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Performance of 30 protocol combinations for the detection of *Cryptosporidium parvum* in stool samples

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ABSTRACT

Background: Intestinal parasitic diseases affect millions of people worldwide. Numerous commercial molecular methods detecting digestive parasites have been developed recently, including multiplex PCR assays able to identify multiple parasites at once. Several studies have demonstrated that the efficacy of these molecular methods is dependent on the specific protocols employed at each stage of the process including pretreatment, extraction and amplification. However, previous studies have exclusively focused on one of these steps, without considering the others. The objective of the present study was to evaluate the performances of molecular tools for *Cryptosporidium parvum* detection in stool samples, considering all steps of the process simultaneously.
Methods: 30 distinct combinations of protocols were evaluated corresponding to three pre-treatment methods, four DNA extraction techniques and six DNA amplification assays. The performances of these combinations were evaluated in terms of detection limit.
Results: We showed that different combinations yielded varying results. The FTD® Stool Parasite technique proved to be the most effective, achieving 100 % detection. Manual extraction methods demonstrated excellent outcomes, although they are time-consuming. The optimal approach for detecting *C. parvum* DNA is a combination of mechanical pretreatment, the Nuclisens® Easymag® extraction method, and the FTD® Stool Parasite DNA amplification method.
Conclusion: This work shows that the molecular diagnosis should consider all stages. A PCR method may not be effective with an unsuitable extraction technique, but can yield optimal results with an appropriate one.

1. Introduction

Cryptosporidiosis is one of the most important diarrheal diseases worldwide, particularly in less developed countries.[1,2] However, its burden remains underestimated, mainly due to the need for specific microscopy techniques, the lack of systematic use of molecular techniques, and the lack of data collection.[2,3] In France, the National Reference Center for Cryptosporidiosis, Microsporidia and other digestive Protozoosis (NRC-CMAP) attempts to reduce this underestimation by evaluating the epidemiology of cryptosporidiosis on the French territory.[4,5] In 2019, the importance of its epidemic potential has been shown by highlighting a contamination of the drinking water network in

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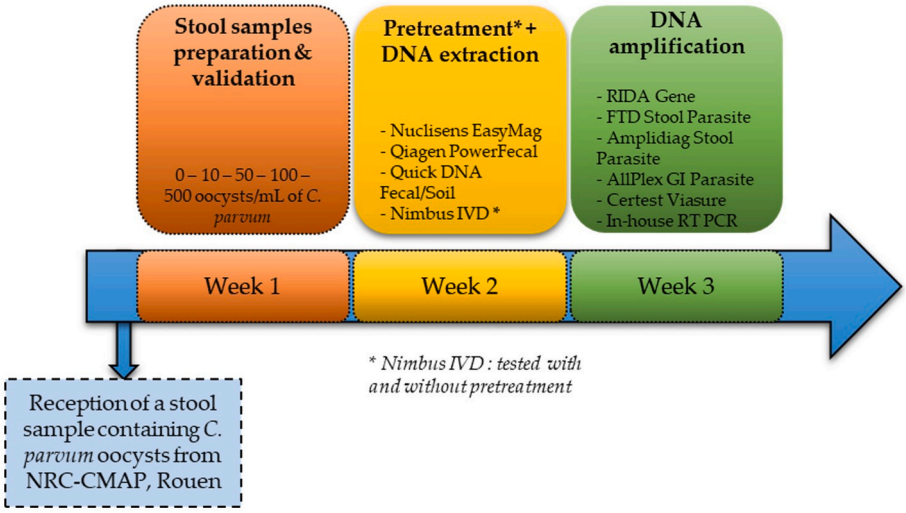

Fig. 1. Protocol flow chart.

south of France.[4] Although cryptosporidiosis causes limited and often self-resolving symptoms, it can lead to severe and recurrent forms in vulnerable people (*i.e.* immunocompromised, elderly and children under five).[6,7] Moreover, therapeutic options are limited, requiring robust prevention and control measures, as well as effective and reliable diagnostic tools.[3]

Nowadays, molecular methods for the detection of *Cryptosporidium* spp. in stool samples are increasingly replacing microscopic techniques, because of higher sensitivity and specificity, time saving, and species identification. The growing availability of multiplex amplification methods also explains the enthusiasm of medical laboratories for molecular biology applied to the diagnosis of parasitic diseases including cryptosporidiosis.[8–10] Many protocols exist for each step of the molecular detection process, including (i) chemical and mechanical pretreatments, (ii) manual or automated DNA extraction techniques, based on various technologies, and (iii) various DNA amplification techniques, targeting different parasites/genes.[11–13] However, disparities in performances exist between all of these available techniques.

As one of the missions of the NRC-CMAP is to evaluate available diagnostic tools, we already studied influencing parameters to optimize *Cryptosporidium* spp. detection. We have previously shown that automated extraction systems, using BOOM® technology, associated with

Table 1
Characteristics of the pretreatment and DNA extraction methods.

Extraction methods	Pretreatment			Extraction				Total
	Mechanical pretreatment	Beads composition	Protocol	Manual/ Automated	Technology	Volumes required *(pretreated stools or fresh samples)*	DNA extract volumes	Time required *(estimated for 8 samples)*
Nuclisens Easymag (EM)	Yes	Lysing Matrix E Tube = Silica (0,1 mm) + Glass (4 mm) + Ceramic (1,4 mm)	• 400 µL stool + 1 mL of Lysis Buffer in a Lysing Matrix E Tube (MP Bio) • Grinding during 1min at 6 m/s Fast Prep 24 (MPBio) • Centrifugation 10min at 10000G	Automated	Magnetic silica/Boom® technology	240 µL of post-pretreatment supernatant	100 µL	1h30
Nimbus IVD without PT (MN)	No	X	X	Automated	Magnetic silica	1 mL of stool suspension	100 µL	2 h
Nimbus IVD with PT (MNP)	Yes	Same as for Nuclisens Easymag	Same as for Nuclisens Easymag	Automated	Magnetic silica	1 mL of post-pretreatment supernatant	100 µL	2h30
PowerFecal pro DNA (QF)	Yes	Dry garnet beads (0,7 mm)	• 250 µL stool + 800 µL of CD1 solution in a PowerBead Pro Tube • Vortex adapter 10min maximum speed • Centrifugation 1min at 15000G	Manual	Silica column	500 µL of post-pretreatment supernatant	100 µL	3 h
Quick DNA Fecal/Soil (ZR)	Yes	0,1 mm & 0,5 mm beads (composition not disclosed)	• 150 µL stool + 750 µL Bashing Bead Buffer in a ZR Bashing Bead Lysis Tube • Grinding during 1min at 6 m/s Fast Prep 24 (MPBio) • Centrifugation 1min at 10000G	Manual	Silica column	Up to 400 µL of post-pretreatment supernatant	100 µL	3 h

Table 2
Characteristics of the DNA amplification methods.

PCR techniques	Genes targeted by PCR reactions	Amplification programs	Cryptosporidium's detection canal	Internal Control detection	Parasites detected
In-house RT PCR	18S rRNA	– 10min at 95 °C – 10s at 95 °C 45 cycles – 15s at 60 °C *, ** – 20s at 72 °C * *Fluorescence reading* ** *decrease of 0.5°C per cycle up to 50°C*	530 nm (LC 2.0)	None	*Cryptosporidium* sp.
RIDA® Gene	ITS1-18S	- 1min at 95 °C – 15s at 95 °C 45 cycles – 30s at 60 °C * * *Fluorescence reading*	Cy5 (CFX-96)	VIC (CFX-96)	*Cryptosporidium* sp. *Entamoeba histolytica* *Giardia intestinalis*
FTD® Stool Parasite	DNA J-like protein gene	– 15min at 50 °C - 1min at 94 °C - 8s at 94 °C 40 cycles - 1min at 60 °C * * *Fluorescence reading*	610 nm (CFX-96)	520 nm (CFX-96)	*Cryptosporidium* sp. *Entamoeba histolytica* *Giardia intestinalis*
Certest® Viasure	18S rRNA	- 2min at 95 °C – 10s at 95 °C 45 cycles – 50s at 60 °C * * *Fluorescence reading*	Cy5 (CFX-96)	HEX (CFX-96)	*Cryptosporidium* sp. *Entamoeba histolytica* *Giardia intestinalis*
Amplidiag®	COWP	– 10min at 95 °C – 15s at 95 °C 45 cycles - 1min at 63 °C * * *Fluorescence reading*	FAM (CFX-96)	Quasar 670 (CFX-96)	*Cryptosporidium* sp. *Entamoeba histolytica* *Giardia intestinalis* *Dientamoeba fragilis*
AllPlex® GI Parasite	Not disclosed	– 20min at 50 °C – 15min at 95 °C – 10s at 95 °C 45 cycles - 1min at 60 °C * – 30s at 72 °C * * *Fluorescence reading*	Quasar 670 (CFX-96)	HEX (CFX-96)	*Cryptosporidium* sp. *Entamoeba histolytica* *Giardia intestinalis* *Dientamoeba fragilis* *Blastocystis hominis* *Cyclospora cayetanensis*

mechanical grinding as pretreatment, display the best performances for the extraction of *C. parvum* DNA.[11] Then, we conducted a complementary study, which evaluated the impact of physicochemical parameters of the grinding beads and showed that best performances were obtained by using a lysis matrix comprising ceramic beads of median size.[12] These previous studies were conducted using the same PCR method, *i.e.* our routinely used real-time PCR method.[14] Parallelly, aiming at evaluating the DNA amplification step, we previously compared eight PCR methods to detect both *C. parvum* and *C. hominis*, whose starting point was DNA extracted by a single technique.[13]

Thus, several studies have investigated the impact of each step of the detection process independently, but, to our knowledge, no study has yet been conducted on different pretreatment/extraction/amplification combinations.[10–13,15–18] Such evaluations are important since inappropriate combination may lead to inefficient detection. In this context, the study aimed to evaluate 30 combinations of protocols (*i.e.* 3 pretreatment, 4 extraction and 6 amplification protocols) for the molecular detection of *C. parvum* in stool samples and provide valuable data for medical laboratories. Overall, we aim to provide medical laboratories with an informed choice when selecting molecular methods for the detection of *C. parvum* from stool samples.

2. Methods

2.1. Design of the study

This study was conducted in December 2021 at the NRC-CMAP at the University Hospital of Dijon. Thirty combinations based on 3 pretreatment, 5 extraction, and 6 amplification methods for the detection of *C. parvum* DNA in stool samples were evaluated. Stool suspensions with concentrations ranging from 0 to 500 oocysts/mL were used, and performances were compared in terms of proportion of positive PCRs and Cycle threshold (CT) values. The flow chart is detailed in Fig. 1.

2.2. Stool samples

A diarrheal stool sample from a young calf, containing 10^5 *C. parvum* oocysts/mL, was used to prepare stool suspensions containing increasing concentrations of *C. parvum* oocysts. Human feces negative for (i) common digestive parasites by microscopy and (ii) *Cryptosporidium* spp. by PCR, were diluted in 0.9 % NaCl and used as a matrix to dilute the initial calf stool sample.[14] *Cryptosporidium*-positive stool suspensions were obtained in sufficient volumes to ensure the whole study and stored at +4 °C. Dilution factors between samples were validated by analysis of the CT values obtained with our in-house Real-Time PCR.[14]

2.3. Pretreatment and DNA extraction

Two automated DNA extraction methods were evaluated: the **Nuclisens EasyMAG®** (BioMérieux, France) with pretreatment step (**EM**); the **Microlab Nimbus IVD** (Hamilton Company, USA) with (**MNP**) or without (**MN**) pretreatment step. Parallelly, two manual DNA extraction methods (including a pretreatment) were tested: the **QIAamp PowerFecal pro DNA kit®** (Qiagen, Germany) (**QF**); the **Quick-DNA Fecal/Soil Microbe Miniprep kit®** (Zymo Research, USA) (**ZR**). When pretreated, all stool suspensions were mechanically pretreated using beads. Characteristics of pretreatment and extraction methods are summarized in Table 1. DNA extracts were stored at +4 °C until PCR amplification *i.e.* for a maximum of 12 days. The quality of the DNA extracts at day 12 was compared to day 0 by examining CT values with

Table 3
DNA extraction and amplification replicates performed during the study.

Oocysts concentration range (oocysts/mL)	Number of extractions done per method	Number of PCR reactions	
		Per extraction	Total
0	1	2	2
10	3	3	9
50	3	3	9
100	2	2	4
500	1	2	2

in-house RT-PCR.[14]

2.4. DNA amplification

Six PCR techniques were compared: our routinely used Real-Time PCR (**in-house RT PCR**) (14); the **Certest® VIASURE** *Cryptosporidium*, *Giardia* & *E. histolytica* Real Time PCR Detection Kit (Certest Biotec, Spain); the **RIDA® GENE** Parasitic Stool Panel II (R-Biopharm, Germany); the **Allplex®** GI-parasite Assay (Seegene, Korea); the **FTD® Stool parasites** (Fast Track Diagnostics, Luxembourg); the **Amplidiag®** Stool Parasites (Mobidiag, France). All PCR runs included two negative controls and one positive control. The in-house RT PCR was performed on a LightCycler 2.0 (Roche Molecular Systems, Switzerland). All the other PCRs were done on a CFX96 (Bio-Rad, France), managed with CFX Manager IVD 1.6 software (Bio-Rad, France), and analyzed with Seegene Viewer® V3 software (Seegene, Korea). All commercial PCRs were realized according to the manufacturer's recommendations, and the in-house RT PCR was carried out as previously described (Table 2).[14]

2.5. Statistical analysis

The number of DNA extractions and amplifications varied with oocysts concentrations, being higher at the lowest concentrations to fit Poisson's law (Table 3).[11] Overall, 785 PCRs were carried out over 5 days. Statistical analyses were performed using the BioStaTGV software. The proportion of positive PCRs were compared at each concentration between the different combinations using the Fisher's exact test. Note that a PCR was considered positive if the CT value was less than or equal to the number of PCR amplification cycles (Table 2). The CT values were compared at each concentration using the Mann-Whitney test. A probability of 0.05 or less was significant.

3. Results

Performances of protocol combinations in term of percentage of positive PCRs.

Percentage of positive PCRs are presented in Table 4 (Statistical data not shown). Overall, **FTD® Stool Parasite** is the only amplification technique that provided 100 % detection for all protocol combinations and oocysts concentrations tested. All other amplification methods displayed acceptable performances with mean positivity rates ranging from 68.6 % to 85.1 %, depending on the combination tested. All negative controls (*i.e.* PCR grade water and 0 oocysts/mL suspensions) were found negative.

Focusing on the DNA extraction step, QIAamp PowerFecal pro DNA kit® (**QF**) and Quick-DNA Fecal/Soil Microbe Miniprep kit® (Zymo Research, USA) (**ZR**) manual methods proved to be suitable for all DNA amplification methods tested, providing mean positive rates reaching 96.2 % and 99.06 % respectively. With RIDA® GENE Parasitic Stool Panel II kit, Nuclisens EasyMAG® (BioMérieux, France) automated extraction method with pretreatment step **(EM)** gave significantly lower detection percentages than manual extraction techniques at 10 oocysts/mL ($p < 0.01$). With other PCR kits, there was no statistical difference between **EM** and manual extraction techniques in terms of percentage of positive PCRs, showing **EM**'s excellent results. Lastly, Microlab Nimbus IVD automated extraction method with (**MNP**) or without (**MN**)

Table 4
Percentage of positive PCRs obtained with the 30 combinations tested.

DNA extraction methods	Oocysts concentration range (oocysts / mL)	DNA amplification methods						Mean percentage of positive PCRs obtained for all DNA amplification methods
		In-house Real Time PCR	Certest® Viasure	Amplidiag® stool parasites	RIDA® Gene parasitic stool panel II	Allplex® GI parasite Assay	FTD® stool parasite	
Nimbus IVD® (MN)	***10***	44,4	66,7	11,1	33,3	44,4	100	**73.2**
	50	83,3	100	66,6	83,3	0	100	
	100	100	100	75	100	50	100	
	500	100	100	100	100	0	100	
Nimbus IVD® (MNP)	***10***	0	0	0	0	0	100	**40.9**
	50	0	11,1	0	22,2	0	100	
	100	0	25	25	50	0	100	
	500	100	100	50	100	100	100	
Nuclisens® EasyMAG (EM)	***10***	88,9	100	33,3	11,1	100	100	**92.5**
	50	100	100	88,8	100	100	100	
	100	100	100	100	100	100	100	
	500	100	100	100	100	100	100	
Quick DNA Fecal/Soil Microbe Miniprep® (ZR)	***10***	77	100	44,4	88,8	100	100	**96.2**
	50	100	100	100	100	100	100	
	100	100	100	100	100	100	100	
	500	100	100	100	100	100	100	
QIAamp PowerFecal Pro DNA Kit® (QF)	***10***	100	100	88,8	100	100	100	**99.06**
	50	100	100	88,8	100	100	100	
	100	100	100	100	100	100	100	
	500	100	100	100	100	100	100	
Mean percentage of positive PCRs obtained for all DNA extraction techniques		**79.6**	**85.1**	**68.6**	**79.4**	**69.7**	**100**	

Percentage of positive PCRs obtained with combinations tested (%)

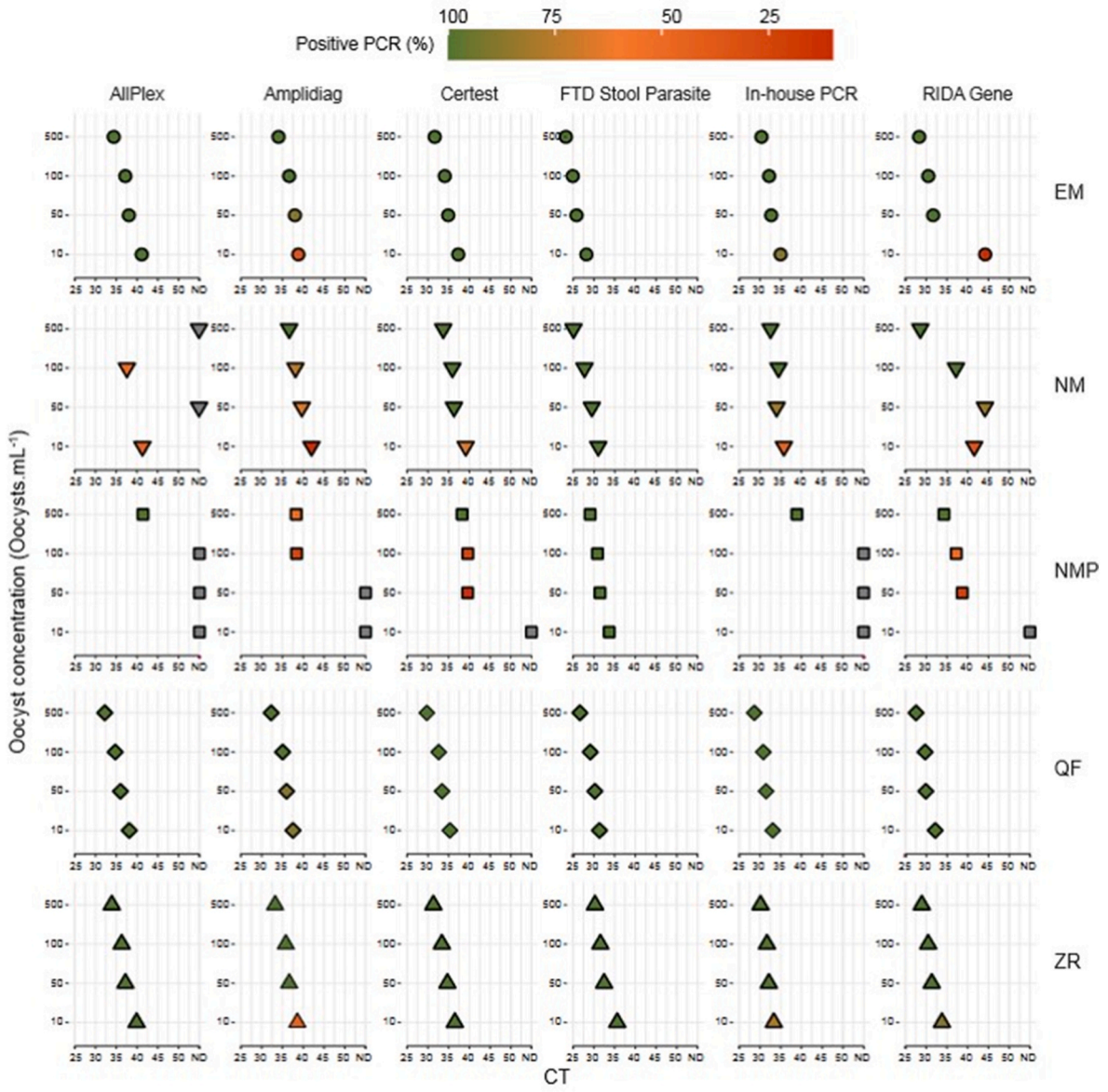

Fig. 2. CT values obtained for the 30 Pretreatment/extraction/PCR combinations. **(a)** 10 oocysts/mL; **(b)** 50 oocysts/mL; **(c)** 100 oocysts/mL. Light grey boxplots correspond to partial detection of replicates (see Table 4).

pretreatment step showed lower performances than other extraction techniques. Indeed, all the PCR techniques (except **FTD® Stool Parasite**) showed their lowest overall positive rates when using **MN** or **MNP**. This is particularly relevant for the **MNP** protocol, which performance is statistically lower, especially at 10 and 50 oocysts/ml. Thus, the addition of a mechanical pretreatment step to the recommended **MN** protocol worsened the performances. Surprisingly, when combined with **MN** protocol, **Allplex**® GI-parasite Assay did detect *C. parvum* DNA at the 10 oocysts/mL concentration, but not at 50 and 500 oocysts/mL, suggesting the presence of PCR inhibitors. Furthermore, it is notable that the results obtained through the **Nimbus IVD®** extraction method demonstrate a considerable degree of variability across different amplification methodologies. For instance, the detection rate was only 63.1 % when combined with the Amplidiag® PCR assay, yet the overall positive rate reached 91.6 % when combined with the Certest® PCR assay.

3.1. Performances of protocol combinations regarding the average CT values

Performances of the 30 protocol combinations tested in this study varied particularly on the CT scale, with many significant differences observed (Fig. 2, Table 5) (statistical data not shown). For samples with a parasite load of 100 oocysts/mL or less, the protocols combined with FTD® Stool Parasite PCR resulted in significantly lower CT values than the combinations using any other DNA amplification method. Furthermore, the protocols comprising **FTD® Stool Parasite** PCR had better reproducibility and lower variability of CT values.

Focusing on the extraction methods, **QF** and **ZR** confirmed their excellent performances. For example, at a parasite load of 100 oocysts/mL, our **in-house RT-PCR** obtained significantly better CT values with both **QR** and **ZR** than with other extraction techniques. The automated extraction **EM** also gave good detection performances, and provided the best combination of the study, when combined with **FTD® Stool Parasite** at a parasite load of 100 oocysts/mL or less. As **MN** and **MNP** extraction protocols mostly provided detection percentages lower than

Table 5
Performances of 30 pretreatment/extraction/PCR combinations regarding the average CT values and standard deviations (SD) at each oocysts' concentration.

DNA extraction methods	Oocysts concentration range (oocysts / mL)	DNA amplification methods: In-house Real Time PCR	Certest® Viasure	Amplidiag® stool parasites	RIDA® Gene parasitic stool panel II	Allplex® GI parasite assay	FTD® stool parasite
Nimbus IVD® (MN)	***10***	35.76 +/- 0.27	39.12 +/- 1.58	41.98	41.59 +/- 0.86	41.32 +/- 0.79	31.00 +/- 1.32
	50	33.99 +/- 0.45	36.30 +/- 0.59	39.65 +/- 1.70	44.17 +/- 0.77	ND	29.44 +/- 0.16
	100	34.38 +/- 0.44	35.92 +/- 0.37	38.12 +/- 0.53	37.20 +/- 6.73	37.58 +/- 0.06	27.66 +/- 0.07
	500	32.53 +/- 0.04	33.71 +/- 0.16	36.61 +/- 1.38	28.59 +/- 0.02	ND	25.01 +/- 0.03
Nimbus IVD® with pretreatment (MNP)	***10***	ND	ND	ND	ND	ND	33.69 +/- 0.47
	50	ND	39.58	ND	38.69 +/- 3.18	ND	31.46 +/- 0.25
	100	ND	39.63	38.41	37.25 +/- 4.32	ND	30.89 +/- 0.17
	500	38.92 +/- 0.55	38.27 +/- 0.29	38.31	34.28 +/- 0.95	41.41 +/- 0.09	29.11 +/- 0.04
Nuclisens® EasyMAG (EM)	***10***	34.96 +/- 1.41	37.32 +/- 1.01	38.83 +/- 0.26	44.23	41.12 +/- 1.01	**28.19 +/- 0.56**
	50	32.70 +/- 0.27	34.90 +/- 0.59	37.99 +/- 1.20	31.68 +/- 0.57	38.02 +/- 0.57	**25.80 +/- 0.40**
	100	32.20 +/- 0.06	34.07 +/- 0.43	36.62 +/- 0.74	30.51 +/- 0.39	37.17 +/- 0.45	**24.90 +/- 0.43**
	500	30.36 +/- 0.22	31.65 +/- 0.60	34.05 +/- 0.13	28.32 +/- 0	34.38 +/- 0.14	**23.23 +/- 0.0**
Quick DNA Fecal/Soil Microbe Miniprep® (ZR)	***10***	33.33 +/- 0.40	36.50 +/- 0.50	38.58 +/- 0.53	33.80 +/- 1.25	39.88 +/- 1.30	35.63 +/- 0.75
	50	32.13 +/- 0.31	34.71 +/- 0.50	36.61 +/- 1.03	31.35 +/- 0.70	37.19 +/- 0.35	32.46 +/- 0.21
	100	31.68 +/- 0.12	33.37 +/- 0.92	35.80 +/- 0.62	30.48 +/- 0.45	36.30 +/- 0.55	31.56 +/- 0.32
	500	30.12 +/- 0.19	31.32 +/- 0.26	33.23 +/- 0.13	28.95 +/- 0.16	33.91 +/- 0.15	30.27 +/- 0.0
QIAamp PowerFecal Pro DNA Kit® (QF)	***10***	33.08 +/- 0.38	35.31 +/- 0.60	37.54 +/- 0.80	32.12 +/- 0.59	38.12 +/- 0.60	31.35 +/- 0.14
	50	31.44 +/- 0.29	33.43 +/- 0.41	36.00 +/- 0.36	29.86 +/- 0.39	36.00 +/- 0.22	30.24 +/- 0.16
	100	30.8 +/- 0.26	32.62 +/- 0.30	35.04 +/- 0.47	29.68 +/- 0.16	34.73 +/- 0.25	29.12 +/- 0.15
	500	28.63 +/- 0.24	29.76 +/- 0.73	32.28 +/- 0.01	27.5 +/- 0.24	32.22 +/- 0.02	26.63 +/- 0.0

Average CT values +/- SD

ND = no detection//red boxes = only one value due to low detection performances, whereas all replicates were performed as described//pink boxes = more than one value, but less than 100 % of detection//green boxes and bold = best extraction/PCR combination.

Table 6
Detection and removal of PCR inhibitors obtained with the AllPlex® PCR when combined with Microlab Nimbus IVD® extraction.

Oocysts concentration range (oocysts/mL)	Results without any dilution		Results after a 1/5th dilution on one replicate
	CT values for *C. parvum* ± SD	*Percentage of positive PCRs obtained (%)*	CT values for *C. parvum*
10	41.32 ± 0.79	*44,4*	Negative (but detection of the internal control)
50	ND	*0*	42.3
100	37.58 ± 0.06	*50*	40.03
500	ND	*0*	37.6

ND = no detection.

100 %, statistical analysis of CT values was limited. However, high CT values observed tend to confirm that a mechanical pretreatment worsened the performances of the **MN** protocol, and this with all the PCR techniques tested.

3.2. Investigation for the presence of PCR inhibitors

Surprising results were obtained when combining **Microlab Nimbus® IVD (MN)** with the **Allplex®** amplification method. Interestingly, internal control was not amplified, suggesting the presence of PCR inhibitors. DNA extracts were diluted 1:5 in PCR-grade water, and submitted again to **Allplex®** amplification. This resulted in amplification of internal controls at all oocysts concentrations, confirming our hypothesis (Table 6).

4. Discussion

Within the NRC-CMAP framework, this study aims to provide data to facilitate medical laboratories in the selection of appropriate molecular combinations for the detection of *C. parvum* in stool samples. Therefore, we evaluated and compared 30 combinations of molecular protocols. The results of our study indicate that the efficacy of a PCR method may be dependent on the choice of extraction method. Specifically, the percentage of positive PCRs varied significantly across different combinations (Fig. 3), suggesting that a PCR method may be considered ineffective when used with an inappropriate extraction method, but ultimately effective when combined with an appropriate extraction method. It is noteworthy that the recommendations provided by manufacturers concerning the use of DNA extraction kits in conjunction with their respective amplification methods are not always effective. For instance, some of the manufacturer-recommended combinations demonstrated satisfactory performance, such as **Nuclisens® Easymag®** and **FTD® Stool Parasite** combination, which exhibited the optimal performance in this study. However, other manufacturer-recommended combinations exhibited limited performance, as observed in the case of the **Amplidiag®** and **Nuclisens® Easymag®** combination (Table 7).

With regard to the extraction step, the manual extraction techniques were shown to be highly efficacious; however, their time-consuming nature may present a limiting factor in their implementation in medical laboratories. It can be reasonably deduced that automated DNA extraction techniques, such as the **Nuclisens® Easymag®** method, represent a satisfactory alternative, given that they are fastest and yield satisfactory results to manual extraction when combined with good DNA amplification methods. Concurrently, despite the capacity of some commercial multiplex PCR assays to detect multiple parasites simultaneously, our findings indicate that these assays may be more susceptible to PCR inhibitors. It is therefore recommended that DNA extracts be diluted in order to avoid any potential inhibition of the PCR process.

Interestingly, our **in-house Real-Time** PCR showed good overall performances, when allowing detection and identification of *Cryptosporidium* species, including rare species, which comforted us to use it as a reference method for comparative studies.[13,14] Indeed, when the herein study focused on *C. parvum*, the main species found in humans, about twenty species can cause human cryptosporidiosis.[1] In France between 2017 and 2019, *C. hominis* represented 24 % of cases. Other species described were rarer but not negligible (*C. felis* (2 %), *C. cuniculus* (>1 %), *C. meleagridis* (<1 %), *C. canis* (<1 %), *C. ubiquitum*

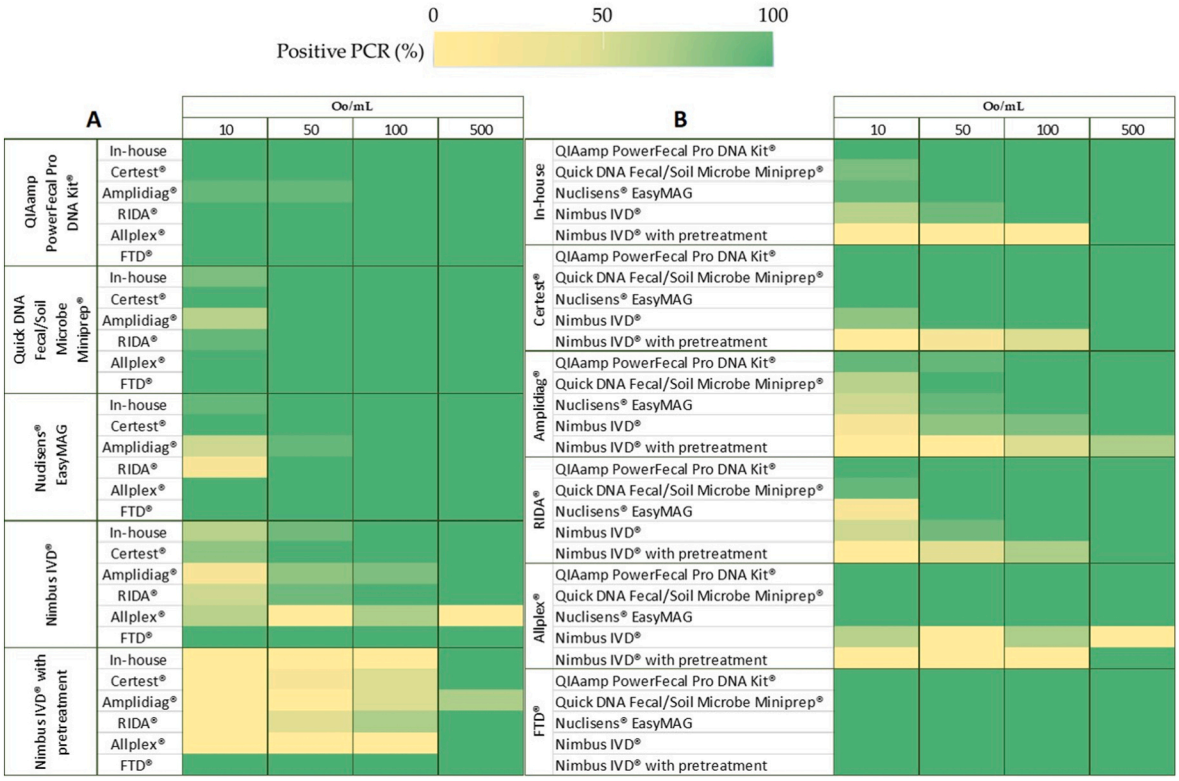

Fig. 3. Heat map representing: (**A**) for a same PCR, the comparison of extraction techniques performances in terms of proportion of positive PCRs and (**B**) for a same extraction technique the comparison of amplification techniques performances in terms of proportion of positive PCRs.

Table 7
Recommended extractions for the five commercial PCR kits evaluated in our study.

PCR techniques	Extractions recommended by manufacturer
In-house RT PCR	Nuclisens® Easymag®(Biomérieux)
RIDA Gene	- RIDA Xtract (R-Biopharm) (Biomérieux) - Maxwell RSC (Promega)
FTD Stool Parasite	Nuclisens® Easymag® (Biomérieux)
Certest Viasure	- RIDA Xtract (R-Biopharm) - QIAamp DNA mini-kit (Qiagen) - QIAamp DNA stool kit (Qiagen) - Maxwell RSC (Promega)
Amplidiag	Nuclisens® Easymag® (Biomérieux)
AllPlex GI Parasite	- Microlab Starlet (Hamilton) - Microlab Nimbus IVD (Hamilton)

(<1 %) and *C. erinacei* (<1 %).[5] Their frequency has increased since then (date unpublished). It is therefore important to be able to detect these species. Interestingly, among the 5 commercial PCRs tested, Launch Diagnostic **(FTD® Stool Parasite)** provide the most complete information about the *Cryptosporidium* species detected by their kit (*i.e.* 20 species). Although Seegene (**AllPlex®**) mentions only 3 species, Autier et *al.* showed that **Allplex®** allows the detection of at least 6 *Cryptosporidium* species.[16] The disclosure of this kind of information is essential in the choice of digestive protozoa amplification kits.

In previous works conducted by our team, we already reported the importance of the pretreatment step for efficient *Cryptosporidium* DNA extraction from stool samples.[11,12] A comparison of our results with those of Valeix et al., who investigated three extraction techniques in combination with our in-house PCR technique (Table 8), revealed that the **QIAamp PowerFecal Pro DNA Kit®** yielded lower CT values in the present study.[11] This difference may be attributed to optimizations made by the Qiagen manufacturer between the two studies: *"The QIAamp PowerFecal Pro DNA Kit builds and improves on our original PowerFecal technology utilizing a novel bead tube and optimized chemistry for more efficient lysis"*.[19] Moreover, our results are consistent with our previously published article.[13] Indeed, we previously (i) showed that, among 8 PCR techniques evaluated, **FTD® Stool Parasite** was the best in term of detection limit for both *C. parvum* and *C. hominis*, and (ii) suggested that the gene targeted could explain these performances.[13] The influence of PCR techniques and extraction methods on the detection of *Cryptosporidium* spp. has also been assessed by other research teams. However, comparison is difficult due to the use of different extraction/PCR techniques than those tested here. For example, Argy et *al.* performed the comparison of multiplex PCRs (including the **RIDA® GENE Parasitic Stool Panel II** and the **Allplex® GI-parasite Assay**) for the detection of *Cryptosporidium* DNA in stool samples but used the **QIASymphony®** (Qiagen) extraction method that was not evaluated in the herein study.[20]

All in all, to the best of our knowledge, our study is the first to evaluate the performance of commercial multiplex PCR kits for the detection of *C. parvum*, considering the entire analytical process, including pre-treatment, extraction and amplification. This work has confirmed that each stage of the analytical process is a crucial step in the overall performance of the detection of *C. parvum* from stool samples. Indeed, variable performance was observed depending on the combinations tested, thereby confirming the importance of the choice of protocols for the detection of *C. parvum*. The discrepancies in performance between the amplification techniques can be attributed to the differences in the targets, particularly the representation of these targets in the parasite genome. The principal disadvantage of manual extractions, despite their optimal detection performance, is that they are time-consuming and unsuitable for private laboratories that are required to process large volumes of samples for analysis. It is important to note that that the extraction/PCR combinations recommended by the manufacturers do not consistently yield the optimal detection performance, further underscoring the lack of available information on this topic. In conclusion, this work represents a preliminary source of data that can

Table 8
Comparison between our results and those obtained by Valeix et *al.* for three extraction techniques combined with our in-house Real-Time PCR.

DNA extractions methods	*Oocysts concentration range (oocysts/mL)*	Valeix et *al.*		Bailly et *al.*	
		Proportion of positive PCRs	CT values	Proportion of positive PCRs	CT values
Nuclisens® EasyMAG (EM)	**10**	66.7 %	X	88.9 %	X
	50	100 %	X	100 %	X
	100	100 %	33.60 ± 0.48	100 %	32.20 ± 0.06
	500	100 %	31.53 ± 0.32	100 %	30.36 ± 0.22
Quick DNA Fecal/Soil Microbe Miniprep® (ZR)	**10**	94.4 %	X	77.0 %	X
	50	100 %	X	100 %	X
	100	100 %	31.91 ± 0.18	100 %	31.68 ± 0.12
	500	100 %	29.86 ± 0.16	100 %	30.12 ± 0.19
QIAamp PowerFecal (Pro) DNA Kit® (QF)	**10**	83.3 %	X	100 %	X
	50	88.9 %	X	100 %	X
	100	100 %	33.54 ± 0.39	100 %	30.80 ± 0.26
	500	100 %	31.48 ± 0.24	100 %	28.63 ± 0.24

assist laboratories in designing their analytical process to achieve optimal performance for the detection of *C. parvum* from stool samples, while adapting it to the challenges encountered in the field (*i.e.*, private laboratories versus hospitals). In order to enhance the efficacy of the detection of digestive parasites in stool samples in private and/or hospital laboratories, future research will be required within the framework of the NRC-CMAP. Therefore, further research is necessary to assess the efficacy of existing and/or novel protocols for the detection of other *Cryptosporidium* species and other digestive protozoa. This will facilitate the enhancement of diagnostic capabilities for digestive protozooses in general within private and/or hospital laboratories.

CRediT authorship contribution statement

Eloïse Bailly: Data curation, Formal analysis, Writing – original draft. **Chloé Baranton:** Data curation. **Stéphane Valot:** Methodology, Writing – review & editing. **Anne Vincent:** Data curation. **Hervé Begue:** Writing – review & editing. **Corentin Beclere:** Data curation. **Alain Bonnin:** Writing – review & editing. **Damien Costa:** Resources. **Philippe Poirier:** Resources. **Louise Basmaciyan:** Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Frédéric Dalle:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

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Conflicts of interest statement

The authors declare no conflict of interest.

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