

FLOTAC for the diagnosis of *Hymenolepis* spp. infection: proof-of-concept and comparing diagnostic accuracy with other methods

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Abstract *Hymenolepis nana* is the most common cestode parasitizing humans, yet it is under-diagnosed. We determined the optimal flotation solution (FS) for the diagnosis of this intestinal parasite with the FLOTAC method, and compared its diagnostic accuracy with an ether-concentration technique and the Kato–Katz method. Zinc sulphate (specific gravity 1.20) proved to be the best-performing FS. Using this

FS, we detected 65 *H. nana* infections among 234 fixed fecal samples from Tajik and Sahrawi children (prevalence 27.8 %). The ether-concentration technique detected 40 infections (prevalence 17.1 %) in the same samples. Considering the combined results as a reference, the sensitivities of FLOTAC and ether-concentration were 95.6 % and 58.8 %, respectively. The Kato–Katz method resulted in a prevalence of only 8.7 %. In terms of eggs per gram of stool, a significantly ($P < 0.05$) higher value was obtained with the FLOTAC and Kato–Katz techniques compared to ether-concentration. In another study carried out in China, the FLOTAC method detected six *Hymenolepis diminuta* infections in 302 fecal samples, whereas five samples were found positive with the Kato–Katz technique. We conclude that FLOTAC is an accurate coprodiagnostic technique for *H. nana* and *H. diminuta*, two species which join a growing list of intestinal parasites that can be reliably diagnosed by this technique.

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Introduction

Hymenolepis nana, also known as dwarf tapeworm, is a small-sized cestode. Adult worms measure 15–40 mm and the oval parasite eggs have a size of approximately $30 \times 50 \mu\text{m}$. Humans and rodents are parasitized by *H. nana*, whereas insects (beetles and fleas) act as intermediate hosts, in which infective cysticeroid larvae develop. Alternatively, the embryonated *H. nana* eggs shed in feces can directly infect humans upon ingestion. Auto-infection with eggs remaining in the intestine and hatching in situ is also possible, allowing infections to become perpetuated despite the short life span of the

adult worm of 4–6 weeks (Schantz 1996). Adults of the related species *Hymenolepis diminuta*, the rat tapeworm, are much longer (20–60 cm), and their eggs have a round shape of approximately 60–80×70–85 µm. The principal final hosts of *H. diminuta* are rodents, but human infection can occur if infected arthropods are ingested (Schantz 1996).

H. nana has a global distribution and is the most common cestode infecting humans (Schantz 1996). Highest prevalence rates are observed in warm semi-arid and arid areas among impoverished children and institutionalized persons (Schantz 1996). However, epidemiological data are scarce and only few studies have investigated the distribution of this parasite in recent years (Steinmann et al. 2010; Matthys et al. 2011). Most scientific publications are case reports among travelers, immigrants (Gualdieri et al. 2011), and adopted children (Di Lernia et al. 2004; Marseglia et al. 2007). *H. diminuta* is less commonly diagnosed but sporadically occurs in Asia and Europe (Wiwanitkit 2004; Patamia et al. 2010). *H. nana* infections often remain asymptomatic. Praziquantel and nitazoxanide are the recommended drugs (Anonymous 2007).

Diagnosis of *H. nana* and *H. diminuta* relies on the microscopic detection of eggs in fecal samples (Schantz 1996). Ethyl acetate or ether-based concentration techniques (García 2007) are recommended to increase sensitivity as egg numbers can be very low. Eggs can also be readily observed in Kato–Katz thick smears (Katz et al. 1972), a widely used diagnostic method in intestinal helminth surveys (Bergquist et al. 2009; Speich et al. 2010). Since *H. nana* eggs are small and eggs of both species are not particularly conspicuous, they might be overlooked by unsuspecting microscopists not familiar with *Hymenolepis* eggs. As an alternative to traditional concentration techniques, the FLOTAC apparatus has been developed (Cringoli 2006; Cringoli et al. 2010). It allows performing a series of multivalent fecal egg count (FEC) techniques for detecting and enumerating parasitic elements in the feces of animals (Rinaldi et al. 2007a, b, 2011; Duthaler et al. 2010; Cringoli et al. 2010, 2011) and humans. In human parasitology, the tool has proved to be more sensitive than the ether-concentration technique and multiple Kato–Katz thick smears for the diagnosis of soil-transmitted helminths (STHs) (Utzinger et al. 2008; Knopp et al. 2009a, b). Promising results using the FLOTAC technique have also been reported for the diagnosis of *Schistosoma mansoni* (Glinz et al. 2010), *Dicrocoelium dendriticum* (Jeandron et al. 2011), and intestinal protozoa (Becker et al. 2011; Gualdieri et al. 2011).

The objectives of the present study were to determine the optimal conditions for *H. nana* diagnosis with FLOTAC, and comparing diagnostic accuracy of FLOTAC with currently more widely used techniques for the detection of *H. nana* and *H. diminuta* eggs in fixed stool samples.

Materials and methods

Study set-up

The study was implemented in two steps. First, in a FLOTAC calibration study using a panel of nine flotation solutions (FS), we determined the FS that yielded the most accurate *H. nana* diagnosis (Cringoli et al. 2010). Second, the diagnostic accuracy of FLOTAC was compared with an ethyl ether-based concentration technique (García 2007; Utzinger et al. 2010) and the Kato–Katz method (Katz et al. 1972). This comparison was done using stool samples fixed in sodium acetate–acetic acid–formalin (SAF) (Marti and Escher 1990).

FLOTAC calibration

The optimal FS for *H. nana* egg detection in SAF-fixed stool samples using the FLOTAC method was determined, as described by Cringoli and co-workers (2010). In brief, two egg-positive SAF-fixed stool samples were pooled, thoroughly mixed and filtered through a wire mesh (aperture=250 µm). The effluent suspension was divided into 54 aliquots of 2 ml each, i.e., six replicates for each of a panel of nine FSs to be tested (see below). Each 2-ml aliquot was placed in a 15-ml tube, centrifuged for 3 min at 170×g, and the supernatant poured off and discarded. The tube with the remaining pellet at the bottom was then filled with the respective FS until the 6-ml level mark, and slowly agitated. The suspension was transferred into one of the two 5-ml chambers of a FLOTAC-100 apparatus using a disposable pipette. The apparatus was centrifuged at 120×g for 5 min, and translated to separate the floating eggs from the sedimented debris. All eggs visible through the ruled window of the reading disc were enumerated under a microscope at 100× magnification.

The following nine aqueous FSs (FS1–FS9), described by Cringoli et al. (2010), were employed: FS1, sucrose and formaldehyde (specific gravity [s.g.] 1.20); FS2, sodium chloride (s.g. 1.20); FS3, zinc sulphate (s.g. 1.20); FS4, sodium nitrate (s.g. 1.20); FS5, sucrose plus mercury II iodide and potassium iodide (s.g. 1.25); FS6, magnesium sulphate (s.g. 1.28); FS7, zinc sulphate (s.g. 1.35); FS8, mercury II iodide and potassium iodide (s.g. 1.44); and FS9, zinc sulphate plus mercury II iodide and potassium iodide (s.g. 1.45). All FSs were prepared at room temperature on the day of the experiment, and their s.g. determined using a hydrometer at 20 °C.

Origin of stool samples

Overall, 234 SAF-fixed fecal samples were available to compare the diagnostic accuracy of the optimal FLOTAC

set-up as determined in step 1 with an ethyl ether-concentration technique. Most of the stool samples ($n=215$) were obtained from schoolchildren, aged 7–11 years, from western Tajikistan in 2009 (Matthys et al. 2011). Of note, 207 of the Tajik children had complete data records, namely FLOTAC, ether-concentration, and duplicate Kato–Katz thick smears from a single stool sample. Additionally, 19 samples were obtained in 2008 from a group of Sahrawi children, with a mean age of 9.5 years, who were hosted for summer holidays in the Tuscany region of Italy. The Sahrawi are a poor population living in the Western Sahara desert. All samples had been fixed in SAF at a ratio of approximately 1 g of feces and 10 ml of fixative (Marti and Escher 1990).

H. diminuta infections were detected in SAF-fixed stool samples collected before and after anthelmintic treatment of 302 individuals of all ages and both sexes from Yunnan province, China (Steinmann et al. 2011). The focus of this diagnostic effort was the detection of STH eggs, but based on Kato–Katz thick smears prepared upon sample submission, the presence in the samples of a few infections with *H. diminuta* had been established.

Diagnostic procedures

The SAF-fixed stool samples for *H. nana* diagnosis were carefully re-suspended, filtered, and divided into two aliquots of 2 ml each. Each aliquot was centrifuged as explained above, the supernatant poured off, and the tubes with the sediment pellets randomly assigned to diagnostic work-up using either the FLOTAC basic technique (Cringoli et al. 2010) or the ethyl ether-based concentration method (Ritchie 1948; Utzinger et al. 2010).

For the FLOTAC basic technique, tubes were filled to the 11 ml level with the best-performing FS according to the preceding FLOTAC calibration (step 1), and slowly agitated. The suspension was then transferred into the two chambers of a FLOTAC-100 apparatus, centrifuged, and read at 100 \times magnification.

For the ethyl ether-based concentration technique, tubes were filled with 7 ml of a 0.8 % sodium chloride solution and 3 ml of ethyl ether, and vigorously shaken. The tubes were then centrifuged at 240 $\times g$ for 5 min, the supernatant poured off, and the pellet re-suspended in a drop of saline solution before transfer onto a microscope slide that was examined at 100 \times magnification.

The samples potentially containing *H. diminuta* eggs were examined with the FLOTAC apparatus according to the same general procedures with a few modifications: FS4 was used as the focus of the study was on STHs (Utzinger et al. 2008; Knopp et al. 2009a, b, 2011) and the second aliquot was used for quality control purposes rather than the ethyl-ether concentration technique.

Ethical considerations and treatment

The study protocol in Tajikistan was approved by the Ministry of Health (reference no. 16/75-92). The protocol for the study of intestinal parasites in Sahrawi children was approved by the Ethical Committee of Azienda Ospedaliera Universitaria (AOU) Pisana, Pisa, Italy. Consent was provided by the children's parents. The study conducted in China was cleared by the ethics committee of Basel (EKBB, reference no. 294/08) and the institutional review board of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention in Shanghai (reference no. 2008091701). The study is registered with Current Controlled Trials (identifier: ISRCTN47375023). Participants and the parents or legal guardians of eligible children signed a written informed consent sheet. Upon study completion, free anthelmintic treatment was offered to study participants.

Statistical analysis

For the calibration, differences between FSs were analyzed using one-way analysis of variance (ANOVA) with post hoc Fisher's least significant difference (LSD). For an assessment of the diagnostic accuracy of different techniques, egg counts were multiplied with the appropriate factor (ten for FLOTAC and ethyl ether-concentration, 24 for Kato–Katz), and the arithmetic mean number of eggs per gram of feces (EPG) calculated for each individual.

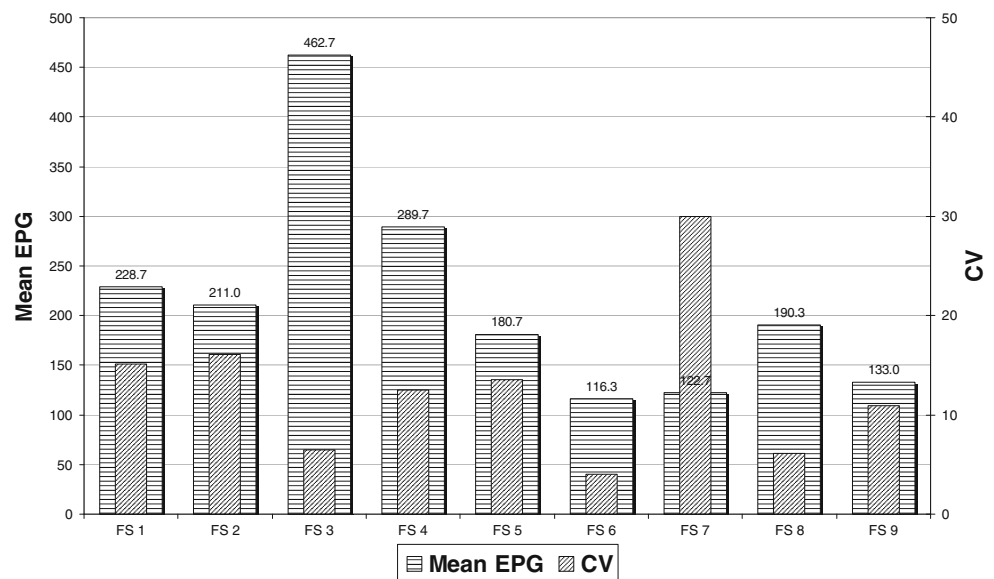
Statistical analysis was carried out using STATA version 10.0 (Stata Corp.; College Station, TX, USA). The statistical differences between the mean EPG values were analyzed using ANOVA (generalized linear model [GLM] for repeated measures) in conjunction with the Bonferroni test for post hoc comparison. In addition, a likelihood ratio test (LRT) of the equality of the coefficients of variation of k normally distributed populations was performed using the software developed by Verrill and Johnson (2007).

Results

FLOTAC calibration

The mean *H. nana* EPG value calculated for each of the nine FSs is shown in Fig. 1, along with the coefficient of variation (CV). *H. nana* eggs were detected with each FS. The best result, in terms of highest mean FEC (462.7 EPG) and lowest CV (6.5 %), was obtained using FS3, i.e., zinc

Fig. 1 Mean number of *Hymenolepis nana* eggs per gram of stool (EPG) and coefficient of variation (CV) in human stool samples when using FLOTAC with nine different flotation solutions (FSs; six replicates per FS)



sulphate (s.g. 1.20). Hence, for the subsequent comparison of diagnostic accuracy, FS3 was used.

Diagnostic accuracy

Using FLOTAC with FS3, *H. nana* eggs were detected in 65 out of the 234 stool samples, translating into a prevalence of 27.8 %. Employing the ethyl ether-concentration technique on the same stool samples, considerably fewer *H. nana* infections were detected ($n=40$, 17.1 %). Both methods combined revealed 68 infections (29.1 %). Considering these combined results as a reference, the sensitivity of the FLOTAC and the ethyl ether-concentration techniques for the diagnosis of *H. nana* were 95.6 % and 58.8 %, respectively (difference $P=0.002$).

The results, stratified by sample origin and diagnostic technique, are shown in Table 1. Among the 215 Tajik samples, 58 (27.0 %) were positive for *H. nana* according to the FLOTAC technique (sensitivity: 95.1 %),

while only 37 (17.2 %) samples were positive using the ethyl ether-based concentration technique (sensitivity: 60.7 %). Among the 207 samples screened with the Kato–Katz method, 18 *H. nana* infections, including four that were missed by the FLOTAC technique, were observed (prevalence: 8.7 %). For the 19 Sahrawi samples, seven (36.8 %) were found positive with the FLOTAC technique (sensitivity: 100 %), but only three (15.8 %) were positive using the ethyl ether-based concentration (sensitivity: 42.9 %).

The mean number of *H. nana* eggs detected with FLOTAC (mean among positives: 617.4 EPG) was significantly higher ($P<0.05$) than the numbers counted after ethyl ether-concentration (mean among positives: 58.2 EPG). Diagnosis with the Kato–Katz method resulted in a mean of 660.7 EPG among the infected (Table 1).

A total of eight *H. diminuta*-positive samples from six individuals were identified with the Kato–Katz, FLOTAC, or both methods combined among the samples from China.

Table 1 *Hymenolepis nana* prevalence and fecal egg count (FEC, expressed as eggs per gram of stool (EPG)) in human stool samples from Tajik and Sahrawi children, stratified by diagnostic technique

Method	No. of positive results (%)	FEC among positives, in EPG*		
		Arithmetic mean	Median	Interquartile range
FLOTAC with FS3				
- Total ($n=234$)	65 (27.8)	617.4 ^a	17.5	147.8
- Tajikistan ($n=215$)	58 (27.0)	n.a.		
- Sahrawi ($n=19$)	7 (36.8)	n.a.		
Ethyl ether-concentration				
- Total ($n=234$)	40 (17.1)	58.2 ^b	11.0	24.0
- Tajikistan ($n=215$)	37 (17.2)	n.a.		
- Sahrawi ($n=19$)	3 (15.8)	n.a.		
Kato–Katz				
- Tajikistan ($n=207$)	18 (8.7)	660.7 ^a	558	459

n.a., not available

*Significant differences for different letters (a, b) ($P<0.05$)

Three cases were tested positive with both methods; eggs were only seen on Kato–Katz thick smears prepared from two samples, the remaining three samples were only positive with the FLOTAC technique.

Discussion

The FS is a key determinant of the sensitivity of any copromicroscopic technique that is based on flotation (Cringoli 2006; Cringoli et al. 2010). In the present study, we found that zinc sulphate (s.g. 1.20; designated FS3 according to a standard protocol) is the optimal FS for detecting *H. nana* eggs with FLOTAC out of a panel of nine tested FSs. Indeed, FS3 resulted in the highest mean FEC (462.7 EPG) and the smallest CV (6.5 %). We also found that FLOTAC shows a higher sensitivity than the widely used ethyl ether-based concentration technique (sensitivity: 95.6 % versus 58.8 %). Perhaps surprisingly, the Kato–Katz method detected only about half as many infections than the ethyl ether concentration technique. FLOTAC not only showed a higher sensitivity than the ether-concentration method, it also resulted in a several-fold higher number of eggs counted in a given amount of stool (FLOTAC, FEC=617.4 EPG; ether-concentration technique, FEC=58.2 EPG). Somewhat higher FECs have been observed when comparing the FLOTAC method with an ether-concentration method for the diagnosis of other nematodes and trematodes (Utzing et al. 2008; Glinz et al. 2010). Interestingly, *H. nana* FECs derived from the FLOTAC and the Kato–Katz techniques were comparable, which is in contrast to previous investigations pertaining to STHs, which revealed considerably higher FECs for Kato–Katz rather than FLOTAC (Utzing et al. 2008; Knopp et al. 2009a, b).

In the light of the available evidence, FS2 and FS4 are the recommended FSs for surveys pertaining to intestinal helminths. Indeed, FS4 has proved to perform best for STH diagnosis in several studies and was the second best-performing FS for *H. nana* diagnosis according to the current calibration study. Moreover, FS4 also shows promise for the diagnosis of *H. diminuta*. The latter parasite is only rarely diagnosed in humans and the detection of six cases among a sample of 302 individuals in Yunnan province, China (prevalence of approximately 2 %), is noteworthy. To date, only around 500 cases of human infections with this parasite have been reported in the scientific literature, albeit this tapeworm is suspected to be considerably more common in humans (Schantz 1996; Wiwanitkit 2004).

Based on the growing evidence of its diagnostic competitiveness with more traditional methods, FLOTAC is gaining in importance as a new or complementary tool in parasitological surveys among humans. Examples include epidemiological surveys and anthelmintic drug efficacy

studies in Zanzibar (Knopp et al. 2009b, 2011), and comparative diagnostic studies in Côte d'Ivoire (Glinz et al. 2010), Ethiopia (Habtamu et al. 2011), and Kyrgyzstan (Jeandron et al. 2011). Moreover, in a recent investigation of intestinal parasites among immigrants in Naples, Italy, it was demonstrated that the FLOTAC was more sensitive than an ethyl acetate-based concentration technique for helminths and intestinal protozoa (Gualdieri et al. 2011). The FLOTAC technique has also been employed for point-of-care diagnosis and results obtained thus far warrant follow-up studies (Coppola et al. 2010).

We conclude that FLOTAC is an efficient coprodiagnostic technique for detecting intestinal parasite infections. Specifically, *H. nana* and *H. diminuta* can be added to the growing list of human intestinal parasites which are reliably diagnosed with the FLOTAC technique. These results should be confirmed by other groups in areas where hymenolepiasis is endemic.

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