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Assessment of the first commercial multiplex PCR kit (ParaGENIE Crypto-Micro Real-Time PCR) for the detection of *Cryptosporidium* spp., *Enterocytozoon bieneusi*, and *Encephalitozoon intestinalis* from fecal samples☆

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A B S T R A C T

Cryptosporidium spp. *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* are opportunistic pathogens responsible for gastrointestinal diseases. We evaluated the ParaGENIE Crypto-Micro Real-Time PCR kit (Ademtech, France), the first CE-IVD compliant PCR assay available for these pathogens. This study was conducted blindly against a reference panel of 115 stool specimens including positive samples for *Cryptosporidium* spp. ($n = 48$) and *E. bieneusi* ($n = 38$) as well as negative or positive samples for other parasites to test for cross-reactivity. An additional set of samples corresponding to 8 rare *Cryptosporidium* species was also included. Discrepancies were evaluated with external in-house PCR tests. The ParaGENIE Crypto-Micro PCR assay displayed a sensitivity/specificity of 91.7%/100% and 97.3%/98.7% for *Cryptosporidium* spp. and *E. bieneusi*, respectively, and was able to detect all 12 *Cryptosporidium* species of the reference panel, including rare species. This new CE-IVD assay will facilitate the diagnosis of intestinal cryptosporidiosis and microsporidiosis, a major concern in immunocompromised patients and travelers.

1. Introduction

Cryptosporidium spp., *Enterocytozoon bieneusi*, and *Encephalitozoon intestinalis* are opportunistic pathogens responsible for diarrhea. Both have a global distribution with prevalence probably varying between countries and are now increasingly recognized as a health concern, as illustrated in transplant recipients (Didier and Weiss, 2011; Galván et al., 2011; Lanternier et al., 2009, 2017). However, their respective prevalence is still poorly known and likely to be both underreported and underestimated at

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least because their detection and identification in the clinical microbiology laboratory require specific techniques and expertise (Cacciò and Chalmers, 2016).

Cryptosporidium spp. are intracellular coccidian parasites whose diagnosis has long relied on various staining methods such as the modified Ziehl–Neelsen technique. Specific PCR assays as well as immunochromatographic strip tests have been developed and evaluated as an alternative to bypass microscopy (Checkley et al., 2015). Nevertheless, recent studies highlighted considerable variations in practices for *Cryptosporidium* detection, ranging from systematic screening to targeted testing according to local procedures, also varying between laboratories (Alexander et al., 2017; Chalmers et al., 2015).

Microsporidia are obligate intracellular fungi capable of infecting a wide range of invertebrate and vertebrate hosts. Among the 15 species described as human pathogens, *E. bieneusi* and to a lesser extent *E. intestinalis* are the major species responsible for intestinal disorders, mostly in immunocompromised patients (Ramanan and Pritt, 2014). Even though microsporidia are genetically related to fungi, they cannot be easily cultured *in vitro*. Diagnosis of these infections therefore relies on alternative methods, mostly direct examination of clinical samples after peculiar staining techniques including the modified trichrome or the optical fluorescent brighteners calcofluor white or Uvitex 2B (Ramanan and Pritt, 2014), or DNA detection tests [reviewed in Ghosh and Weiss (2009) and Verweij and Stensvold (2014)]. In addition to being time consuming and insensitive, microscopic detection of *E. bieneusi* and *E. intestinalis* spores in stools is challenging due to their small size (1–3 μm). These spores can be also confused with bacteria or small yeasts, even for well-trained microscopists, making PCR the method of choice, being the most sensitive and specific (Verweij and Stensvold, 2014). PCR assays also provide species identification which is mandatory as therapy differs between species (Didier et al., 2005).

In the last 10 years, a countless number of commercial multiplex PCR kits for the detection of gastrointestinal pathogens, including *Cryptosporidium*, have been developed and evaluated (Friesen et al., 2018; Laude et al., 2016; Madison-Antenucci et al., 2016; Mengelle et al., 2013; Morio et al., 2018; Ramanan et al., 2018). However, none of these multiplex schemes include the detection of microsporidia.

Recently, the ParaGENIE Crypto-Micro Real-Time PCR kit (AdemTech, Pessac, France), a CE-IVD–compliant multiplex real-time PCR assay designed for the diagnosis of cryptosporidiosis and intestinal microsporidiosis due to *E. bieneusi* and *E. intestinalis*, has been developed. Here, our aim was to evaluate the sensitivity and specificity of this new assay for the detection of *Cryptosporidium* spp. and microsporidia and to assess its performances on a wide range of *Cryptosporidium* species.

2. Methods

2.1. Sample collection

A reference panel of 115 stool samples prospectively collected during routine clinical procedures was used to evaluate the new multiplex PCR assay. All samples were processed in the same way. Detection of *Cryptosporidium* oocysts was performed by microscopic examination after a Bailenger's biphasic concentration method (Para-selles/Kop-color; Fumouze Diagnostics, Levallois Perret, France) using the modified Ziehl–Neelsen staining. When positive, *Cryptosporidium* species identification was obtained by amplification and sequencing of the polymorphic region of the 18S rRNA, as described previously (Laude et al., 2016). Detection of *E. bieneusi* and *E. intestinalis* was performed using 2 in-house species-specific real-time PCR assays after DNA extraction using the QIAamp DNA stool kit (Qiagen, Courtaboeuf, France), as described previously (Espern et al., 2007). All samples were also investigated for other parasites according to standard microscopic methods. In all, this reference panel was composed of samples positive for *Cryptosporidium* spp. ($n = 48$), *E. bieneusi* ($n = 38$) as well as for other gastrointestinal parasites ($n = 20$) or negative ($n = 9$). All samples were stored at −20 °C while awaiting for multiplex PCR testing. Finally, 9 other samples, preserved in ethanol, were kindly provided by the Public Health Agency of Sweden (Solna, Sweden) to further evaluate the performance of this assay against rare *Cryptosporidium* species. These samples belonged to *Cryptosporidium meleagridis* ($n = 2$), *Cryptosporidium cuniculus* ($n = 1$), *Cryptosporidium erinacei* ($n = 1$), *Cryptosporidium* horse genotype ($n = 1$), *Cryptosporidium* chipmunk genotype ($n = 1$), *Cryptosporidium suis* ($n = 1$), *Cryptosporidium ubiquitum* ($n = 1$), and *Cryptosporidium viatorum* ($n = 1$).

2.2. Multiplex detection of Cryptosporidium and microsporidia

2.2.1. Stool DNA extraction

DNA extraction was performed on an automated DNA extraction platform (Automag, AdemTech) using the ParaGENIE Stool DNA Extraction kit (AdemTech) according to the manufacturer's protocol. Briefly, a calibrated amount of stool (approximately 150–200 mg) was first homogenized in 800 μL of resuspension buffer (supplied in the kit) for 5 min using grinding beads (supplied in the kit). The stool suspension was then centrifuged at 12,000*g* for 3 min. Finally, 250 μL of the supernatant was incubated with 400 μL of Stool Lysis Buffer for 10 min at room temperature before being transferred in the DNA extraction plate duly prepared according to the manufacturer's recommendations and filled in the Automag system. For each sample, 20 μL of internal control was added to the lysate before running the automated extraction procedure to detect possible PCR inhibition. DNA extracts were stored at −20 °C before PCR analysis.

2.2.2. Multiplex real-time PCR

PCR was performed on a CFX96 instrument (Bio-Rad, Richmond, VA) using the CE-IVD–marked (meaning approved by the EU for *in vitro* diagnosis) ParaGENIE Crypto-Micro Real-Time PCR kit (AdemTech). Besides the internal control, this 4-plex PCR assay specifically detects *Cryptosporidium* spp., *E. bieneusi*, and *E. intestinalis* by targeting small subunit rRNA gene sequences using 3 distinct fluorescence channels. Amplification was carried out in a 25-μL reaction volume containing 20 μL of reconstituted UTP-containing master mix and 5 μL of DNA extract. To avoid carryover contamination between PCRs, the ParaGENIE Crypto-Micro mix contains uracil-DNA glycosylase (UDG). After a preincubation step at 37 °C for 10 min for UDG activation and 10 min at 95 °C for Taq activation, the amplification was performed over 45 cycles as follows: denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 60 s. Negative (DNase/RNase-free water) and positive controls (1 for each targeted pathogen, supplied in the kit) were included in each assay. Internal control reaction was considered inhibited if its signal Ct (threshold cycle) was >35 cycles. In this case, eluates were 10-fold diluted in DNase/RNase free water and reanalyzed. Samples were considered positive for the targeted pathogens if Ct was <45 cycles.

2.3. Data analysis

Results of the PCR were analyzed blind to the results of the reference panel, considered as the reference. Samples showing evident PCR inhibition were considered uninterpretable and were therefore excluded from the analysis. Discrepancies were investigated at Clermont–Ferrand Parasitology and Medical Mycology Laboratory using an in-house real-time PCR assay targeting *Cryptosporidium* spp., *E. bieneusi*, and *E. intestinalis* [respectively adapted from Mary et al. (2013) and Menotti et al. (2003a, 2003b)]. This protocol has been approved by our local ethic committee (Nantes University Hospital).

3. Results

This new multiplex PCR assay was evaluated blindly against the 115 samples of the reference panel. Only 1 sample (0.9%) yielded strong PCR inhibition when tested using the ParaGENIE Crypto-Micro Real-Time PCR assay. Excluding this sample (positive for *Cryptosporidium* spp. by microscopy), the validation study was therefore performed on the 114 remaining samples.

Regarding *Cryptosporidium* spp., the new PCR assay successfully detected 43 out of the 47 (91.5%) positive samples from the reference panel (Table 1). Though this collection did not cover a large range of species (when species identification was available), all that were included were detected, i.e., *C. parvum* ($n = 31$), *C. hominis* ($n = 5$), *C. meleagridis* ($n = 1$), *C. felis* ($n = 1$), and *C. cuniculus* ($n = 1$). Four *Cryptosporidium*-positive samples were therefore not detected by the ParaGENIE assay (i.e., false negative). These samples belonged to *C. parvum* ($n = 2$), *C. hominis* ($n = 1$), or unidentified species ($n = 1$). Interestingly, the latter sample as well as a *C. parvum*–positive sample, both containing a few oocysts per slide, was also negative by the external assay but positive by antigen detection (Xpect *Cryptosporidium* immunochromatographic assay, Remel, Inc., Lenexa, KS). Finally, 1 negative sample from the reference panel was positive by the ParaGENIE assay. Positivity for *Cryptosporidium* spp. was also confirmed by the external PCR assay (i.e., true positive by the ParaGENIE assay). Taken together, adjusted sensitivity and specificity for *Cryptosporidium* spp. were 91.7% and 100%, respectively. Interestingly, all 9 additional samples used to evaluate the performance of the ParaGENIE assay against unusual *Cryptosporidium* species ($n = 8$) were detected positive. Considering these extra samples, the sensitivity for *Cryptosporidium* spp. was further increased to 93%.

Regarding microsporidia, the new ParaGENIE assay successfully detected 36 of the 37 *E. bieneusi*–positive samples from the reference panel. The remaining sample, not detected by the new assay, being confirmed positive by the external PCR was therefore categorized as a false negative of the ParaGENIE assay. Conversely, 1 negative sample from the reference panel was detected positive for *E. bieneusi* by the ParaGENIE assay. However, being also negative when tested by the external PCR, this sample was finally considered as a false positive of the ParaGENIE assay. Taken together, adjusted sensitivity and specificity of the ParaGENIE assay for *E. bieneusi* were 97.3 and 98.7%, respectively (Table 1). No sample was detected positive for *E. intestinalis* using the ParaGENIE assay. However, as no sample positive for *E. intestinalis* was included in the reference panel, the sensitivity for this species was not determined.

Finally, as expected, all samples positive for other gastrointestinal parasites (listed in Table 2) that included various helminths, amoebas, flagellates, or other coccidias were negative for the targeted pathogens when tested by the ParaGENIE assay, confirming the high specificity of this multiplex PCR.

Table 2
Gastrointestinal parasites included in the study to test for possible cross-reactions (not targeted by the multiplex PCR assay).

Genus/species	*n*
Entamoeba dispar	3
Entamoeba histolytica	1
Entamoeba coli	5
Endolimax nana	2
Entamoeba hartmanni	2
Iodamoeba butschlii	1
Blastocystis spp.	3
Hymenolepis nana	1
Schistosoma mansoni	1
Chilomastix mesnili	1
Dientamoeba fragilis	1
Pentatrichomonas intestinalis	1
Cystoisospora belli	1
Sarcocystis spp.	1
Giardia intestinalis	1
Hookworm eggs	1
Trichuris trichiura	1
Taenia spp.	1

4. Conclusions

In the present study, we aimed to evaluate the first commercially available multiplex CE-IVD PCR real-time PCR assay targeting *Cryptosporidium* spp., *E. bieneusi*, and *E. intestinalis*, being important and still frequently underestimated causes of diarrhea and intestinal disorders in immunocompromised patients. However, in a 1-year prospective study conducted at Nantes University Hospital, France, among kidney and/or pancreas transplant recipients with diarrhea, both diseases rank first after bacterial and viral infections (Deltombe et al., 2019). In addition, both cryptosporidiosis and microsporidiosis are also known to cause acute gastroenteritis in immunocompetent individuals and travelers, but their diagnosis is rarely performed in this context, reinforcing the underestimation of the real burden of these pathogens (Checkley et al., 2015; Didier and Weiss, 2011).

The present evaluation was conducted on a reference panel of positive samples for these pathogens as well as negative samples or containing unrelated parasites to test for possible cross-reactions. We demonstrate that this new multiplex assay provides robust sensitivities and specificities for the detection of *Cryptosporidium* spp. and *E. bieneusi*. We were not able to determine its performances on clinical samples positive for *E. intestinalis* as this species is less prevalent than *E. bieneusi* and was not included in our reference panel (Galván et al., 2011; Lanternier et al., 2009). Further studies are therefore required to specifically address this question. Nevertheless, as for *E. bieneusi* and *Cryptosporidium* spp., PCR efficiency, linearity, and appropriate limits of detection were previously verified for *E. intestinalis* using DNA from *in vitro* culture (data not shown). Anyhow, the relatively high number of samples positive for *Cryptosporidium* spp. and *E. bieneusi* can be considered as a strength of this study. One major advantage of this multiplex assay relies on its ability to detect a wide range of *Cryptosporidium* spp., at least the 12 following species tested in the present study (*C. parvum*, *C. hominis*, *C. parvum*, *C. felis*, *C. meleagridis*,

Table 1
Performances of the ParaGENIE Crypto-Micro Real-Time multiplex PCR assay for the detection of *Cryptosporidium* spp., *E. bieneusi*, and *E. intestinalis* ($n = 114$).

	Positive for both reference and ParaGENIE PCR	Positive for reference but negative by ParaGENIE PCR	Negative for reference but positive by ParaGENIE PCR	Negative for both reference and ParaGENIE PCR	Sensitivity (Se) Specificity (Sp)
Cryptosporidium spp.	43	4[a]	1[b]	66	Se =91.7% Sp = 100%
Enterocytozoon bieneusi	36	1[c]	1[d]	76	Se = 97.3% Sp = 98.7%
Encephalitozoon intestinalis	0	0	0	114	Se = ND Sp = 100%

Note: “Reference” refers to the findings of the reference panel and includes the modified Ziehl–Neelsen for *Cryptosporidium* spp. or PCR assay for microsporidia.

[a] Two of these samples positive by the modified Ziehl–Neelsen staining could not be confirmed by the external PCR assay but were positive by antigen detection (Xpect *Cryptosporidium*, Remel).

[b] This sample initially considered negative in the reference panel was also confirmed to be positive by the external PCR assay (false negative in the reference panel).

[c] This sample was confirmed positive by the external PCR assay (false negative by the ParaGENIE multiplex PCR assay).

[d] This sample was confirmed negative by the external PCR assay (false positive by the ParaGENIE multiplex PCR assay).

C. cuniculus, *C. viatorum*, *C. suis*, *C. ubiquitum*, *C. erinacei*, chipmunk, and horse genotypes). This is relevant for the clinical practice as unusual species can infect humans and are also more likely to occur in immunocompromised patients when compared with immunocompetent individuals (Cacciò et al., 2005; Costa et al., 2018; Guyot, 2010). Unfortunately, we could not assess the performance of this assay on *C. canis* as no sample was available. Compared with previously published multiplex PCR methods targeting these pathogens (Huibers et al., 2018; Lee et al., 2010; Rubio et al., 2014), this assay combines both an automated DNA extraction, which allows simultaneous processing of multiple samples (approximately 2.5 h from sampling to PCR results), and the CE-IVD marking, facilitating its implementation in the clinical lab.

Though we observed satisfactory performances for both pathogens, it should be noted that most false-negative results were observed for *Cryptosporidium* spp. A reliable explanation is that *Cryptosporidium* DNA release from oocysts is probably a critical issue when addressing the diagnosis of these pathogens by PCR. Some authors have assessed various extraction protocols, but the most efficient is not necessarily applicable in the real life of routine laboratory diagnosis (Le Govic et al., 2016; Mary et al., 2013).

This new CE-IVD–compliant real-time PCR assay combined with an automated DNA extraction process is a sensitive and specific molecular diagnostic tool for the diagnosis of cryptosporidiosis and intestinal microsporidiosis infections, with a good agreement when compared with standard methods. Taken together, besides the inherent costs associated with commercial tests, this method has the potential to facilitate the diagnosis of these pathogens, especially in laboratories having limited experience in microscopic examination, having a high number of samples to analyze, and/or not able to set up in-house PCR assays.

Declaration of interest

None.

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