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Full Length Research Papers

***Cryptosporidium* immunolabeling in paraffin-embedded ileum of CD-1 neonatal mice**

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Cryptosporidiosis is a well-recognized zoonotic disease and represents both a major public health and veterinary problem. There are currently no available commercial antibodies designed for *Cryptosporidium* labeling in histological sections, and no protocol has been suggested. The aim of this study was to establish a standardized protocol using immunofluorescence techniques to detect *Cryptosporidium* in paraffin-embedded intestine sections. Neonatal CD-1 mice were experimentally infected with 10⁵ *Cryptosporidium parvum* Iowa strain oocysts. At peak infection (8 days post-infection), intestines were removed, fixed in formalin buffer and included in paraffin. Three commercial anti-*Cryptosporidium* antibodies (Sporo-GloTM, Crypt-a-GloTM, and Merifluor[ ]) were tested on tissue sections using standard immunofluorescence methods. Both parasite colonization and non-specific immunolabeling in the terminal ileum were evaluated. Our results showed that all antibodies detected intracellular developmental stage *C. parvum*. Qualitative differences were noted between antibodies, particularly in relation to background staining and artefacts, which were more present with Crypt-a-GloTM and the Merifluor[ ] antibodies. Our results indicated that Sporo-GloTM was the most effective antibody for *Cryptosporidium* detection in paraffin-embedded intestine. This technique proved to be an efficient tool for histological studies of cryptosporidiosis and could be recommended in future studies investigating infectivity of *Cryptosporidium* isolates, as well as testing efficacy of new therapeutic agents.

Keywords: Immunofluorescence histology, CD-1 neonatal mouse model, *Cryptosporidium parvum*, paraffin-embedded tissue section, anti-*Cryptosporidium* commercial antibody

INTRODUCTION

Cryptosporidiosis is a common health problem of both public and veterinary interest worldwide. Livestock (cattle, goats, and sheep) are significant carriers of the predominant species, *C. parvum*, which is the most frequently implicated in zoonotic transmission (Shahiduzzaman and

Dauguschies, 2012). In cattle, the disease is clinically indicated by diarrhea, especially in young animals. The resultant delayed growth and mortality in livestock are thus responsible for considerable economic losses (de Graaf et al., 1999).

The well-characterized neonatal CD-1 mouse strain has been used to evaluate the anti-cryptosporidial efficacy of different compounds in several studies (Finch et al., 1993; Korich et al., 2000). Many tools are currently available to determine the presence of *Cryptosporidium*. The most frequently-used technique in routine diagnosis is the direct detection of *Cryptosporidium* oocysts in feces (Chalmers and Katzer, 2013), whereas immunolabeling enables the evaluation of infection intensity. Today, no specific commercial antibody is recommended to stain *Cryptosporidium* in histological sections. Thus, the present study was performed to establish a standardized *Cryptosporidium* immunolabeling protocol in paraffin-embedded ileum tissue sections using classic immunofluorescence (IF) techniques. Three commercial available antibodies were tested: Sporo-Glo™ and Crypt-a-Glo™ (Waterborne™ Inc., New Orleans, USA), and Merifluor® (Meridian Bioscience Inc., Italy).

MATERIALS AND METHODS

Parasite

C. parvum oocysts (Iowa strain) were purchased from Waterborne Inc. (New Orleans, Louisiana, USA). This strain is considered to be the benchmark for the study of *C. parvum* (Puiu et al., 2004) and was originally collected from experimentally-infected neonatal calves, purified, and suspended in phosphate-buffered saline (PBS), then stored at 4°C until use.

Animals

CD-1 outbred mice used in this study were obtained from Charles River Breeding Laboratories (France). The neonatal CD-1 mouse strain used in this study is well characterized and has previously been used to evaluate the anti-cryptosporidial efficacy of several compounds (Finch et al., 1993; Korich et al., 2000). Animals were housed in isolators in dedicated facilities under aseptic conditions throughout the experiment. Bedding was changed every two days. Suckling mice were housed with their mothers throughout the study in an experimental housing system which consisted of plastic cages measuring 60 × 40 cm with filter tops (Charles River, France) containing wood shavings as bedding. All animals had access to standard pelleted diet and water *ad libitum*. Temperature was maintained at approximately 21°C.

Experimental infection

Two litter groups were used in this study: one uninfected litter group (*Cp* -, n=5) and one infected litter group (*Cp*

+, n=2). In the infected litter, 5-day-old neonatal mice were experimentally inoculated by esophageal gavage with 10⁵ *C. parvum* oocysts, while each neonatal mouse in the uninfected litter only received an equal volume of phosphate-buffered saline (PBS). Gavage was performed using 22-25 plastic feeding tubes (Phymep, France), and by maintaining the head of neonatal mice in an extended position. All animals were euthanized by cervical dislocation at 8 days post-infection, the peak infection date (data not shown). Immediately after euthanasia, the distal 5 cm of the ileum was removed, fixed and embedded in paraffin, and sectioned for histology.

Ethics statement

All animal experiments were conducted according to the European Guidelines for animal welfare (2010/63/EU) with approval from the commission for animal experiments of the Anses/ENVA/UPEC French Regional Ethical Committee 16 (Registration number 14/04/15-4B), and by the French Ministry of National Education, Higher Education and Research (project APAFIS#2186-2015100618295444v2).

Cryptosporidium immunolabeling

The direct IF technique was carried out using three commercial antibodies: (a) Sporo-Glo™ (A600FL-20X, Waterborne, New Orleans, Louisiana, USA), a rat polyclonal antibody for the detection of *C. parvum* intracellular dividing stages; (b) Crypt-a-Glo™ (A400FLR-1X, Waterborne, New Orleans, Louisiana, USA USA), a murine monoclonal antibody to detect *C. parvum* oocysts in water and other environmental samples; and (c) Merifluor® *Cryptosporidium Giardia* kit (Meridian Biosciences, Inc., Italy), containing a mice monoclonal antibody to detect oocysts of both *Cryptosporidium* and *Giardia* in stool samples. All of these primary antibodies were directly conjugated to fluoresce in isothiocyanate (FITC, green) to enable visual localization of *C. parvum* intracellular stages. To identify cell nuclei, the sections were also stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, France) that binds to DNA (blue).

Standard fixation and ileum paraffin inclusion techniques were used as described in a previous modified protocol (Neckel et al., 2016). The ileum tissue was fixed in formalin buffer for 48h (4% formaldehyde in 0.1 M phosphate buffer). Then 1 cm-long sections were dehydrated with increasing alcohol gradients and paraffin-embedded according to standard procedures. Subsequently, 5 µm-thick sections were obtained. Sections were affixed on Superfrost™ Plus (Thermo Scientific, France) slides to improve attachment, dried overnight at 37°C, and acetone-fixed at room temperature.

Slides were dewaxed and rehydrated by successively passing the slides through the following baths: Xylene for 6 minutes; Xylene 1:1 vol/vol with 100% ethanol for 3 minutes; 100% ethanol for 6 minutes; 95% ethanol for 3 minutes; and then 70% ethanol for 3 minutes. Slides were then washed with distilled water. Antigen unmasking was performed with 10 mM sodium citrate buffer (Sigma, France) containing 0.05% Tween 20 (Montanox, Seppic, France) in a microwave oven at 700 W for 1 min, then slides were cooled in the same buffer used for heating. After washing with distilled water, slides were incubated with 0.5% trypsin solution (Sigma, France) and placed in a humid oven at 37°C for 10 minutes, then washed again with distilled water. Auto-fluorescence was eliminated by incubation in a blocking solution of 1% bovine serum albumin (BSA, Sigma, France) in 1X PBS (Invitrogen, France) for 2 hours with gentle stirring. After washing with distilled water, slides were incubated with commercial antibodies (diluted 1/20 in PBS) in a dark humid room at room temperature for 60 min. Slides were incubated with DAPI (5 µg/mL in PBS) for 10 minutes under similar conditions. Final mounting used ProLong Gold Antifade Reagent (Invitrogen, France).

Sections were observed with a Leica DMI 3000B fluorescent microscope using the Leica Application Suite software (version 4.5.0; Leica Microsystems, Inc., Switzerland).

The most effective antibody was determined by evaluating the ease with which intracellular forms of the parasite could be observed, as well as the absence of any artifact or non-specific immunolabeling, particularly in uninfected animals. To determine which antibody detected parasite development with the greatest sensitivity, infection intensity was quantitatively evaluated via several different measurements in infected animals. These included the mean number of intracellular stage *C. parvum* contiguous with the epithelium that also co-stain with DAPI, relative to the circumference of the histological section as previously described (Tamburrini and Pozio, 1999). For each antibody test, 10 sections were randomly selected. The number of intracellular stages/cm was compared between the three antibodies by the non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparison post-test, using GraphPad Prism software (version 5, USA). Statistical significance was accepted at $P < 0.05$.

RESULTS

Uninfected animals did not display any evidence of parasitic infection. All newborn mice remained clinically healthy during all experimental trials.

Our results showed that all three antibodies detected *Cryptosporidium* at the intracellular stage of development. Differences were noted between antibodies, particularly regarding the observation of internal parasite morphology and background staining. Technical IF assessment with clearly-defined paraffin-embedded tissue morphology was clearer with the Sporoglo™ antibody (Figure 1). The use of DAPI, which non-specifically stains DNA, enables the detection of *C. parvum* nuclei during the development stage.

Intracellular stages were also detected with Merifluor® and Crypt-a-Glo™ monoclonal antibodies. However, difficulties arose when observing internal forms, and the presence of non-specific background labeling was noticed in 6/10 and 5/10 slides with Merifluor® and Crypt-a-Glo™ antibodies, respectively.

The number of intracellular-stage *C. parvum* labeled along 1 cm of ileum section circumference indicates that the three commercial antibodies all enable simple quantification of intracellular stages. No significant differences in parasite count were observed with the three antibodies (Table 1).

DISCUSSION

In this study, we used an ileum IF staining method with three different commercial anti-*C. parvum* antibodies in two experimental groups of neonatal CD-1 mice: i) uninfected animals, which eliminated non-specific and background labeling; and ii) infected animals, where the parasite multiplied on the surface of the intestinal epithelium, particularly in the ileum area.

There are two major types of IF staining methods: direct IF and indirect IF. Direct IF is less sensitive than the indirect technique because the signal is not amplified but direct IF does have greatly reduced fluorescent background staining in paraffin-embedded tissues (Kajimura et al., 2016). Thus we chose to use the direct IF technique. These study results demonstrate that the direct IF assay is of potential value in investigating cryptosporidiosis, by enabling the detection of *C. parvum* intracellular developmental stages, even though the antibodies used were not originally intended for IF in histological sections by the manufacturers. No differences in infection intensity were observed between the experimental groups. However, there were clear differences in labeling results between the three antibodies. We report that IF using the Sporoglo™ antibody more clearly illustrated the internal structure of *C. parvum* intracellular stages, thus facilitating their identification, and presented less background staining (non-specific signal) than Crypt-a-Glo™ or Merifluor® antibodies. This could be explained by the fact that Sporoglo™ was produced in rat while the two other antibodies were produced in mice.

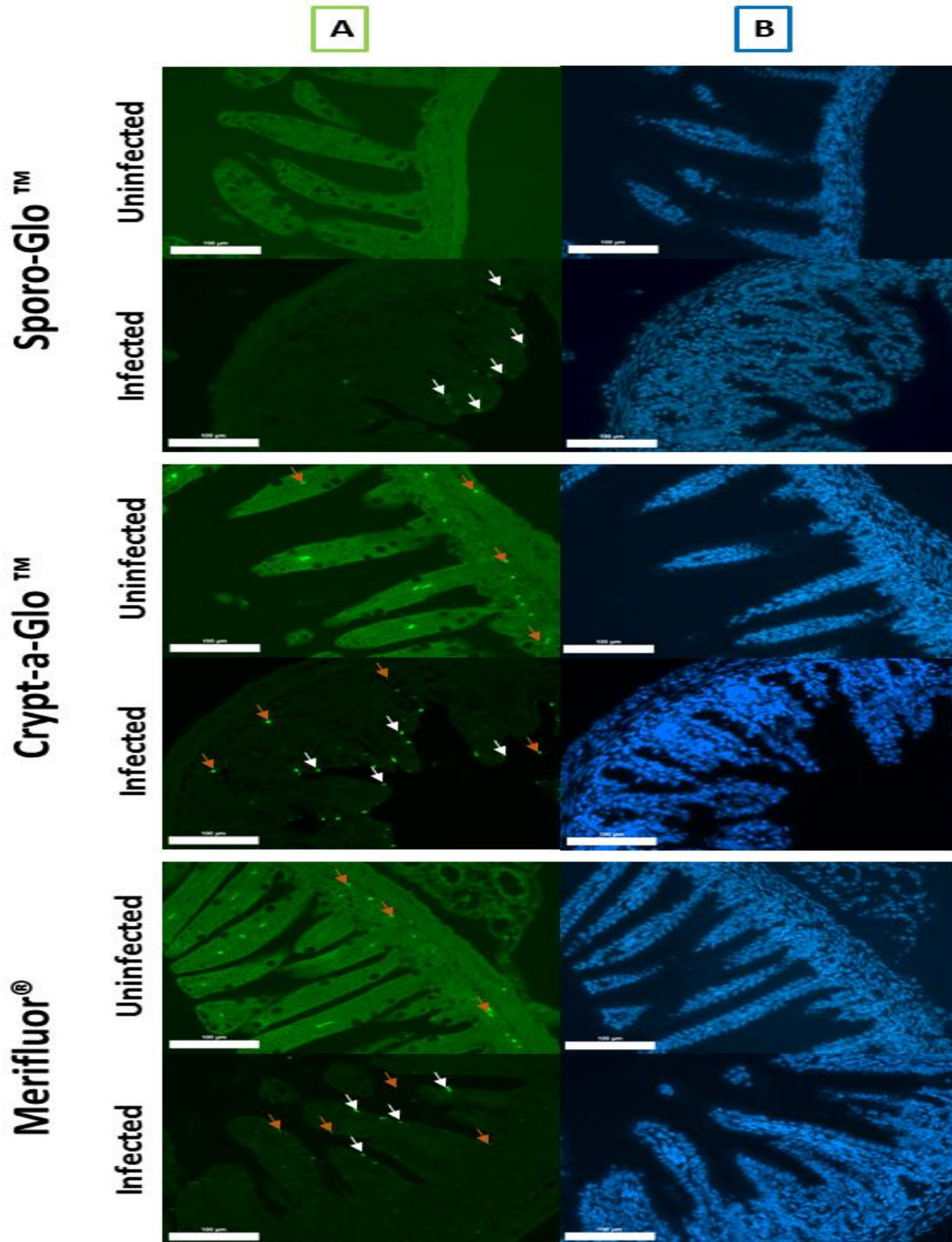


Figure 1: Representative IF staining images of ileum from infected and uninfected CD-1 neonatal mice. A. Staining with three commercial antibodies labelled with FITC. B. Staining with DAPI. The white arrows represent *C. parvum* intracellular stages. The orange arrows represent artifacts and non-specific colorations. Magnification bars: 100 μ m.

Table 1: Infection level in 10 histological ileum sections from two infected neonatal mice, using three commercial antibodies.

Antibody	Sporo-Glo™	Crypt-a-Glo™	Merifluor® <i>Cryptosporidium/Giardia</i>
Neonatalmouse1	116,40 ± 15,75 ^{NS}	124,70 ± 46,48 ^{NS}	96,63 ± 11,95 ^{NS}
Neonatalmouse2	111,90 ± 13,62 ^{NS}	103,50 ± 13,28 ^{NS}	109,90 ± 9,97 ^{NS}

NS: Non Significant

Results are presented as the Mean number of *Cryptosporidium parvum* intracellular stages per 1 cm of ileum ± standard deviation. For each antibody tested on mouse 1 and mouse 2, infection level was compared between the three antibodies by the non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparison post-test.

DAPI DNA staining did not enable clear morphological identification of *C. parvum* developmental stages, but was important to confirm real antibody staining. Using IF in combination with DAPI is a recommended strategy to confirm the presence of endogenous *C. parvum* developmental stages. In another study (Boxell et al., 2008), Sporo-Glo™ antibody was proven to detect both extracellular stages (sporozoites and merozoites) and intracellular stages (trophozoites, merontes, and gamontes) in cell-free culture. Sporo-Glo™ is specific for *C. Parvum* and has not yet been described to detect other *Cryptosporidium* species in histological sections. In our laboratory, this antibody was successfully used to perform direct immunofluorescence in cultured cells (HCT-8 and Caco-2 cells) infected with *C. ubiquitum* isolated from an infected lamb (data not published). It would therefore be of interest to test this antibody with other *Cryptosporidium* species.

In our study, the most effective of the three tested antibodies was Sporo-Glo®, and we recommend its use in routine experimental *Cryptosporidium* diagnostic practice. The staining protocol used to detect *Cryptosporidium* in paraffin-embedded sections follows recommendations from the academic and pharmaceutical-based histopathology researchers consortium (Howat et al., 2014), and could be shared with the scientific community to avoid the need for repetition.

This technique can be recommended to confirm *Cryptosporidium* proliferation and amplification when routine clinical diagnosis methods are unable to detect the parasite (especially in animals that excrete low levels of parasite), and to determine oocyst infectivity during water quality surveillance. In fact, fecal matter is most often used for *Cryptosporidium* oocyst detection, but success depends largely on the sensitivity of the utilized technique. In studies where infectious doses are low, mice take longer to excrete oocysts in their fecal pellets (Yang et al., 2000), and thus in these cases, histology becomes the preferred method.

There is a need for the development of fully effective compounds to prevent cryptosporidiosis, thus this

methodology will likely be very useful in studies investigating novel active anti-cryptosporidial compounds.

CONCLUSION

In conclusion, the present study clearly demonstrates that a direct IF method with the commercial Sporo-Glo™ antibody is effective when investigating cryptosporidiosis in a neonatal mouse model. The experimental procedure is described in detail, and may aid laboratories planning to introduce this standardized *Cryptosporidium* immunolabeling protocol in paraffin-embedded tissue sections.

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Conflict of interests: The authors declare that they have no conflicting interests.

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