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Blinded, Externally Controlled Multicenter Evaluation of Light Microscopy and PCR for Detection of Microsporidia in Stool Specimens

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The quality parameters for the detection of microsporidia in identical sets of 50 stool samples were determined for six laboratories where technicians used light microscopy and for six laboratories where technicians used PCR. The average overall sensitivities were 67% (89% for patient samples only) for the PCR laboratories and 54% (80% for patient samples only) for the light microscopy laboratories. Specificities were 98 and 95%, respectively. Differences in results were most apparent between the individual laboratories rather than between the two major methods used.

In-house evaluations of PCR protocols, especially by those who developed them, are generally satisfactory to excellent. In contrast, among the few blinded, externally controlled evaluations of PCR protocols outside the field of virology, there have been rather contrasting results. Impressive examples are the studies by Noordhoek et al. of the detection of *Mycobacterium tuberculosis* (17, 18). For parasites, too, there often exist several PCR protocols for each of the more frequently and many of the less frequently occurring human-pathogenic species, including microsporidia (2–6, 8–10, 13–16, 20, 21, 23, 24). However, none of these protocols has been validated in a blinded, externally controlled fashion. The only exception is a study by five laboratories of solutions of *Toxoplasma gondii* DNA (12). The various results from the different laboratories were attributed to the possible incompatibility of using DNA solutions instead of whole cells with some of the DNA preparation methods. It was concluded that these artificial samples were not appropriate for determining the PCR tests' sensitivities and specificities for clinical specimens.

Hence, while one may recognize the potential of the PCR technique, the probability of a positive result being correctly positive and a negative result being from a parasite-free specimen is quite uncertain. In an effort to assess the performance of this method for the detection of a parasite, we have conducted a blinded, externally controlled, multicenter study for the detection of microsporidia by PCR and compared the results to those obtained by light microscopy. We have chosen microsporidia (*Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, and *Encephalitozoon cuniculi*), intracellular, spore-forming parasites responsible for diarrhea and other manifestations in immunocompromised patients, because the small size of the spores makes an alternative to their detection by light microscopy especially advantageous.

Study design. All technicians from 12 participating laboratories were informed of and agreed to the following conditions. Aliquots of 50 stool samples, approximately 1 g each, had to be analyzed by each laboratory within 3 months. Only stool samples were tested. Approximately 10 to 30 of the samples were to be negative controls, most of them originating from healthy, immunocompetent persons, and the other samples had to be stool samples with confirmed parasites other than microsporidia. Some positive samples were to be from healthy persons and spiked with cultured spores of *E. intestinalis*, *E. hellem*, or *E. cuniculi* at different concentrations. At least one-third of all samples had to be duplicates, and all samples were assigned a random number. Specimens were tested by light microscopy at six laboratories (laboratories M1 to M6), and specimens were tested by PCR at the other six laboratories (laboratories P1 to P6). The results were to be made public independently of the outcome, with the participating laboratories remaining anonymous.

Stool samples containing spores identified as *Enterocytozoon* spp. by light or electron microscopy or both were from seven immunocompromised patients diagnosed with microsporidiosis. The species was confirmed to be *Enterocytozoon bieneusi* by sequencing of the internal transcribed spacer of the rRNA gene (19). The samples were stored at −20°C until they were

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TABLE 1. Techniques used by light microscopy laboratories[a]

Laboratory	Stool sample fixative	MT staining temp (°C)	MT staining time (min)	Avg no. of slides/ sample	Avg microscopy time before negative result declared (min)	Control used
M1	Formalin	37	30	1	10	Reference slides
M2	Formalin	RT	90	2	10	Reference slides
M3	Formalin	RT	90	2	10	Not specified
M4	Formalin	RT	90	5	20	Reference slides
M5	MIF	RT	90	1	15	Reference slides
M6	None	RT	90	2	10	Reference slides

[a] MT, modified trichrome (22); RT, room temperature; MIF, merthiolate-iodine-formalin fixation.

divided into aliquots. Spores from *Encephalitozoon* species (*E. cuniculi* IPZ:CH-H14, *E. hellem* IPZ:CH-H3, and *E. intestinalis* IPZ:D-H11) were isolated, cultivated on human embryonic lung fibroblast (MRC-5) cells, characterized both phenotypically and genotypically as described by Deplazes et al. (7), and stored at −20°C until used for spiking. Negative stool samples, including those to be spiked, were from immunocompetent, healthy persons without travel histories for the prior 3 months. Four stool samples were from immunocompetent outpatients suffering from diarrhea who had confirmed infections with (i) *Blastocystis hominis*, *Endolimax nana*, and *Entamoeba hartmanni*; (ii) *Giardia lamblia* only; (iii) *Heterophyes heterophyes* only; or (iv) *Entamoeba histolytica* and *B. hominis*. Aliquots were prepared, coded, and distributed by and the results were received at the Robert Koch Institute in Berlin, Germany, which did not participate in the sample analyses. On the day of the sample preparation, the negative controls were prepared first, the patient samples were next, and the spiked samples were last. For the spiked samples, the aliquots were spiked individually. Utmost care was taken to avoid cross-contamination during sample preparation. All samples were shipped without fixatives and at ambient temperature by mail or overseas courier service.

Laboratories were asked to decide on the presence or absence of microsporidia in every sample only by the method assigned to them (either light microscopy or PCR) and to use their own protocols for analysis. If possible, they should specify the genus and the species of the microsporidia present. The methods used are summarized in Table 1 for laboratories M1 to M6 and in Table 2 for laboratories P1 to P6.

Sensitivity. For the six PCR laboratories, the average sensitivity with all samples was 67% (89% for the patient samples only and 44% for the spiked samples only), with a range of 36 to 96%. For the six light microscopy laboratories, the average sensitivity with all samples was 54% (80% for the patient samples only and 27% for the spiked samples only), with a range of 25 to 71%. In this study, differences in sensitivity were more dependent on the individual laboratory than on the analysis method (Table 3). All things considered, technicians at the PCR laboratories achieved, on average, a higher sensitivity (67%) than those at the light microscopy laboratories (54%), scoring 9 percentage points better with the patient samples and 17 percentage points better with the spiked samples. But these averages obscure the wide variations in results between the individual laboratories and must not be taken as the sole argument for favoring one method over the other. For example,

TABLE 2. Techniques used by PCR laboratories

Laboratory	Amt of stool/ analysis (g)	DNA isolation methods[a]	Time needed for DNA isolation (h)	Target rDNA[b]	No. of repetitions of PCR runs/ sample	Method[c] of: PCR type (no. of tubes)	Positive control	Negative control	Check for PCR inhibitors[d]	Method[e] of: Verification of PCR products	Species determination	No. of separate PCRs needed for species determination	Estimated time needed from sampling to result (days)	Time spent by technician to produce result (h)
P1	1	P, C, GBD, SMA	3	SSU	1	Single	PCR	None	None	SB, RFLP	GSP-SSP	4	7	10
P2	0.1	AL, SGA	1	ITS	0	Nested (2)	PCR	Isol. + PCR	None	RFLP	RFLP	1	2	3
P3	0.1	GT, SGA	1.5	SSU	0	Nested (2)	PCR	PCR	None		NP-SSP	4	2	3.5
P4	0.1	P, GBD	1.5	SSU	2	Single	PCR	PCR	Dil. + Msp DNA		SSP	4	3	5
P5	0.2	GT, SGA	2	SSU	0	Single	PCR	PCR	None	RFLP		NA[f,g]	2	4
P6	0.1	P, SMA	16	SSU	0	Single	PCR	Isol. + PCR	Dil. + Bac DNA		NP-SSP, RFLP	NA[h]	2	2

[a] AL, alkaline lysis (13); C, chitinase digestion; GBD, glass bead disruption; GT, guanidinium thiocyanate lysis (1); P, proteinase K digestion; SGA, silica gel adsorption (GeneClean kit; Bio 101, La Jolla, Calif.); SMA, silica membrane adsorption (QIAamp tissue kit; Qiagen, Santa Clarita, Calif.).

[b] rDNA, rRNA gene; ITS, internal transcribed spacer; SSU, small subunit.

[c] Isol., DNA isolation.

[d] Bac DNA, addition of bacterial DNA; Dil., DNA dilution; Msp DNA, addition of microsporidial DNA.

[e] GSP-SSP, genus-specific PCRs followed (if sample was positive for *Encephalitozoon*) by species-specific PCRs of original DNA; NP-SSP, nested PCR with species-specific primers; SB, Southern blotting.

[f] NA, not applicable.

[g] Species determination was impossible due to persistent failure of an intended species-determing nested PCR.

[h] Exclusive use of *Enterocytozoon bieneusi*-specific primers.

TABLE 3. Quality parameters of microsporidian detection (without species differentiation)

Laboratory	No. of true positives			No. of true negatives (n = 22)	No. of false positives	No. of false negatives			% Sensitivity			% Specificity
	All samples (n = 28)	Patient samples[a] only (n = 14)	Spiked samples[b] only (n = 14)			All samples	Patient samples only	Spiked samples only	All samples	Patient samples only	Spiked samples only	
P1	27	13	14	21	1	1	1	0	96	93	100	95
P2	23	14	9	22	0	5	0	5	82	100	64	100
P3	19	11	8	21	1	9	3	6	68	79	57	95
P4	17	14	3	22	0	11	0	11	61	100	21	100
P5	16	13	3	22	0	12	1	11	57	93	21	100
P6	10	10	0	21	1	18	4	14	36	71	0	95
M1	20	14	6	21	1	8	0	8	71	100	43	95
M2	18	14	4	22	0	10	0	10	64	100	29	100
M3	18	14	4	22	0	10	0	10	64	100	29	100
M4	14	10	4	22	0	14	4	10	50	71	29	100
M5	13	13	0	17	5	15	1	14	46	93	0	77
M6	7	2	5	21	1	21	12	9	25	14	36	95

[a] *Enterocytozoon bieneusi.*
[b] *Encephalitozoon* spp.

100% sensitivity with the patient samples was attained at five laboratories, three of which were light microscopy laboratories. With samples spiked at the highest concentration, 10^6 spores/g, technicians from all but one microscopy laboratory detected four or more of the six samples, results which could be matched at only three of the PCR laboratories (Table 4). Light microscopy therefore appears to be the more robust method for high concentrations of spores, while PCR is more sensitive for low concentrations of spores.

The average sensitivity was higher at laboratories where nested PCRs were performed (75%) than at those where single PCRs were performed (63%), but again, variation between individual laboratories was high and the best sensitivity was attained at a laboratory where only single PCRs were performed (P1). However, the most extensive sample preparation, including a spore concentration step starting with 1 g of stool, occurred at this laboratory. Except for this laboratory, a possible dependency on the method of DNA isolation was not obvious. For example, technicians at laboratory P2, which had the second-best overall score and a higher specificity than that attained at P1, used a very simple sample preparation requiring not more than 1 h to complete (Table 2). Similarly, there was no correlation between the number of repetitions of analyses per sample and the achieved sensitivities. Surprisingly, the same was true for checking for PCR inhibitors (Table 2), although this was just one of several differences between the PCR protocols, but probably not the most decisive one.

Specificity. Specificity was high at all laboratories, i.e., either 95 or 100%, except at one microscopy laboratory which scored 77% (Table 3). With the exception of this laboratory, no laboratory reported more than one false-positive result, regardless of the method used. This came as a surprise at least for the PCR technique, where cross-contaminations are an imminent danger. In contrast to results of earlier surveys (17, 18), PCR contaminations were infrequent in this study and occurred both in laboratories where single PCRs were performed (P1 and P6) and in one where nested PCRs were performed (P3). Still, more than one such result was never encountered in any laboratory.

Detection limit. A uniform detection limit between 10^4 and 10^6 spores per g of stool was apparent for light microscopy only (Table 4). While technicians at two PCR laboratories detected concentrations as low as 10^2 spores/g, most of the individual results of PCR were varied and increasing spore concentrations did not per se lead to higher sensitivities, except at one laboratory, P1, where all spiked samples were correctly identified but where one sample was also falsely determined to be positive. While samples containing 10^4 or fewer spores per g could be detected, with a single exception, by PCR only, four or more of the six samples spiked at high concentrations of 10^6 spores/g were detected by technicians at all but one light microscopy laboratory. Only three PCR laboratories could match this result. Therefore, detection limits were lower but incon-

TABLE 4. Numbers of correctly identified spiked samples in relation to spore concentration[a]

Laboratory	No. of correctly identified samples spiked with:			
	10^2 *E. hellem* spores/g (n = 2)[b]	10^3 *E. intestinalis* spores/g (n = 2)[c]	10^4 *E. hellem* or *E. cuniculi* spores/g (n = 2 each)[d]	10^6 *E. hellem*, *E. cuniculi*, or *E. intestinalis* spores/g (n = 2 each)[e]
M1	0	0	0	6
M2	0	0	0	4
M3	0	0	0	4
M4	0	0	0	4
M5	0	0	0	0
M6	0	0	1	4
P1	2	2	4	6
P2	1	1	3	4
P3	0	2	0	6
P4	0	0	1	2
P5	0	1	0	2

[a] Without species identification and without laboratory P6.
[b] The average percentages of correctly identified samples at this concentration were 0% at the light microscopy laboratories and 30% at the PCR laboratories.
[c] The average percentages of correctly identified samples at this concentration were 0% at the light microscopy laboratories and 60% at the PCR laboratories.
[d] The average percentages of correctly identified samples at this concentration were 4% at the light microscopy laboratories and 40% at the PCR laboratories.
[e] The average percentages of correctly identified samples at this concentration were 61% at the light microscopy laboratories and 67% at the PCR laboratories.

TABLE 5. Reliability of species differentiation at PCR laboratories

Laboratory[a]	Identification method(s)	No. of samples with species:		No. of false positives	% Accuracy of reported results	% of positive samples correctly identified (n = 28)
		Correctly identified	Incorrectly identified			
P1	SSP[b]	25	2	1	89	89
P2	RFLP	20	3	0	87	71
P3	SSP	19	0	1	95	68
P4	SSP	17	0	0	100	61
P6[c]	SSP, RFLP	10	0	1	91	36
Avg		18.2	1	0.6	92	65

[a] Laboratory P5 did not determine species.
[b] SSP, species-specific primers.
[c] *Enterocytozoon bieneusi*-specific primers were used exclusively.

sistent by PCR, and light microscopy proved to be the more robust method for detecting high concentrations of spores.

In view of these data, it can be speculated that a substantial number of microsporidial infections currently go undetected, as has been previously suspected for *E. intestinalis* infections (11), especially with, but not limited to, the moderate-to-low concentrations of spores which might be found in less severely immunocompromised patients. The high sensitivity obtained with the patient samples does not necessarily contradict this explanation, since these samples were screened by light microscopy and are therefore biased towards high concentrations of spores.

Genus and species differentiation. Genus determination, which was attempted by technicians at two of the six light microscopy laboratories and at five of the six PCR laboratories, was correctly done in all cases. This finding is therapeutically relevant, because only *Encephalitozoon* spp. are susceptible to benzimidazole treatment. Morphologically, the spores can be differentiated by size; *Enterocytozoon bieneusi* spores measure between 1 and 1.5 μm, and *Encephalitozoon* spp. measure between 2 and 3 μm. The species cannot be distinguished by spore morphology. Technicians at five of the six PCR laboratories differentiated the microsporidia to the species level by analyzing the PCR products and were successful in 87 to 100% of all attempts. Accuracy was highest (89 to 100%) when species-specific primers were used (Table 5). The use of restriction fragment length polymorphism (RFLP) at laboratory P2 was slightly less accurate (87%). However, the percentage of samples correctly identified to the species level by RFLP (71%) was almost identical to the average percentage (73%) from laboratories P1, P3, and P4, where species-specific primers for all four species were used (Table 5).

Practical conclusions. The apparent differences in the quality of results between laboratories rather than between the methods employed might be considered an argument for establishing centralized reference centers to guarantee a high level of quality in the detection of microsporidia in stool samples. Until that time, individual laboratories are encouraged to improve their respective techniques as much as possible but not to give up one method in favor of the other. It will certainly be helpful to have a second technique available when a confirmation of the result of the first is desired. While microscopy is known to be highly dependent on the expertise of the examiner, it is not certain if, on the other hand, the generation of a standardized PCR test kit will be a more promising way to guarantee reliable detections of microsporidia by a larger number of laboratories. From the only prior bacteriological study which addressed this question, an unexpected result was that the use of commercial PCR kits did not produce better results than in-house PCR protocols (18). If the reliable detection of microsporidia is not to be restricted to a limited number of reference centers, techniques other than light microscopy and PCR, e.g., coproantigen enzyme-linked immunosorbent assays, might have to be developed.

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