cassettes [138], in which three separate PCR reactions and a final fusion PCR are required. This method is simple, rapid and relatively inexpensive. In addition, there is the freedom of attaching long flanking regions to any selectable marker cassette. However, all primers should be designed to have almost the same Tm value, which is crucial for annealing and amplification of the final PCR product in fusion PCR. Furthermore, high fidelity DNA polymerase is required, and the purification of the three PCR products should be done without any contamination of the primers.

The MultiSite Gateway® based approach, although not as simple as the One-Step-PCR strategy, is far less time-consuming than the conventional method. Once

products are obtained from the *att*- PCR amplifications, the knockout DNA constructs can be generated within as few as five days. The reactions involved are robust and have very high success rate. Typically, at least 9 colonies are screened to be positive from a total of 10 colonies from BP and LR reactions. Using the knockout constructs, we successfully obtained *ECH*$^{+/-}$, *ECH2*$^{+/-}$ and *ECH2*$^{-/-}$ parasites, proving that constructs generated based on the MultiSite Gateway® system is able to efficiently target genes and result in the replace of target gene by drug selectable markers. In addition, *DHFR-ts* gene and a gene encoding a putative protein (*G6PT*) were also successfully knocked out using this MultiSite Gateway® based approach (data not shown).

Notably, in *ECH1*$^{+/-}$*ECH2*$^{-/-}$ and *ECH2*$^{-/-}$ parasites, although both drug selectable marker genes are inserted into the *ECH* locus in the genome, *ECH2* gene can still be amplified from the genomic DNA of these parasites using *ECH2* specific primers. This amplification is found in both uncloned population and a clone of *ECH1*$^{+/-}$*ECH2*$^{-/-}$ (Figure 3). One reason of the amplification of *ECH2* gene in *ECH1*$^{+/-}$*ECH2*$^{-/-}$ and *ECH2*$^{-/-}$ parasites is probably that *ECH2* gene is located in one of those chromosomes that have three allelic copies, and the third copy still exists in the *ECH1*$^{+/-}$*ECH2*$^{-/-}$ and *ECH2*$^{-/-}$ parasites. Alternatively, it is possible that, although the WT parasites only have two copies of *ECH2*, an extra copy is generated in some drug resistant parasites. In fact, our preliminary real-time quantitative PCR results reveal that the copy number of *ECH2* gene in WT CL is approximately twice of that in the clone of *ECH1*$^{+/-}$*ECH2*$^{-/-}$, suggesting the latter to be more likely. Nevertheless, it is possible that no copy of *ECH2* exists in some other clones, and screening for more individual clones might enable us to prove this possibility.

Previous studies have demonstrated that *T. cruzi* virtually lack mechanisms to control gene expression at the transcriptional level, and primarily control protein synthesis by post-transcriptional regulation processes [24], such as mRNA stabilization and degradation [25]. Protein-coding genes are generally arranged in long clusters of tens-to-hundreds of genes on the same DNA strand, and all mRNAs are modified post-transcriptionally by the addition of a spliced leader RNA to their 5'-ends [26, 27]. To ensure the expression of drug selectable markers after they are integrated into *T. cruzi* genome, the UTR or intergenic region of a gene that is constitutively expressed in all stages of *T. cruzi* is usually cloned into the downstream of the drug selectable marker to provide signals for RNA processing and stability. *GAPDH* is one of those constitutively expressed genes [139]. With One-Step-PCR strategy, we generated knockout DNA cassettes to knockout the Aspartate Amino Transferase gene (*AsAT*) in a way that the coding sequence of drug selectable marker is upstream of a 700 nts *GAPDH* intergenic region (*GAPDH IR*). Transfection of this DNA into WT CL produced parasites that survived drug selection (Xu *et al*, unpublished). However, inverse PCR analysis [140] revealed that, in these drug resistant parasites, the drug selectable marker gene is inserted into the *GAPDH IR* of *T. cruzi* genome while the *AsAT* gene locus is left intact. This result suggests that the 700 bp *GAPDH IR* guided the homologous recombination in the *GAPDH* locus instead of the AsAT locus and enabled the expression of the drug selectable marker. This result is not surprising because previous studies have reported that the solely presence of an 810 bp a ribosomal promoter sequence leads to the integration of pRIBOTEX and pTREX vectors into the ribosomal locus [141]. Therefore, when designing knockout constructs, one should be aware that the integration of *GAPDH*

UTR or *GAPDH IR* might compete with the gene specific region in the knockout construct for homologous recombination with the genomic DNA.

In our study, the constructs used to generate *ECH2*$^{-/-}$ have neither *GAPDH UTR* nor *GAPDH IR* downstream of the drug selectable markers. This indicates that the signal for RNA stability and processing provided by the regulation factors of *ECH2* is sufficient for the drug resistance gene at the *ECH2* locus to be expressed at a level that can confer drug resistance, even though *ECH2* gene is indicated to have a low level of expression in epimastigote stage. Therefore, a *GAPDH UTR* or *GAPDH IR* might not be required for the expression of a drug selectable marker, even when the target gene has low expression level in epimastigote stage. Hence, when the length of target-gene specific region is less than that of *GAPDH UTR* or *GAPDH IR*, one might consider not including the *GAPDH UTR* or *GAPDH IR*, in order to eliminate the interference of homologous recombination guided by those sequences.

Applying this MS/GW based gene knockout strategy to target deletion of other genes will not only facilitate reverse genetic study of *T. cruzi* genes, but also contribute to vaccine studies of Chagas disease. There has been renewed interest in whole-organism vaccine approaches using genetically attenuated parasite as vaccines [97, 99, 100, 142]. Genetic attenuation disrupts specific genes, creating homogeneously arrested parasites, potentially expressing the full repertoire of protective antigens. Live parasites might also be superior for the induction of protection in that they are more immunogenic as compared to subunit vaccines. It is conceivable that, if genes that are involved in processes that are unique and essential to amastigotes are deleted in *T. cruzi* epimastigotes, the resulting mutant parasites can be converted to metacyclic

trypomastigotes *in vitro*, and used to vaccine hosts. These knockout parasites would be expected to convert to amastigotes, but be unable to develop further, and eventually die in the host cell. The stimulation of a wide spectrum of immune responses by the defective parasites might optimally protect the host.

In fact, the *ECH2*$^{-/-}$ parasites that we generated based on MultiSite Gateway® system, according to our results, already exhibit attenuated ability to infect host Vero cells and are defective to replicate inside the host cells, as compared to WT parasites (Figure 6). This result is in agreement with our hypothesis that the fatty acid metabolism that the ECH gene products are involved in is important for *T. cruzi* amastigotes. The *in vivo* infectivity study of this parasite line is being carried out in animal model and our preliminary data suggests that the infected C56BL/6 mice display a loss of parasite load during the acute phase of infection. Our future study will be continuing the *in vivo* infectivity study, testing if parasite load is eventually cleared in the infected mice, evaluating whether the infection with this parasite line is able to provide protection to challenge of virulent parasite, and investigating if this protection is related to the memory function of $CD8^+$ T cells generated in the host mice.

Table 1. Oligonucleotides for generation of knockout constructs based on MS/GW system.

Name	Sequence
attB4_ECH5'UTR_f	GGGGACAACTTTGTATAGAAAAGTTGTTGCTCGCCTTGTTCGAA
attB1_ECH5'UTR_r	GGGGACTGCTTTTTTGTACAAACTTGTACTGGAAGAAGAAGAAAA
attB2_ECH3'UTR_f	GGGGACAGCTTTCTTGTACAAAGTGGTTGGGACATTCTTTATTT
attB3_ECH3'UTR_r	GGGGACAACTTTGTATAATAAAGTTGTTCCTGCCGCCGCGGTAA
attB1_Neo_f	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGGATCGGCCATTGAACA
attB2_Neo_r	GGGGACCACTTTGTACAAGAAAGCTGGGTCACACGGCTAGCATACTCTA
attB1_Hyg_f	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAAAAAGCCTGAACTCAC
attB2_Hyg_r	GGGGACCACTTTGTACAAGAAAGCTGGGTCAAATTCTGTTCAATGTAAT
attB4_ECH2H5'_f	GGGGACAACTTTGTATAGAAAAGTTGAAGCTTATGGGGAGGTGTGCTTG
attB1_ECH2_5'_r	GGGGACTGCTTTTTTGTACAAACTTGTCAATTATAACCAGGACCCTC
attB2_ECH2_3'_f	GGGGACAGCTTTCTTGTACAAAGTGGTCCTGCGCGCCTTTTGC
attB2_ECH2E3's_r	GGGGACAACTTTGTATAATAAAGTTGATATCGTCTTCTTCAAAAATTCC
attB1_KNeo_f	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCCGCCACCATGGGATCGGCCATTGAA
attB2_1F8Neo_r	GGGGACCACTTTGTACAAGAAAGCTGGGTTCAGAAGAACTCGTCAAGAAG
attB1_KHyg_f	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCCGCCACCATGGCAATGAAAAAGCCTGAACTCAC
attB2_1F8Hyg_r	GGGGACCACTTTGTACAAGAAAGCTGGGTACTCTATTCCTTTGCCCTCG

Table 2. Oligonucleotides for One-Step-PCR

Name	Sequence
LP_ECH_Neo_f	ATGACTCAAAGCTTTGACAGAGTTCCGCACGACACAAAGG GGGAGAGTATAGGGACGCTGCATGTTGTGAACTTTCCCAT GGGATCGGCCATTGAACA
LP_ECH_Neo_r	TTAGTACGGCACGGTCGGATGCCACTGGCGAGCTTGATTCC CCATAGGCTTAAAGTACGCCTCCACATAATCCGCAGTTCAG AAGAACTCGTCAAGAAG
LP_ECH_Hyg_f	ATGACTCAAAGCCTTGACAGGGTTCCGCACGACACAAAGG GGGAGAGTATAGGAACGCTGCATGTTGTGAACTTTCCCAT GAAAAAGCCTGAACTCACC
LP_ECH_Hyg_r	TTAGTACGGCAAAGTCGGATGCCACTGGCGAGCTTGATTCC CCAAAGGCTTAAAGTACGCCTCCACATAATCCGCAGTTACT CTATTCCTTTGCCCTC
LP_dhfr_Neo_f	GAATCCACACAAACAGGCCGGCAAGCGGAAAAGAGGTCG TACGTACTTTTACACTTTTCTCTCACTTGTGCGTCCACCATG GGATCGGCCATTGAAC
LP_dhfr_Neo_r	CGCCTTTTTACGGTCCCCACGTACTACTTTCGGCTCCCCGTT TCTGGCGCGCACGATTTGAAAGGTTTTCCTAAATGATCAGA AGAACTCGTCAAGAAG

Table 3. Oligonucleotides for PCR analysis

Name	Sequence
B	ATACTTTCTCGGCAGGAGCA
C	CGCAAGGAATCGGTCAATAC
D	ACATTGTTGGAGCCGAAATC
E	ATGAAAAAGCCTGAACTCACC
F	TACTCTATTCCTTTGCCCTC
G	ATGGGATCGGCCATTGAACA
H	TCAGAAGAACTCGTCAAGAAG
f2	CACATGCGTTATTATGGTCC
r2	TATTACCCGAGACATTGACG
r3	CTCGAGTTAGTACGGCAAAGTCGGATGC
M13_f	GTAAAACGACGGCCAG
M13_r	CAGGAAACAGCTATGAC
ECH2_f	CAAAGGGGGAGAGTATAGG
ECH2_r	TTGCCTCCATCATTGTTTCC
170_f	TGGCGTGAATGCTTTGCT
170_r	GGAAACAATGATGGAGGCAA

Table 4. Oligonucleotides for probe generation of Southern blot analysis

Name	Sequence
ECH1_pb_f	ATGGTGAAAGCCATGCAACG
ECH1_pb_r	CCCCAAAGGCTTAAAGTACG
Hyg_f	ATGAAAAAGCCTGAACTCACC
Hyg_r	TACTCTATTCCTTTGCCCTC
Neo_f	ATGGGATCGGCCATTGAACA
Neo_r	TCAGAAGAACTCGTCAAGAAG

Figure 1. Consecutive *ECH1* and *ECH2* genes are simultaneously replaced by Hyg^R-containing construct generated based on MS/GW system.

A) *ECH1*, *ECH2* and *HygR-GAPDH-IR* genomic loci in $ECH^{+/-}/Hyg^R$ parasites.

B) Plasmid map of pDEST/ECH_Hyg-GAPDH. Hyg^R and a downstream *GAPDH-IR* was inserted in between *ECH2* 5'UTR and *ECH1* 3'UTR. The plasmid was digested with restriction enzymes Pci I and Pvu II to obtain linearized DNA for transfection.

C) PCR genotyping analysis. Templates used for PCR: (-): no template control; (sko), gDNA of the transfected line ($ECH^{+/-}/Hyg^R$) and (WT), gDNA of WT CL.

D) Genomic DNA Southern blot analysis. 2 and 4 are two individual clones of $ECH^{+/-}/Hyg^R$. Left panel, gDNA digested with EcoR I and hybridized with labeled *ECH1* CDS; right panel, gDNA digested with Ban I and hybridized with Hyg^R CDS.

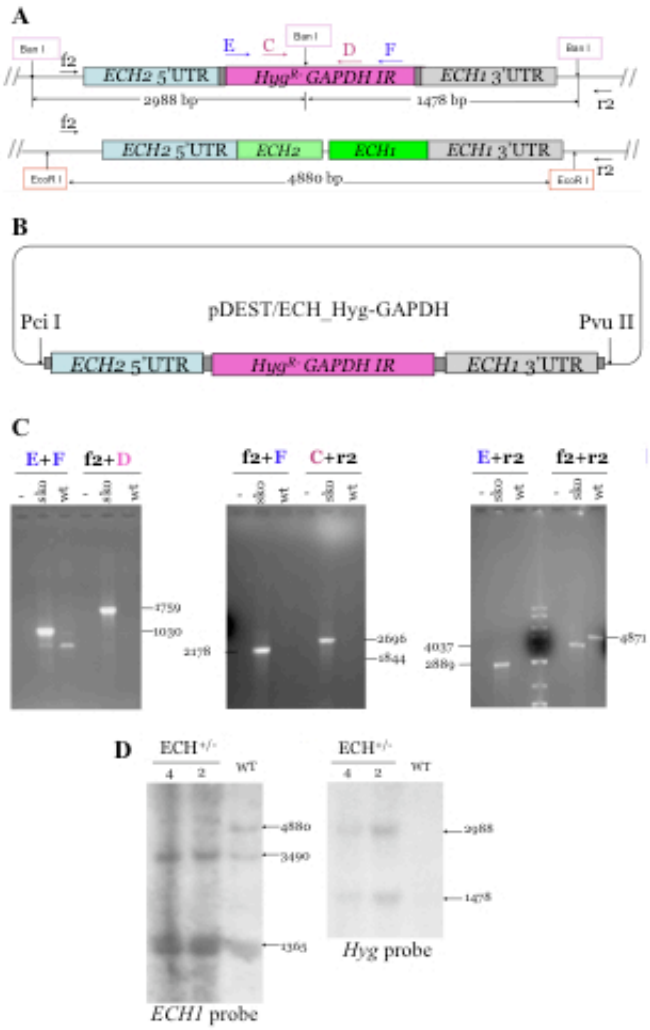

A
Ban I
E
C
Ban I
D
F
Ban I
f2
ECH2 5'UTR
Hyg^R GAPDH IR
ECH1 3'UTR
2988 bp
1478 bp
r2
f2
ECH2 5'UTR
ECH2
ECH1
ECH1 3'UTR
r2
EcoR I
4880 bp
EcoR I
B
pDEST/ECH_Hyg-GAPDH
Pci I
Pvu II
ECH2 5'UTR
Hyg^R GAPDH IR
ECH1 3'UTR
C
E+F
f2+D
f2+F
C+r2
E+r2
f2+r2
- ko wt
1759
1030
2178
2696
2544
4037
2889
4871
D
ECH+/-
4 2
WT
4880
3490
1365
ECH1 probe
2988
1478
Hyg probe

Figure 2. Consecutive *ECH1* and *ECH2* genes are simultaneously replaced by Neo^R containing construct generated based on MS/GW system.

A) Plasmid map of pDEST/ECH_Neo-GAPDH. Neo^R with a downstream *GAPDH 3'UTR* was inserted in between *ECH2* 5'UTR and *ECH1* 3'UTR. The plasmid was digested with restriction enzymes Pci I and Pvu I to obtain linearized DNA for transfection.

B) *ECH1*, *ECH2* and *NeoR-GAPDH 3'UTR* genomic loci in $ECH^{+/-}/Neo^R$ parasites.

C) PCR genotyping analysis. Templates used for PCR: (-): no template control; (sko), gDNA of the transfected line ($ECH^{+/-}/Hyg^R$) and (WT), gDNA of WT CL.

D) Genomic DNA Southern blot analysis. gDNA of WT CL and transfected parasite gDNA digested with Ban I and hybridized with Neo^R CDS.

E) Transcriptome profile of *ECH1* and *ECH2*. *ECH1* is most abundant in epimastigote stage while *ECH2* is up-regulated in amastigote stage. A, amastigote; T, trypomastigote; E, epimastigote; M, metacyclic trypomastigote.

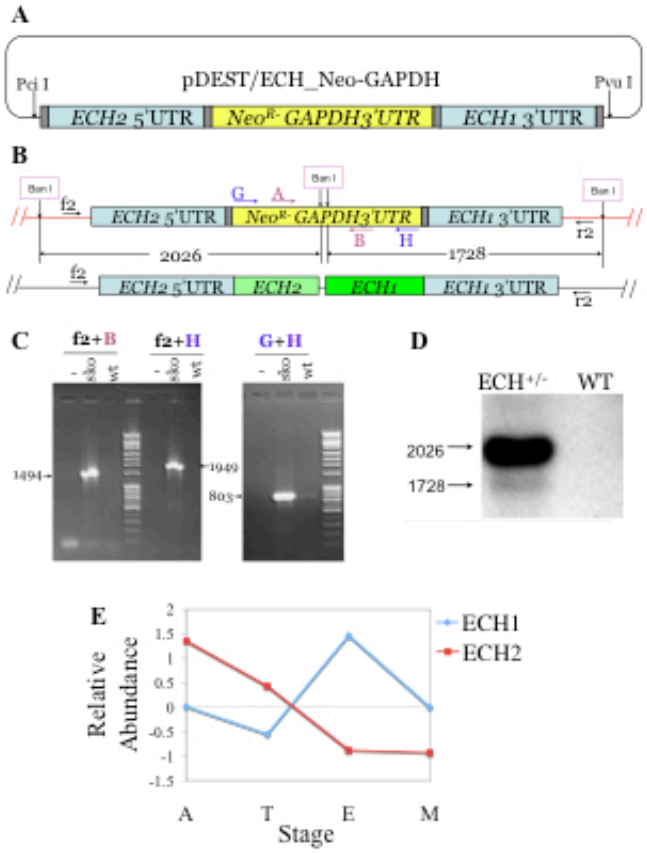

A
pDEST/ECH_Neo-GAPDH
Pci I
Pvu I
ECH2 5'UTR
Neo^R-GAPDH3'UTR
ECH1 3'UTR
B
Ban I
f2
G
A
B
H
r2
2026
1728
ECH2
ECH1
C
f2+B
f2+H
G+H
sko
wt
1494
1949
803
D
ECH+/-
WT
2026
1728
E
Relative Abundance
ECH1
ECH2
A
T
E
M
Stage

Figure 3. *ECH*$^{+/-}$*/Neo*^{R} transfected with a knockout cassette from pDEST/ECH2_Hyg generated through MS/GW approach.

A) Plasmid map of pDEST/ECH2_Hyg. *Hyg*^{R} is flanked by 500 bp 5'UTR and 340 bp 3'UTR of *ECH2*.

B) *ECH1*, *Neo-GAPDH-IR* and *Hyg*^{R} genomic loci in *ECH1*$^{+/-}$*ECH2*$^{-/-}$ parasites.

C) PCR genotyping analysis. Templates used for PCR: (-), no template control; (*ECH*$^{+/-}$*/Neo*), gDNA of *ECH*$^{+/-}$*/Neo*^{R}; (*ECH1*$^{+/-}$*ECH2*$^{-/-}$), gDNA of the transfected line; (WT), gDNA of WT CL; (plasmid N), pDEST/ECH_Neo-GAPDH; (plasmid H), pDEST/ECH2_Hyg; (plasmid N1), an irrelevant plasmid control.

D) PCR analysis. ECH2_f and ECH2_r primers only amplify *ECH2* gene specificly. (-), no template control; (clone 4), gDNA of a clone of *ECH1*$^{+/-}$*ECH2*$^{-/-}$; (uncloned), gDNA of uncloned population of *ECH1*$^{+/-}$*ECH2*$^{-/-}$; (WT), gDNA of WT CL.

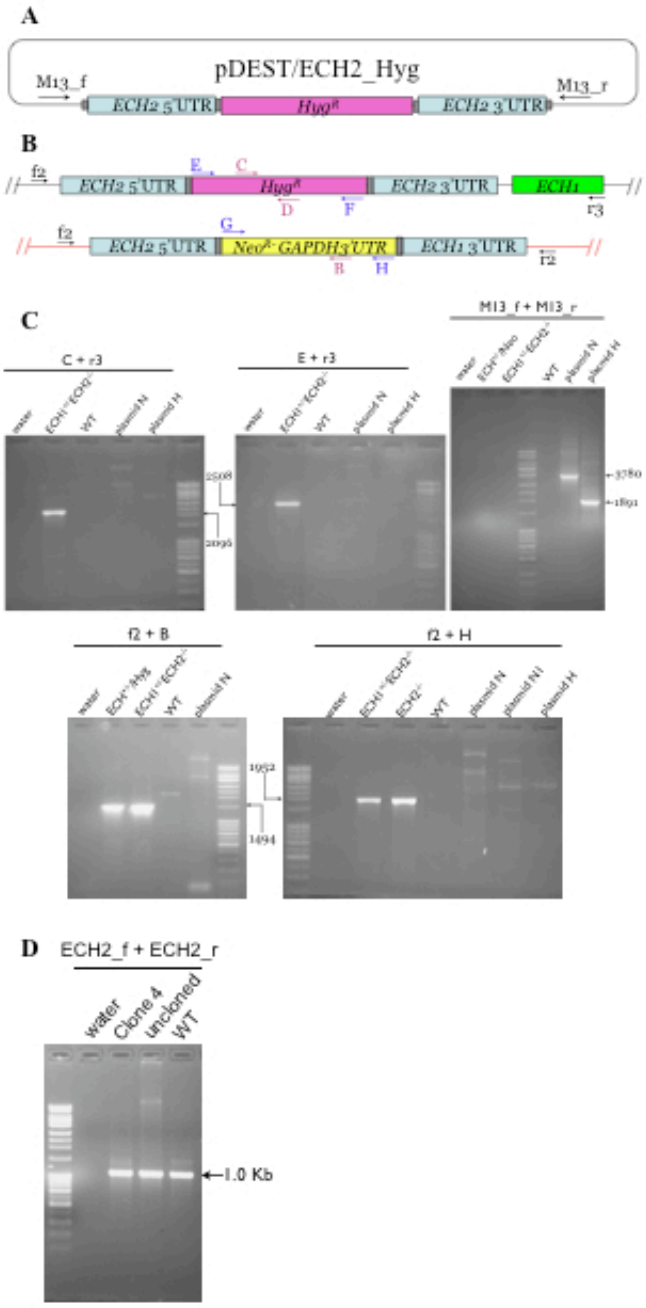

A
pDEST/ECH2_Hyg
M13_f
M13_r
ECH2 5'UTR
Hyg^R
ECH2 3'UTR
B
f2
E
C
ECH2 5'UTR
Hyg^R
ECH2 3'UTR
ECH1
D
F
r3
G
Neo^R-GAPDH3'UTR
ECH1 3'UTR
B
H
r2
C
C + r3
E + r3
M13_f + M13_r
water
ECH1^-/Hyg
ECH1^-/ECH2^-
WT
plasmid N
plasmid H
2508
2095
3780
1891
f2 + B
f2 + H
ECH1^-/Hyg
ECH2^-
plasmid N1
1952
1494
D
ECH2_f + ECH2_r
water
Clone 4
uncloned
WT
1.0 Kb

Figure 4. WT CL transfected with constructs produced via MS/GW approach to replace both allelic copies of *ECH2* gene.

A) Plasmid map of pDEST/ECH2_Neo. Neo^R was flanked by 500 bp 5'UTR and 340 bp 3'UTR of *ECH2*.

B) ECH1, Neo^R and Hyg^R loci in $ECH2^{-/-}$ parasites.

C) PCR genotyping analysis. (water), no template control; ($ECH2^{+/-}$), gDNA of $ECH2^{+/-}/Hyg^R$; ($ECH2^{-/-}$), gDNA of transfected line ; (WT), gDNA of WT CL; (plasmid N), pDEST/ECH2_Neo; (plasmid H), pDEST/ECH2_Hyg.

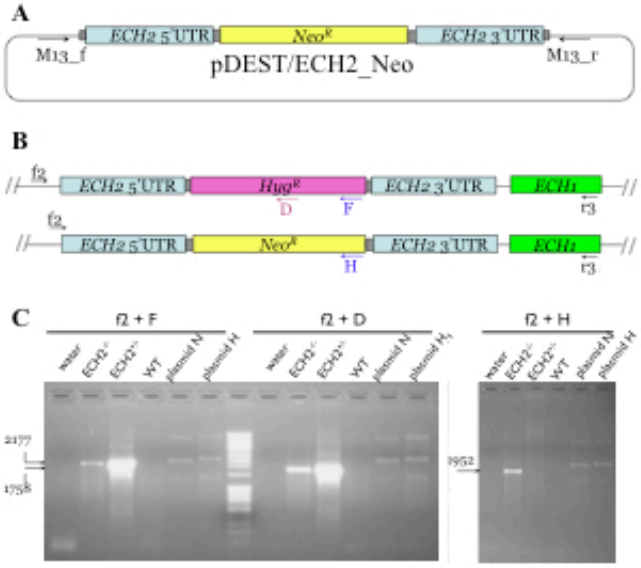

A
ECH2 5'UTR
Neo^R
ECH2 3'UTR
M13_f
pDEST/ECH2_Neo
M13_r
B
f2
ECH2 5'UTR
Hyg^R
ECH2 3'UTR
ECH1
D
F
r3
f2
ECH2 5'UTR
Neo^R
ECH2 3'UTR
ECH1
H
r3
C
f2 + F
f2 + D
f2 + H
water
ECH2-/-
ECH2+/+
WT
plasmid N
plasmid H
2177
1758
1952

Figure 5. One-Step-PCR based strategy for knocking out *ECH* genes and *DHFR-ts* gene.

A) Constructs were made using One-Step-PCR based strategy for knocking out both *ECH1* and *ECH2* genes simultaneously, LP-ECH-Neo and LP-ECH-Hyg**.** The constructs have drug selectable markers (Neo^R and Hyg^R) flanked by 78 nts of 5' end CDS of *ECH2* and 78 nts of 3' end CDS of *ECH1*.

B) LP-DHFR-ts-UTR-Neo, a knockout cassette with Neo^R flanked by 78 nts of 5' UTR of *DHFR-ts* and 78 nts of 3' UTR of *DHFR-ts*.

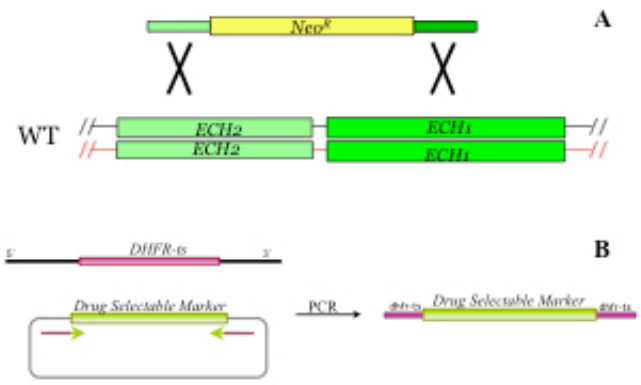
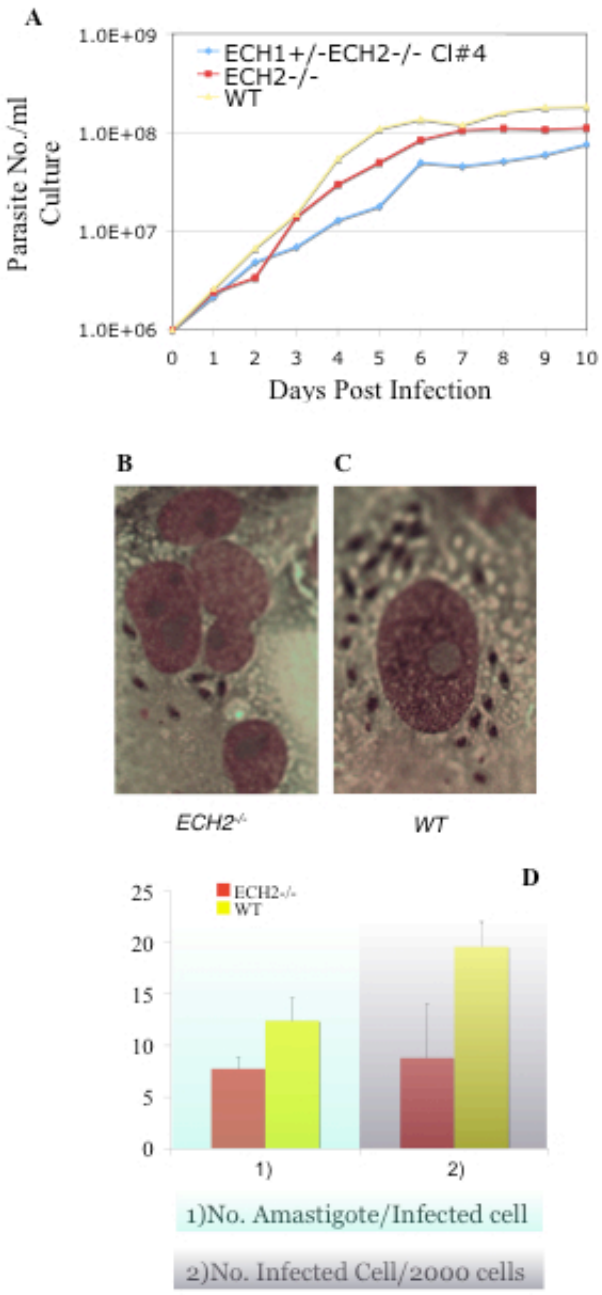

A
Neo^R
WT
ECH2
ECH2
ECH1
ECH1
B
DHFR-ts
Drug Selectable Marker
PCR
Drug Selectable Marker

Figure 6. Phenotype of *ECH2*$^{-/-}$ parasites generated based on MS/GW system.

A) Growth curve of *ECH2*$^{-/-}$, a clone of *ECH1*$^{+/-}$*ECH2*$^{-/-}$ (*ECH1*$^{+/-}$*ECH2*$^{-/-}$ Cl #4) and WT CL epimastigotes. Both parasites were diluted from mid-log phase (10^6 cells/ml) and counted on a hemocytometer every 24 hrs for 10 days. No statistical difference is observed between *ECH2*$^{-/-}$ and WT CL ($P = 0.24$), whereas *ECH1*$^{+/-}$*ECH2*$^{-/-}$ parasites grow significantly slower than WT CL ($P < 0.05$).

Vero cells were infected with the same number of *in vitro* converted metacyclic trypomastigotes (in a mixture with epimastigotes) of *ECH2*$^{-/-}$ (B) or WT (C) at a 1:8 ratio (Vero cells: metacyclic trypomastigotes). Extracellular parasites were washed off at 24 hrs after infection. Cells were Dif-quick stained 3 days later, and amastigotes and infected Vero cells were counted. D) 1) Number of Amastigotes/Infected Vero cell, $P<0.05$; 2) Number of infected Vero cells/(2000 Vero cells), $P<0.05$.


A
1.0E+09
1.0E+08
1.0E+07
1.0E+06
Parasite No./ml Culture
ECH1+/-ECH2-/- Cl#4
ECH2-/-
WT
0 1 2 3 4 5 6 7 8 9 10
Days Post Infection
B
C
ECH2-/-
WT
D
25
20
15
10
5
0
ECH2-/-
WT
1)
2)
1)No. Amastigote/Infected cell
2)No. Infected Cell/2000 cells

CHAPTER III

CONCLUSION

This study has discovered that 78 nucleotides gene-specific regions utilized in the One-Step-PCR strategy are likely to be insufficient for homologous recombination in *T. cruzi*, which might shed light on the underlying mechanism of homologous recombination in this protozoan. The results of this study also confirmed that Multisite Gateway® based system can be applied to gene knockout study in *T. cruzi*, which undoubtedly will serve as an extremely valuable tool for rapid gene knockout in *T. cruzi*. Not only will this powerful tool enable high-throughput gene knockout and facilitate reverse genetic study of *T. cruzi*, the resulting knockout parasites that exhibit attenuated infectivity can also be tested as experimental vaccines for Chagas disease. The observation that *ECH2*$^{-/-}$ obtained using MS/GW based system display attenuated infectivity and defective ability to replicate as amastigotes suggests that knocking out genes that are involved in processes that are essential to *T. cruzi* amastigotes is a very promising approach to generate live attenuated vaccines for Chagas disease. We have also identified multiple other genes that are involved in some pathways that might be essential for *T. cruzi* amastigotes, such as fatty acid transporter gene, fatty acid elongase gene, oligosaccharyl transferase gene and inonsine-5-monophosphate dyhydrogenase gene. Using MS/GW based system, the knockout studies of these genes are currently being carried out and the knockout parasites will be tested for their potential as experimental vaccines for Chagas disease.

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11. Brisse, S., J.C. Dujardin, and M. Tibayrenc, *Identification of six Trypanosoma cruzi lineages by sequence-characterised amplified region markers.* Mol Biochem Parasitol, 2000. **111**(1): p. 95-105.

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