Fluorescent ester dye-based assays for the in vitro measurement of *Neospora caninum* proliferation

Caroline M. Mota, Marcela D. Ferreira, Lourenço F. Costa, Patrício S.C. Barros, Murilo V. Silva, Fernanda M. Santiago, José R. Mineo, Tiago W.P. Mineo *

Laboratório de Imunoparasitologia “Dr. Mário Endfeldz Camargo”, Instituto de Ciências Biomédicas, Universidade Federal de Uberlândia, Av. Amazonas s/n, Bloco 4C, Sala 4C01, Campus Umuarama, 38405–320 Uberlândia, Minas Gerais, Brazil

**ABSTRACT**

Techniques for the measurement of parasite loads in different experimental models have evolved throughout the years. The quantification of stained slides using regular cytological stains is currently the most common technique. However, this modality of evaluation is labor-intensive, and the interpretation of the results is subjective because the successes of the assays mainly rely on the abilities of the professionals involved. Moreover, the novel genetic manipulation techniques that are commonly applied for closely related *Toxoplasma gondii* have not yet been developed for *Neospora caninum*. Thus, we aimed to develop a simple protocol for parasite quantification using pre-stained *N. caninum* tachyzoites and fluorescent probes based on ester compounds (i.e., CFSE and DDAO). For this purpose, we employed a quantification procedure based on flow cytometry analysis. Pre-stained parasites were also examined with a fluorescent microscope, which revealed that both dyes were detectable. Direct comparison of the numbers of CFSE+ and DDAO+ cells to the values obtained with classical cytology techniques yielded statistically comparable results that also accorded with genomic DNA amplification results. Although the fluorescence emitted by DDAO was more intense and provided better discrimination between the populations of parasitized cells, CFSE+ tachyzoites were detected for several days. In conclusion, this study describes a simple, fast, low-cost and reproducible protocol for *N. caninum* quantification that is based on parasite pre-staining with fluorescent ester-based probes.

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1. Introduction

*Neospora caninum* is a coccidian parasite that is closely related to *Toxoplasma gondii* and is responsible for reproductive failure in cattle worldwide that leads to substantial economic losses in the beef and dairy cattle industries (Dubey et al., 2007; Goodswen et al., 2013). This obligatory intracellular protozoan was only characterized in 1988 (Dubey et al., 1988). Since then, *N. caninum* has been described as a common cause of infections in a wide range of domestic and wild animals (Vitaliano et al., 2004; Kul et al., 2009; Martins et al., 2011; Mineo et al., 2011; Darwich et al., 2012; Moreno et al., 2012; Nasir et al., 2012). Although this parasite is widely distributed across all continents and found in most warm-blooded animal species, its sexual reproduction, which leads to environmental contamination by oocysts, occurs only in the gastrointestinal tracts of different canid species, such as dogs (McAllister et al., 1998), crab-eating foxes (Dubey, 2003), coyotes (Gondim et al., 2004), Australian dingoes (King et al., 2010) and gray wolves (Dubey and Schara, 2011).

* Corresponding author. Tel.: +55 34 3218 2058; fax: +55 34 3218 2333.
  E-mail addresses: tiagomineo@ichim.ufu.br, tiagomineo@gmail.com (T.W.P. Mineo).
The diagnosis of *N. caninum* from routine samples is typically performed via the combination of several laboratorial approaches that include histologic, serologic, immunohistochemical and molecular methods (Dubey and Schares, 2006) and constitute a labor-intensive aspect of parasite investigation. While histological evaluation requires professional experience for precise diagnoses, parasite-specific antibody detection by itself is not conclusive and exhibits questionable specificity due to possible serological cross-reactions with closely related protozoa (Bjorkman and Lunden, 1998; Uzêda et al., 2013).

Within this context, parasite detection and quantification in research protocols is still incipient and requires urgent development to be applicable for studies of the immune mechanisms involved in infection, drug development and parasite biology. Traditional protocols, such as parasite quantification from stained slides using light microscopy, are the most commonly applied techniques due to their feasibilities and low-costs. However, these methods are labor-intensive and subjective. PCR-based methods improve *N. caninum* quantification because of their high specificities and sensitivities (Collantes-Fernández et al., 2002; Okeoma et al., 2005; Ghalmi et al., 2008), but these methods do not provide parasite visualization, require trained personnel and are relatively expensive. Currently, the best results are obtained via the fluorescent detection of parasitic cellular membranes and organelles with monoclonal antibodies (Sohn et al., 2011; Uzêda et al., 2013). However, these primary antibodies are not commercially available, which hampers the widespread use of such techniques. In this context, we aimed to evaluate in vitro protocols for parasite quantification that are based on the pre-staining of *N. caninum* tachyzoites with fluorescent ester dyes.

### 2. Materials and methods

#### 2.1. In vitro *N. caninum* maintenance

*N. caninum* tachyzoites (Nc-1; Dubey et al., 1988; kindly supplied by Prof. Solange M. Gennari) were maintained by continuous passages in a diploid immortalized cell line derived from cervical cancer (HeLa; CCL-2, ATCC, Manassas, VA, USA). Briefly, HeLa cells were cultured in RPMI-1640 medium (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 25 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% heat-inactivated calf fetal serum (CFS; Life, Thermo Scientific) in an incubator with controlled temperature and atmosphere (37°C, 5% CO2, 95% relative humidity; Fisher, Thermo Scientific). Extracellular parasites were washed twice (720 × g, 10 min, 4°C) with phosphate buffered saline (PBS, 0.01 M, pH 7.2), and the resulting pellet was resuspended in RPMI. Finally, the parasites were suspended in RPMI-1640 medium, and the numbers of viable tachyzoites were determined by Trypan

![Fig. 1](image-url)  
**Fig. 1.** Microscopic images of HeLa cells infected with *Neospora caninum* illustrating the different detection techniques. HeLa cells were infected with the Nc-1 isolate of *N. caninum* (parasite:cell MOI 1) and incubated under a controlled temperature and atmosphere for 24 h. Wells containing tachyzoites labeled with CFSE (A) and DDAO (B) were visualized and photographed with an inverted fluorescent microscope. The nuclei are stained in blue with Hoescht. The HeLa cells were cultured and infected on round glass coverslips, stained with toluidine blue (C) or Diff-Quick (D) and mounted on glass slides for evaluation with a regular light microscope. Scale bar = 10 μm.
blue exclusion (Sigma–Aldrich Co. LLC, St. Louis, MO, USA) in a regular slide hemocytometer (Neubauer chamber).

2.2. Parasite proliferation assays using fluorescent esters

The HeLa cells were seeded in six-well culture plates (1 × 10⁶ cells/well; Nunc, Thermo Scientific) and subsequently underwent an incubation step for cell adherence (3 h, 37 °C, 5% CO₂). Approximately 3 × 10⁶ tachyzoites/mL were pre-stained with 5 μM/mL of either CFDA-SE or DDAO-SE (CFSE and DDAO; Life, Thermo Scientific). After 10 min at 37 °C, the tachyzoites were washed with 10 mL of RPMI-1640 with 10% FCS and centrifuged at 800 × g for 10 min at 4 °C. Viable tachyzoites were determined with the Trypan blue exclusion test and used to infect HeLa cells at different parasite:cell proportions (multiplicity of infection – MOI: 10, 5, 1, 0.5 and 0.25). After 18–24 h, the cell monolayers were visualized and photographed with an inverted fluorescent microscope (EVOsFL, Thermo Scientific). In some experiments, the cell nuclei were stained with Hoescht 33342 (Life, Thermo Scientific). For parasite quantification, the infected cell monolayer was harvested using EDTA (10 μM), washed in PBS, centrifuged (500 × g, 10 min, 4 °C), resuspended, and immediately read in a flow cytometer (FACSCount, Becton, Dickinson and Company – BD, Franklin Lakes, NJ, USA) with at least 50,000 events acquired per tube. The laser power and filter threshold were set with the aid of appropriate reaction controls, i.e., unstained parasite and cell suspensions and an aliquot of the extracellularly stained parasites, preserved by fixation in PBS with 4% formaldehyde and maintained at 4 °C. Flow cytometry gating was performed using dedicated software (FlowJo X, Tree Star Inc., Ashland, OR, USA).

2.3. Parasite proliferation assays using rapid histological stains

The HeLa cells were cultured on 13-mm round glass coverslips in 24-well plates (1 × 10⁵ cells/well; Nunc, Thermo Scientific) for 24 h at 37 °C and 5% CO₂. The cells were infected with different MOIs (10, 5, 1, 0.5 and 0.25), and after 18–24 h, the cells were washed with PBS, fixed in 10% buffered formalin for 2 h and stained with 1% toluidine blue and Diff-Quick (Sigma–Aldrich) for 5 s. The coverslips were mounted on glass slides, and 200 cells were examined under light microscopy to determine the *N. caninum* infection index (i.e., the percentage of infected cells/100 examined cells) (Oliveira et al., 2009). Three slides from each treatment condition were assessed by two independent observers. The stained cells and parasites were visualized and photographed using an automated inverted microscope (FSX100, Olympus, Japan).

2.4. Parasite proliferation assays using real-time PCR (qPCR)

To quantify the *N. caninum* tachyzoites in the infected cell cultures, the DNA was extracted from the pelleted cells with a commercial kit (Wizard Genomic DNA purification Kit, Promega Co., Madison, WI, USA). The PCR protocol was performed using the primers and conditions described by Collantes-Fernández et al. (2002). A standard curve was established by a 10-fold serial dilution of 100 ng of the DNA and was included in the reaction. All samples were standardized for 200 ng of DNA and run in triplicate with the negative controls. The amplification, data collection and analysis were performed with a real-time PCR thermal cycler (StepOne Plus, Life, Thermo Scientific) using the SYBR green system (GoTaq qPCR Mater Mix, Promega).

2.5. Statistical analyses

The numbers of positive cells detected via pre-staining of the tachyzoites with CFSE and DDAO or Diff-Quick and toluidine blue passed the D’Agostino and Pearson omnibus normality test and were subsequently compared with one-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test. Each experiment was independently conducted at least three times, and each condition was analyzed in triplicates. A P value <0.05 was used as the cut-off for statistical significance. Prism 6 (GraphPad

![Fig. 2. Quantification of *Neospora caninum* parasite load in dose-dependent assays. HeLa cells were infected with different infection proportions of tachyzoites from the Nc-1 isolate of *N. caninum* (parasite:cell, MOI: 0.25, 0.5, 1, 5, and 10) and incubated under a controlled temperature and atmosphere for 24 h. (A) Comparisons of the different quantification methods based on cell counts. (B) Genomic Nc5 gene DNA contents evaluated by quantitative PCR (qPCR). The data are representative of three independent experiments and are presented as the means ± SEMs.](image)
3. Results

The first step for defining the practicability of the assay was to examine whether the labeled parasites were visible with a fluorescent microscope. CFSE has an emission peak at 517 nm, while DDAO presents a far-red emission that peaks at 660 nm. Although these peaks are well defined, the fluorescent signals of these dyes can be captured with a broad range of filters. We used filters that were designed to capture wavelengths emitted by FITC and PE fluorochromes to detect CFSE and DDAO, respectively. As shown in Fig. 1, the parasites that were stained with both ester-based probes could be detected through a fluorescent microscope, although the cell membranes of the parasites were not well defined. Additionally, the CFSE-labeled parasites (Fig. 1A) emitted a brighter signal than did the DDAO+ tachyzoites (Fig. 1B). Undoubtedly, the labeled parasites were far easier localized in cell monolayers than

they were with conventional slide staining with toluidine blue (Fig. 1C) or Diff-Quick (Fig. 1D).

The potential utility of tachyzoite labeling for the quantification of parasite burden within cell monolayers was compared to that of conventional cytological stains. As shown in Fig. 2A, significant dose-dependent increases were detected in all assays ($P=0.8817$). Additionally, the increasing parasite burden observed in these assays exhibited a profile similar to that obtained by qPCR (Fig. 2B).

There were notable differences in the emitted fluorescent signals from the two dyes that were detected via flow cytometry and gated with the aid of uninfected cells (Fig. 3, black boxes). The gating of the cells infected with the CFSE-stained parasites (CFSE+) exhibited a lower signal-to-noise ratio than did that of the DDAO-stained parasites; the differences in the mean intensities of fluorescence (MIFs) of the infected and uninfected cells varied between three- to five-fold (Fig. 3A). The fluorescence emitted by the DDAO+ cells was notably brighter, and the MIF values of the infected cells were 50- to 100-fold greater than those for the uninfected cells (Fig. 3B). Notably, autofluorescence

![Fig. 3](image-url)

Fig. 3. Comparison of *Neospora caninum* quantifications based on parasites that were pre-stained with CFSE or DDAO. The HeLa cells were infected by tachyzoites from the Nc-1 isolate of *N. caninum* that had previously been labeled with CFSE or DDAO (MOI 0.5). The cells were incubated under a controlled temperature and atmosphere for 24 h. Representative dot plots of CFSE+ (A) and DDAO+ (B) cells obtained by flow cytometry analyses. Each figure indicates the values of the mean intensity of fluorescence (MIF), the overall positive cell percentages for each dye, and the populations with high staining intensities for the CFSE and DDAO (Nc+high). The dot plots indicate the concentrations of cells with color; blue represents lower cell concentrations, and red indicates high cell densities. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
was irrelevant at the DDAO emission wavelength, which supported a clearer distinction that allowed for the easier identification of parasite-associated cells compared to the CFSE staining. Additionally, it was possible to observe a population of cells with brighter intensities that possibly reflected cells with high parasite burdens; this population is referred to as \( \text{Nc}^{+}_{\text{high}} \) (orange boxes, Fig. 3).

In contrast, the strong fluorescent signal emitted by DDAO is a short-term signal that lasts for only 24–48 h, but the CFSE+ tachyzoites were detected as late as 12 days after staining under continuous passages in monolayers of HeLa cells (Fig. 4). These parameters are reflected in the proportions of infected cells (Fig. 4A) and in the MIFs (Fig. 4B).

4. Discussion

Infections with the protozoan \( N. \text{caninum} \) cause billions of dollars of damages to cattle ranchers and pet owners (Reichel et al., 2013). However, the development of biotechnological tools for the identification of this protozoan is still incipient. In contrast, genetic tools for the closely related parasite \( T. \text{gondii} \) have rapidly evolved (Huynh and Carruthers, 2009; Andenmatten et al., 2013) and have enabled the acquisition of substantial knowledge about the cell biology and host interactions of this parasite (Beck et al., 2013). Attempts to develop similar tools for \( N. \text{caninum} \) have been scarce, and only a few reports on this subject are available (Pereira et al., 2014; Howe et al., 1997; Zhang et al., 2010; Collantes-Fernandez et al., 2012).

Given the need to develop and apply new tools to acquire a broad and comprehensive knowledge of \( N. \text{caninum} \), we proposed an alternative method for parasite quantification that uses fluorescent ester-based probes (CFDA-SE and DDAO-SE). The assays utilizing both probes and the protocols utilizing cytological stains and PCR assays revealed increasing trends in parasite detection in cells that were infected at higher MOIs. Because the interpretations of conventional staining techniques are subjective, quantification with flow cytometry is expected to represent a more precise and consistent alternative for assays that aim to quantify cellular parasite burdens. The fluorescence emitted by DDAO was notably brighter than that of CFSE and thus resulted in clear quantification of the infection proportions. However, long-term infection experiments utilizing DDAO might not be reliable due to the quick decay of the fluorescence signal; CFSE could be adopted for such purposes.

Altogether, the CFSE and DDAO dyes were shown to be reliable and minimally time-consuming, while the interpretation of results from traditional slide staining protocols might be subjective. Similar procedures have been reported to be successful in at least one other protozoan model (Leite et al., 2012). Additionally, we have previously reported the detection of CFSE-labeled \( N. \text{caninum} \) tachyzoites after seven days in vivo (Mineo et al., 2010). Considering the advantages of these quantification protocols and the lack of tools that have been developed for the genetic manipulation of \( N. \text{caninum} \), we recommend these protocols for the quantification of parasite loads in studies seeking to elucidate parasitic cell biology mechanisms, host–parasite interactions and drug development studies.

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