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# Article Review: Editing genome of *Toxoplasma gondii* by CRISPR/Cas9 system

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**A B S T R A C T**

It is well known that finding out the genetic information of any organism helps in understanding how performs its metabolism, and knowing the extent of the effect that gene modification affects the performance of these functions. Many techniques have been used to editing genome of parasites such as Zinc fingers and Transcription activator-like effector nucleases (TALENs), But the development of clustered regularly interspaced short palindromic repeats/ CRISPR-associated (CRISPR Cas9) system created the quantum leap in this area, as this technique relies on identifying the portion to be modified using a guide from Guide RNAs (gRNA) that identifies this part very accurately. Not only that, but the enzyme present with it called CAS9 works as a molecular scissor to cut DNA at this part and allow the required modification.Since 2014, the date on which the first article about use CRISPR/Cas9 in the editing of *Toxoplasma gondii* genome was published, after that continued, developed new methods and protocols that facilitate the researchers work, we will attempt in this paper to review some of these major achievements.

## 1. INTRODUCTION

### CRISPER/Cas9 in review

The CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/ CRISPR-associated gene 9) system consist of single-guide RNA (sgRNA) and associated endonuclease protein (CRISPR associated protein 9 (cas9) to creating double-strand breaks (DSBs) at the same DNA site [1]. This system was first discovered in *E. coli* (1980) as a defense mechanism against Plasmid or viruses invading [2], in 2007, Barrangou and colleagues demonstrated that *Streptococcus thermophilus* have the ability to resistance bacteriophage when incorporating a fragment of the virus genome in their CRISPR locus [3]. There are three types of CRISPR mechanism have been identified, in most studies, type II was the prefer [4]. CRISPR/Cas system mechanism induces deletion (Knockdown) or insertion (knock-in) mutation in the target DNA locus, small guide RNA (sg RNA) that driven from trans-acting crispr RNA binds to specific repeat in the target

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DNA that named protospacer adjacent motif (PAM), then Cas9 cuts an aim chain (3 bp upstream PAM) in the double DNA strands [1]. Efficient PAM site for Cas9 mostly is 5'-NGG-3' (N: any nucleotide). sgRNA flanked with the primer of target DNA [5]. Repair processing Followed DSBs that be either homology-directed recombination (HDR) or non-homologous end-joining (NHEJ) that use DNA as a template by replacement nucleotides as DNA donor type or random nucleotides respectively [5]. Mostly uniform mutations were generated by CRISPR/Cas9- based genome editing in many diploid organisms (i.e., nonchimeric mutations, such as bi-allelic, heterozygous and homozygous mutations) [6]. Many functional applications rely on the CRISPR-Cas9 system besides creating single or double-strand breaks at the target sites, such as specific transcriptional control [7] and targeted base editing [8]. Also, the Cas9 protein has been recreated to admit the changing PAM sequence and enhance targeting accuracy [9]. These innovations have increased the upgrading of the CRISPR-Cas9 system as a huge and resourceful tool for genetic engineering [1].

CRISPR /Cas9 system has widely used in the editing of the genome in zebra fish, plants, rats, and human cell lines [10–14]. The number of investigations into the

generation of mutants by CRISPR/CAS9 has risen significantly in modern years, especially for generally changed screening, due to the rapidly rising predominance of genome editing in biological research [2].

Although first reported of CRISPR / Cas9 genomic editing system in apicomplexan parasites just from 3 years ago, this technique rapidly became a crucial part in apicomplexa research [15].

## 2. T. GONDII GENOME

The distingue sexual cycle characterizes of *T. gondii* gave it a significant genetic variance among its isolates. The first study to genetic diversity using isoenzymes and recoded presence of 12 distinct groups for Tachyzoite protein Zymodemes (Z1- Z12) [16], later found corresponding of Z1, Z2 and Z4, and Z3 to type I, II and II respectively [17]. Howe and Sibley (1995) showed lower genetic diversity in clonal populations, they suggested that proximally 95% of clonal followed main genotypes (I, II, III), while (5%) results of mixture the three main types [18]. Other phylogenetic studies [19;20] have found that there are only two distinct types (I and II) and suggested that type III was a subgroup of type II [20], or from successful recombination between the two main groups [21], recently [22] and other studies have found fourth clonal type the genomes of *T. gondii* -same as many eukaryotic organisms- contain both of DNA (nuclear and organelles). *T. gondii* consists of about 87 Mb nuclear genome, mitochondrial genome (6 kb), and plastid-like genome (35 kb) [23]. *T. gondii* RH strain has 111 unique genes in 69.35Mb [24]. All genes distributed in 11[25], 13 or 14 chromosomes [26]. The nuclear genome is haploid in most utmost parasite's life cycle, without for a small diploid phase in the cat intestine before meiosis [27]. The haploid tachyzoite genome allows for genetically modification of genes with relative ease [28].

### 2.1. Apicoplastid Genome

One of the Apicomplexa characteristics is the presence of Apicoplastide. Electron micrographs scanning showed existing of circular distinct DNA, along with another linear DNA, This DNA was double strand with 35 kb in size, with limiting coding capacity made it rely on the nuclear genome [29]. This genome dividing autonomously early when Parasite multiple [30]. It is composed of a large inverted repeat of two rRNA copies one a small subunit (SSU) rRNA and one large subunit (LSU) rRNA, directed tail to tail, this rRNA similar to prokaryotic RNA but different from Plastid and mitochondria RNA, DNA also encodes to a eubacterial-like RNA polymerase subunit that transcripts all plastid genome [31].

Apicoplastid is essential in Tachyzoite replicons and the emergence from the parasitophorous vacuole [32], has the main role in heme biology and mitochondria metabolism [33], and some fatty acids biosynthesis [30].

### 2.2. Mitochondrial Genome.

Mitochondria were derived in an endosymbiotic event from α-proteobacteria last common ancestor, it was present in all eukaryotes [34], mitochondrial DNA is linear 6kb in size, with products of three genes, cytochrome b (*Cyb*), cytochrome c oxidase III (*Cox3*), and cytochrome c oxidase I (*Cox1*), along with interspersed rRNA elements [35]. *T. gondii* genome (ME49) consists of many contigs 139 consists of portions or -in some cases- complete cytochrome genes, also 140 mitochondrial rRNA gene fragments [36].

Mitochondria is powerhouses of the cell, it produces most of (ATP) through oxidative phosphorylation [37], also synthesis of many molecules such as lipids, amino acids, coenzyme Q, iron-sulfur clusters, and hem, so catabolism of molecules such as amino acids, fatty acids and monocarboxylates, regulation of apoptosis, and the production of ATP and NADH, finally, storage of ions and signaling molecules such as $Ca^{2+}$[38].

## 3. EDITING OF *TOXOPLASMA GONDII* GENOME

The prosperity of genetic devices to editing genomes is important to guess the biology of any organism. such instruments have been most exhaustive applied in Apicomplexa phylum (T. *gondii*, *Plasmodium* spp. and more recently *Babesia* spp) [15]. Since Elmer Pfefferkorn (1970) has been applied, the first genetic manipulations to *Toxoplasma* by using chemical mutagenesis [31], many approaches as CRISPR/CAS9 system, transcription activator-like effector nucleases (TALENs) and Zinc-finger nucleases (ZFNs) has been performed [39].

CRISPR/CAS9 system became Succeed by increasing off-target effects through tolerating small mismatches, insertions and other mutations in the target sequence[40], T. *gondii* has a great consequence of molecular devices augmented to this organism genetic editing [41], knocking out the KU80 gene, the deficient in the Non-homologous end joining (NHEJ) pathway (ΔKU80 strain)[42], Presence of numerous selectable markers, the rapid lack of exogenous (non-integrated) DNA, and no detectable exogenous DNA 7 days post-transfection making precision integration of specific sequences at specific loci through homologous recombination, rather than the naturally random integration that observed in this parasite[43].

In 2014, editing of T. *gondii* genome by CRISPER/ Cas journey has been begun by Shen and co-workers when

used specific sgRNA under U6 promoter and unique expression vector fusing with specific loci with a green fluorescent protein(Cas9-NLSGFP, wild-type strain (RH) genes that encode for uracyl phosphoribosyl transferase (UPRT) was targeted due to its lack the resistance to fluoro-deoxyribose (FUDR), the authors concluded that local recombination at the sgRNA target region by CRISPR/Cas9 has occurred, therefore, performing gene inactivation. Finally, authors successfully editing of the genome of a T. *gondii* wild isolate by disrupted the gene that encoding serine-threonine kinase (ROP18) in the GT1 strain and delivered its gene knock-in. In this way, demonstrating that the difference between the ΔKU80 strain and GT1, when used this system [44].

After one month, Sidik *et al*., (2014) established the second study. This work focused on the devices of DNA repair in the mutant cell lines generations. This group utilized a 1-vector approach to provoking Homology-directed repair (HDR) and (Non-homologous end joining) NHEJ-mediated DNA repair by cloning a Cas9-FLAG-NLS fusion protein (expressed under T. *gondii* tubulin promoter (pTgTUB)) and unique sgRNA (driven by TgU6 promoter) to transfect parasites with or without DNA donor, respectively. They used immunofluorescence microscopy to prove the nuclear localization of Cas9-FLAG-NLS. In parasites, where (SAG1) protein was targeted, they recorded 20% knockout performance in RH strain, while 10-fold fewer knockout cells in ΔKU80. They observed 30% homologous recombination in ΔKU80 strain with a DNA donor molecule [41].

In murine hosts, innate immune mechanisms activated by interferon-gamma (IFγ) inhibited by Rhoptry proteins (ROP5 and ROP18) [45]. Behnke and co-workers employed a hybrid between type 2 (ME49) strain (intermediately virulent) and type 10 (VAND) strain (highly virulent) to map the genetic data for differences in virulence in the mouse. They used two single guides RNA CRISPR plasmids to perform double-CRISPR plasmid that used to knockout the ROP5 locus [45].

Zheng and colleagues (2015) revealed a new approach that uses the influence of the CRISPR/Cas9 system. When took a Cas9 target site in leucine aminopeptidase gen and then used a knock-out vector containing Cas9 and single-guide RNA. The outcomes showed that knockout of T. gondii leucine aminopeptidase required blocking attachment/invasion and replication, so, concluded that leucine aminopeptidase may be beneficial for a specific adjunctive drug just in T. *gondii* [46].

The calcium-dependent protein kinases (CDPKs) relates to the superfamily of kinases, play different purposes in the life cycle of *T. gondii* such as gliding motility, cell invasion, and egress as well as some other important developmental processes [47]. Long et al., (2016) created twenty-four mutants cell lines in 1 and 2 types, and developed a more effective approach for disturbance of CDPKs genes using CRISPR /Cas9 and a selectable marker (Cre-loxP), they used single plasmid to express two gRNA from 5' and 3' region of CDs, they submit that complex CDPK6 and the majority of CDPKs had indistinguishable as a phenotype for growth *in vitro* or infection model [48].

CRISPR based genome widescreen developing by Sidik et al., (2016) to identification of basic T. *gondii* genes. They used essential expression of Cas9 endonuclease with a decoy sgRNA. This group identified more than 200 essential genes, these genes called as crucial conserved apicomplexan protein (ICAP) encoding genes. Authors have created Ko parasites from ICAPS and have also described their role in the frequency of parasites in the laboratory. The screenings indicated the role of ICAPI2 (among ICAPs) for parasite invasion), The decrease in the level expression of CLAMP making inhibition in an invasion because of the defects in plaque formation. Due to homology with claudin-19 and claudin-15.

In mammalian, The ICAP12 was renamed as claudin-like apicomplexan microneme protein (CLAMP [49].

CRISPR/Cas9 used to intrude all nuclear protein-coding genes in T. *gondii* using a combined format, depend on transfecting a guide RNA library into parasites constitutively expressing Cas9[ 50]. This Cas9 strain coupled this strain with the plasmid pU6-DHFR, which encodes a sgRNA of choice and confers resistance to pyrimethamine through a resistant version of the dihydrofolate reductase thymidylate synthase (DHFR-TS) gene [50]. That study the effect of culture conditions in parasite's genetic requirements, which will enable studies of their metabolic needs, host-specificity, and drug-resistance mechanisms [50]. The use of tiny scale CRISPR libraries targeting gene subsets doesn't resolve the problem [51]. Young et al. produced an approach for speedy creation of style guide RNA (gRNA) libraries using arrayed single-stranded oligonucleotides for reproducible combined cloning of CRISPR/Cas9 libraries [51]. They used this system to create mutant pools of varying sizes in the protozoan parasite (T. *gondii)* and outline optimized examination approach for tiny scale libraries. This study showed a dormant trans-rescue of individual knock-out parasites in pools of mutants associated with homogenous knock-out lines of the virulence factor MYR1 [51].

## 4. CONCLUSION

CRISPR/Cas9 system is effective mechanisms for editing and modelling genome, its ease and availability making it be the first. Recently this technique has been improved to editing Eukaryotic (parasites) genome, especially *T. gondii*. It has been succeeded to editing many genes, and improve some new modulating method.

### Ethical statement

The research was conducted according to the recommendations of the Ethics Committee at the College of Science - University of Qadisiyah.

### Competing interest

The author declare that he has no conflict of interest that affects this study.

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# تحرير جينوم طفيلي المقوسة القندية بنظام CRISPR/Cas9

مسافر هندي العارضي

المديرية العامة للتربية في القادسية – وزارة التربية.

**الخلاصة:**

ومن المعروف جيدا أن معرفة المعلومات الوراثية عن أي كائن يساعد في فهم كيفية قيامه بعملياته الحيوية، ومعرفة إلى أي مدى يؤثر تعديل الجينات على أداء هذه الوظائف.

استخدمت العديد من التقانات في تحرير جينوم مختلف الطفيليات مثل تقانة اصابع الزنك ونسخ الانزيمات مماثلة المنشط (TALENs)، ولكن تقانة CRISPR/Cas حظيت بالاهتمام الاكبر في الآونة الأخيرة، تستخدم هذه التقانة دليل من الحامض الرايبي (gRNA) لمهاجمة اهداف محددة في الجينوم وليس هذا فحسب بل إن إنزيم القطع الداخلي (cas9) المستخدم في هذه التقانة يمكنه قطع الحامض النووي من المكان المحدد ويسمح بتحويره.

استخدمت هذه التقانة مع طفيلي المقوسة القندية في عام 2014، ثم طورت بروتوكولاتها وطرق عملها مما يسهل عمل الباحثين في هذه المجال، البحث الحالي محاولة لمراجعة للإنجازات في هذا المجال.

**الكلمات المفتاحية:**

المقوسة القندية، تقانة كريبر\ كاس، تحرير الجينوم/ التلاعب بالجينوم.