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The chloramphenicol acetyltransferase vector as a tool for stable tagging of *Neospora caninum*

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ABSTRACT

Neospora caninum is an obligate intracellular Apicomplexa, a phylum where one of the current methods for functional studies relies on molecular genetic tools. For *Toxoplasma gondii*, the first method described, in 1993, was based on resistance against chloramphenicol. As in *T. gondii*, we developed a vector constituted of the chloramphenicol acetyltransferase gene (CAT) flanked by the *N. caninum* dihydrofolate reductase-thymidylate synthase (DHFR-TS) 5′ coding sequence flanking region. Five weeks after transfection and under the selection of chloramphenicol the expression of CAT increased compared to the wild type and the resistance was retained for more than one year. Between the stop codon of CAT and the 3′ UTR of DHFR, a Lac-Z gene controlled by the *N. caninum* tubulin 5′ coding sequence flanking region was ligated, resulting in a vector with a reporter gene (Ncdhfr-CAT/NcTub-tetO/Lac-Z). The stability was maintained through an episomal pattern for 14 months when the tachyzoites succumbed, which was an unexpected phenomenon compared to *T. gondii*. Stable parasites expressing the Lac-Z gene allowed the detection of tachyzoites after invasion by enzymatic reaction (CPRG) and were visualised macro- and microscopically by X-Gal precipitation and fluorescence. This work developed the first vector for stable expression of proteins based on chloramphenicol resistance and controlled exclusively by *N. caninum* promoters.

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1. Introduction

Neospora caninum is an apicomplexan parasite that has been closely related to abortion and loss of fertility in cattle [1]. One of the key events to deeply investigate the invasion/replication system of the parasite is based on molecular exploitation. Although highly developed for *Toxoplasma gondii*, molecular tools are rare for *N. caninum*. The genetic manipulation in *N. caninum* with exclusive promoters was initiated by our group through the mutation of DHFR-TS and insertion of the Lac-Z gene [2]. Another possible alternative is the development of parasites that are resistant to chloramphenicol.

Chloramphenicol is an antibiotic that inhibits translation through the binding to a peptidyltransferase enzyme on the 50S ribosome protein, both in Gram-negative and -positive bacteria [3]. It probably acts in a manner similar to that in the apicomplexan apicoplast, a non-photosynthetic plastid-like organelle [4]. In bacteria, resistance against chloramphenicol is achieved by chloramphenicol acetyltransferase (CAT). CAT transfers an acetyl group of Acetyl-S-CoA to chloramphenicol to yield the initial product acetoxychloramphenicol, which is devoid of significant antibiotic activity [5]. *T. gondii* has sensitivity to chloramphenicol; however, the tachyzoites acquire resistance after transfection with the gene of resistance, CAT, flanked by a 5′ coding sequence flanking region and a 3′ downstream region of an active and non-lethal tachyzoite gene [6].

The development of a gene-controlling system in *T. gondii*, based on tetracycline transactivation [7,8], allowed the evaluation of genes related to the gliding and invasion processes. For *N. caninum*, there is a lack of options for the control of gene expression, particularly those responsive to a drug addiction. In this work, we performed the insertion of Lac-Z and tagged the tachyzoites through chloramphenicol resistance. The tachyzoites were able to receive and express the resistance gene, and the expression of the reporter gene was responsive to the concentration of the drug applied. Our findings will contribute to the development of more elaborate experiments for the genetic manipulation of *N. caninum*.

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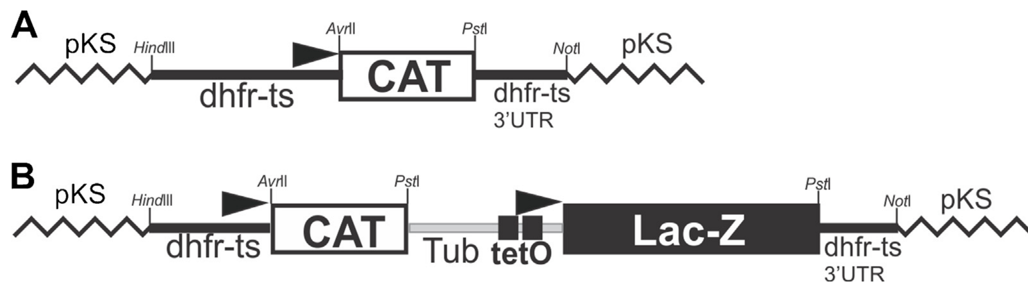

Fig. 1. The *Neospora caninum* Ncdhfr-CAT and Ncdhfr-CAT/NcTub-tetO/Lac-Z construct. (A) The vector for CAT expression was constructed by successive ligations of NcDHFR 5′ coding sequence flanking region, chloramphenicol coding sequence (CAT) and 3′ downstream region of NcDHFR in the TgtubYFP-TetR/sag-CAT [8]. (B) The vector for transient expression of Lac-Z in *Neospora caninum*, NcTub-tetO/Lac-Z, was ligated to Ncdhfr-CAT after treatment with PstI.

2. Materials and methods

2.1. N. caninum culture

Vero cell cultures were maintained in RPMI-1640 medium (Sigma) supplemented with 5% foetal calf serum (Gibco/Invitrogen), 50 μg/ml of kanamycin at 37 °C and 5% CO_2 in T-25 cm^2 or 75 cm^2 tissue culture flasks. *N. caninum* tachyzoites of Nc-1 isolate were maintained in Vero cell monolayers and purified [9].

2.2. Construction of Ncdhrf-CAT and Ncdhfr-CAT/NcTub-tetO/Lac-Z

The *T. gondii* construct, TgtubYFP-TetR/sag-CAT [8], was designed from the pCAT-GFP, which was originally derived from a pKS backbone plasmid [10]. TgtubYFP-TetR/sag-CAT was successively treated with restriction enzymes and ligated as described below.

The vector Ncdhfr-CAT was constituted of the CAT coding sequence (663 bp), amplified from the TgtubYFP-TetR/sag-CAT [8] with forward (CCCAGATCTATGCATGAGAAAAAAATC) and reverse (CCCCATATGTTATGCCCCGCCCTGCCA) primers, controlled by the 5′ coding sequence flanking region of the dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene of *N. caninum* (Fig. 1A); this was amplified (1428 bp) using the forward (TTTAAGCTTTGGGCATCACTGAGGGACTT) and reverse (TTTCCTAGGCATGTTTCGCTGCACAACTC) primers and the 3′ UTR sequence of the same gene (886 bp) was amplified using the forward (CCCCTGCAGTGGAAAAATCTGAAATATATA) and reverse (TCCGCGGCCGCCTTTCTCGCAAGTCTCCTG) primers (Fig. 1A).

The Lac-Z expression was obtained with ligation of the Lac-Z gene downstream of the *N. caninum* tubulin 5′ coding sequence flanking region. Treatment of Ncdhrf-CAT with PstI allowed ligation between the CAT coding sequence and the 3′ UTR region of NcDHFR-TS gene, generating the Ncdhfr-CAT/NcTub-tetO/Lac-Z construct (Fig. 1B). The tetO region is a site with affinity to the TetR protein extracted from a *T. gondii* construct [8], but only the β-galactosidase expression was used in this manuscript.

2.3. Stably transfected tachyzoites and CAT-ELISA

The plasmids (25 μg) were inserted in freshly purified *N. caninum* tachyzoites (1×10^8) by the enzyme restriction mediated integration (REMI) with HindIII, in cytomix buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 25 mM Hepes, 2 mM EDTA, 5 mM $MgCl_2$, pH 7.6) supplemented with 5 mM glutathione, as described by [11]. Cells were transferred to a 0.4 cm gap cuvette, electroporated with 1.8 kV at 25 mFd and 100 Ω in a BioRad GenePulser Xcell and incubated for 18 h at 37 °C, 5% CO_2, when 30 μM of chloramphenicol was added. After the lytic cycle, tachyzoites were purified and cultured with 20 μM of chloramphenicol until the development of resistant tachyzoites in the third week. Two weeks later, stable transfected tachyzoites (1×10^7) were compared with the wild type strain for the expression of chloramphenicol acetyltransferase using the CAT-ELISA kit (Roche Applied Science, USA), following the manufacturer's instructions. The reaction was read at an absorbance of 405 nm (Synergy H1, BioTek, software Gen5 2.01) and absorbance of the Vero cells was used as blank; values were analysed by one-way ANOVA with the software Prism 5.1, and post-analysed by Tukey test.

2.4. Selection of Lac-Z tachyzoites under chloramphenicol

Two equal and independent aliquots of purified tachyzoites (3×10^7) were transfected, in separated cuvettes, with 5 μg of plasmid Ncdhfr-CAT/NcTub-tetO/Lac-Z, previously treated with HindIII, and incubated in 5% CO_2 at 37 °C for 18 h, at which point four concentrations of chloramphenicol (5, 10, 20 and 40 μM) were added. After each lytic cycle, the tachyzoites were purified in Sephadex G-25 (PD-10 columns, GE) and 1×10^4 tachyzoites were inoculated in a new Vero cell culture until the next lytic cycle. The purified tachyzoites were counted in a haemocytometer and 1×10^3, 1×10^4, 1×10^5 and 1×10^6 (only after the third cycle) were incubated with 5 mM of CPRG (chlorophenolred-β-D-galactopyranoside, Roche) for 18 h at 37 °C. The samples were transferred to a 96-well plate in duplicate and the absorbance was measured at 570 nm (Synergy H1, BioTek). The absorbance equivalent to 1×10^5 tachyzoites with the standard error was calculated and plotted against each lytic cycle.

2.5. Rescue of plasmid from transfected tachyzoites

Genomic DNA from stable tachyzoites (5×10^7), selected using 20 μM of chloramphenicol for 2 months, was extracted (Wizard® Genomic DNA Purification kit, Promega), treated with PstI (0.5 U) for 18 h, purified (Illustra GFX PCR DNA and Gel Band Purification Kit, GE) and eluted in 30 μl of deionised water. A volume of 17 μl was submitted to a ligation reaction with 0.4 U Weiss of T4 ligase (Fermentas) for 18 h at 16 °C. The ligated *N. caninum* DNA was electroporated in *E. coli* Top10 (2.5 kV; 25 μF; 200 Ω), and the ampicillin-resistant colonies were either PstI- or EcoRI-treated for visualisation of the whole plasmid or for verification of the Ncdhfr-CAT insertion, respectively.

2.6. Semi-quantification of rescued plasmids

Genomic DNA from stable tachyzoites (1×10^7) selected with 5, 10, 20 and 40 μM of chloramphenicol from the seventh lytic cycle were extracted (Wizard® Genomic DNA Purification kit, Promega) and 50 ng (GeneQuant Pro, The GE Healthcare Lifesciences, formerly Amersham Biosciences) was amplified using the PCR primers Forward Ncdhfr (TTTAAGCTTTGGGCATCACTGAGGGACTT)

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