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Exploring challenges in *Giardia* cyst visualisation by common microscopy methods

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ABSTRACT

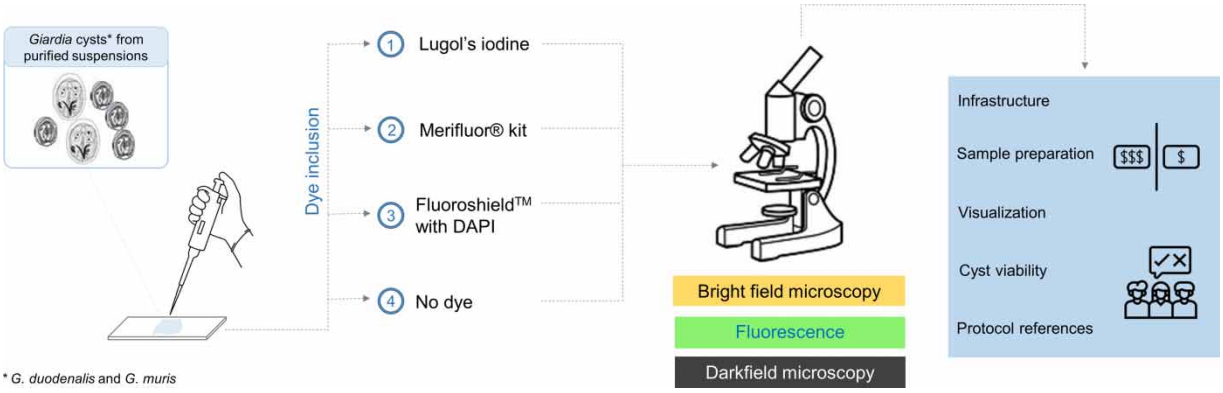
Giardia spp. is an intestinal parasite responsible for worldwide disease outbreaks. Guiding researchers and practitioners to choose among current methods for microscopy detection of the infectious forms may be directly beneficial to public health and the environment. This study provides an overall comparison of brightfield (BF), fluorescence and darkfield (DF) microscopies for detecting *Giardia duodenalis* and *Giardia muris* cysts, by illustrating micrographs of such protocols applied to purified samples, as well as discussing advantages and constraints based on secondary information and collected data. BF analysis included Lugol's iodine staining. In fluorescence microscopy, samples were processed by immunofluorescence assay (IFA) with DAPI and by standalone DAPI dye. Cyst suspensions were also analysed by DF microscopy using a recently developed low-cost system. The three techniques enabled detecting *Giardia* spp. cysts, although they did not provide species identification by morphology. The overview of each method points out some relevant aspects to consider when selecting common optical microscopy techniques, and includes challenges and advantages regarding each of them.

Key words: darkfield, immunofluorescence, Lugol's iodine, optical microscopy, waterborne protozoa

HIGHLIGHTS

- Comparisons of BF, DF, standalone DAPI and IFA-combined are provided.
- BF and DF may be alternatives for low-cost detection of Giardia cysts.
- Combinations of at least two diagnostic methods are recommended to minimise inherent errors.

GRAPHICAL ABSTRACT



1. INTRODUCTION

For a long time, water, sanitation, and hygiene (WASH) practitioners and regulating bodies have relied heavily on indicator microorganisms for many of their decisions, particularly in developing nations. However, recent studies have indicated that total reliance on faecal indicator bacteria may be misleading, since this group is often less persistent and more easily inactivated during treatment processes than other organisms, including the protozoa *Giardia* spp. (Mraz *et al.* 2021).

Giardia duodenalis is one of the commonest intestinal parasites that infects humans worldwide (Yason & Rivera 2007). From 2011 to 2016, 37% of worldwide waterborne protozoa outbreaks were caused by this zoonotic protozoan (Efstratiou *et al.* 2017). The chlorine-resistance characteristic combined with cysts' ability to permeate filtration units because they are small (6–15 μm) can explain the occurrence of giardiasis epidemics after consumption of treated drinking water in several countries (Smith 1998; Omarova *et al.* 2018). This illustrates the importance of considering this pathogen when making decisions regarding water and sanitation, as well as including it in regular monitoring of water systems, either conventional or point-of-use. The latter, particularly, is very important in low-income and self-supplied regions, which are more likely to be contaminated than others (Genter *et al.* 2021).

Because of the typical cyst morphology that avoids mistaking *Giardia* for other organisms, optical microscopy is still the main methodology used routinely to detect this organism in water, food, and faecal samples (Adeyemo *et al.* 2018). In addition, as it is low-cost (compared to molecular-based tools) and relatively easy to run, this sort of technique is the most suitable for developing countries (Tangtrongsup & Scorza 2010).

Brightfield (BF) microscopy associated with Lugol's iodine stain is considered the gold standard methodology for detecting cysts in stool samples (Hooshyar *et al.* 2019). It enhances the internal structures of cysts, which stand out from the other materials on the slide (Tangtrongsup & Scorza 2010). Nevertheless, in the field of water and sanitation, the standard protocol is direct immunofluorescence assay (IFA). This technique consists of adding fluorescent-labelled antibodies that will link specifically to the cysts' wall producing a bright fluorescence that can be observed in the microscope (USEPA 2012, 2014). For confirmation, it has been suggested that DAPI (4′,6-diamidino-2-phenylindole) must be applied at the same time as the IFA test (USEPA 2012). The stain highlights the nuclei when it binds to DNA (USEPA 2012).

More recently, Belini *et al.* (2018) showed that darkfield (DF) microscopy was effective in detecting *Giardia* spp. in purified suspensions. This illumination technique is based on the formation of a hollow cone of light that encloses the objective lens, causing the light to bypass them. Thus, only diffracted/scattered light from objects in the mounting medium enters the objective lens, forming bright white structures against a dark background (Murphy 2001).

Despite the variety of microscopy techniques for *Giardia* cyst detection, selecting the most suitable method still poses a challenge, especially for practitioners. Therefore, this study aimed to explore, under controlled conditions, common methods for detecting *Giardia* spp. cysts, shedding light onto the possibilities for choosing protocols for detecting this parasite.

2. METHODS

2.1. *Giardia* cysts

Purified suspensions of *Giardia duodenalis* (200 cysts μL^{-1}) and *Giardia muris* cysts (149 cysts μL^{-1}) (Waterborne, Inc., New Orleans, USA) were used for microscopy. Although *G. duodenalis* is the only species considered pathogenic to humans, the rodent-exclusive pathogen, *G. muris* has been used as a model for *G. duodenalis* since their morphologies are similar (Haas & Kaymak 2003), making it also relevant to environmental, water and sanitation studies.

Aliquots of 5 μL of each suspension were spiked together onto flat glass microscopy slides for three different preparations – see below. The procedure was repeated for each species on an individual slide.

2.2. Microscopy visualisation and imaging

For BF microscopy, one drop of Lugol's iodine was added to the slides. *G. duodenalis* and *G. muris* identity were confirmed based on characteristics of cyst size (7–10 μm long × 8–12 μm wide) and internal structures, according to classic literature description (Filice 1952; Jakubowski 1984).

As for fluorescence microscopy, spiked samples were kept dry overnight. Then, as recommended in Method 1623.1 (USEPA 2012), IFA was performed using the Merifluor® kit (Meridian Bioscience, Inc.), according to the manufacturer's recommendations. When using this kit, *Giardia* cysts appear as brilliant apple green fluorescence objects under ultraviolet light, with a typical size and an oval to round shape (USEPA 2012).

To visualise the cysts' nucleus, two drops of Fluoroshield™ with DAPI (Sigma-Aldrich®) were added and incubated for 10 min at room temperature. This reagent stains the cysts a light blue internally, staining up to four distinct nuclei with a green rim or highlight (USEPA 2012). A second fluorescence microscopy process involved a simpler preparation method using only Fluoroshield™ with DAPI.

An epifluorescence microscope (BX51, Olympus®) was used for both BF and fluorescence microscopy. Use of the former involved conventional staining with Lugol's iodine and observation of the organisms' structures under visible light using a 40× objective lens. The latter involved the use of FITC and DAPI as optical filters (Table 1) for visualising cysts and nuclei, respectively. Physical dimensions determined on Image-Pro® 6.3 were compared to reported patterns for *Giardia* cysts, according to USEPA (2012).

Table 1 | Optical filter specifications used in the Olympus BX51 microscope

Optical filter	Wavelength (nm)	Excitation wavelength (nm)	Emission wavelength (nm)
FITC	570	460–490	520
DAPI	400	330–385	420

DF images were acquired by adapting the standard microscope using an inexpensive, custom-made DF setup developed by Belini *et al.* (2018). A dry objective lens (Olympus BX41TF) and a charge-coupled device camera (Samsung® SDC313) were used. Image capture was carried out on PixelView®. Knowledge of the cysts' characteristic morphological features improved *Giardia* detection accuracy (Belini *et al.* 2018).

3. RESULTS AND DISCUSSION

3.1. Lugol's iodine staining under BF

As shown in Figure 1, this technique simplifies reliable identification of *Giardia* cysts by determining additional criteria besides size and shape. Using Lugol's iodine, *Giardia* spp. cysts, their double walls (thick arrows) and one internal structure (axoneme indicated by the narrow arrow) can be seen (Figure 1(a) and 1(b)). Occasionally, when comparing *Giardia* cysts, their size appears to be a consistent indicator for identification against different organisms. However, as *G. duodenalis* and *G. muris* share the same dimension spectra they cannot be differentiated between themselves.

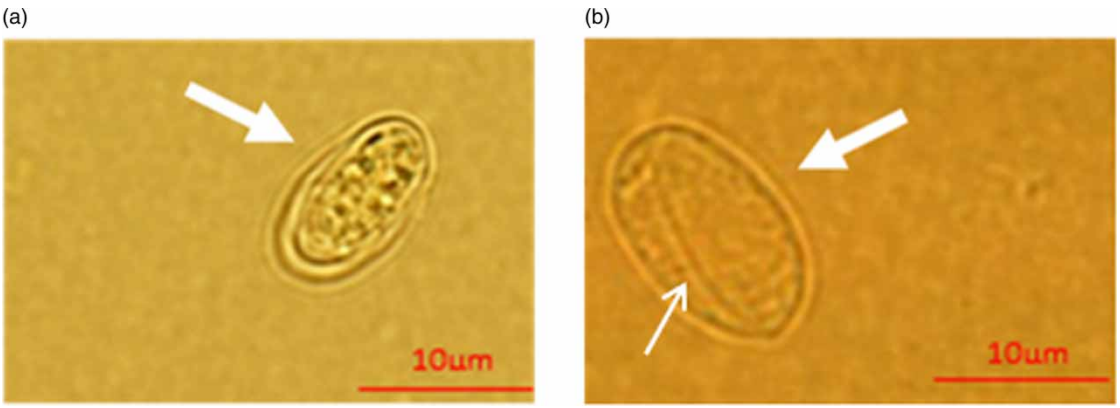


Figure 1 | Microscopic images of (a) *Giardia muris* and (b) *Giardia duodenalis* with Lugol's iodine acquired under BF. Thick arrows point to cyst walls and the narrow arrow to an axoneme.

Although Lugol's iodine is the most used dye in routine laboratory diagnostics, its use requires an expert to identify organisms and their typical structures (Charakova 2010). Furthermore, internal quality control of the working iodine solution must be performed regularly on known reference organisms to ensure the performance of the stain (Dalynn Biologicals 2005). As staining is not selective, this method hinders cyst detection in environmental samples, which are likely to contain debris and therefore require concentration and purification steps, and deep reliance on the microscopist's subjective analysis.

3.2. Immunofluorescence assay and DAPI staining

Figure 2(a) displays a clear *Giardia* cyst (narrow arrow) visualised under FITC filter block. This result was confirmed by DAPI, which exhibited intense blue internal staining (Figure 2(b)). Thicker arrows in this micrograph also suggested the presence of cysts, but differences in the fluorescence signal led to doubt. As DAPI staining is not as specific as FITC-fluorescence, considering interferences, separate samples were prepared to evaluate the particularities of each species.

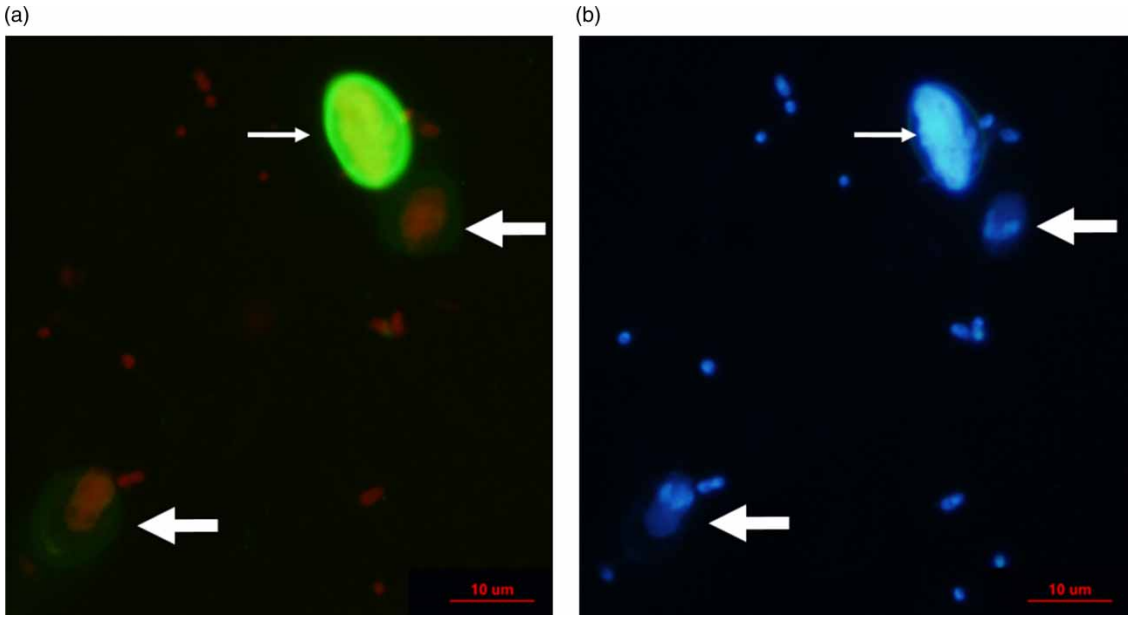


Figure 2 | Visualisation of a mixed sample under (a) FITC and (b) DAPI. Narrow arrows point to *Giardia duodenalys* cysts visualised as expected and thick arrows to possible *Giardia muris* cysts.

Figure 3 shows *G. duodenalis* cysts under FITC (a) and DAPI (b), compared to *G. muris* under the same filters (c and d, respectively). *G. muris* fluorescence intensity was clearly lower than that in *G. duodenalis* (Figure 3(a) and 3(c)), suggesting that observations indicated by thick arrows in Figure 2 referred to *G. muris*.

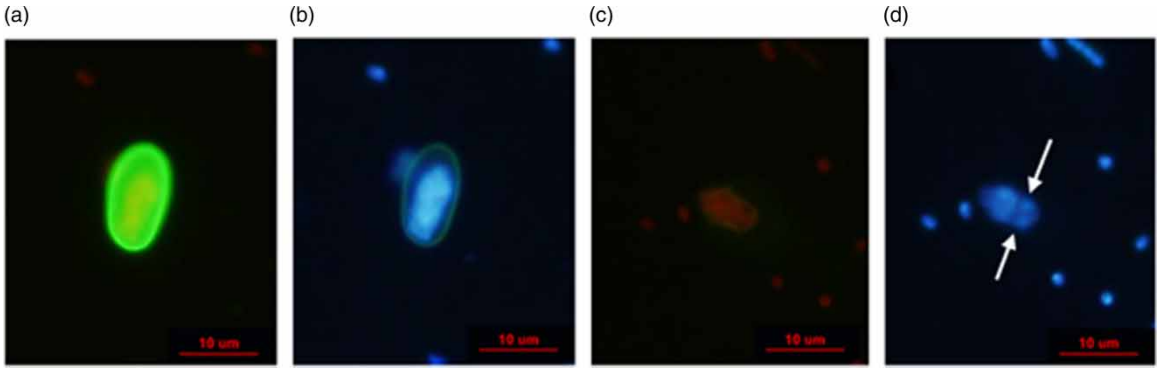


Figure 3 | Difference in *Giardia* cyst fluorescent dye uptake: *Giardia duodenalis* visualised under (a) FITC and (b) DAPI; *Giardia muris* under (c) FITC and (d) DAPI. Narrow arrows indicate the nuclei of *Giardia muris*.

Despite the high sensitivity and specificity of Merifluor®, its datasheet indicates that the kit is recommended for use on faecal human samples, in which *G. duodenalis* is, possibly, the only species present. Therefore, the monoclonal antibodies of the kit are likely to be specific to *G. duodenalis* antigens. This assumption could explain the difference in fluorescence intensity between the two species but could also be a limiting factor for the kit's use in environmental matrices, since there is no assurance which species might be present in the sample (Edelstein & Edelstein 1989; Alderisio *et al.* 2017). From a public health perspective, this is particularly important, as differences in fluorescence staining may mislead reported cyst occurrences and concentrations in environmental samples (Alderisio *et al.* 2017).

Still in that sense, although FITC-characteristic fluorescence was unclear for *G. muris* (Figure 3(c)), two nuclei were observed clearly (Figure 3(d)), implying that Fluoroshield™ with DAPI exclusive staining could represent an alternative for distinguishing *G. duodenalis* from *G. muris*. However, when this hypothesis was tested, it was found that DAPI fluorescence declines over time when applied as a standalone dye (Figure 4(a)–4(c)). This confirms that a successful IFA protocol must include fluorescent-labelled antibodies and DAPI (USEPA 2012). That should also be taken into account for different matrices. If IFA readings in purified suspensions are misleading, this challenge is likely to be even harder for environmental samples.

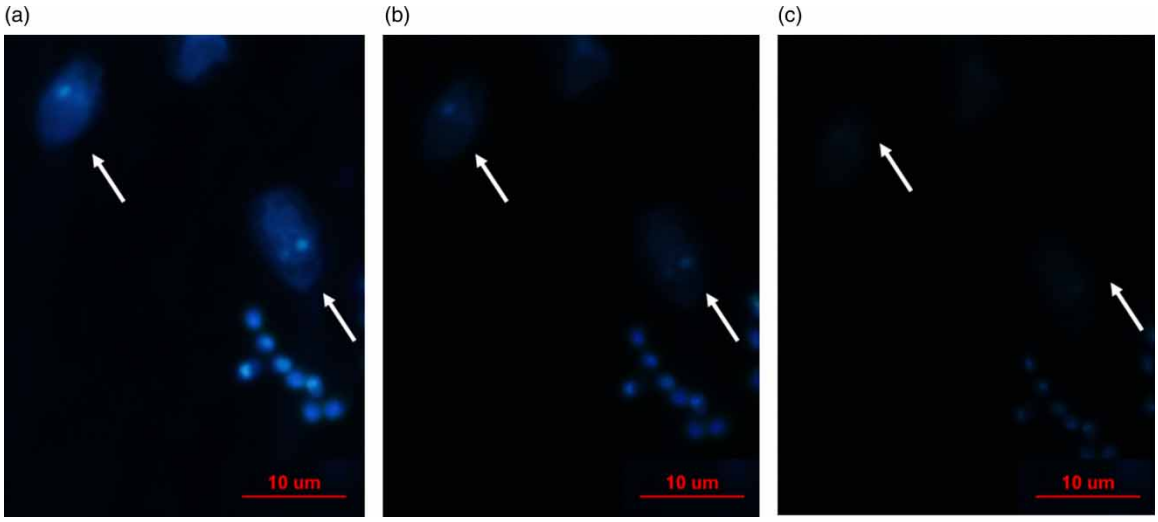


Figure 4 | Staining loss of *Giardia muris* cysts labelled with Fluoroshield™ with DAPI (a) immediately after UV exposure, (b) 6 min after UV exposure, and (c) 12 min after UV exposure. Narrow arrows point to cysts.

It is noted that IFA refers to the current protocol established by USEPA methods 1623.1 and 1693 for analysis of water and disinfected wastewater (2012, 2014), thus it is assumed to be the first resource WASH practitioners would rely on for periodically monitoring quality considering protozoan parasites. In fact, several studies on environmental samples that do not have a standardised detection method include IFA (Olson *et al.* 1999; Greinert *et al.* 2004; Graczyk *et al.* 2008; Grit *et al.* 2012; Giglio & Sabogal-Paz 2018; Sammarro-Silva & Sabogal-Paz 2020, 2021a, 2021b; Ogura & Sabogal-Paz 2021, 2022). The motive for choosing this protocol is assumed to be its high sensitivity, as older references that consider complex matrices were also immunofluorescence-based, as in Olson *et al.* (1999), which used IFA to quantify the survival of cysts seeded into soil and cattle waste.

3.3. Darkfield

Image results obtained for transmitted DF illumination demonstrated that, despite being unable to differentiate *G. duodenalis* from *G. muris*, DF microscopy allowed them to be distinguished by their typical oval shape from other illuminated elements. These interferers are particles that are smaller than the diffraction resolution limit for conventional light microscopy (~200 nm) (Murphy 2001).

An example retrieved DF micrograph is shown in Figure 5. The arrows point to *Giardia* cysts but it is not possible to identify their species. (The poor resolution is due to the imaging equipment, as different setups were used for BF and DF in this study.)

Figure 5 | *Giardia* cysts under transmitted darkfield illumination. Arrows point to cysts.

DF imaging can be accomplished in conventional light microscopes equipped with commercially available accessories or, as in the case of Belini *et al.* (2018), with a low-cost, easy-to-use ring illuminator built with standard light-emitting diodes. By using transmitted DF illumination, the authors observed well-defined cyst walls and intracellular structures, including nuclei and retracted cytoplasm, while axonemes were only imaged in the reflected DF mode. These results encourage further use of transmitted DF microscopy in *Giardia* spp. visualisation.

DF microscopy is supported mainly by its low-cost and lack of need for reagents, dyes, or specific equipment (Belini *et al.* 2018). So, it might be an alternative for detecting *Giardia* spp. cysts in resource-poor settings. However, in a general scenario contemplating environmental samples, scattering of other particles may hinder visualisation, particularly if the slide contains debris, thus purification methods would apply just as they do in all protocols mentioned above. Like Lugol's iodine in BF, DF effectiveness depends on well-trained observers. Due to possible microscopist subjectiveness, it is recommended that at least two professionals analyse samples for quantification.

3.4. Overview of cyst visualisation techniques under optical microscopy

Table 2 summarises general properties of BF, DF, and fluorescence protocols. These may be either advantages or limitations depending on circumstances – e.g., laboratory settings, available resources and level of urgency for cyst detection/analysis, personnel, matrix quality, etc. On the basis of both the micrographs obtained in this study and experience, the combination of techniques is believed to be an alternative to deal with the constraints inherent in individual methods. If more than one observer is available, that is also important to avoid bias in slide interpretation, as well as human error, as all the procedures cited above are observer-dependent.

4. CONCLUSIONS

Selecting a microscopy imaging method for *Giardia* cyst identification depends on several factors, including available laboratory infrastructure and human resources, professional expertise, the matrix to be analysed, and time. Still, protocols that require bulky equipment and costly reagents may be challenging.

An exploratory approach was used to demonstrate that BF, DF, and IFA (combined or not with DAPI) enable *Giardia* spp. cyst detection in purified samples, despite not providing species identification This suggests that simple and inexpensive techniques such as BF and DF microscopy may be useful in low- and middle-income countries when IFA protocols cannot be performed, if validation is carried out.

Table 2 | Overview of three microscopy techniques for *Giardia* detection considering sample processing, visualisation, and additional features

Category	Brightfield	Darkfield	Fluorescence (IFA; DAPI)
Infrastructure[a]	Conventional light microscope	Low-cost adaptation of conventional light microscope[b]	Epifluorescence microscope equipped with FITC and DAPI optical filters; incubator for 37 °C
Sample preparation	Easy to run; inexpensive reagent; rapid preparation; samples need to be analysed immediately after preparation	Label-free; immediate reading	Needs to be performed in absence of direct light; easy to run, but preferably performed by experts; commercial kits are available; requires up to 4 h for sample drying and 30 min incubation
Visualisation	Not selective; allows visualisation of internal structures under 1,000× magnification; hindered by debris; requires experts	Not selective; allows visualisation of internal structures under 1,000× magnification; highly hindered by debris; requires experts	IFA: selective staining; easy to identify against background; hardly affected by debris; may underestimate concentration due to low signal of *G. muris*[c]; staining lasts up to 7 days if stored in absence of light at 4 °C; does not require experts. DAPI: not selective; may be useful as confirmatory method; quickly loses fluorescence; requires experts
Cyst viability	Cyst viability may be assessed by additional staining[d]	N/A	Cyst viability may be assessed by a variety of fluorescent probes commercially available[d]
Protocol references	Standard analysis for stool samples[e]	New method[b]	USEPA reference method for water and disinfected wastewater analysis[f]

Note: DAPI, 4′,6-diamidino-2 phenylindole; N/A, information not available.
[a]Considered within the scope of sample preparation and analysis, i.e., it consider neither concentration nor purification.
[b]Belini *et al.* (2018).
[c]Low signal found in this study and by Alderisio *et al.* (2017).
[d]Sammarro-Silva & Sabogal-Paz (2021a, 2021b).
[e]Hooshyar *et al.* (2019).
[f]USEPA (2012, 2014).

Ideally, analysis for *Giardia* cyst detection should include a combination of at least two techniques, so that constraints are compensated for. Additionally, microscopy samples should preferably be analysed by different professionals, with repetitions, for more reliable results. This is important, particularly in environmental analyses, in which interferents and different species of pathogens may be present, nonetheless, it would increase sample processing costs considerably depending on the method.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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