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

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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.

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 Schares, 2011).

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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% CO_2, 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

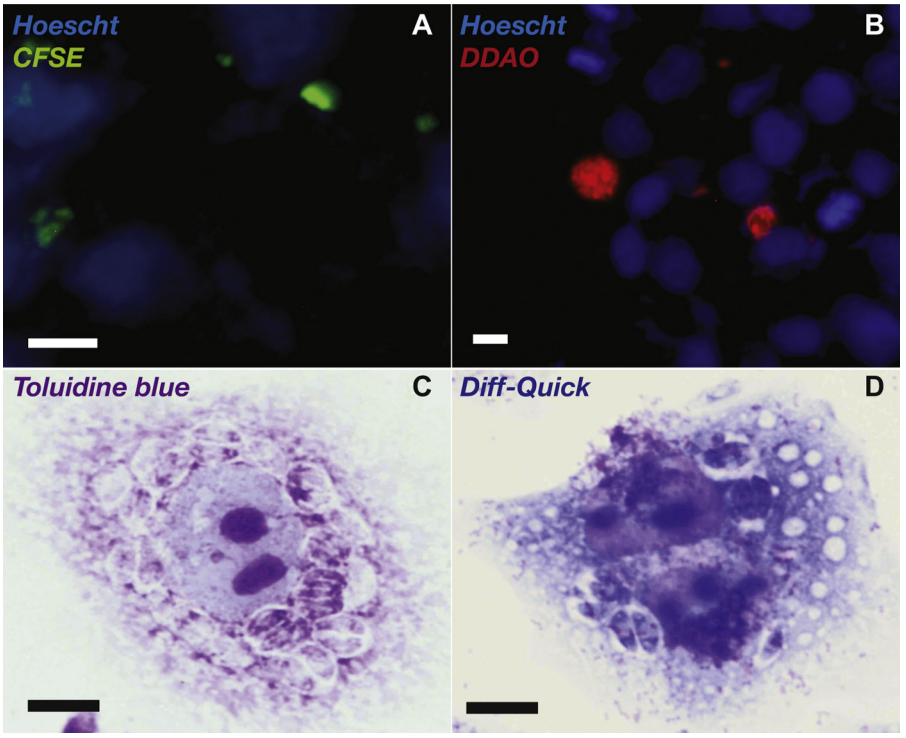

Fig. 1. Microscopic images of HeLa cells infected with *Neospora caninum* illustrating the different detection techniques. HeLa cells were infected with the Nc-1 isolate of *N. caninum* (parasite:cell MOI 1) and incubated under a controlled temperature and atmosphere for 24 h. Wells containing tachyzoites 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.