Improvement to Gene Editing in *Neospora Caninum*: Knockout *ku80* to Increase the Efficiency of Homologous Recombination

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Abstract

Background: The CRISPR/Cas9 technology based on homologous recombination has been widely used for gene editing in organism. Gene deletion of *ku80* complex can increase the efficiency of homologous recombination(HR), or gene editing by CRISPR/Cas9 in *Arabidopsis thaliana* and *Toxoplasma gondii*, which has remained elusive in *Neospora caninum*.

Methods: Here, we knocked out the *ku80* in Nc1 strain, named $\Delta ku80$, using CRISPR/Cas9. In vitro, phenotype assays, including plaque formation, invasion, replication and egress were carried out to determination of the growth of $\Delta ku80$. In vivo, we respectively injected BALB/c mice with different doses of NcKU80 and Nc1, and measured the survival curves and parasites burdens of mice infected. To test the efficiency of HR, $\Delta ku80$ and Nc1 strains were transfected with the same HA-tagged plasmids, and the percentage of parasites with HA-tagged was investigated.

Results: It is showed that the growth and virulence of $\Delta ku80$ kept unaffected, while the efficiency of gene targeting via double-crossover at several genetic loci was increased 2 to 3 fold in $\Delta ku80$.

Conclusions: It is concluded that $\Delta ku80$ can be used as an effective strain for rapid gene editing in *N. caninum*.

Background

The global prevalence of Neosporosis poses serious harm, causing abortion in cattle, neuromuscular disorder in calves, neuromuscular injury and hind limb paralysis in puppies. Its causative pathogen is *Neospora caninum*, an obligate intracellular apicomplexan parasite, which is capable of parasitizing nucleated cells of legion mammalian species. *N. caninum* is highly similar to *Toxoplasma gondii* in life cycle, morphological and biological characteristics.

To prevent and control Neosporosis, in-deep analysis *N. caninum* gene function study closely associated with gene editing technology is required. However, most gene editing methods have common problems in practice application, such as off-target binding and low efficiency of spontaneous homologous recombination. This is also an existing issue in *N. caninum*' study, which is the reason why numerous researches focus on technological optimization. Lei et al. constructed the overexpressing strain of NcROP18, but failed to knock out it [1]. Ma et al. completely knocked out ROP5 and ROP16 genes of *N. caninum* using homologous recombination, although the screening efficiency was only 1.4% [2, 3]. In recent years, CRISPR/Cas9 technology has become the first choice of genome editing as a transformative DNA genome editing system. Arranz-Solís and Yang successively applied CRISPR/Cas9 technology in *N. caninum* to improve the effectiveness of genome editing successfully, promoting the study of this parasite[4, 5]. In present, several studies have applied CRISPR/Cas9 technology to disrupt gene in *N. caninum*.

The CRISPR/Cas9 nuclease complex consists of guide sgRNA and Cas9 endonuclease. Cas9 can generate DNA double-strand breaks (DSB) at a specific target gene site under sgRNA guidance. Once a DSB is produced, it must be repaired because a single unrepaired DSB can result in cell death. Three pathways contribute to DSB repair in eukaryotes: homologous recombination (HDR or HR), classical nonhomologous end joining (c-NHEJ) and alternative end joining (alt-NHEJ). Among these, HR is the primary DSB repair pathway in yeasts and prokaryotes, while higher eukaryotes including *N. caninum* use c-NHEJ primarily. However, CRISPR/Cas9 technology is based on HR, which generates gene insertion and point mutations by rearranging DNA sequences in the presence of homologous DNA flank sequences. These factors lead to the low efficiency of CRISPR/Cas9 gene editing in *N. caninum* [6, 7]. To improve the HR efficiency of CRISPR/Cas9 technology in *N. caninum*, we committed to block c-NHEJ.

KU protein binds on DSB ends to initiate c-NHEJ. Studies have shown that knocking out *ku80* significantly improves the efficiency of gene editing in *Toxoplasma gondii* without changing its biological peculiarities [8, 9]. In this study, *ku80* knockout strain of *N. caninum* was constructed to inhibit c-NHEJ using CRISPR/Cas9 technology, and the probability of HR during DNA repair was increased.

Methods

Cell and Parasites Culture

Vero cells and HFF cell lines were transduced and cultured with 6% and 10% fetal bovine serum (FBS, Gibco, USA) DMEM (Dulbecco's Modified Eagle's Medium). *N. caninum* was cultured in the above two cells with 1% fetal bovine serum DMEM. The culture conditions are 37°C and 5% CO₂.

Plasmid and Transgenic Parasite Construction

Primers for PCR amplification are shown in Table 1. The gRNA targeting site for NcKU80 (5'-GTTTCAGCCTTCAGCTCTCCG-3') was designed in the EuPaGDT Library of ToxoDB, and pCRISPR-Cas9-NcKU80 was constructed based on pSAG1-Cas9-NLS-GFP-SAG1 plasmid preserved in the Key Laboratory of Animal Parasitology (Beijing, China). The method is as described by Yang [5]. The gRNA was integrated into the plasmid downstream of the 3' Flank sequence of the U6 promoter by ligation transformation (Vazyme, Nanjing, China).

The knockout plasmid was constructed on the basis of plasmid pTCR-CD retained in the laboratory. 5' and 3' homologous flanks of NcKU80 were amplified using Nc1 genome as a template. To facilitate screening, the chloramphenicol resistance gene and red fluorescent protein gene (RFP) were inserted between 5' and 3' homologous flanks, and ligated into plasmid backbone pTCR-CD with flanks. The plasmid CRISPR and the plasmid pTCR-NcKU80 were co-transfected into Nc1. And used the limited dilution method to clone NcΔku80 after chloramphenicol treatment.

The lab-built plasmid CRISPR and pTCR of NcGrxC5-oe, NcGrxC5-HA, NcGrxS14-oe, NcGrxS14-HA was constructed by the method as above. The difference was that these pTCR plasmids were integrated the

DHFR gene inserted and the strains were treated with pyrimethamine after co-transfect.

Plaque Assay

200 tachyzoites were added into 12-well plate HFF cells and fixed with 4% paraformaldehyde after 8 days, and then stained with 0.2% crystal violet dye. The plaque area was calculated using Photoshop CC 2018 software (Adobe, United States). The calculation was repeated 3 times to take the average. Three independent trials were carried out.

Immunofluorescence Assay

The 12-well plates were fixed with 4% paraformaldehyde, permeated with 0.25% Triton X-100 for 20 minutes and were blocked with 3% BSA for 30 minutes. sealed with 3%BSA. The primary antibodies were rabbit anti-SRS2 (1:200) and mouse anti-HA (Sigma-Aldrich, Saint Louis, MO, USA; 1:100). While the secondary antibodies were the goat anti-mouse IgG1 (FITC-labeled) and goat anti-rabbit IgG1 (CY3-labeled). And nucleus was labelled by DAPI. All antibodies were incubated for 1 hour.

Invasion assay

A total of 1×10^5 tachyzoites were added to the HFF cell that cultured on 12-well plates. After 1 hour, the parasites that didn't attach or invade to cells were washed with PBS. Immunofluorescence assay was performed after cultured for 24 hours. The 12-well plates were observed using a fluorescence microscope, and the number of HFF cells and PV were calculated by randomly selecting 5 visual fields from the top, bottom, left, right and middle positions respectively. The invasion capacity of the parasite is the number of PV in the field divided by the number of HFF cells. The calculation was repeated 3 times to take the average. Three independent trials were carried out.

Replication assay

Cultivate parasites and IFA in the same way as invasion assay. 100 PVs were randomly calculated using a fluorescence microscope, and then the number of PVs containing different amounts of tachyzoites was calculated. The calculation was repeated 3 times to take the average. Three independent trials were carried out.

Egress assay

Cultivate parasites in the same way as invasion assay and treated with calcium ionophore A23187 (Sigma-Aldrich, USA) for 3 minutes after 24 hours. IFA was then utilized. The escaping ability of tachyzoites is expressed as the proportion of escaping PVs to the total PVs. Calculate 100 PVs and the calculation was repeated 3 times to take the average. Three independent trials were carried out.

Virulence tests in mice

The 6-week-old female BALB/c mice were purchased from Jinmuyang Laboratory Animal Breeding Co., Ltd. (Beijing, China) and were kept in a sterile cage for a week, ad libitum access to clean food and water.

Five per group mice were intraperitoneally injected with different doses of tachyzoites and were observed for 30 days.

Quantitative Real-Time PCR

Parasite quantitative detection in mice by qPCR was carried out following the method Collantes-Fernández reported [10]. The mice were humanely sacrificed at 30 days, then collected brains and extracted DNA using the DNA Extraction Kit (Beijing Aide Lai Biotechnology Co. Ltd., Beijing, China). Primers were designed to quantitatively detect 28S RNA of mice and *Nc5* gene of *N.caninum* by qPCR. Established the standard curve of 28S RNA and *Nc5*. The parasite load was expressed as the content of *N.caninum* *Nc5* per nanogram of mouse 28S rRNA.

Data Analysis

All data were analyzed using GraphPad Prism 8.0.2 (San Diego, CA, USA), and the results were displayed as mean $\pm$ standard deviation and tested by two-way ANOVA and the Student's *t* test. *P*-values are indicated as asterisks: **p* < 0.05, ***p* < 0.01, ****p* < 0.001 and *****p* < 0.0001. We consider all *p* < 0.05 to be significant.

Results

Deletion of *ku80* in *N. caninum*

The KU domain-containing protein of the *N. caninum* was searched in the ToxoDB genomic resource database, and two genes were found (NCLIV_056045 and NCLIV_064150). BLASTp analysis of these two genes showed that NCLIV_056045 had a higher percent identity with KU80 protein of *Arabidopsis* and *Toxoplasma gondii*, respectively 24.06% and 64.81% (E-value of 3e-10 and 0.0), named *Ncku80*.

The CRISPR/Cas9 technology platform constructed by Yang et al. [5] was used to delete *ku80* in the *N. caninum* wild-type (WT) strain (Nc1) parasites (Fig. 1A). Identification of *ku80* genes was carried out using PCR. Two primer pairs on the upstream and downstream of *ku80* gene were designed to identify the absence of the target gene. Further, two primer pairs were designed to verify the integration in 5' flank and 3' flank (Fig. 1B). Two gene-deletion strains, named $\Delta ku80$, were obtained by screening. A red fluorescent protein (RFP) coding sequence was added to the 5' end of the chloramphenicol acetyltransferase (CAT) gene, fluorescent knockout strains were captured with an inverted fluorescence microscope (Fig. 1C).

Knocking out *ku80* did not influence the phenotype of *N. caninum* in vitro

The ideal result of this study is to construct a parent strain that can be used in practical study. Therefore, in addition to increase the efficiency of HR, the morphological, growth rate and virulence of the constructed strain cannot be changed. In terms of morphological characteristics, $\Delta ku80$ and Nc1 were observed under optical microscope and fluorescence microscope after IFA. Both $\Delta ku80$ and Nc1 strains can form elliptic parasitophorous vacuole (PV), and the tachyzoites have a classical crescent-shaped the same as Nc1 (Fig. 2A).

In terms of growth, *N. caninum* has a complete lytic cycle in which the tachyzoite first invades the host cell, and the tachyzoites maintained proliferating in the host cell until sufficiently large PV is produced[11]. Subsequently, the PV bursts, and the released tachyzoites invaded other cells. In order to analyze whether knocking out *KU80* affects *N. caninum*' lytic cycles and virulence, phenotype assays, including plaque formation, invasion, replication and egress should be carried out. Plaque formation assays can comprehensively reflect the parasites develop ability in vitro. Both Nc1 and $\Delta ku80$ led to uniform, clear morphological rules and plaques (Fig. 2B). We randomly selected 50 plaques and calculated their area. T-test analysis showed that $\Delta ku80$ kept the parallel capability of plaque formation from its parental strain. ($P=0.4029$, Fig. 2C).

For invasion assay, IFA showed $\Delta ku80$ invade to HFF cells normally, which suggests there is no barrier or delay to invasion in the knockout strain ($P=0.3478$, Fig. 2D). For replication assay, 100 vacuoles were randomly selected to calculate the number of tachyzoites inside each vacuole. There were no difference between Nc1 and $\Delta ku80$ on the numbers of vacuoles carrying 2, 4, 8, 16 or more tachyzoites ($P=0.9814$, Fig. 2D). For the egress assay, Nc1 and $\Delta ku80$ were stimulated with the calcium ionophore A23187 at the same time. It was observed that $\Delta ku80$ had similar speed of escaping from HFF cells as Nc1. ($P=0.2748$, Fig. 2D). Overall, the above experiments proved that compared with Nc1, knocking out NcKU80 had no effect on the *N. caninum* phenotype in vitro.

Knocking out ku80 did not influence the virulence in mice of *N. caninum*

To estimate the effect of knocking out *ku80* on the virulence of *N. caninum* in mice, we respectively injected BALB/c mice with different doses of $\Delta ku80$ and Nc1. With the injection of 1.5×10^7 tachyzoites, it was found that two of the five mice died on 7dpi and one died on 10dpi in Nc1 group. Similarly, five mice have two deaths on 7dpi and one death on 18dpi in $\Delta ku80$ group. Statistical analysis using Graph Pad 8.0 found no significant difference between the two groups ($P=0.9119$, Fig. 3A). With the injection of 2×10^7 tachyzoites, two of five mice respectively died on 7dpi and 13dpi in $\Delta ku80$ group. While three of the five mice respectively died on days 7, 9, and 18 in Nc1 group. Statistical analysis demonstrated that the two groups also share the same survival status after injection with same doses ($P=0.6576$, Fig. 3B). The mice injected with 1×10^7 tachyzoites in the two groups survived 30 days of continuous observation. On the 31st day, brain tissues of the surviving mice were collected for qPCR to detect the parasite burden in the brain, and the result showed there was no difference in the parasite burden between the two groups ($P=0.1363$, Fig. 3C).

Results 2 and 3 illustrated that the growth and the virulence of $\Delta ku80$ kept unaffected.

Comparison of homologous recombination efficiency between $\Delta ku80$ and Nc1

Since KU80 plays an important role in NHEJ, it is hypothesized that knocking out $\Delta ku80$ can block NHEJ, thus increasing the probability of HR. In the studies of *N. caninum*, overexpression and mutation are the main editing technology. The efficiency of the gene replacement events in the *ku80* knockout background can be evaluated by introducing HA-tag into plasmids permits. The HA-tagged mutation (HA) and

overexpression (oe) plasmids of glutaredoxins C5(*GrxC5*) and S14(*GrxS14*) were constructed previously. Previous experiments had shown apicoplast localization of *GrxC5* and *GrxS14*, and neither of their overexpression nor gene deletion affected the growth of the *N. caninum* (unpublished data). Therefore, to determine if the gene replacement efficiency is increased in the *ku80* knockout background, we chose the lab-built HA-tagged overexpression to targeted into UPRT locus, and HA-tagged mutation plasmids to *GrxC5* and *GrxS14* locus. 1×10^7 $\Delta ku80$ and Nc1 tachyzoites were transfected with an equal volume of HA-tagged plasmid and CRISPR/Cas9 plasmid at the same time. All strains (*NcGrxC5-oe*, *NcGrxC5-HA*, *NcGrxS14-oe*, *NcGrxS14-HA*) were verified by PCR for the insertion of endogenous HA-tags correctly. IFA was used to measure the percentage of HA-tagged in 300-tachyzoites after 10 days of cultivation (Fig. 4A). Each plasmid was transfected 5 times.

The statistical results showed that the gene replacement events of $\Delta ku80$ was remarkably increased more than twice or even three times that of Nc1, showing a significant difference (Fig. 4B-4E). It is worth noting that using $\Delta ku80$ as parent strains success 90.75% gene replace, while that is 63.25% in Nc1, although no significant difference was found in the two groups (Fig. 4F, $P = 0.0678$). Above all, our results proved that the knockout of *ku80* could significantly increase the efficiency of HR in *N. caninum*.

Discussion

In this study, *Ncku80* knockout strain was developed using CRISPR/Cas9 technology, and the phenotype and virulence experiment indicated that the deletion of *Ncku80* had no significant impact on the growth or the virulence of *N. caninum*. Since KU80 is an important factor of c-NHEJ DNA repair pathway, the frequency of the gene replacement events of $\Delta ku80$ was explored by transfecting HA-tagged plasmids. It is proved that the efficiency of HR in $\Delta ku80$ has raise considerably. Therefore, this strain can be applied to the study of *N. caninum* to advance the efficiency of gene edition.

CRISPR/Cas9 technology has been introduced into the apicomplexan parasite, such as *Toxoplasma*, *Plasmodium*, *Cryptosporidium*[12] as well as *N. caninum*, which has considerably promoted the study on these parasites. However, due to the priority of c-NHEJ, the efficiency of HR is relatively low when CRISPR/Cas9 is used for gene insertion or deletion in many organisms. That is the reason why researchers attempted to construct *ku* gene deletion strains, and some results have been achieved. *ku* gene homologous sequence deletion strains have been constructed in *various fungi* [13] by blocking c-NHEJ, the efficiency of HR was increased to nearly 100%. In *Arabidopsis*, mutations in *ku70* can result high-frequency gene targeting [14]. In *Toxoplasma*, *ku80* gene deletion strains have also been constructed, markedly increased gene replacement efficiency to 300 to 400 fold compared to wild-type strains[9]. While little efforts were made on *ku80* knockout strain in *N. caninum*. We successfully filled in this gap, and proved the efficiency of HR was significantly increased in $\Delta ku80$.

The efficiency of CRISPR/Cas9 technology has always been an obstacle[15]. Several parameters affect the efficiency of gene targeting in CRISPR/Cas9 technology, including the fitness score of the target gene, drug selectable markers, plasmid, the targeting DNA flank lengths, the life force of parasite and so on.

Some methods have been reported to recover the defect, including substituting the *N. caninum* specific promoter and terminator[5, 15]. In this study, deletion of *ku80* was proved to work well.

Although KU70/ KU80-mediated c-NHEJ plays a role in DSB repair in many protozoa, the c-NHEJ factor is often lost in parasites[16]. For example, in *Plasmodium*, *Trypanosoma brucei*, and *African trypanosomes*, the repair of DSB is mediated by a Ku-independent microhomology-mediated end Joining (MMEJ) [17–19]. While in *N. caninum*, three conserved factors of c-HNFJ were retained: KU70, KU80 and Lig4[16]. Our study confirmed the existence of KU80 protein in *N. caninum*, and it was revealed that the deletion of $\Delta ku80$ decreases the rate of c-NHEJ, thus increasing HR's. An important issue is the virulence of $\Delta ku80$, we determined that $\Delta ku80$ can invade into the host cell, multiply in and release from it, pass across the blood-brain barrier and cause a same injury in the mice as Nc1. It means *ku80* knockout background did not lead to develop defect in *N. caninum*, which are similar to fungi and *Toxoplasma*[9, 13].

Constructing $\Delta ku80$ without any drug tagging has been tried originally but ended in failure. In order to broaden the application range of this strain, the knockout of the chloramphenicol acetyltransferase gene is underway. It may be easier to successfully generate in the *ku80* knockout background. When using this strain for gene editing, selectable markers other than chloramphenicol could be used for positive or negative selection. Positive selection including DHFR (resistance to pyrimethamine), HXGPRT (resistance to Mycophenoloic acid/xanthine), while negative selection including UPRT (resistance to 5-fluorodeoxyuracil), SNFR1 (resistance to Sinefungin) and HXGPRT (resistance to 6-thioxanthine) [20].

When four plasmids were used for transfecting in the *ku80* knockout background, the success rate of gene replacement was unchanged, additionally the efficiency was more than doubled. It suggested that HR might play a less important role in the success rate of transfection than other factors, such as the target gene locus, the mass of the plasmid and drug screening marker. The dramatic improvement of HR efficiency in $\Delta ku80$ makes it easier to screen the monoclonal strains. Moreover, the success rate of overexpression was higher than that of mutation, which may be due to different drug markers used (floxuridine, FUDR in oe and DHFR in HA). It demonstrated the value of pharmacological markers in gene editing.

Conclusions

A strain was constructed with *ku80* deletion, which improved the efficiency of homologous recombination and could improve the efficiency of gene editing in the study of *N.caninum*.

Abbreviations

HR

homologous recombination, c-NHEJ: classical nonhomologous end joining (c-NHEJ), DSB: DNA double-strand breaks, PCR: polymerase chain reaction, IFA: immunofluorescence Assay, qPCR: Quantitative Real-Time PCR, Vero cells: African green monkey kidney cell, HFF: human foreskin fibroblast,

PV:parasitophorous vacuole, HA:hemagglutinin, oe:overexpression, FUDR:floxuridine, CAT:chloramphenicol acetyltransferase, DHFR: dihydrofolate reductase, BSA:bovine albumin, PBS:phosphate buffered saline, FITC:fluorescein isothiocyanate isomer, DAPI:2-(4-Amidinophenyl)-6-indolecarbamide dihydrochloride

Declarations

Ethics approval and consent to participate

The raising and handling of animals are in compliance with the recommendations in the Guide for the Care and Use of Laboratory Animals in China. All experiments were approved by the Institutional Animal Care and Use Committee of China Agricultural University (under the certificate of Beijing Laboratory Animal employee ID: 18049). The surviving mice infected with 1×10^7 doses were injected with atropine (0.02mg/kg) at 30dpi, and were humanely sacrificed by cervical dislocation.

Consent for publication

Not applicable

Availability of data and materials

Data supporting the conclusions of this article are included within the article.

Competing interests

The authors declare that the research was conducted without conflict of interest.

Author' contributions

QL and JL conceived the study. KW performed the experiments. QL and KW analyzed the data and drafted the manuscript. XS helped in animal experiments. YW and XY helped in manuscript writing. All authors read and approved the final manuscript.

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Figures

Fig1:Deletion of *ku80* gene in *N. caninum*

A graphical representation of substitute *ku80* gene with a chloramphenicol resistance gene(a). The gRNA targeting site for *Ncku80* (GTTTCAGCCTTCAGCTCTCCG) was designed in the EuPaGDT Library of ToxoDB. The recombinant plasmid includes a chloramphenicol resistance gene that was inserted between 5' and 3' homologous flanks of *Ncku80*.The deletion of *ku80* gene and the chimerism of CAT were identified(b). Primers F1/R1 and F2/R2 were designed to verify the integration in 5' flank and 3' flank.Primers F3/R3 and F4/R4 were designed to identify the deletion of the *ku80* gene.The recombinant plasmid contained a weak red fluorescence, which is trailed off after subculture(c).

Figure 1

See image above for figure legend

Fig2: Determination of the phenotype of $\Delta ku80$ in vitro.

IFA using anti-NcSRS2 antibodies was performed to investigate the morphological characteristics of $\Delta ku80$ (a). Plaque formation assays(b).50 plaques were randomly selected and their area were calculated by Photoshop CC software (Adobe, United States) using Pixel point. The statistical test used was *t*-test(c). Invasion assay (d), Replication assay (e) and Egress assay (f) showed no significant differences between $\Delta ku80$ and Nc1 in the ability of growth. The statistical test used in three assay was *t*-test, two-way anova and *t*-test separately (d). Three independent trials were carried out in each assay. ns = no significant difference.

Figure 2

See image above for figure legend

Fig3: Determination of the virulence of $\Delta ku80$ in mice.

Growth curve of BALB/c mice injected with 1.5×10^7 (a) and 2×10^7 (b) doses of tachyzoites. qPCR was used to detect the parasite burden in the brain of mice injected with 1.5×10^7 doses of tachyzoites(c). The survival curve and the Statistical analysis were performed with Graph Pad Prism (SAS Institute Inc., United States), showing no significant differences virulence between $\Delta ku80$ and Nc1 in mice. Data were identified by *t*-test. ns = no significant difference.

Figure 3

See image above for figure legend

Fig4: Comparison of homologous recombination efficiency between $\Delta ku80$ and Nc1 s

$\Delta ku80$ and Nc1 were transfected with an equal volume of HA-tagged plasmid(a)(b): NcGrxC5-oe; c:NcGrxC5-HA;d:NcGrxS14-oe; e:NcGrxS14-HA) and CRISPR/Cas9 plasmid at the same time. The statistical results showed that the HR of $\Delta ku80$ was remarkably increased. Each plasmid was transfected 5 times. f: The homologous recombination ratio in 5 transfections of 4 type of plasmids, showing no significant differences between $\Delta ku80$ and Nc1 background. Data were identified by *t*-test. *** $p < 0.001$, **** $p < 0.0001$, ns = no significant difference.

Figure 4

See image above for figure legend

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