FLOTAC: a new sensitive technique for the diagnosis of hookworm infections in humans

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Kato—Katz;
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Sensitivity;
Côte d’Ivoire

Summary Hookworms infect more than 10% of the world’s population, but current diagnostic tools have drawbacks. Our objective was to compare the diagnostic performance of three methods (Kato—Katz, ether concentration and FLOTAC techniques) for hookworm diagnosis. Stool samples were obtained from 102 schoolchildren in Côte d’Ivoire. First, a duplicate 41.7 mg Kato—Katz thick smear was prepared. Next, a small portion of stool (mean weight 1.8 g) was preserved in sodium acetate—acetic acid—formalin and forwarded to a European laboratory. These samples were split in three parts, one processed by an ether concentration technique and two by the FLOTAC technique. All samples were examined by experienced technicians for hookworm eggs using light microscopy. The observed hookworm prevalences as assessed by the FLOTAC, Kato—Katz and ether concentration techniques were 65.7%, 51.0% and 28.4%, respectively. Considering the combined results as the diagnostic ‘gold’ standard, the FLOTAC technique had a sensitivity of 88.2% compared with 68.4% for the Kato—Katz and 38.2% for the ether concentration techniques. The Kato—Katz method resulted in a significantly higher mean number of eggs per gram of stool (155.8 EPG) compared with the FLOTAC (37.7 EPG) and ether concentration (5.7 EPG) methods. The FLOTAC method shows promise as an important new tool for individual hookworm diagnosis and for rigorous monitoring of helminth control programmes.

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FLOTAC for hookworm diagnosis

1. Introduction

There is growing recognition of the huge public health significance of soil-transmitted helminthiases (Bethony et al., 2006; Lammie et al., 2006; WHO, 2002). Two-thirds of the world’s population are at risk of contracting an infection with one or more of the main soil-transmitted helminths, including Ascaris lumbricoides, the hookworms (Ancylostoma duodenale and Necator americanus) and Trichuris trichiura (Horton, 2003; Utzinger and Keiser, 2004). The collective burden of soil-transmitted helminthiases may be as high as 39 million disability-adjusted life-years, similar to that due to malaria or tuberculosis (Katz et al., 1972) is widely used in epidemiological surveys and control programmes (WHO, 1991). The ether-based concentration method (Allen and Ridley, 1970) is also widely used for diagnosis of helminths and intestinal protozoa and has the advantage of fixing the eggs or cysts (Cheesbrough, 2005). The latter issue is important in remote settings where microscopy may not be readily available.

Several methods are available for determining the presence and intensity of hookworm infections. Because of its simplicity and relatively low cost, the Kato—Katz technique (Katz et al., 1972) is widely used in epidemiological surveys and is recommended by the WHO for monitoring helminth control programmes (WHO, 1991). The ether-based concentration method (Allen and Ridley, 1970) is also widely used for diagnosis of helminths and intestinal protozoa and has the advantage of fixing the eggs or cysts (Cheesbrough, 2005). The latter issue is important in remote settings where microscopy may not be readily available.

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2. Materials and methods

2.1. Study area and stool samples

Stool samples were obtained from schoolchildren aged 6–14 years who were recruited from five primary schools in the district of Toumodi, located approximately 180 km northwest of Abidjan, the economic capital of Côte d’Ivoire. The region is situated in the ‘V-Baoué’, the transition zone from rainforest to savannah. The average annual temperature is 27 °C and the mean annual precipitation is 1060 mm (N’Goran et al., 1998, 1999; Rohner et al., 2007). Helminth infections, particularly hookworms and Schistosoma haematobium, are highly endemic in this part of Côte d’Ivoire (N’Goran et al., 1998, 2001; Rohner et al., 2007).

Stool sample collection was carried out in December 2006, in the middle of the dry season. Overall, 116 schoolchildren were randomly selected from 640 schoolchildren who were found to be mildly to moderately anaemic (defined as haemoglobin level >80 g/l and <115 g/l for children aged 6–12 years and >80 g/l and <120 g/l in children 12–14 years).

2.2. Ethical considerations

The study was embedded in a randomised controlled trial (No. ISRCTN21782274) assessing the effect of different interventions for anaemia prevention, and the stool collection

particularly in cases of low infection intensities (Booth et al., 2003; Utzinger et al., 2002). Considering the pooled results from 3578 stool examinations among schoolchildren in Côte d’Ivoire, using the Kato—Katz and ether concentration methods as the diagnostic ‘gold’ standard, the sensitivities of the Kato—Katz technique and the ether concentration method were 77.8% and 58.6%, respectively (Raso et al., 2006). Comparison of 988 stool samples from Malawi subjected to the Kato—Katz and ether concentration method revealed respective prevalences of 27.3% and 19.3% (Dacombe et al., 2007). Stool samples from 354 Zanzibari infants (aged 5–11 months) were examined by five different methods; the hookworm prevalence based on a duplicate Kato—Katz thick smear was 6.1%, whereas two ether concentration methods revealed prevalences of 5.0% and 5.8% (Goodman et al., 2007).

A novel multivalent faecal egg count method, the so-called FLOTAC technique, has been described recently both for human and veterinary medicine (Cringoli, 2006). This method is facilitated by the FLOTAC® apparatus, which has been designed to carry out flotation in a centrifuge, followed by a transversal cut (i.e. translation) of the apical portion of the floating suspension. The FLOTAC technique allows quantification of eggs and/or larvae of nematodes and trematodes as well as cysts and oocysts of intestinal protozoa in up to 1 g of faeces. It should therefore considerably improve the sensitivity of the aforementioned diagnostic tools. The present paper reports use of the FLOTAC technique for hookworm diagnosis in human stool samples, with results being compared with the standard Kato—Katz and ether concentration methods. Implications and arising research needs are discussed.
reported here was done during the baseline survey. Written informed consent was obtained from the parents or legal guardians of participating children. At the end of the trial, all children with a hookworm or other soil-transmitted helminth infection were treated with a single 500 mg oral dose of mebendazole.

2.3. Field and laboratory procedures

Stool samples were transferred to the nearby laboratory and processed as follows. First, a duplicate Kato—Katz thick smear was prepared from each stool sample on microscope slides using 41.7 mg punched plastic templates (Katz et al., 1972). After a clearing time of 30—45 min, the slides were examined under a light microscope by experienced laboratory technicians. The numbers of hookworm eggs and other soil-transmitted helminths (i.e., A. lumbricoides and T. trichiura) were counted and recorded separately. For quality control, 10% of the slides were re-examined by the senior technician. The mean number of hookworm eggs was calculated from the two slides examined per child and was multiplied by a factor 24 to obtain the number of eggs per gram of stool (EPG).

Second, a hazelnut-sized portion of each stool sample was placed into a small tube containing 10 ml of sodium acetate—acetic acid—formalin (SAF) solution (Bogoch et al., 2006; Marti and Escher, 1990). Each aliquot was weighed (PB303-S Delta Range; Mettler Toledo, Greifensee, Switzerland). The SAF-preserved stool samples were forwarded to the Laboratory of Veterinary Parasitology, University of Naples ‘Federico II’ (Naples, Italy) and simultaneously processed by an ether concentration method (Allen and Ridley, 1970) and the FLOTAC technique (Cringoli, 2006). Once at the laboratory, 25 small glass balls were put into each SAF-fixed stool sample and the tubes were vigorously shaken until all the faecal matter was broken down. Each mixture was poured through a wire mesh screen with an aperture of 350 μm and the strained suspension was equally split into three tubes.

Each tube (containing one-third of the original weight of each stool sample) was centrifuged for 1 min at 2000 rpm and then randomly assigned to a method. Specifically, the first tube was used to perform the ether concentration method (Allen and Ridley, 1970), whose resulting slides were examined under a light microscope for the presence of helminths by experienced laboratory technicians. The remaining two tubes were filled to 5 ml with the following two flotation solutions: (i) sodium nitrate (specific gravity = 1.20) for detection and counting of nematode eggs (e.g., hookworms) and eggs of cestodes; and (ii) zinc sulphate plus potassium iodomercurate (specific gravity = 1.45) for detection and counting of nematode larvae (e.g., Strongyloides stercoralis) and trematode eggs (e.g., Schistosoma mansoni and Fasciola hepatica). These two flotation solutions were chosen from a battery of 14 solutions with specific gravities ranging between 1.20 and 1.45 (Cringoli et al., 2004), after pre-testing with eight stool samples selected at random. Egg counts commenced following centrifugation (5 min at 1000 rpm) and translation, and they were performed under both grids (volume of each = 5 ml), corresponding to each of the two flotation solutions used (Cringoli, 2006).

For both methods, EPG was calculated as follows:

$$\text{EPG} = \frac{\text{no. of eggs counted}}{\text{weight of stool sample in g}} \times 3.$$  

2.4. Statistical analyses

Data were double-entered and cross-checked and statistical analyses were performed using version 13 of SPSS software for Windows (SPSS Inc., Chicago, IL, USA). The final analysis focused on those stool samples where complete data records (i.e., Kato—Katz, ether concentration and FLOTAC results) were available.

Agreement between two methods (i.e., Kato—Katz versus FLOTAC, ether concentration versus FLOTAC, and Kato—Katz versus ether concentration) for hookworm diagnosis was assessed using the κ statistic (Thrusfield, 1995). The sensitivity (proportion of true positives in the population) and negative predictive value (proportion of individuals with a negative test result who are correctly diagnosed) were calculated for each of the three methods, considering the combined results from the individual methods as the diagnostic ‘gold’ standard. This approach maximised sensitivity values, which is argued as follows: the detection of a hookworm egg by any of the methods employed was considered as a true-positive result. Following this approach, however, specificity and positive predictive values could not be calculated (Bogoch et al., 2006). Furthermore, mean, standard error (SE), and 25th, 50th and 75th percentiles of EPG values were calculated for the three different techniques. Since EPG values were overdispersed, as revealed by analysis of the normality tests of Shapiro–Wilk (P < 0.05) and the normal Q–Q plots, the values were transformed utilising the natural logarithm (ln) of EPG + 1. The reason for adding 1 to each EPG value is to avoid the mathematically non-defined logarithm of zero. Statistical differences between the mean EPG values (using the In-transformed data) were analysed using ANOVA, in conjunction with the Bonferroni test for post-hoc comparison.

3. Results

3.1. Operational results

Overall, 116 stool samples were collected and duplicate Kato—Katz thick smear results were available from 115 of them. Sufficient quantities were available to preserve a small portion in SAF solution from 110 stool samples. These SAF-preserved samples were forwarded to Italy, where, as mentioned previously, eight samples were used for identification of the most suitable flotation solutions for the FLOTAC technique. Complete data records were available for 102 stool samples; all subsequent analyses pertain to this cohort. The mean weight of the SAF-preserved stool samples was 1.80 g (range 1.00—2.18 g).

3.2. Comparison of methods

Tables 1–3 show the results of the comparison between the different diagnostic approaches by means of two-way contingency tables of frequencies. Agreements between two methods compared with each other are reported, including κ statistics. Of the 102 stool samples examined for hook-
Table 1  Two-way contingency table of frequencies showing agreement between the Kato–Katz and FLOTAC techniques for the diagnosis of hookworms in human stool samples

<table>
<thead>
<tr>
<th>FLOTAC</th>
<th>Kato–Katz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>43</td>
</tr>
<tr>
<td>Negative</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
</tr>
</tbody>
</table>

\( \kappa \) agreement = 0.349 (\( P < 0.001 \)).

Table 2  Two-way contingency table of frequencies showing agreement between the ether concentration and FLOTAC techniques for the diagnosis of hookworms in human stool samples

<table>
<thead>
<tr>
<th>FLOTAC</th>
<th>Ether concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>29</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
</tr>
</tbody>
</table>

\( \kappa \) agreement = 0.344 (\( P < 0.001 \)).

Table 3  Two-way contingency table of frequencies showing agreement between the Kato–Katz and ether concentration techniques for the diagnosis of hookworms in human stool samples

<table>
<thead>
<tr>
<th>Ether concentration</th>
<th>Kato–Katz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>21</td>
</tr>
<tr>
<td>Negative</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
</tr>
</tbody>
</table>

\( \kappa \) agreement = 0.242 (\( P = 0.006 \)).

Table 4  Sensitivity and negative predictive values (NPV) of the FLOTAC, Kato–Katz and ether concentration techniques for the diagnosis of hookworms in human stool samples when considering the pooled results from the three methods as the diagnostic ‘gold’ standard

<table>
<thead>
<tr>
<th>Technique</th>
<th>Sensitivity (95% CI)</th>
<th>NPV (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLOTAC</td>
<td>88.2% (78.2–94.1%)</td>
<td>79.5% (64.2–89.7%)</td>
</tr>
<tr>
<td>Kato–Katz</td>
<td>68.4% (56.6–78.3%)</td>
<td>67.6% (55.6–77.7%)</td>
</tr>
<tr>
<td>Ether concentration</td>
<td>38.2% (27.5–50.1%)</td>
<td>60.8% (51.5–69.5%)</td>
</tr>
</tbody>
</table>

4. Discussion

Notwithstanding significant advances in our understanding of the epidemiology of hookworm infections, growing appreciation of the ‘true’ public health impact of this neglected tropical disease, development of new tools and progress made in the control of hookworm infection in different settings (Brooker et al., 2004, 2006; Hotez et al., 2006b), little was found between FLOTAC and the ether concentration method (0.344). An even weaker agreement was found between the Kato–Katz and ether concentration methods (0.242).

The pooled data revealed an overall hookworm prevalence of 74.5% (95% CI 64.7–82.4%). The sensitivity and negative predictive values of the individual methods, compared with the pooled results, which was considered our diagnostic ‘gold’ standard, are summarised in Table 4. The sensitivities of the FLOTAC technique, the duplicate Kato–Katz thick smears and the ether-based concentration method were 88.2% (95% CI 78.2–94.1%), 68.4% (95% CI 56.6–78.3%) and 38.2% (95% CI 27.5–50.1%), respectively.

Table 5 summarises hookworm EPG values (mean, SE and percentiles) according to the diagnostic approach taken. The mean EPG revealed by the FLOTAC (37.7) and Kato–Katz (155.8) techniques were significantly higher than that obtained by the ether concentration method (5.7) (\( P < 0.001 \) for both comparisons). The mean EPG produced by the Kato–Katz technique was higher than that produced by FLOTAC and the difference showed statistical significance (\( P = 0.042 \)).

Other helminth eggs (i.e. A. lumbricoides and T. trichiura) and helminth larvae (e.g. S. stercoralis) were also detected in the stool samples examined; however, these results were not included in the present paper because of very low observed prevalences. No S. mansoni eggs were detected using all three methods considered.
emphasis has been placed on the development of novel diagnostics or the standardisation of existing copromicroscopic techniques. Our findings provide important new information on the performance of available methods for the in vivo diagnosis of hookworm infection in humans. Strengths and weaknesses of the different methods are discussed, along with implications and arising research needs.

The first issue pertains to sensitivity. Whilst the FLOTAC technique identified 67 of the 102 stool samples as hookworm-positive, a duplicate Kato—Katz thick smear reading and an ether concentration method found 52 and 29 of the samples to be positive, respectively. Thus, the observed prevalence of hookworm infection based on the FLOTAC technique was 29% higher compared with the Kato—Katz technique and 131% in the case of the ether concentration method. It follows that the sensitivity of the FLOTAC technique for hookworm diagnosis was considerably higher compared with the other two diagnostic approaches. Importantly, in 38 cases FLOTAC was positive for hookworms whereas the ether concentration method gave a negative result, and 24 times the FLOTAC technique found hookworm eggs whereas the Kato—Katz thick smear readings were negative. All of the 29 hookworm cases diagnosed by the ether concentration method were also found to be positive by FLOTAC. With regard to hookworm-positive Kato—Katz thick smear readings, there were nine cases where FLOTAC produced a negative result. Interestingly, in these cases one out of the two Kato—Katz readings was negative whereas only a few eggs (i.e. 1—3) were found on the other slide, suggesting that only light hookworm infections were missed by FLOTAC. This observation might be explained by the following reasons. First, we used only one of the two chambers of the FLOTAC® apparatus for the diagnosis of hookworms (the other was used for diagnosis of trematodes). Second, the hookworm eggs are very fragile. Results of a recent study performed on faecal samples obtained from dogs, comparing either fresh or SAF-preserved stool samples, showed that in the SAF-conserved samples more than 30% of hookworm eggs were broken or altered and hence not detected (unpublished data). Third, the highly aggregated distribution of helmint eggs in stool samples may lead to the absence of eggs in cases where only one small sample is examined (Anderson and Schad, 1985; Utzinger et al., 2002).

Our results thus underscore previous observations made for various parasites of veterinary importance: FLOTAC has a higher sensitivity than other more widely used diagnostic approaches (Cringoli, 2006; Rinaldi et al., 2007). Moreover, our study confirms that the prevalence of human hookworm infections, and conceivably also other soil-transmitted helminths and S. mansoni, may be underestimated by routinely used techniques such as the Kato—Katz or ether concentration methods. An important explanation is the small amount of faecal matter analysed by the former method; 41.7 mg of stool in a single Kato—Katz thick smear reading (Katz et al., 1972). Examination of multiple Kato—Katz thick smears from one or ideally from several stool specimens enhances the sensitivity of helmint diagnosis but is operationally challenging, costly and more time consuming (Booth et al., 2003; Engels et al., 1996; Raso et al., 2006; Utzinger et al., 2002). The FLOTAC technique examines a larger amount of faeces. In the present study, a mean of 0.6 g of stool was subjected to hookworm diagnosis by FLOTAC, but the amount could be doubled (using both chambers) or even higher. Future studies should investigate whether indeed the sensitivity can be further enhanced by examination of larger stool samples.

The second issue pertains to infection intensity. The mean hookworm EPG among children according to the Kato—Katz results (155.8 EPG, i.e. a mean of 6 eggs counted per microscope slide multiplied by a factor of 24 to obtain the number of EPG) was significantly higher when compared with the FLOTAC technique (37.7 EPG, i.e. a mean of 23 eggs counted in the apparatus multiplied by a factor of 1.4—3). At first sight, this result raises concern about the ability of the FLOTAC method for quantification of infection intensity. Different factors might have contributed to the discrepancy of EPG between Kato—Katz and FLOTAC. Importantly, it has been shown before that the observed helminth infection intensity is a function of sampling effort; the more samples subjected to the Kato—Katz method, the lower the infection intensity. For example, the mean S. mansoni egg count among 253 schoolchildren from Côte d’Ivoire was 161 EPG based on a single Kato—Katz thick smear, whereas the observed infection intensity was 111 EPG after examination of four Kato—Katz thick smears derived from different stool samples (Utzinger et al., 2000). Whilst this issue actually challenges the accuracy of the Kato—Katz method for estimating helmint infection intensity, the point mentioned before that preservation of the stool samples in SAF might result in some hookworm eggs being broken or altered suggests that the mean EPG has been underestimated. This issue should be addressed in future investigations comparing mean EPG revealed by FLOTAC using either fresh or SAF-conserved stool samples.

It is important to note that the Kato—Katz technique and the FLOTAC method resulted in significantly higher EPG values than the ether concentration method (5.7 EPG). A 27.3-fold difference was found between the Kato—Katz and ether concentration methods, whereas the corresponding factor between FLOTAC and the ether concentration method was 6.6. Given the low sensitivity and the significant underestimation of infection intensity by the ether concentration method, this technique when used alone should be discouraged for hookworm diagnosis.

If the high sensitivity of the FLOTAC technique is confirmed by other groups in different settings and further improvements are made with regard to quantifying infection intensity (e.g. studies on fixatives), widespread use of the Kato—Katz technique for epidemiological surveys and monitoring of helmint control programmes is put in question. Particularly in settings where the overall prevalences and intensities of helmint infections are low, for example after repeated rounds of chemotherapy, results obtained by the Kato—Katz method must be analysed with care. New data suggest that the Kato—Katz technique has drawbacks for screening stool samples from infants, as helmint infection intensities tend to be low and stool samples are more liquid than those collected from school-aged children or adults (Goodman et al., 2007). This issue is of considerable relevance, as recent studies suggest that infants and pre-school children are at higher risk of helmint infections and associated morbidity than previously assumed (Goodman et al., 2007; Stothard and Gabrielli, 2007).
Three further aspects are worth highlighting. First, since the FLOTAC technique is able to examine stool quantities up to 1 g or more, there is no need for high multiplication factors for determining EPG. In the present study, the mean weight of stool samples subjected to hookworm diagnosis by FLOTAC was 0.6 g, and hence a multiplication factor ranging between 1.4 and 3 was used. Note that for a single Kato–Katz thick smear reading, the corresponding multiplication factor for the EPG calculation is 24, or in other words 8–17 times higher than for the FLOTAC technique. The negative implications of high multiplication factors for EPG calculations have been discussed elsewhere (Cringoli et al., 2004; Mes et al., 2001). Second, the FLOTAC technique is multivalent. Hence, the technique can be used for the simultaneous diagnosis of different helminths as well as intestinal protozoa. This issue is of relevance because multiple-species parasitic infections are the rule rather than the exception in the developing world and hence a combination of different diagnostic approaches is recommended (Brooker and Utzinger, 2007; Raso et al., 2004). Third, subsequent studies should address the feasibility of the FLOTAC technique when applied in epidemiological studies in resource-poor settings, including technicians’ satisfaction with this method and issues of costs and cost effectiveness.

In conclusion, our data suggest that the FLOTAC technique may improve the ability to diagnose human hookworm infections accurately. These findings need to be confirmed in other epidemiological settings where hookworm, other soil-transmitted helminths, S. mansoni and intestinal protozoa are prevalent. The FLOTAC method shows promise as an important new tool for individual hookworm diagnosis and for rigorous monitoring of helmint control programmes.

Authors’ contributions: JU, LR, MBZ and GC designed the study protocol; LKL, FR, ABT and EKN designed and carried out the field work; LKL prepared the stool specimens for the Kato–Katz and the ether concentration methods and examined the slides under a light microscope; LR, LKL and GC prepared the stool specimens for the FLOTAC method and examined the slides; JU, LR and GC drafted the manuscript. All authors read, revised and approved the final manuscript. GC is guarantor of the paper.

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Ethical approval: The study was embedded in a randomised controlled trial entitled ‘Iron fortification and parasite control to reduce anaemia among schoolchildren in Côte d’Ivoire’, which was approved by the institutional review boards of the Swiss Federal Institute of Technology (Zurich, Switzerland) and the Centre Suisse de Recherches Scientifiques (Abidjan, Côte d’Ivoire). The global study received ethical clearance from the Ethics Committee of the University and the State of Basel (reference number 224/06) and the ‘Comité National d’Ethique des Sciences de la Vie et de la Santé’ (reference number MSHP/CAB/CNESVS/06). The trial is registered as an International Standard Randomised Controlled Trial (reference number ISRCTN21782274).

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