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Multicentric Evaluation of a New Real-Time PCR Assay for Quantification of *Cryptosporidium* spp. and Identification of *Cryptosporidium parvum* and *Cryptosporidium hominis*

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***Cryptosporidium* is a protozoan parasite responsible for gastroenteritis, especially in immunocompromised patients. Laboratory diagnosis of cryptosporidiosis relies on microscopy, antigen detection, and nucleic acid detection and analysis. Among the numerous molecular targets available, the 18S rRNA gene displays the best sensitivity and sequence variations between species and can be used for molecular typing assays. This paper presents a new real-time PCR assay for the detection and quantification of all *Cryptosporidium* species associated with the identification of *Cryptosporidium hominis* and *Cryptosporidium parvum*. The sensitivity and specificity of this new PCR assay were assessed on a multicentric basis, using well-characterized *Cryptosporidium*-positive and -negative human stool samples, and the efficiencies of nine extraction methods were comparatively assessed using *Cryptosporidium*-seeded stool samples and phosphate-buffered saline samples. A comparison of extraction yields showed that the most efficient extraction method was the Boom technique in association with mechanical grinding, and column extraction showed higher binding capacity than extraction methods based on magnetic silica. Our PCR assay was able to quantify at least 300 oocysts per gram of stool. Satisfactory reproducibility between laboratories was observed. The two main species causing human disease, *Cryptosporidium hominis* and *Cryptosporidium parvum*, were identified using a duplex real-time PCR assay with specific TaqMan minor-groove-binding ligand (MGB) probes for the same amplicon. To conclude, this one-step quantitative PCR is well suited to the routine diagnosis of cryptosporidiosis since practical conditions, including DNA extraction, quantification using well-defined standards, and identification of the two main species infecting humans, have been positively assessed.**

Cryptosporidium is a protozoan parasite involved in waterborne gastrointestinal infections. The disease usually is mild in immunocompetent patients but can have serious consequences in immunocompromised patients (1). To date, no treatment has been able to eradicate *Cryptosporidium*, especially in immunocompromised patients (2).

Several species are causative agents of diarrhea in humans and animals. In humans, *Cryptosporidium parvum* and *Cryptosporidium hominis* are the most frequently detected species, accounting for almost 90% of cases of diagnosed cryptosporidiosis but displaying different prevalence rates in different areas. The two species are almost equally represented in Europe and the United States (3–8), whereas *C. hominis* is predominant in tropical regions, reaching a prevalence of up to 88% of identified cases in some countries (9–12). Several other species also have emerged as causes of cryptosporidiosis in immunocompromised and immunocompetent patients, but at much lower prevalence rates than *C. parvum* or *C. hominis* (5, 10, 11).

Laboratory methods for detecting *Cryptosporidium* were first based on microscopy in combination with various staining methods, the most widely used being the modified Ziehl-Neelsen staining method for oocysts, the sensitivity of which has been estimated at 75% (13, 14). A direct fluorescent antibody assay (DFA) improved the sensitivity of conventional microscopy, as its sensitivity is about 1,000 oocysts per g of stool (15).

Antigen detection methods have been developed and marketed as immunochromatographic assays (16, 17), possibly coupled with *Giardia* antigen detection. These methods are easier to use as rapid detection tests but do not allow for quantification or typing of the parasites. Higher sensitivity of detection in environmental and clinical samples can be achieved by performing molecular diagnosis of cryptosporidiosis using PCR. The most commonly used targets are the 18S rRNA gene (18), the *Cryptosporidium* oocyst wall protein (19), the 60-kDa glycoprotein (20), heat shock protein 70, the Laxer locus, and microsatellite loci (reviewed in reference 21). Several studies agree on the higher sensitivity of PCR targeting the 18S rRNA gene, in relation to its copy number (22). Nested PCR has been put forward as a means of improving the sensitivity of detection (23) and reverse transcription-PCR as a method for studying viability in environmental samples (24). Real-time PCR presently is the most extensively used method,

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TABLE 1 *Cryptosporidium* sequence alignment of the polymorphic region of the amplicon (ClustalW2 multiple sequence alignment file)

Species	Sequence	GenBank accession no.
C. hominis	TTTACGGATCACAATT----------AATGTGACA	EU675853.1
C. parvum	TTTACGGATCACATAA----------ATTGTGACA	AF112570.1
C. felis	TTTACGGATCACAATAATTTATTTTGTGACA	AF112575.1
C. bovis	TTTACGGATCACATTA----------TGTGACA	EF514234.1
C. cuniculus	TTTACGGATCACAATT----------AATGTGACA	HQ397716.1
C. canis	TTTACGGATCACATTT----------TATGTGACA	AF112576.1
Cryptosporidium chipmunk genotype	TTTACGGATCACATTTTG---------ATGTGACA	EF641026.1

allowing for both accurate quantification of the molecular target and typing under technical conditions that avoid cross contamination and DNA carryover (25, 26). However, DNA extraction remains a limiting condition for PCR technologies, since DNA polymerase inhibitors (which often are found in environmental and clinical samples) are copurified with nucleic acids and the yield of extraction depends on the technical conditions (27, 28).

In this context, four university hospital laboratories of medical parasitology and one veterinary laboratory of the French Agency for Food, Environmental, and Occupational Health and Safety (ANSES) (Niort, France), all belonging to the French ANOFEL *Cryptosporidium* National Network (5), set up a multicentric evaluation of a new sensitive real-time quantitative PCR (qPCR) assay capable of easily discriminating and quantifying the main *Cryptosporidium* species involved in human pathology. This organization made it possible (i) to share well-defined clinical samples and positive controls, (ii) to compare the extraction rates of nine methods using a single independent quantification process, and (iii) to assess comparatively the performance of a new qPCR assay among laboratories using the same DNA samples (extracted in the coordinating laboratory), primers, fluorescent probes, and standards.

MATERIALS AND METHODS

Study design. The Marseilles parasitology laboratory coordinated the study. The Lyons, Paris, and Nice parasitology laboratories and the ANSES veterinary laboratory were participating laboratories. Well-characterized positive stool samples were provided by the French ANOFEL *Cryptosporidium* National Network. The ANSES laboratory provided *C. parvum* oocysts. The coordinating laboratory prepared the natural stool samples and the spiked samples, performed the DNA extractions for testing of the different PCR methods, and prepared the natural and plasmid DNA standards. All of the participating laboratories received identical sets of materials, including 20 samples to test the DNA extraction methods and 100 DNA solutions from positive and negative biological samples to test the diagnosis/quantification and typing PCR assays.

Stool samples. Forty human stool specimens (N1 to N40) were selected on the basis of negative microscopic results for *Cryptosporidium* with the Ziehl-Nielsen modified staining method and for other protozoa or helminths with the formalin-ether concentration method. All of these specimens served as negative controls, and one of them was used to prepare samples seeded with *Cryptosporidium* oocysts. DNA from human stool specimens containing *Cystoisospora belli* ($n = 3$), *Cyclospora cayetanensis* ($n = 1$), *Enterocytozoon bieneusi* ($n = 3$), *Entamoeba histolytica* ($n = 2$), *Giardia intestinalis* ($n = 6$), and *Candida* spp. ($n = 10$) was used for the specificity assessment.

Sixty *Cryptosporidium*-positive human or animal stool specimens (P41 to P100) were provided by the ANOFEL *Cryptosporidium* National Network. The diagnosis was established by microscopy, and stool samples were preserved in 2.5% potassium dichromate (final concentration). *Cryptosporidium* species were determined by PCR sequencing at the 18S rRNA gene locus (29) and included *C. hominis* ($n = 23$), *C. parvum* ($n = 20$), *Cryptosporidium felis* ($n = 8$), *Cryptosporidium bovis* ($n = 4$), *Cryptosporidium cuniculus* ($n = 2$), *Cryptosporidium canis* ($n = 2$), and *Cryptosporidium* chipmunk genotype ($n = 1$). Ten additional fresh stool samples that were positive by microscopy for *Cryptosporidium* (7 with *C. parvum* [E1, E3, E4, E5, E6, E9, and E10] and 3 with *C. hominis* [E2, E7, and E8]) were provided by the participating laboratories in order to test the DNA extraction protocols.

Artificial positive samples were prepared with *C. parvum* oocysts that had been purified from heavily infected calf stools, after concentration using ethyl acetate and purification on a discontinuous Percoll gradient (30). Ten samples were prepared by adding 100, 200, 500, 1,000, or 10,000 purified *C. parvum* oocysts to either 200 mg of a negative human stool sample (E11 to E15) or 200 μl of phosphate-buffered saline (PBS) (E16 to E20), which resulted in final parasite loads of 500, 1,000, 2,500, 5,000, and 50,000 oocysts per g, respectively. The 20 samples numbered E1 to E20 were sent to the participating laboratories, which were asked to perform the DNA extraction upon receipt.

DNA extraction. DNA from the 40 negative samples (N1 to N40) and the 60 positive samples (P41 to P100) was extracted in the coordinating laboratory using a NucliSENS easyMAG device (bioMérieux, Marcy l'Etoile, France), based on the method described by Boom et al. (31). The extraction protocol was adapted for stool processing as follows: 400 mg of stool samples was added to 1 ml of NucliSENS lysis buffer in a tube containing ceramic beads (lysing matrix D; MP Biomedicals, Illkirch, France) and then disrupted in a FastPrep-24 grinder (MP Biomedicals) at maximum power for 1 min. After 10 min of incubation at room temperature to allow complete lysis, tubes were centrifuged at 10,000 × *g* for 10 min and three extractions were performed, each with 250 μl of supernatant. Each extraction included five washing steps with NucliSENS extraction buffer 2 (reference no. 280131; bioMérieux). Elution was performed at 70°C with 100 μl of elution buffer; 1 μl of the resulting eluate corresponded to 1 mg of starting material. The DNA eluates from each sample then were pooled, aliquoted into five microtubes, frozen, and sent to the participating laboratories. Using a similar extraction procedure, a *Cryptosporidium* DNA standard solution was prepared from purified *C. parvum* oocysts resuspended in PBS (10^6 oocysts/extraction); the resulting eluate contained 10,000 equivalent oocysts of DNA per microliter.

One hundred DNA samples to be tested (60 positive and 40 negative) and DNA standard solutions were sent to each participating laboratory and stored at −20°C. These samples were tested within 4 months after receipt.

***Cryptosporidium* qPCR.** Two new real-time PCR assays were developed in the coordinating laboratory. The first assay was designed to detect the presence of *Cryptosporidium* DNA and amplified a DNA fragment located in the 18S rRNA gene (GenBank accession no. EU675853.1, positions 33 to 211). The direct and reverse primer sequences were CATGGA TAACCGTGGTAAT and TACCCTACCGTCTAAAGCTG, respectively. Amplification resulted in a 178-bp amplicon that included a polymorphic region (nucleotides 157 to 162) (Table 1). A TaqMan probe homologous to a conserved region of the sequence (Pan-crypto, FAM-CTAGAGCTA

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ATACATGCGAAAAAA-MGB-BHQ [FAM, 6-carboxyfluorescein; MGB, minor-groove-binding ligand; BHQ, black hole quencher]) was designed to detect all *Cryptosporidium* species. The second assay (duplex PCR) was designed to differentiate *C. hominis* and *C. parvum* by means of two hybridization probes, i.e., FAM-ATCACAATTAATGT-MGB-BHQ (*C. hominis*) and 6-carboxyrhodamine (VIC)-ATCACATTAAATGT-MGB-BHQ (*C. parvum*).

Primers and probes were used at 0.2 μM and 0.1 μM (final concentrations), respectively. One microliter of sample DNA was added to the reaction tubes (final volume, 25 μl). The amplification consisted of activation of the *Taq* DNA polymerase for 10 min at 94°C, followed by 45 cycles of 94°C for 10 s, 54°C for 30 s, and 72°C for 10 s, with thermal transitions between denaturation and primer annealing of <1.2°C/s, in order to enable probe hybridization. Multiplex assays including the typing probes were performed under the same technical conditions, in order to confirm that there was no interference between probes.

Control DNA and plasmids. Control DNA was prepared from purified *C. parvum* oocysts as described above and by cloning of the *C. hominis* sequence in a plasmid using a TOPO TA cloning kit (Invitrogen reference no. K4520-01; Life Technologies SAS, Saint Aubin, France). This plasmid was amplified, purified from bacteria, and quantified by spectrophotometry. In the qPCR assays, these controls were used to establish a standard curve using a dilution range of 0.1 to 10,000 equivalent oocysts and/or 1 to 10^7 copies of plasmid DNA. A set of plasmid dilutions was included in each quantification experiment for calculation of the number of target copies for one oocyst and assessment of the sensitivity of the assay. *C. parvum* and *C. hominis* control DNAs were included in each typing assay as positive controls.

An M13 plasmid containing nonrelevant DNA (reference no. 360364; Applied Biosystems, Villebon-sur-Yvette, France) was used to detect inhibitors in extracted DNA. Twenty copies of this plasmid were added to each reaction tube and then a real-time PCR targeting this plasmid was performed, using M13 universal primers and a TaqMan probe specific to the inserted DNA. A positive result was considered indicative of the absence of inhibitors in the sample.

Interlaboratory comparison of nine extractions methods. Nine extraction protocols were applied to 10 natural (E01 to E10) and 10 seeded (E11 to E15 in stool and E16 to E20 in PBS) samples. The extraction processes were applied to the entire contents of the tubes (200 mg or 200 μl), and the elution volume was set at 100 μl; the different protocols used are summarized in Table 2. Three extraction kits (NucliSENS easyMAG, QIAamp DNA Mini, and NucleoSpin Tissue) were used with and without preliminary mechanical grinding; the others (QIAamp DNA Stool mini-kit, UltraClean fecal DNA isolation kit, and Maxwell 16 Tissue DNA purification kit) were used according to the manufacturer's instructions.

Following DNA extractions by the participating laboratories, the eluates were sent to the coordinating laboratory to be tested by qPCR. All eluates were tested in duplicate in a single qPCR run, for more accurate comparison of the extraction rates. Results were expressed as the number of oocysts per gram of starting material by using serial dilutions of the *Cryptosporidium* DNA standard solution as a reference.

Interlaboratory qPCR assessment. Primers, probes, and DNA controls were sent to the participating laboratories. Each laboratory was asked to perform quantification assays in duplicate with stool sample DNA using the Pan-crypto TaqMan probe and then to genotype the positive samples using the *C. hominis* and *C. parvum* probes. The participating laboratories performed all of the tests blind to the microscopic and qPCR results of the coordinating laboratory. The threshold cycle (C_T) values and quantities were recorded and analyzed.

Statistical analysis. A regression analysis of the quantitative results was performed with raw data to examine the relationships between the two series of quantification assays in each center. Analysis of variance (ANOVA) was carried out to examine the influence of the extraction methods on the results and to identify a possible center effect on quantification. ANOVA was performed after logarithmic transformation of

TABLE 2 Comparison of the main characteristics of the DNA extraction methods

Abbreviated name	Manufacturer	Commercial name	Mechanical breakage beads	Automation protocol	Direct lysis	Proteinase K digestion	Specific adsorption of inhibitors	Silica support	Elution temp (°C)
easyMAG	bioMérieux	NucliSENS easyMag	No	Yes, specific B	Yes	No	No	Magnetic silica	70
easyMAG + grinding	bioMérieux	NucliSENS easyMag	FastPrep-24 (MP Biomedicals) with lysing matrix D	Yes, specific B	Yes	No	No	Magnetic silica	70
Maxwell	Promega	Maxwell 16 Tissue DNA purification kit	No	Yes, tissue	Yes	No	No	Magnetic silica	70
MO BIO	MO BIO Laboratories	UltraClean fecal DNA isolation kit	Yes, included	No	Yes	No	Yes	Silica column	Room temp
NucleoSpin	Macherey-Nagel	NucleoSpin Tissue	No	No	After washing	Yes	No	Silica column	Room temp
NucleoSpin + grinding	Macherey-Nagel	NucleoSpin Tissue	MagNA Lyser (Roche) Green beads	No	After washing	Yes	No	Silica column	Room temp
QIAamp DNA	Qiagen	QIAamp DNA Mini	No	No	Yes	Yes	No	Silica column	Room temp
QIAamp Stool	Qiagen	QIAamp DNA Stool Mini	No	No	Yes	Yes	Yes	Silica column	Room temp
QIAamp DNA + grinding	Qiagen	QIAamp DNA Mini	TissueLyser (Qiagen) with steel beads	No	Yes	Yes	No	Silica column	Room temp

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TABLE 3 Quantification of *Cryptosporidium* DNA from 10 natural fecal samples (E1 to E10), five seeded fecal samples (E11 to E15), and suspensions of oocysts in PBS (E16 to E20)

Sample	No. of oocysts added per g of sample	No. of oocysts detected per g of sample with:								
		easyMAG	easyMAG + grinding	Maxwell	MO BIO	NucleoSpin	NucleoSpin + grinding	QIAamp DNA	QIAamp Stool	QIAamp DNA + grinding
Natural fecal samples										
E01		2.5	6	4.5	0	1.5	0.5	0	0	1
E02		284.5	655	293	45	372	278	252	0.5	221
E03		56.5	377	18.5	3	565	31	323	2.5	280.5
E04		37.5	222.5	42.5	25	378.5	459	178	0.5	265.5
E05		139	1,165	192.5	0.5	422	85	560	0.5	550
E06		60.5	265.5	256.5	1.5	187	1,030	327.5	3.5	151
E07		2,695	1,430	1,430	202	3,495	1,000	5,300	198	7,200
E08		263	175	16.5	3	100	6	56.5	11	168.5
E09		2,805	7,450	2,140	575	5,100	496	27,700	174	20,000
E10		1,745	5,950	1,935	318	20,600	3,150	59,000	427	27,550
Seeded fecal samples										
E11	0.5	0	2	5	0.5	0.5	0.5	0.5	0	2.5
E12	1	0	0.5	0	0	5.5	0	2	0	4
E13	2.5	0.5	4.5	2	0	11	0.5	15	0	13
E14	5	2.5	8.5	18.5	0.5	8.5	2	12.5	0	16.5
E15	50	135	148.5	76	3	116.5	2.5	36	3	26
Seeded PBS samples										
E16	0.5	0	0	0	0	0	0	2	0	2.5
E17	1	1	0	1	0	0	0	3.5	0	1
E18	2.5	0	2.5	0.5	0	1.5	0.5	7.5	0	9.5
E19	5	4	0.5	0	0	1.5	0.5	29	0	19
E20	50	43	20.5	38.5	0.5	10	1.5	134	0	147.5

quantitative data. All statistical analyses were conducted using StatView 5 software (SAS Institute, Cary, NC).

RESULTS

DNA extraction methods present variable yields. All extraction products obtained with the nine extraction methods from the 15 *Cryptosporidium*-positive fresh fecal samples (E01 to E15) were positive by PCR, except for five obtained with QIAamp DNA Stool (E01, E11, E12, E13, and E14), two with NucliSENS easyMAG without grinding (E11 and E12), one with QIAamp DNA without grinding (E01), one with NucleoSpin with grinding (E12), one with Maxwell (E12), and three with UltraClean (E01, E12, and E13) (Table 3). The results of the ANOVA performed on the 15 sets of data were not conclusive because of a large variance due to the results for three samples with high parasite loads (E7, E9, and E10). These loads were estimated by qPCR to be around 10^6 oocysts/g following NucliSENS easyMAG extraction, $>10^8$ oocysts/g with QIAamp DNA, and $<5 \times 10^5$ oocysts/g with the other extraction protocols. This variation is probably related to the saturation of silica rather than to other components of the extraction process. Consequently, these samples were excluded from the ANOVA extraction rate comparison.

The ANOVA performed on the results of the 12 remaining samples showed that the extraction protocol significantly influenced the quantification results ($P = 0.006$). The effect of the method on the extraction rate is summarized in Fig. 1, where the mean values of parasite loads are presented. With a significance level of 5%, the post-ANOVA Tukey-Kramer test showed that the mean difference was greater than the critical difference for three pairs of results, i.e., QIAamp DNA Stool compared with NucliSENS easyMAG, QIAamp DNA Stool compared with easyMAG plus grinding, and QIAamp DNA Stool compared with QIAamp DNA plus grinding. For these 12 fecal samples with low-

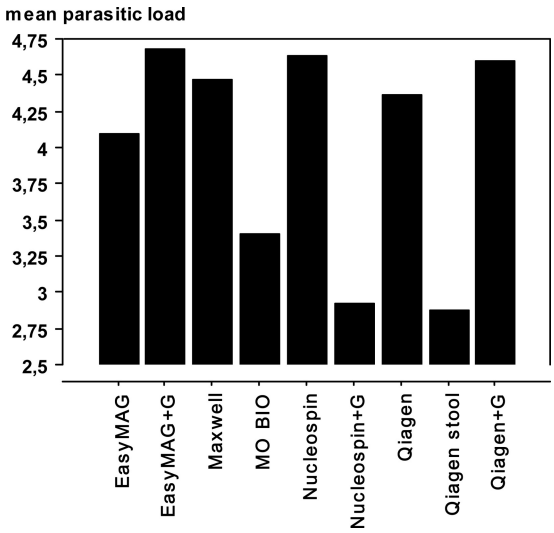

FIG 1 Comparison of the mean log values for oocyst quantification (no. of oocysts per gram of stool) obtained from 12 samples, with medium or low parasite burdens, after nine different extractions protocols. ANOVA detected a significant influence of the technique on quantification results ($P = 0.0059$). G, grinding.

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TABLE 4 Correlation matrix for the parasite loads found in two independent series of quantifications (a and b) of 60 positive samples in four participating laboratories

Assay designation[a]	Correlation coefficient							
	1.a	1.b	2.a	2.b	3.a	3.b	4.a	4.b
1.a	1.00	0.896	0.911	0.905	0.914	0.896	0.926	0.879
1.b	0.896	1.00	0.906	0.900	0.807	0.849	0.896	0.909
2.a	0.911	0.906	1.00	0.999	0.945	0.968	0.991	0.986
2.b	0.905	0.900	0.999	1.00	0.944	0.968	0.991	0.986
3.a	0.914	0.807	0.945	0.944	1.00	0.944	0.951	0.917
3.b	0.896	0.849	0.968	0.968	0.944	1.00	0.959	0.937
4.a	0.926	0.896	0.991	0.991	0.951	0.959	1.00	0.974
4.b	0.879	0.909	0.986	0.986	0.917	0.937	0.974	1.00

[a] Laboratory designations were as follows: 1, Lyons; 2, Marseilles; 3, Nice; 4, Paris.

to-medium parasite burdens, the highest extraction rate was observed with NucliSENS easyMAG plus grinding, whereas the lowest extraction rates were observed with QIAamp DNA Stool and UltraClean (<2%, compared with NucliSENS easyMAG).

Surprisingly, NucliSENS easyMAG plus grinding was more efficient with fecal specimens than with saline suspensions of oocysts, as the mean values for the samples seeded with identical quantities of oocysts were 32,800 and 4,700 oocysts/g, respectively. The extraction yield from PBS suspensions was 39%, leading to overestimated quantification results as the standard curve was established on the basis of DNA extracted from saline suspensions of oocysts. Taking this correction into account, we found a mean of 12,650 oocysts/g in spiked fecal samples, and comparison with the actual quantities added (mean, 11,800 oocysts/g) showed that the extraction yield from fecal samples was about 100%.

Mechanical grinding improves DNA extraction. Three extraction kits (NucliSENS easyMAG, QIAamp DNA, and NucleoSpin) were tested with and without mechanical grinding. To estimate the effects of mechanical grinding, we performed DNA extraction (in duplicate) from 15 fecal samples with and without grinding, and the differences between the mean C_T values were calculated. Mechanical grinding using a FastPrep instrument significantly improved the NucliSENS easyMAG extraction yield by 2.17-fold ($P < 0.0001$), while no significant difference was observed with the QIAamp DNA or NucleoSpin Tissue kits ($P = 0.6$ and 0.21, respectively).

Sensitivity and specificity. Using the Pan-crypto probe and plasmid dilutions, the sensitivity of detection was estimated at 10 gene copies per reaction tube, since this quantity was always detected, while a single copy was amplified in two of 12 experiments. DNA corresponding to 0.3 oocysts was amplified in all cases. By taking the quantity of starting material and the elution volume into account, the practical sensitivity was estimated as 300 oocysts per g of stool. The number of target copies per oocyst was estimated by comparing the standard curves obtained with plasmid and oocyst DNA dilutions. Using data from six independent experiments, the mean ratio was estimated at 25 copies of the 18S rRNA gene per oocyst.

In silico analysis of this amplicon revealed no significant sequence homology with other parasitic or mammalian DNA. The absence of cross-amplification was confirmed by the negative results obtained from human, parasitic, and fungal DNA, as well as DNA from microorganisms of the normal intestinal flora.

Quantitative results for the 60 positive samples. The first step involved examining the 60 positive stool samples for the presence of *Cryptosporidium* DNA by means of a SYBR green PCR in the coordinating laboratory. All samples contained amplifiable DNA, and melting point analysis revealed a single melting peak at 77°C. These samples were then tested by the participating laboratories with qPCR, using the Pan-crypto probe. All laboratories except the ANSES laboratory performed two independent experiments. All samples except one (P59, *C. parvum* with a low parasite burden by microscopy) were amplified in all experiments, with parasite loads ranging from 300 to 35×10^6 oocysts per gram of stool.

The intralaboratory reproducibility was estimated through the correlation between the C_T values and the deducted oocyst counts from duplicate experiments. R^2 values ranged from 0.92 to 0.96 for C_T values and from 0.80 to 0.99 for oocyst counts, attesting to the variability induced by the standard curve for oocyst quantification.

The interlaboratory variability with respect to quantification of the parasite burdens in stools (mean values from the two sets of data) was examined for four laboratories by using a correlation matrix and ANOVA. The correlation coefficients ranged from 0.807 to 0.999 (Table 4), with a median of 0.931, attesting to the good reproducibility of quantification between laboratories. ANOVA showed no laboratory effect ($P = 0.313$).

Molecular discrimination between *C. hominis* and *C. parvum*. The 60 positive DNA samples were tested by means of a duplex PCR using the *C. hominis* and *C. parvum* probes. DNA extracted from samples containing *C. hominis* or *C. parvum* hydrolyzed the corresponding probes (23 and 19 samples, respectively). The two samples containing *C. cuniculus* DNA hydrolyzed the *C. hominis* probe, since the two species share the same sequence (Table 1). No signal was observed with samples containing *C. bovis* (4 isolates), *C. felis* (8 isolates), *Cryptosporidium* chipmunk genotype (1 isolate), or *C. canis* (2 isolates).

As these specific probes hybridize to the amplicon produced by the detection PCR, we tested the possibility of performing duplex PCR using the Pan-crypto probe and one species-specific probe. No negative interaction between the different probes and no loss of sensitivity were observed, as shown by the similar y intercepts and standard curve slopes obtained with the different combinations tested, compared with PCR using a single probe (Table 5).

DISCUSSION

Real-time quantitative PCR (qPCR) is a unique tool for the sensitive detection, quantification, and specific characterization of *Cryptosporidium* species of medical and veterinary importance. However, qPCR is a multiple-step procedure, each step of which is

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TABLE 5 Influence of multiplexing on the main characteristics of the qPCR

DNA species	Probe(s)[a]	Slope	*y* intercept
C. hominis	Pan-crypto	−3.32	40.27
C. hominis	Pan-crypto + *C. parvum*	−3.44	40.60
C. hominis	*C. hominis* + *C. parvum*	−3.54	40.01
C. hominis	*C. hominis*	−3.46	39.65
C. parvum	Pan-crypto	−3.41	39.63
C. parvum	Pan-crypto + *C. parvum*	−3.53	39.46
C. parvum	*C. hominis* + *C. parvum*	−3.4	39.72
C. parvum	*C. parvum*	−3.42	39.45

[a] The combination of the Pan-crypto and *C. hominis* probes was not tested because the probes used the same fluorescent ligand.

sensitive to several technical parameters and needs to be optimized to improve accuracy. In this context, our multicenter study enabled a thorough assessment of several crucial steps in the qPCR procedure.

The first crucial step is DNA extraction. By comparing nine DNA extraction protocols applied to the same samples, we found marked variations, in the range of 2 log units, in extraction rates. The UltraClean and QIAamp DNA Stool kits displayed poor extraction rates. For the latter, our results coincide with those obtained for water samples by Jiang et al. (27), who concluded that the QIAamp DNA Stool Mini kit was not able to remove inhibitors efficiently despite the use of a specific adsorption system. The extraction rates for the other methods were significantly better, but each method had distinctive features depending on the principle of purification and the parasite burden. The two kits using silica columns, i.e., QIAamp DNA Mini and NucleoSpin kits, were characterized by higher saturation limits than the methods based on magnetic silica, as already observed for *Mycobacterium* (32), while NucliSENS easyMAG gave higher extraction yields with fecal samples containing low parasite burdens. Curiously, NucliSENS easyMAG demonstrated a better extraction rate with fecal samples than with saline suspensions of oocysts. This difference was not observed with the Qiagen or Maxwell systems.

Mechanical grinding improved DNA extraction with NucliSENS easyMAG but not with the QIAamp DNA Mini or NucleoSpin Tissue kits. This means that proteinase K digestion was effective enough to alter the oocyst wall and allowed DNA release with simple cell lysis. By comparing NucliSENS easyMAG with mechanical grinding with the QIAamp DNA Stool kit, Masny et al. found that chemical lysis plus grinding was more effective than enzymatic lysis (33). Our results are in line with such observations; Elwin et al. recently reported that semipurification of oocysts associated with column extraction was more effective than direct extraction with mechanical grinding in guanidinium thiocyanate buffer (3). These studies emphasize the importance of sample pretreatment.

A second determining step in qPCR is the use of reliable standards for quantification. Like other laboratories, we encountered difficulties in relation to the purification of oocysts, their preservation, and the variable extraction yield. In order to overcome these limitations, we estimated the number of target copies per oocyst (i.e., around 25 copies per oocyst) and proposed standardizing positive controls using plasmid DNA. We checked that the use of oocyst or plasmid DNA standards resulted in similar sensitivity limits in our system. The actual sensitivity was estimated to be about 300 oocysts per g of stool but might be lower with an extraction protocol leading to a more-concentrated DNA solution. Other PCR methods based on the same target sequence displayed comparable sensitivity values (22, 26). The sensitivity of the 18S PCR determined by Stroup et al. (34) was 100 to 1,000 oocysts/200 mg of stool; that technique used the QIAamp DNA Stool kit and Scorpion probes. From our experience, this lower sensitivity would be related mainly to the DNA extraction method, rather than the molecular probe used.

Compared to microscopy, the sensitivity threshold of our PCR assay is probably lower than that of the conventional DFA, but a comparison between the two methods was not performed. Previously, Xiao and Herd (15) and Valdez et al. (35) found a detection limit of 1,000 oocysts per gram using a DFA. More recently, Pereira et al. (36) estimated a limit of detection for a DFA of 1,000 to 6,000 oocysts per gram in bovine feces. However, those authors showed that concentrating oocysts by immunomagnetic separation before the DFA resulted in a 2-log-unit increase in sensitivity (36), thus achieving the sensitivity of PCR.

Designing appropriate targets and probes for the concomitant quantification and identification of infecting species is a third step that might improve *Cryptosporidium* qPCR. Several authors have already developed or combined species-specific qPCRs but have found it difficult to ensure that the sensitivity of typing matches the sensitivity of detection (22). The authors were thus confronted with the risk that a sample with a low parasite load would be detected as positive but the parasites could not be typed. This difficulty has been resolved in our qPCR assay by using the same amplicon for detection and typing. Indeed, the polymorphism of the selected target made species discrimination possible with the use of different TaqMan probes with a single set of primers, which makes multiplexing easier. In order to facilitate species-specific hybridization at the polymorphic region, the minor-groove-binding ligand (MGB) was used as a melting point enhancer in order to shorten the nucleotide sequence.

With this method, all cases of cryptosporidiosis were reliably detected using the Pan-crypto probe and *C. hominis* and *C. parvum* were identified using the specific probes. The participating laboratories were consistent in this regard, since 59 of the 60 positive samples were detected by PCR in all of them. Some individual variations were found in the estimation of oocyst counts, but the coefficient of variation between laboratories was <2%. The delay between DNA extraction in the coordinating laboratory and performance of PCR in the participating laboratories (1 week to 4 months) did not significantly influence the results, since ANOVA showed no laboratory effect on the quantification of parasite burdens in stool specimens. This observation confirms the robustness of the assay and indicates that variability depends essentially on DNA extraction rather than the PCR process itself.

However, other species that are uncommon in humans could not be characterized, because of either sequence homologies or lack of hybridization with the *C. hominis* or *C. parvum* probes. This constitutes the main limitation of our assay, although the presence of such uncommon species might be suspected with the discrepancy between hydrolysis of the Pan-crypto probe and the negative results of the typing assay.

A set of PCRs targeting another part of the 18S rRNA gene has been proposed by Hadfield et al. (26). In that assay, detection is based on amplification of the rRNA gene and typing is performed at another locus (LIB13), using two MGB probes for species iden-

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tification. As these two species-specific probes are not compatible, a first multiplex assay was required for detection of all species and *C. parvum* identification and a second assay was necessary for *C. hominis* identification. Application of this technique to routine analysis seems complicated because the specificity of the detection/quantification PCR has to be confirmed by sequencing since the yeast 18S rRNA gene (GenBank accession no. JN940588.1) shares sequence homologies with the selected primers and probe, which might lead to nonspecific amplification. Moreover, neither the 18S nor LIB13 PCR displayed the same sensitivity as shown by Elwin et al. (28). Recently, specific assays have been proposed in order to identify uncommon species. Hadfield and Chalmers (37) proposed a *C. cuniculus* real-time PCR that can overcome the inability to discriminate between *C. hominis* and *C. cuniculus*. New specific PCRs and probes are required to differentiate between other zoonotic species.

In conclusion, through our multicentric evaluation, we have been able to assess the performance of a new sensitive one-step qPCR for the diagnosis of cryptosporidiosis, discrimination between the two major species infecting humans, and quantification of parasite burdens. This assay is well suited to routine use as practical conditions have been improved, including DNA extraction and the use of well-defined standards.

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