Improvement of routine diagnosis of intestinal parasites with multiple sampling and SAF-fixative in the Triple-Faeces-Test

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Abstract

Background and study aim : **To perform optimal laboratory diagnosis of intestinal parasites is demanding. Because intestinal parasites are intermittently shedded, examination of multiple stools is imperative. For reliable detection of vegetative stages of protozoa, fresh stools should be examined direct after production, or stools should be preserved in a fixative. These aspects in routine practice are often neglected with as a result lower sensitivity of the diagnostic procedure. With application of the Triple-Faeces-Test (TFT) protocol, where both multiple sampling and a SAF-fixative are included, these practical problems could be overcome.**

The aim of this study was to compare the recovery of intestinal parasites in faecal specimens using TFT protocol versus the conventional diagnostic method (ether-sedimentation of one fresh stool sample).

Methods : **During a three years period, results obtained in routine practice with the TFT protocol were compared with results from examination of sediment obtained with the ethyl-acetate-sedimentation technique of one unpreserved faeces specimen.**

Results : **From 2,776 patients, 28.1% were positive for one or more intestinal parasites after examination of the TFT test, compared to 10.3% positivity with the conventional method ($P < 0.05$). Pathogenic species and non pathogenic species were observed respectively 191 and 449 times with TFT and 105 and 152 times with conventional method ($P< 0.05$).**

Conclusions : **The application of the Triple-Faeces-Test in routine clinical practice significantly increased recovery of intestinal parasitic infections.** (Acta gastroenterol. belg., **2006**, 69, **361-366**).

Introduction

Infections with enteric parasites, although considered to be rare, are an important cause of gastrointestinal illnesses in European countries (1,2). In Belgium, for 2004, the national incidence of clinical infections with *Giardia lamblia* and *Cryptosporidium parvum,* has been reported by a network of geographically distributed sentinel laboratories, as 12.5 and 2.8 cases per 100,000 population respectively (3). Moreover, emerging coccidia like *Cyclospora cayetanensis* or *Isospora belli* are regularly reported. Other parasites such as *Dientamoeba fragilis* and *Blastocystis hominis* are increasingly recognised as potential enteric human pathogens in industrialized countries (4,5).

In our country, intestinal parasitic diseases are however still under-diagnosed because laboratory tests for intestinal parasites often are not requested by health care providers and because of technical difficulties with laboratory diagnosis (6). In general, one fresh stool sample is delivered to the laboratory for parasitological examinations. Because there is often considerable delay between production of the sample and examination in the laboratory, vegetative stages of protozoa, which cannot withstand conditions outside the intestine longer than 30 min to one hour, disintegrate and as a result are unrecognisable. As a result these stages are not detected by laboratories. To optimalize technical aspects of laboratory diagnosis of intestinal parasites, there is therefore a need for both a simple device which enables multiple sampling, to counteract intermittent shedding, and also a method to conserve vegetative stages of protozoa.

Recently a new test was described, the Triple-Faeces-Tests (TFT) protocol, which combined these requirements and which strongly improved diagnosis of intestinal parasites (7). The aim of this study carried out in Belgium, is to compare the yield from using the TFT protocol with that obtained using the conventional method (ethyl-acetate concentration of one fresh stool sample) in the diagnosis of intestinal parasites.

Materials and methods

From January 2002 to April 2005, all stool samples from outpatients visiting the Saint-Pierre University Hospital located in the centre of the Brussels' area and clinically suspected to have gastrointestinal infection caused by parasites were examined according to the Triple-Faeces-Test (TFT) procedure (7).

The TFT-set is a collection kit, containing 1 empty tube and 2 others filled with sodium acetate acetic acid formalin (SAF) preservative (Fig. 1). The kit has to be filled with stool on three consecutive days : on day 1, stool in a tube with SAF (TFT 1), on day 2 in an empty tube (TFT 2) and on day 3 in a tube filled with SAF (TFT 3). Each patient received guidelines printed in both Dutch and French.

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Fig. 1. — Triple-Faeces-Test-collection kit used by patients in Saint-Pierre University Hospital and by general practitioners.

SAF fixed stool specimens are examined with direct wet preparations for vegetative stages of protozoa, cysts and helminth eggs. From the unpreserved stool specimen (TFT 2, collected on day 2) sediment obtained with the routine ethyl-acetate-sedimentation technique, was studied with a direct wet smear for protozoan cysts and helminth eggs. In addition, a Rhodamine-auramine O staining for *Cryptosporidium* was performed, with confirmation with Kinyoun carbolfuchsin acid-fast staining (Fig. 2) (8).

From TFT 1 and TFT 3 (SAF-fixed stools from the first and third collection day) direct smears were studied with "Kop-Color" (Laboratories Fumouze, Levallois Perret, France) which stains vegetative and cyst stages of protozoa yellow and the background blue. An area equivalent of a cover slip of 22 by 22 mm was examined by light microscopy at 100× and 400× magnifications. When parasites were observed but direct identification was not possible on the wet smear, a permanent stain was performed with Chlorazol Black for confirmation (9). These preparations were screened with 200 oil-immersion fields using bright-field microscopy at 600× and 1000× magnification.

Results obtained after examination of the complete TFT (all three samples) were regarded as the result of the new test. They were then compared with results of the examination of the sediment obtained with the ethyl-acetate-sedimentation technique of the non fixed second sample of the TFT (TFT 2), which was the old routine practice in our laboratory.

In unpreserved stools from HIV infected patients and travellers, a fluorescence technique with Uvitex 2B staining for diagnosis of *Microsporidium spp* was performed (10).

During the study, *Giardia lamblia*, *Entamoeba histolytica/dispar, Isospora belli*, *Cyclospora cayetanensis, Dientamoeba fragilis*, and *Enterocytozoon bieneusi* were classified as pathogenic intestinal protozoa, *Blastocystis hominis*, *Entamoeba hartmanni, Entamoeba coli, Iodamoeba bűtschlii, Chilomastix mesnilli* and *Endolimax nana* as non-pathogens. Differential diagnosis of *Entamoeba histolytica* (pathogenic) and *Entamoeba dispar* (non pathogenic), which are two morphologically identical species of amoebae, was done with the *Entamoeba histolytica* II enzyme-linked immunosorbent assays test (TechLab Blackburg, Virginia) according to the manufacturer's instructions.

Comparisons were made with the McNemar test for paired observations, using the Statistical Package for Social Sciences 11.5 for Windows (SPSS Inc. ; Chicago, IL, USA) and EpiInfo v6.04c PLUS for DOS (Centers for Disease Control and Prevention ; Atlanta, GA, USA) software.

Results

From 1 January 2002 through 30 April 2005, 7,404 stool specimens from 2,776 patients were analyzed for parasites. TFT-sets were filled with stool precisely according to instructions by 2,091 / 2,776 (75.3%) of the patients. Among the 685 patients who filled incomplete TFT sets, 11.1% of them filled all three tubes but added too much stool in one or two of the SAF tubes, 43.4% filled two out of three tubes and 45.5% only one tube.

From patients delivering a complete TFT-set, 28.1% (587 out of 2091) had one or more intestinal parasites (Table 1). One or more pathogenic protozoa were observed in 153 cases (7.3%).

G. lamblia and *D. fragilis* were detected in 77 (3.7%) and 42 (2.0%) patients respectively, *Cryptosporidium parvum* in 23 cases (1.1%) and *Entamoeba histolytica / dispar* in 9 cases (0.4%). Among the nine patients with *Entamoeba histolytica / dispar,* only one was confirmed *E. histolytica* by ELISA method (11.1%). Infection with one or more non-pathogenic protozoa was observed in 19.5% (407/2091) patients and helminth infections in 1.3% (27/2091) (Table 1). Among the non-pathogenic protozoa, *Blastocystis hominis* (11.0%) and *Entamoeba coli* (5.6%) scored most frequently. Helminths such as *Trichuris trichiura* (0.3%), *Strongyloides stercoralis* (0.1%), *Taenia spp* (0.05%), *Ascaris lumbricoides* (0.3%) were also recovered, but in small numbers.

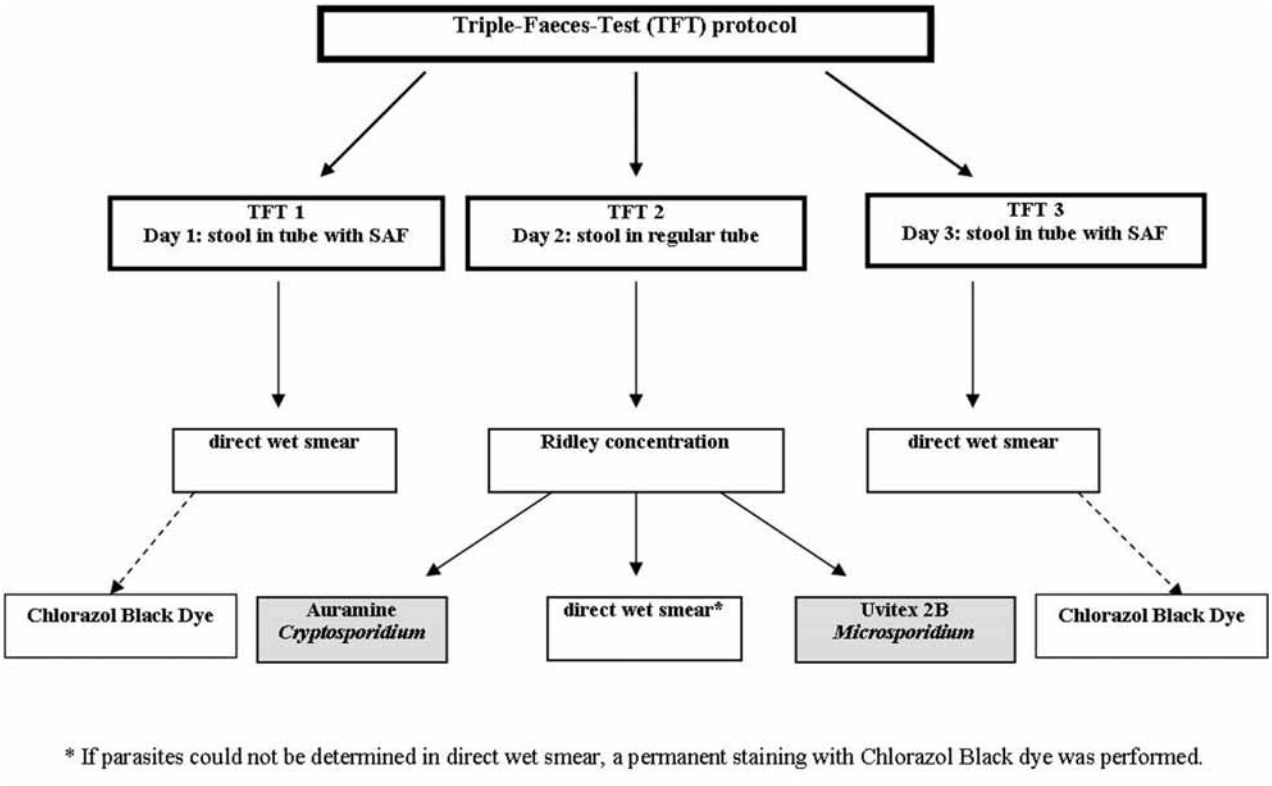

Fig. 2. — Diagnosis of intestinal parasitic infections with the Triple-Faeces-Test (TFT) - protocol in Saint-Pierre University Hospital

With the conventional method, examination of the sediment after ethyl-acetate-sedimentation of one unpreserved stool sample, 10.3% (216 out of 2091 patients) were diagnosed with an intestinal parasitic infection. *G. lamblia, D. fragilis* and *E. histolytica /dispar* were observed in 45 (2.15%), 1 (0.05%) and 2 (0.05%) patients respectively. Infection with non-pathogenic protozoan species was observed in 5.7% (119/2091) of cases and helminth infections in 0.9% (19/2091) (Table 1).

Both, *G. lamblia, D. fragilis, E histolytica/dispar, B. hominis, E. coli, I. bűtschlii, E. nana* and *E. hartmanni,* were significantly more observed with TFT when compared to the conventional procedure ($P < 0.05$). Recovery of *C. parvum, Isospora belli, Cyclospora cayetanensis, Enterocytozoon bieneusi, C. mesnili* and helminths was similar with TFT and conventional procedure.

Fluctuation in shedding of protozoa was demonstrated in the various stool samples of one TFT-set (Table 2) (numbers of findings in the table do not represent patients).

Discussion

Application of the Triple-Faeces-Test (TFT) strongly increased detection of intestinal parasitic infections. Using the TFT, 28.1% of the patients were diagnosed with parasitic infections, compared to 10.3% when using our conventional diagnostic method. Detection was significantly improved both for pathogenic protozoa (*G. lamblia, E histolytica/dispar* and *D. fragilis*) as well as non-pathogenic protozoa (*B. hominis, E. coli, E. hartmanni, I. bűtschlii,* and *E. nana*).

The recovery rate observed was however relatively low compared to findings with a similar sudy in The Netherlands (7). This could be due to the different populations examined. In the absence of national guidelines for stool parasite examination, the physicians who submitted samples for ova & parasites examination did not systematically follow ASM recommendations to request parasite examination only in clinically suspected cases (11). The lack of guidelines leads to a very heterogeneous studied population.

Rational approach to the stool ova and parasite examination is still controversial and different authors support the hypothesis that the examination of only one single stool specimen is sufficient enough for most patient (12). However, from literature there is convincing evidence that examination of multiple stool samples and use of a fixative, strongly enhances sensitivity of stool examination for intestinal parasites, suggesting a role for both components in routine diagnosis (13-14). In routine daily practice however, these demands are often not fulfilled. Examination of unpreserved stool specimens is also still routine practice in many Belgian laboratories. Despite repeated requests by physicians for patients to deliver more samples, compliance to deliver these to the laboratory often is low.

Table 1. — **Recovery of intestinal parasites with TFT-sets compared to results with the conventional method (one fresh sample). Total number examined with both methods was 2,091**

	No positive cases with				Increase with TFT compared to one fresh sample	
	One fresh sample		TFT			
	N°	%	N°	%	N°	p
Potentially pathogenic protozoa						
Giardia lamblia	45	2.15	77	3.68	32	0.04
Dientamoeba fragilis	1	0.05	42	2.01	41	0.01
Cryptosporidium parvum	23	1.10	23	1.10	0	NS
Microsporidium	3	0.14	3	0.14	0	NS
Isospora belli	5	0.24	5	0.24	0	NS
Entamoeba histolytica / dispar	2	0.05	9	0.43	7	0.01
Cyclospora cayetanensis	1	0.05	1	0.05	0	NS
Non pathogenic protozoa						
Blastocystis hominis	29	1.39	231	11.05	202	0.01
Entamoeba coli	85	4.07	117	5.60	32	0.02
Iodamoeba bûtschlii	11	0.53	33	1.58	22	0.01
Endolimax nana	17	0.81	43	2.06	26	0.01
Chilomastix mesnili	5	0.24	11	0.53	6	N S
Entamoeba hartmanni	5	0.24	14	0.67	9	0.04
Helminths						
Ancylostoma	4	0.19	4	0.19	0	NS
Trichuris trichiura	4	0.19	7	0.33	3	NS
Ascaris lumbricoides	6	0.29	7	0.33	1	NS
Strongyloides stercoralis	2	0.10	2	0.10	0	NS
Taenia spp.	1	0.05	1	0.05	0	NS
Schistosoma mansoni	1	0.05	1	0.05	0	NS
Enterobius vermicularis	1	0.05	3	0.14	2	NS
Others helminths	6	0.29	6	0.29	0	NS
Total No positive cases	216	10.33	587	28.07	371	0.01

Conventional diagnostic procedure for intestinal parasites in our laboratory was formerly also based on examination of unpreserved stool samples. Most patients delivered only one sample. Moreover, the time between production of stools and delivery to the laboratory, in most cases, was delayed for hours or even days, including periods of refrigeration. As a result, most vegetative stages of protozoa are destroyed or unrecognisable for microscopic examination and sensitivity of parasitic stool examination essentially relied on recovery of cysts of protozoa and eggs of helminths in sediment after ethyl-acetate concentration of an non fixed stool sample (15). With the non fixed second sample of the TFT (TFT 2), the same ethyl-acetate concentration procedure was performed.

In the present study we therefore compared results of the complete TFT (results of the three samples combined) with the second sample of the TFT (TFT 2, unfixed stool), results of which were essentially similar to our former routine procedure. From literature, examination of the first (TFT 1) and third (TFT 3) stool sample of the TFT, both fixed-stools, should improve detection of vegetative stages of protozoa and also cysts which, due to fluctuation of shedding, could be absent in sample 2. Also helminth eggs, when present in sufficient numbers, could be detected. Examination of TFT 1 and TFT 3 indeed strongly increased sensitivity of the diagnostic procedure, as was shown by the large increase of the number of cases observed with a parasitic infection (increase from 216 to 587 patients). These findings are in agreement with data from literature and strongly support use of both a fixative and multiple sampling in routine diagnostic examination for parasitic infections (16).

Combining results of examination of only two of the three samples of the TFT-set, TFT 1 (SAF-fixed sample) and TFT 2 (fresh concentrate sample) did show significantly less recovery of *G. lamblia, D. fragilis* and *B. hominis* ($P < 0.05$) as compared to results obtained using the complete TFT with three samples (data not shown).

To increase compliance of filling and delivery of 3 stool samples by patients, is an important aspect of the TFT. In this study 2167 out of 2776 (78.1%) patients filled all three tubes, suggesting a high compliance by patients. A patient instruction-form detailing how to handle the TFT is included in each collection kit, and is important for good compliance. It is possible that the form in the present study (in Dutch and French) was inadequate because the patient-population participating in the study is multiethnic. Introduction of additional translations (Arabic and Russian), has further increased compliance for filling the TFT-sets (data not shown).

B. hominis was the most frequently observed protozoan species (231 out of 2091 patients, 11%). Because of the ongoing debate about its pathogenicity, we classified this species as a non-pathogen (17). However, there are also several reports suggesting an association between (heavy) *B. hominis* infection and symptoms of

Table 2. — **Fluctuation in the shedding of intestinal parasites as observed with the TFT. Total no. examined was no. 2,091**

	N° of positive findings with			
	TFT1 (SAF, direct)	TFT2 (Fresh, concentrated)	TFT3 (SAF, direct)	TFT (TFT1, TFT2, TFT3)
Potentially pathogenic protozoa				
Giardia lamblia	47	45	47	77
Dientamoeba fragilis	32	1	33	42
Cryptosporidium parvum	0	23	0	23
Microsporidium	0	3	0	3
Isospora belli	3	5	2	5
Entamoeba histolytica / dispar	7	2	5	9
Cyclospora cayetanensis	0	1	0	1
Non pathogenic protozoa				
Blastocystis hominis	223	29	180	231
Entamoeba coli	79	85	79	117
Iodamoeba bûtschlii	29	11	31	33
Endolimax nana	30	17	29	43
Chilomastix mesnili	9	5	6	11
Entamoeba hartmanni	10	5	7	14
Helminths				
Ancylostoma	2	4	2	4
Trichuris trichiura	1	4	3	7
Ascaris lumbricoides	6	6	5	7
Strongyloides stercoralis	1	2	2	2
Taenia spp.	1	1	0	1
Schistosoma mansoni	0	1	2	1
Enterobius vermicularis	3	1	2	3
Others helminths	4	6	4	6
Total No positive cases	381	216	323	587

diarrhoea, abdominal pain, nausea and anorexia (5). When present in large numbers and no other causes for symptomatology are found, specific therapy could be considered (18). Because *B. hominis* degenerates relatively quickly outside the body, correct diagnosis is only possible through direct examination of freshly passed stool specimen or by using a preservative (5). Use of ethyl-acetate or ether concentration of unpreserved stool is insufficient for diagnosis of *B. hominis* infection (18). As reported in literature, we also observed a strong variation in day to day shedding (Table 2) underlining the need for multiple sampling for proper diagnosis (19).

With an incidence of 3.7%, *G. lamblia* was the most commonly pathogenic parasite observed in our study. Other surveys conducted in Europe have reported *G. lamblia* prevalence rates ranging from 1% to 30%, depending on the location and ages of the patients studied (20,21). Application of the TFT resulted in a significant increase of recovery of *G. lamblia* ($p < 0.05$)

D. fragilis was the second most common potential pathogenic parasite found in our study (2%), which is reported as a cause of symptomatic disease in 20-58% of infected cases (4). Symptoms include a large range of gastrointestinal signs, including diarrhoea, loose stools, abdominal pain or abdominal cramps. Recent studies indicate that especially in children *D. fragilis* can be an important cause of abdominal complaints (22-24) Because its survival time outside the body is most limited, diagnosis of *D. fragilis* infection depends on examination of freshly passed stools, stools preserved in a fixative and permanently stained smears. Formol-ether concentrate is not a suitable procedure because vegetative stages are destroyed (11). With the TFT, we for the first time observed *D. fragilis* frequently in patients in our routine practice. Prevalence of *D. fragilis* infection varies strongly, from 1.5% to 52.5%, among different human populations (4). Prevalence in our study, 2%, was relatively low. Higher rates have especially been observed in individuals living in crowded conditions (e.g., institutions, communal living), or conditions of poor hygiene (25).

For recovery of protozoan (oo)cysts and helminth eggs, ethyl-acetate concentration of unpreserved stool specimens was very effective and we did not find a significant increase in the recovery with the complete TFT. With TFT1 and TFT3 an additional of 8 cases with a helminth infection were diagnosed compared to examination of only one stool sample (TFT 2).

With our specific protocol for HIV-infected patients and travellers, we were able to recover 3 cases with microsporidiosis, one of them being an immunocompetent traveller. All three cases were *Enterocytozoon bieneusi*, the most common microsporidian species observed among humans (26). Similar results were also obtained by Muller *et al.* who found 9 positives cases of microsporidium infection using light microscopy and PCR on stool specimens of 148 returning travellers (27).

Results of this study suggest that the Triple-Faeces-Test protocol is an effective method for diagnosis of intestinal parasites. Application of the method greatly improves collection of multiple stools and provides the laboratory with the proper material for a thorough parasitological examination, which strongly enhances sensitivity of the diagnostic procedure.

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