



Parasitology

Rapid diagnostic tests relying on antigen detection from stool as an efficient point of care testing strategy for giardiasis and cryptosporidiosis? Evaluation of a new immunochromatographic duplex assay[☆]



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ABSTRACT

Microscopy is the gold standard for the diagnosis of gastrointestinal parasites but is time-consuming and dependent on operator skills. Rapid diagnostic tests represent alternative methods but most evaluations have been conducted on a limited number of samples preventing their implementation in the clinical setting. We evaluated a new CE-IVD marked immunochromatographic assay (Crypto/Giardia K-SeT®, Coris Bioconcept) for the detection of *G. intestinalis* and *Cryptosporidium* spp. in 2 phases (retrospective and prospective) on a set of 482 stool samples including rare *Cryptosporidium* species. Besides *G. intestinalis*, this test could represent a rapid and reliable alternative to the modified Ziehl-Neelsen staining for the diagnosis of cryptosporidiosis (sensitivity/specificity were 89.2%/99.3% and 86.7%/100% for *G. intestinalis* and *Cryptosporidium* resp.), reducing diagnostic delays. Such strategy would also be time-saving by avoiding wet mount microscopy and concentrations steps, being particularly appropriate for laboratories having little expertise in microscopy or not able to implement molecular diagnostic methods.

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1. Introduction

Giardiasis and cryptosporidiosis, caused respectively by a protozoan flagellate, *Giardia intestinalis*, and a coccidian parasite belonging to the genus *Cryptosporidium*, are 2 major parasitic diseases responsible for

diarrhea worldwide. Both parasites are transmitted via the fecal-oral route, through consumption of contaminated food and water (Davies and Chalmers, 2009). Laboratory diagnosis of these infections is usually achieved by microscopic stool examination which is still considered as the gold standard in spite of being time-consuming and highly dependent on operator's skills (Manser et al., 2014). To overcome these limitations, there is currently a growing interest for alternative methods such as multiplex PCR or antigen-based detection tests (RDTs) mostly relying on immunochromatographic methods. Compared with PCR assays, RDTs, sometimes used as rapid point of care tests (POCs) are

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easy to use being technically less complex, requiring a limited training. RDTs also offer a shorter hands-on time, reducing diagnostic delays, so that the results are usually available within 15–20 minutes. Although a growing number of RDTs for *Cryptosporidium* spp. and *G. intestinalis* have been developed in the last years and are now commercially available, their performances are often difficult to assess in part due to the low number of positive samples included in validation studies, that could therefore prevent their implementation in the clinical laboratory (Agnamey et al., 2011; Bouyou-Akotet et al., 2016; Goñi et al., 2012; Minak et al., 2012). In addition, some RDTs have a dramatically reduced sensitivity with unusual *Cryptosporidium* species that is of concern in the clinical setting (Agnamey et al., 2011).

Here, we aimed to evaluate a new CE-IVD marked immunochromatographic test (Crypto/Giardia K-SeT®, Coris Bioconcept, Belgium) for the simultaneous detection of both *G. intestinalis* and *Cryptosporidium* spp. from human stool samples. This study was performed in 2 distinct steps: i) a validation study on a large reference panel (previously determined by microscopy as the reference method) of positive and negative stool samples for various parasites including the aforementioned targeted parasites; ii) a 4-month prospective study at our laboratory, in parallel with standard microscopy, to evaluate its utility in the real-life setting.

2. Materials and methods

2.1. Immunochromatographic detection of *G. intestinalis* and *Cryptosporidium* spp.

Crypto/Giardia K-SeT® is a commercial solid-phase rapid qualitative immunochromatographic test designed for simultaneous detection of *G. intestinalis*- and *Cryptosporidium*-specific antigens in stool samples. Briefly, the stool sample is first transferred to the specimen dilution tube using a fecal sampling system (included in the kit and allowing standardization of the inoculum). For liquid or semi-liquid samples, 80 µL can be pipetted using a micropipette and directly added to the dilution tube as recommended in the manufacturer's instructions. After homogenization, 3 drops of the diluted fecal material are dispensed into the test device. Results are read after 15 minutes incubation at room temperature. A clinical sample is considered negative if a reddish-purple line appeared at the control line position without any other band. Samples showing a band at both the control line and the *Cryptosporidium* or *Giardia* test line positions are considered positive for *Cryptosporidium* spp. or *G. intestinalis*, respectively. Samples showing bands at all 3 lines are considered as mixed infections. Samples displaying no visible band at the control line are considered uninterpretable/invalid.

2.2. Phase 1 study: performance on a reference panel of stool samples

During this phase, the new RDT was evaluated blindly against a reference panel of 184 frozen stool samples (184 patients), collected from 12 hospital laboratories distributed across France (Laude et al., 2016). This collection included 134 stool specimens positive for various gastrointestinal parasites together with 50 negative samples. Each sample, stored at –20 °C while awaiting the present study, had been previously investigated using a standardized protocol relying on 2 well-trained microscopists, including direct examination followed by concentration's methods and modified Ziehl-Neelsen staining (Laude et al., 2016). In all, this panel was composed of 37 samples positive for *G. intestinalis*, 30 samples positive for *Cryptosporidium* species together with 67 other samples positives for 17 other gastrointestinal parasites representing the diversity of pathogens that can be recovered from stool samples (Table 1). To gain further insights into the performance of this assay, 9 additional samples positive for 8 rare *Cryptosporidium* species were also included (provided by the Public Health Agency of Sweden, Solna, Sweden) (Table 1).

Table 1

Complete list of the gastrointestinal parasites included in the reference panel (Phase 1 study).

Genus and species	N
<i>Giardia intestinalis</i>	37
<i>Cryptosporidium</i> spp.	39
<i>C. hominis</i>	5
<i>C. parvum</i>	22
<i>C. felis</i>	1
<i>C. meleagridis</i> *	3
<i>C. viatorum</i> *	1
<i>C. ubiquitum</i> *	1
<i>Cryptosporidium</i> horse genotype*	1
<i>Cryptosporidium</i> chipmunk genotype 1*	1
<i>C. cucinulus</i> *	2
<i>C. erinacei</i> *	1
<i>C. suis</i> *	1
Other	
<i>Entamoeba coli</i>	35
<i>Endolimax nana</i>	20
<i>Entamoeba dispar</i>	12
<i>Entamoeba hartmanni</i>	4
<i>Entamoeba histolytica</i>	4
<i>Iodamoeba bütschlii</i>	3
<i>Blastocystis</i> spp.	15
<i>Cystoisospora belli</i>	2
<i>Sarcocystis hominis</i>	1
<i>Dientamoeba fragilis</i>	1
<i>Chilomastix mesnili</i>	1
<i>Pentatrichomonas intestinalis</i>	1
<i>Schistosoma mansoni</i>	4
Hookworms	2
<i>Trichuris trichiura</i>	1
<i>Hymenolepis nana</i>	1
<i>Taenia</i> sp.	1
<i>Enterocytozoon bieneusi</i>	1

* For these species, additional samples (preserved in ethanol) were kindly provided by the Public Health Agency of Sweden, Solna, Sweden.

2.3. Phase 2 study: prospective study

During this phase, all stool specimens received for parasitological investigation at our laboratory (Nantes University Hospital, France) from January 7th to 27th April 2017 were systematically included. According to our local protocol, upon reception, each sample was first examined microscopically by fresh mounting and after a Baillenger's concentration step (Fumouze diagnostic, Levallois Perret, France). In patients with history of travel abroad and/or suspicion of helminths infection, an additional concentration step was performed (merthiolate iodine formaldehyde technique, Fumouze diagnostic, Levallois Perret, France). Additional methods were performed upon request, including the modified Ziehl-Neelsen staining for cryptosporidiosis or agar-plate culture according to Arakaki for strongyloidiasis (Arakaki et al., 1990). Twice weekly, each stool sample included in the study protocol was tested using the RDT test Crypto/Giardia K-SeT®, blindly to the results of microscopy (samples were stored at +4 °C, without preservative while awaiting the RDT). Samples showing insufficient quantity to perform both methods (microscopy and RDTs) were excluded ($n = 13$).

2.4. Data analysis

During each study phase, RDT results were analyzed blindly to the results of microscopic examination. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were determined. When required, discrepancies between both diagnostic approaches (RDT vs. microscopy) were investigated at Dijon Parasitology and Medical Mycology Laboratory by 2 in-house specific PCR assays targeting *G. intestinalis* and *Cryptosporidium* spp., as described previously (Laude et al., 2016). This study protocol has been approved by our local review committee (CHU de Nantes).

Table 2Results of the Crypto/Giardia K-SeT® assay against the reference panel ($n = 184$ samples, Phase 1 study).

			Microscopy	
			Positive	Negative
Antigen detection (Crypto/Giardia K-SeT®)	<i>G. intestinalis</i>	Positive	33	1
		Negative	4	146
	<i>Cryptosporidium</i> spp.	Positive	26	0
		Negative	4	154

3. Results

As explained above, during the first study phase, all 184 stool specimens of a reference panel previously determined by microscopy were processed blindly with the Crypto/Giardia K-SeT®. Results are summarized in Table 2. In all, 33 of the 37 positive samples for *G. intestinalis* by microscopy were also detected by the RDT (89.2%). All 4 remaining *G. intestinalis* that were RDT-negative contained only a few cysts per microscopic slide. One sample was positive for *G. intestinalis* by the RDT assay but negative by microscopy. Being negative by the external PCR assay, the sample was thus considered as a false-positive. Finally, compared with microscopy, analytical sensitivity and specificity of the RDT assay for *G. intestinalis* were 89.2% and 99.3%, respectively (Table 3). Among the 30 samples positive for *Cryptosporidium* spp., 26 (including one *C. felis* and one *C. meleagridis*) were also detected by the RDT (86.7%) (Table 2). The 4 samples positive by microscopy but negative by the RDT were either *C. hominis* or *C. parvum*. All 4 specimens were weakly positive at microscopy (a few oocysts per slide). Taken together, analytical sensitivity and specificity of the RDT for *Cryptosporidium* spp. were of 86.7% and 100%, respectively. Testing additional samples of rare *Cryptosporidium* species (not *C. parvum* nor *C. hominis*) revealed that most (7 of 9 samples) were also positive (*C. meleagridis*, *C. viatorum*, *C. ubiquitum*, *Cryptosporidium* horse genotype, *C. suis*, *C. erinacei*, *Cryptosporidium* chipmunk genotype I). One *C. meleagridis* sample and the *C. cuniculus* samples were negative. Overall, of the 11 different *Cryptosporidium* species, all but one (*C. cuniculus*) were detected by the RDT. No cross-reactivity was observed with any of the other gastrointestinal parasites included in the reference panel.

Phase 2 study allowed us to include 339 consecutive samples ($n = 233$ patients), during a 16 weeks periods. In all, 273 samples (80.5%, $n = 203$ patients) were negative by microscopy. Sixty-six samples (19.5%, $n = 30$ patients) were positive for at least one gastrointestinal parasite (Table 4). Twenty-two displayed multi-parasitism (i.e. more than one species in a single sample). During this prospective period, 3 samples were positive for *G. intestinalis* by microscopy (0.88%, $n = 3$ patients). By comparison, 4 samples were detected positive for *G. intestinalis* by the RDT: all 3 samples positive by microscopy, as well as an additional microscopy-negative sample (1.2%, $n = 3$ patients). This *G. intestinalis* antigen-positive samples was from one of the patients previously diagnosed with giardiasis by microscopy during the study period. For *Cryptosporidium* spp. a perfect agreement was noted between microscopy and the RDT, with 7 samples positive by both methods (2.1%, $n = 3$ patients), even though the modified Ziehl-Neelsen was not performed on all samples ($n = 81$ of 339, 24%).

Fifty-five samples ($n = 25$ patients) were positive for parasites other than *G. intestinalis* and *Cryptosporidium* spp., by routine microscopy

Table 4

Comparative findings of microscopy-based approach vs. RDT assay (Phase 2 study).

Status of the samples	Detected by microscopy	Detected by the RDT assay
Negative	273	328
Positive	66	11
<i>Giardia intestinalis</i>	3	4
<i>Cryptosporidium</i> spp.	7	7
<i>Entamoeba coli</i>	32	0
<i>Endolimax nana</i>	13	0
<i>Entamoeba dispar/E. histolytica</i>	5	0
<i>Entamoeba hartmanni</i>	4	0
<i>Iodamoeba bütschlii</i>	1	0
<i>Blastocystis</i> spp.	11	0
<i>Cystoisospora belli</i>	2	0
<i>Schistosoma mansoni</i>	3	0
<i>Dicrocoelium dendriticum</i>	1	0
Hookworms	3	0
<i>Strongyloides stercoralis</i>	3	0

(Table 4). All were negative by the RDT. Notably, 23 of these 25 patients had specific risk factor for other parasites (i.e. eosinophilia, immunosuppressive condition and/or history of travel abroad). Globally, performances of the Crypto/Giardia K-SeT® according to Phase 1 and Phase 2 studies in terms of sensitivity, specificity, PPV and NPV for giardiasis and cryptosporidiosis are given in Table 3.

4. Discussion

In most microbiology laboratories worldwide, diagnosis of giardiasis and cryptosporidiosis still relies on routine microscopic examination of fecal samples, however this approach has numerous limitations: i) a low turnaround time, ii) various complementary methods are required including stainings, concentration steps and more specific techniques (such as the Baermann extraction method for strongyloidiasis), and iii) the need for skillful microscopists and continuous microscopic education. Finally, microscopy has a moderate sensitivity leading to the recommendations that at least 3 samples collected over a few days are required to increase its performances, although this is currently under debate (Branda et al., 2006; van Gool et al., 2003). As a consequence, alternative methods such as PCR assays and RDTs have been increasingly developed. Compared with PCR, RDTs are not prone to DNA carryover contamination issues leading to false-positive results, and offer, with minimal training required, a shorter turnaround time without the need for specialized equipment. According to our findings, the Crypto-Giardia K-SeT® displays attractive analytical performances when compared with other antigen-based tests, in particular, thanks to its potential to detect a wide range of *Cryptosporidium* species including rare ones (Agnomey et al., 2011; Llorente et al., 2002; Van den Bossche et al., 2015). This finding is of clinical relevance as human cryptosporidiosis is not only due to *C. parvum* and *C. hominis* even though both species predominate in the clinical setting (Ryan et al., 2014). Performances were also satisfactory for *G. intestinalis*. We can assume the few microscopy-positive but antigen-negative samples, were likely due to the very low parasite burden in these specimens and/or to a possible alteration during storage. Besides, these findings were not observed during the prospective part of our study. Finally, no cross-

Table 3

Performances of the Crypto/Giardia K-SeT® assay according to Phase 1 and Phase 2 study periods.

	Phase 1 study ($n = 184$)				Phase 2 ($n = 339$)			
	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV
<i>G. intestinalis</i>	89.2%	99.3%	97.1%	97.3%	100%	100%	100%	100%
<i>Cryptosporidium</i> spp.	86.7%	100%	100%	97.5%	100%	100%	100%	99.2%

reactivity was observed with any other gastrointestinal parasites suggesting a high specificity of this immunochromatographic test.

Another aim of this study was to evaluate the utility of such RDT assay under real-life conditions to search for a possible improvement in the diagnostic workflow given the low prevalence of parasite-associated diarrhea in non-endemic countries (illustrated here during the retrospective study) and the low turnaround time of microscopy-based diagnosis. For instance, performing the modified Ziehl-Neelsen staining for *Cryptosporidium* oocysts takes approximately 60 min (from concentration, fixation, staining and reading) compared with 15–20 min using a RDT. Based on our findings we can be confident about the performance of this new RDT assay. Hence replacing Ziehl-Neelsen staining by an efficient RDT assay would be time-saving. Implementation of such test, would be useful to improve the global workflow allowing a rapid screening (often referred as “point-of-care” testing), as both parasites are usually performed together as the primary parasitology examination. Such strategy, allowing to detect the most common parasites in non-endemic countries, with reduced diagnostic delays, has been shown to be even more interesting with PCR assays that can detect a wider range of gastrointestinal parasites allowing to propose clinically relevant diagnostic algorithms (Shimelis and Tadesse, 2014; Strand et al., 2008; van Lieshout and Roestenberg, 2015). Finally such approach should also facilitates the diagnosis of both parasitic infections of public health importance that can be underestimated, as recently illustrated by others (Alexander et al., 2017; Currie et al., 2017).

To conclude, the present study argues that, in addition to *G. intestinalis*, this RDT could represent a rapid and reliable alternative to the modified Ziehl-Neelsen staining for the diagnosis of cryptosporidiosis allowing to reduce diagnostic delays. Such strategy would also be time-saving by avoiding wet mount microscopy and concentrations steps, being particularly appropriate for laboratories having little expertise in microscopy or not able to implement molecular diagnostic methods.

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Conflicts of interest

Authors have no conflict of interest to disclose.

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