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# Assessment of a Multiplex PCR for the Simultaneous Diagnosis of Intestinal Cryptosporidiosis and Microsporidiosis

## Epidemiologic Report from a French Prospective Study

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Microsporidiosis and cryptosporidiosis are associated with chronic diarrhea in immunocompromised patients. The objectives of this study were to: i) assess a multiplex quantitative PCR assay targeting *Cryptosporidium* spp and the microsporidian *Enterocytozoon bienewi* and *Encephalitozoon* spp, and ii) provide an update on the epidemiology of these pathogens. A prospective study was conducted from January 2017 to January 2019. Performance of the assay was assessed, and all cryptosporidia and microsporidia isolates were genotyped. The sensitivity of the multiplex PCR method reached 1 copy/μL for each targeted pathogen. The sensitivity of co-proantigen testing in the diagnosis of cryptosporidiosis was 73%. The sensitivity of microscopy in the diagnosis of cryptosporidiosis was 64%, and microsporidiosis, 50%. Among the 456 patients included, 14 were positive for *Cryptosporidium* spp (4 different species); 5, for *E. bienewi*; and 2, for *Encephalitozoon intestinalis*. The overall prevalence of cryptosporidia was 3.1%, and of microsporidia, 1.5%; in kidney transplant recipients ( $n = 82$ ), corresponding values were 7.3% and 2.4% (6 and 2 patients), respectively. Two cases of *E. intestinalis* infection were diagnosed in children who had traveled to the tropics. This study is the first to assess a multiplex quantitative PCR method for the simultaneous diagnosis of intestinal microsporidiosis and cryptosporidiosis. The highest prevalences of both pathogens were observed in kidney transplant recipients. (*J Mol Diagn* 2021, 23: 417–423; <https://doi.org/10.1016/j.jmoldx.2020.12.005>)

Microsporidia and cryptosporidia are intracellular eukaryotic pathogens involved in diarrhea, and less frequently in extraintestinal disease, in a wide range of animals. Among the 38 species of *Cryptosporidium*, 20 are causative agents of diarrhea in humans, with *Cryptosporidium parvum* and *Cryptosporidium hominis* representing about 90% of isolates.<sup>1,2</sup> The most frequent microsporidian species found in humans are *Enterocytozoon bienewi* (>90% of cases) and *Encephalitozoon intestinalis*. Both intestinal microsporidiosis and cryptosporidiosis are associated with chronic diarrhea in immunocompromised patients. They were a

frequent cause of opportunistic diarrhea in AIDS patients until the availability of antiretroviral therapies.<sup>3</sup> Today, these infections emerge in solid organ transplant recipients and in patients with hematologic malignancies.<sup>3,4</sup> Immunocompetent patients may also be infected, although most of these cases are asymptomatic or resolve spontaneously.<sup>3,4</sup>

In medical laboratories, microsporidiosis and cryptosporidiosis are mainly diagnosed using light microscopy

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with staining-based techniques. However, these methods are time-consuming, are difficult to read, harbor variable performance, and may lack specificity and sensitivity. Several PCR assays targeting *Cryptosporidium* spp, or the microsporidian *E. bienersi* and/or *E. intestinalis*, have been developed.<sup>5–8</sup> However, physicians frequently request the diagnosis of cryptosporidiosis and microsporidiosis simultaneously, given that the epidemiology, symptoms, and risk factors are the same. Therefore, the present study aimed to develop multiplex quantitative real-time (q)-PCR targeting, in a single reaction, *Cryptosporidium* spp, *E. bienersi*, and *Encephalitozoon* spp. All isolates positive for microsporidia and/or cryptosporidia were investigated using epidemiologic analysis, species identification, and genotyping.

## Materials and Methods

### Clinical Samples

Between January 2017 and January 2019, stool samples were collected from different wards of the teaching hospital at Clermont-Ferrand (France). Diagnosis was performed in immunocompromised patients presenting with soft or liquid stools, systematically in children, and in non-immunocompromised adults when prescribed by a clinician. Specificity assays of the multiplex qPCR were performed using samples positive for 16 other intestinal parasites (Supplemental Table S1).

### Microscopic Examination and Co-Proantigen Testing

From January to December 2017, cryptosporidia were identified using microscopy (Bailenger concentration method followed by Ziehl staining), the RIDAQuick *Cryptosporidium* co-proantigen test (R-Biopharm, Pfungstadt, Germany), and multiplex qPCR.<sup>9</sup> For the identification of microsporidia, Trichrome staining and multiplex qPCR were performed.<sup>10</sup>

### DNA Extraction

Two hundred milligrams of stool was introduced in vials containing 800  $\mu$ L of easyMAG lysis buffer (bioMérieux, Craponne, France) and 100  $\mu$ L of 0.5 mm glass beads (Next Advance, Vincennes, France). Then, a bead beating step was performed for 3 minutes at 3000 Hz (TissueLyser; Qiagen, Hilden, Germany), followed by a centrifugation for 10 minutes at  $20,000 \times g$ . Two hundred microliters of supernatant spiked with 5  $\mu$ L of DiaControlDNA DNA virus (Diagenode, Liège, Belgium) was used for DNA extraction using the ELITE InGenius system (ELITechGroup, Puteaux, France), with a final elution volume of 50  $\mu$ L.

### Multiplex Quantitative PCR

The detection of *Cryptosporidium* spp, *E. bienersi*, and *Encephalitozoon* spp was performed using primers and probes previously described (Table 1).<sup>5,6,11</sup> PCR was performed in a final volume of 25  $\mu$ L containing 5  $\mu$ L of DNA, primers/probes targeting *Cryptosporidium* spp, *E. bienersi*, and *E. intestinalis* (Table 1), 2.5  $\mu$ L of DiaControlDNA primers/probe mix (Diagenode), and 12.5  $\mu$ L of Amplidiag master mix (Mobidiag, Espoo, Finland). Real-time PCR was performed on the ELITE InGenius system. The amplification consisted of an initial denaturation step of 5 minutes at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing/elongation at 60°C for 1 minute.

### Plasmid Preparation for qPCR Sensitivity Assessment

Briefly, *Cryptosporidium* spp, *E. bienersi*, and *E. intestinalis* target sequences were cloned into pGEM-T Easy Vector (Promega, Madison, WI) and transfected into *Escherichia coli* DH5 $\alpha$ . For each plasmid containing target sequence, serial dilutions containing  $10^2$  to  $10^6$  copies/ $\mu$ L were tested in triplicate. Templates containing mixed targets were analyzed using plasmid mixtures in different proportions: 10/100/1000-fold greater than each other. Each reaction was performed in triplicate.

### Species Identification and Genotyping of *Cryptosporidium* Species

Species identification was performed by the French National Reference Center for Cryptosporidiosis (Dijon, France) using sequencing of the gp60 encoding gene, as previously described (Table 1).<sup>8,12</sup>

### Genotyping of *Enterocytozoon bienersi* Isolates and Identification of the *Encephalitozoon* Species

*Encephalitozoon* spp were identified and *E. bienersi* genotyped by sequencing the internal transcribed spacer region as previously described (Table 1).<sup>13,14</sup>

### DNA Sequence Analyses

PCR products were sequenced in both strands (GATC Biotech/Eurofins Genomics, Brussels, Belgium). The sequences were submitted to the NCBI database using the nucleotide Basic Local Alignment Search Tool (<http://www.ncbi.nlm.nih.gov/BLAST>, last accessed April 30, 2020) and genotypes/species were identified using determination of the exact match or closest similarity against deposited sequences.

**Table 1** Primers and Probes Used in This Study

Method	Target (gene)	Primers and probes	Final concentration, $\mu\text{mol/L}$	Reference
Multiplex qPCR	<i>Cryptosporidium</i> spp (small subunit rRNA)	Forward	5'-CATGGATAACCGTGGTAAT-3'	Mary et al <sup>11</sup>
		Reverse	5'-TACCCTACCGTCTAAAGCTG-3'	
		Probe	5'-FAM-CTAGAGCTAATACATGCGAAAAA-MGB-BHQ1-3'	
	<i>Enterocytozoon bienewisi</i> (small subunit-rRNA)	FEB1	5'-CGCTGTAGTTCTGCAGTAACTATGCC-3'	Menotti et al <sup>5</sup>
		REB1	5'-CTTGCGAGCGTACTATCCCCAGAG-3'	
		Probe	5'-YY-ACGTGGGCGGGAGAAATCTTAGTGTTCGGG-BHQ1-3'	
	<i>Encephalitozoon</i> spp (small subunit-rRNA)	FEI1	5'-GCAAGGGAGGAATGGAACAGAACAG-3'	Menotti et al <sup>6</sup>
		REI1	5'-CACGTTTCAGAACCCATTACACAGC-3'	
		Probe	5'-TR-CGGGCGGCACGCGCACTACGATA-BHQ2-3'	
<i>Cryptosporidium</i> spp identification	<i>Cryptosporidium</i> spp (small subunit-rRNA)	CRU18SF	5'-GAGGTAGTGACAAGAAATAACAATACAGG-3'	Hadfield et al <sup>8</sup>
		CRU18SR	5'-CTGCTTTAAGCACTCTAATTTTCTCAAAG-3'	
		CRU18STM	5'-FAM-TACGAGCTTTTAACTGCAACAA-MGB-NFQ-3'	
	<i>Cryptosporidium parvum</i> (LIB13)	CRULib13F	5'-TCCTTGAAATGAATATTTGTGACTCG-3'	0.9
		CRULib13RCp	5'-TTAATGTGGTAGTTGCGGTTGAAAC-3'	0.9
		CRULib13TMcp	5'-VIC-TATCTCTTCGTAGCGGCGTA-MGB-NFQ-3'	0.15
	<i>Cryptosporidium hominis</i> (LIB13)	CRULib13F	5'-TCCTTGAAATGAATATTTGTGACTCG-3'	0.9
		CRULib13RCh	5'-AAATGTGGTAGTTGCGGTTGAAA-3'	0.3
		CRULib13TMCh	5'-VIC-CTTACTTCGTGGCGGCGT-MGB-NFQ-3'	0.1
Genotyping of <i>Cryptosporidium</i>	<i>Cryptosporidium</i> spp ( <i>gp60</i> )	AL3531	5'-ATAGTCTCCGTGTATTC-3'	Alves et al <sup>12</sup>
		AL3535	5'-GGAAGGAACGATGTATCT-3'	
		AL3532	5'-TCCGCTGTATTCTCAGCC-3'	
		AL3534	5'-GCAGAGGAACCAGCATC-3'	
Genotyping of <i>E. bienewisi</i>	<i>E. bienewisi</i> (ITS)	AL4037	5'-GATGGTCATAGGGATGAAGAGCTT-3'	Sulaiman et al <sup>13</sup>
		AL4039	5'-ACGGATCCAAGTGATCCTGTATT-3'	
		AL4038	5'-AGGGATGAAGAGCTTCGGCTCTG-3'	
		AL4040	5'-AGTGATCCTGTATTAGGGATATT-3'	
<i>Encephalitozoon</i> spp identification	<i>Encephalitozoon</i> spp (ITS)	MSP-1	5'-TGAATGKGTCCCTGT-3'	Katzwinkel-Wladarsch et al <sup>14</sup>
		MSP-2A	5'-TCACTCGCCGCTACT-3'	
		MSP-3	5'-CACACCGCCCGTCTRYTAT-3'	
		MSP-4A	5'-CTATGTTAAGTYMAARGGT-3'	

BHQ, black hole quencher; FAM, 6-carboxyfluorescein; ITS, internal transcribed spacer; MGB, minor groove binding; NFQ, nonfluorescent quencher; qPCR, quantitative PCR; rRNA, ribosomal RNA; TR, Texas Red; VIC, VIC fluorescent dye; YY, Yakima Yellow.

## Results

### Performance of the Multiplex qPCR Assay

The lower limit of detection of the multiplex qPCR assay was set at 1 copy/ $\mu\text{L}$  for each target. Reproducibility was assessed from 30 independent experiments, with CVs of 4.14% for *Cryptosporidium* spp, 4.35% for *E. bienewisi*, and 4.82% for *E. intestinalis*. A target amount as low as  $10^2$  copies/ $\mu\text{L}$  was still detected for each of the three targets when mixed with  $10^5$  copies/ $\mu\text{L}$  of both other targets, even though an increased Ct value was observed (Supplemental Table S2). No cross-reaction was observed with the other

intestinal parasite tested (Supplemental Table S1). Interestingly, the primers and probe used for detecting *E. intestinalis* also detected the DNA of *Encephalitozoon cuniculi* and *Encephalitozoon hellem*. Such cross-reactions were expected given that DNA alignment revealed that the target region was highly conserved between the three *Encephalitozoon* spp (Supplemental Figure S1).

### Positive Sample Analysis

Over the 2 years (from January 2017 to January 2019), 595 stool samples, corresponding to 456 patients, were tested for

**Table 2** Epidemiologic Features of the Studied Population

Feature		Value	
Number of patients		456	100%
Age	Median (range), years	55 (0–95)	
	<10 years	64	14.0%
	>75 years	54	11.8%
Sex	Male	260	57.0%
	Female	196	43.0%
Immunocompetent patients		187	41.0%
Immunocompromised patients		269	59.0%
SOT	Total	107	23.5%
	Kidney	82	18.0%
	Heart	10	2.2%
	Lung	3	0.7%
	Liver	11	2.4%
	Kidney/liver	1	0.2%
	Total	24	5.3%
HIV	CD4 count median (range), cells/mm <sup>3</sup>	239 (3 to 1473)	
	Viral load median (range), copies/mL	0 (0 to 10 <sup>6</sup> )	
Hematologic malignancy	Total	92	20.2%
	HSCT	37	8.1%
	AML	28	6.1%
	Lymphoma	28	6.1%
	ALL	7	1.5%
	MDS	7	1.5%
	Other	22	4.8%
Other immunosuppression		46	10.1%
Travelers		43	9.4%

ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; HIV, human immunodeficiency virus; HSCT, hematopoietic stem cell transplantation; MDS, myelodysplastic syndrome; SOT, solid organ transplant recipients.

both microsporidia and cryptosporidia. Epidemiologic features are summarized in Table 2. The male/female ratio was 1.33 and the median age was 55 years, with 14.0% of children aged <10 years old. Immunocompromised patients represented 59.0% of the cohort, with three main groups: solid organ transplant recipients (23.5%), patients with hematologic malignancies (20.2%), and HIV patients (5.3%). Thirty-six stool samples were found positive on multiplex qPCR, including 24 for *Cryptosporidium* spp, 10 for *E. bieneusi*, and 2 for *E. intestinalis* (species confirmed on internal transcribed spacer sequencing), representing 14 (3.07%), 5 (1.10%), and 2 (0.44%) patients, respectively (Table 3). Therefore, 4.17% of patients were found to be positive for infection with at least one of the three pathogens. One patient was first positive for *E. bieneusi* and later for *Cryptosporidium* spp; and another was positive for both pathogens at the same time. Among positive patients, 13 (68%) were immunocompromised, including 9 solid organ transplant recipients (47%; 7 kidney and 2 liver), 2 with hematologic malignancies (11%) who underwent hematopoietic stem cell transplantation, and 2 HIV patients (11%) with a CD<sub>4</sub> count of <200 cells/mm<sup>3</sup> and a high viral load (Table 4). Among the 6 immunocompetent, positive patients, 4 were children aged 5 months to 13 years and had

traveled to Mayotte (1 case with *C. hominis*), Mayotte and Burkina Faso (2 cases with *E. intestinalis*), and Burundi (1 case with both *Cryptosporidium* spp and *E. bieneusi*). No travel history or other relevant information was found in the 2 other immunocompetent patients positive for *C. parvum* and *C. hominis*.

### Comparison of Diagnostic Methods

Over the year 2017, stool samples ( $n = 80$ ) were tested using microscopy, co-proantigen testing, and multiplex qPCR. During this period, 11 samples were found to be

**Table 3** Results of the Multiplex qPCR Diagnosis During the 2-Year Study

	Patients, number (%)	Stool sample, number (%)
Total	456 (100)	595 (100)
Positive	19 (4.17*)	36 (6.05)
<i>Cryptosporidium</i> spp	14 (3.07)	24 (4.03)
<i>Enterocytozoon bieneusi</i>	5 (1.10)	10 (1.68)
<i>Encephalitozoon</i> spp	2 (0.44)	2 (0.34)

\*Two patients were positive for both *Cryptosporidium* spp and *E. bieneusi*. qPCR, quantitative PCR.

**Table 4** Epidemiologic Features of Positive Patients for *Cryptosporidium* spp and/or Microsporidia

Features		Overall values		<i>Cryptosporidium</i> spp		<i>Enterocytozoon</i> <i>bieneusi</i>		<i>Encephalitozoon</i> spp	
Number of patients	19	100%	14*	100%	5*	100%	2	100%	
Age	Median (range), years	47 (0.5–83)	n.a.	49 (0.5–74)	n.a.	47 (2–83)	n.a.	8.5 (4–13)	n.a.
	<10 years	4	21%	2	14%	2	40%	1	50%
Sex	Male	8	42%	6	43%	2	40%	1	50%
	Female	11	58%	8	57%	3	60%	1	50%
Immunocompetent patients	6	32%	4	29%	1	20%	2	100%	
Immunocompromised patients	13	68%	10	71%	4	80%	0	0%	
SOT	Total	9	47%	6	43%	4	80%	0	0%
	Kidney	7	37%	6	43%	2	40%	n.a.	n.a.
	Liver	2	11%	0	0%	2	40%	n.a.	n.a.
HIV	Total	2	11%	2	14%	0	0%	0	0%
	CD <sub>4</sub> count (median), cells/mm <sup>3</sup>	33 (7–58)	n.a.	33 (7–58)	n.a.	n.a.	n.a.	n.a.	n.a.
	Viral load (copies/mL)	41,088 (892 –81,283)	n.a.	41,088 (892 –81,283)	n.a.	n.a.	n.a.	n.a.	n.a.
HM	Total	2	11%	2	14%	0	0%	0	0%
	AML	1	5%	1	7%	n.a.	n.a.	n.a.	n.a.
	Lymphoma	1	5%	1	7%	n.a.	n.a.	n.a.	n.a.
	HSCT	2	11%	2	14%	n.a.	n.a.	n.a.	n.a.
Travelers	7	37%	5	36%	1	20%	2	100%	

\*Two patients were positive for both *Cryptosporidium* spp and *E. bieneusi*.

AML, acute myeloid leukemia; HM, hematologic malignancies; HSCT, hematopoietic stem cell transplantation; n.a., not applicable; SOT, solid organ transplant.

positive for *Cryptosporidium* spp, and 4, for *E. bieneusi*. With the multiplex qPCR as a reference, co-proantigen testing and microscopy for the diagnosis of cryptosporidiosis had sensitivity values of 73% and 64%, respectively. The sensitivity of microscopy for microsporidiosis was 50%. No microscopy-positive/qPCR-negative samples were recorded. Co-proantigen testing for *Cryptosporidium* was positive in 2 samples that tested negative on microscopy. However, *Cryptosporidium* co-proantigen testing failed to detect 1 microscopy-positive stool sample (further identified as *C. parvum*).

### Species Identification and Genotyping

Among the 14 *Cryptosporidium* spp–positive isolates, 8 *C. parvum*, 2 *C. hominis*, 1 *Cryptosporidium felis*, and 1 *Cryptosporidium meleagridis* were identified. The latter 2 isolates were not identified at the species level (Supplemental Table S3). Genotyping of the 8 *C. parvum* isolates indicated that they were of subtype families IIa and IId, including 3 of genotype IIa A15 G2 R1, and 1 of each of the following genotypes: IIa A16 G3 R1, IIa A17 G2 R1, IId A15 G1, IId A22 G1, and IId A22 G1b. The 2 *C. hominis* isolates were subtyped as genotypes Ia A14 R3 and Ib A10 G2. Genotyping of the 5 *E. bieneusi* isolates revealed the presence of 3 of genotype C and 2 of genotype IV. The 2 *Encephalitozoon*

spp isolates were confirmed as *E. intestinalis* on sequencing of the internal transcribed spacer region.

### Discussion

This study was the first to assess an in-house multiplex qPCR method for simultaneous detection of *E. bieneusi*, *Encephalitozoon* spp, and *Cryptosporidium* spp, which are mainly involved in opportunistic chronic diarrhea. Moreover, it provides rapid discrimination between *E. bieneusi* and *Encephalitozoon* spp, which is essential information for the initiation of targeted therapy. Multiplex qPCR showed high sensitivity at a threshold of 1 copy/μL for each target. Interestingly, cross-reactions between *Encephalitozoon* spp were observed. Although these cross-reactions require the confirmation of species by sequencing, the advantage is the detection of the two other *Encephalitozoon* spp involved in human diseases. Moreover, the co-occurrence of the three pathogens in the same sample had no impact on the results, even if one was overrepresented compared to the others. This finding was further confirmed in the prospective study by the concurrent detection of *Cryptosporidium* spp. and *E. bieneusi* in the same stool sample.

The advantage of molecular tools for the diagnosis of microsporidiosis and cryptosporidiosis was previously



demonstrated in studies showing increased sensitivity and rapid species identification.<sup>5,6,15–17</sup> The results of the present study confirm these previous works in multiplex conditions, with a higher sensitivity of multiplex qPCR compared to that of microscopic examination. As previously reported, *Cryptosporidium* antigenic testing was superior to microscopy but inferior to multiplex qPCR in cases of low parasite load, with a sensitivity of 73%.<sup>16</sup>

Among the 595 analyzed stool samples, 6.1% were positive for at least one of the three targeted pathogens, corresponding to 4.2% of the patients. The overall prevalence of cryptosporidiosis and microsporidiosis were 3.07% and 1.54%, respectively. Most of positive samples were diagnosed in immunocompromised patients, including solid organ transplant recipients and patients with hematologic malignancy or HIV. Two of the HIV patients were infected with cryptosporidia, whereas no cases of microsporidia infection were diagnosed. The same was observed in patients with hematologic malignancy, with the prevalence of cryptosporidiosis reaching 2.2% in hematopoietic stem cell transplantation recipients. Most of the cryptosporidiosis and *E. bienersi* cases were diagnosed in kidney transplant recipients (KTRs), with prevalences of 7.3% and 2.4%, respectively. These results are consistent with those from a recent study from the French National Reference Center for Cryptosporidiosis,<sup>18</sup> which reported that KTRs constituted the main population at risk for cryptosporidiosis. Given that antiretroviral therapies are available, most cases of microsporidiosis in developed countries are diagnosed in KTRs. While few data are available from the literature, the prevalence of microsporidia seems to range from 5.8% to 11.6% in KTRs.<sup>19–22</sup>

Among the 14 cases of cryptosporidiosis, 8 isolates were identified as *C. parvum* (5 of genotype IIa, and 3 of genotype IIId); 2 isolates, as *C. hominis* (1 of genotype Ia, and 1 of genotype Ib); 1 isolate, as *C. meleagridis*; and 1 isolate, as *C. felis*. Two isolates had no species identification because of low parasite load. *C. parvum* IIa and IIId are commonly found in Europe, especially the bovine IIa genotype.<sup>2</sup> In contrast, *C. hominis* Ia is usually seen in developing countries, which is consistent with the history of one patient who had travelled to Mayotte. The patient carrying *C. hominis* Ib had traveled to Spain, where that genotype has recently been described.<sup>23</sup> The *C. felis* isolate was identified in a HIV patient who had traveled to Mali. However, these findings are not sufficient for concluding imported cases because this species is the third most common zoonotic species encountered in France, behind *C. parvum* and *C. hominis*.<sup>18,24</sup> Interestingly, 1 isolate, from a hematopoietic stem cell transplantation recipient who had traveled to Indonesia, was identified as *C. meleagridis* and can be considered as a probable imported case, because this zoonotic species is rarely reported in France, with only four cases between 2006 and 2009.<sup>24,25</sup>

Genotyping of *E. bienersi* is based on the internal transcribed spacer region sequence, with >250 genotypes

reported to date.<sup>26</sup> *E. bienersi* genotypes can be classified into nine groups, with group 1 containing most of the genotypes infecting humans.<sup>26</sup> To date, few data regarding *E. bienersi* genotypes in France are available. In the present study, two isolates of genotype IV (frequent synonym: genotype K) and belonging to zoonotic group 1c<sup>27</sup> were identified in a pediatric liver transplant recipient and in an immunocompetent child who had traveled to Burundi. Interestingly, that genotype has been previously reported in African countries, such as Uganda, Cameroon, Gabon, and Malawi.<sup>28–31</sup> Two isolates of genotype C (group 1f) were identified in adult KTRs, and one, in a liver transplant recipient. This genotype seems to be exclusively of human origin and is often isolated from solid organ transplant recipients, especially KTRs.<sup>19,27</sup> In a retrospective study conducted in Eastern France, the presence of isolates of genotypes A, C, D, and S9, all belonging to group 1, was reported.<sup>19,32</sup> Interestingly, the three isolates of genotype C identified in this study were from KTRs. However, updated data on genotypes involved in other clinical contexts are required for confirming a potential link between KTR and genotype C. Finally, the two cases of *E. intestinalis* infection were detected in immunocompetent children who had traveled to tropical areas.

To conclude, this multiplex qPCR method allowed the concurrent detection of major opportunistic eukaryotes involved in chronic diarrhea. Based on the results from this 2-year prospective study, this tool has a place in the first-line investigation of diarrhea in immunocompromised patients, especially in KTRs, but also in immunocompetent patients.

## Author Contributions

M.M. performed literature search and wrote the manuscript; M.M. and C.F. collected the data; M.M., L.F., F.D., and P.P. performed experiments; C.N., F.D., C.G., and P.P. provided critical input in writing the manuscript; P.P. conceived the study; all of the authors read and approved the final manuscript. P.P. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

## Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.jmoldx.2020.12.005>.

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