Diagnosis, transmission and immunology of human Oesophagostomum bifurcum and hookworm infections in Togo

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Chapter 3

Diagnosis of *Oesophagostomum bifurcum* and hook-worm infection in humans: Day-to-day and within-specimen variation of larval counts.

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SUMMARY

O. bifurcum, as well as hookworm infections are hyperendemic among humans in northern Togo and Ghana. For parasite-specific diagnosis a coproculture is obligatory, because only the infective larvae, and not the eggs, can be distinguished morphologically.

The sensitivity of duplicate coprocultures from a single stool sample was found to be above 90% in comparison to a gold standard of ten coprocultures made from a single stool specimen. Prevalence of infection with *O. bifurcum* and hookworm further increased with the number of coprocultures made from each individual stool. Notwithstanding the high sensitivity, intensity of infection per individual varied considerably from day-to-day and the number of larvae found in different samples out of one stool also varied highly, both showing a heterogeneous distribution. Surprisingly, daily fluctuation and within-specimen variation could not be differentiated from each other, probably because of the variation created by the coproculture technique.

To estimate the intensity of infection, it is sufficient to make repeated coprocultures from only one individual stool sample. Laborious collection of stool samples on subsequent days does not give better estimates of the individual infection status.

INTRODUCTION

Oesophagostomum bifurcum is considered a common nematode of monkeys (Weinberg, 1908). In northern Togo and Ghana, however, it is highly prevalent among humans and a cause of significant morbidity (Polderman *et al.*, 1991). Encapsulated immature worms of *O. bifurcum* may cause tumour-like nodules leading to intestinal occlusion and abcedation (Gigase *et al.*, 1987; Polderman *et al.*, 1995).

Diagnosis of *Oesophagostomum* infections is hampered by the fact that the morphology of *O. bifurcum* eggs is identical to the eggs of hookworm (Blotkamp *et al.*, 1993), which is also highly endemic in the same region. Therefore, diagnosis based on egg identification, i.e. thick smears, will not be parasite-specific. Only after coproculture of eggs for a week, during which the larvae will hatch, is it possible to reliably differentiate the two parasites using characteristic morphological features present in the infective larvae (Little, 1981; Blotkamp *et al.*, 1993). The likelihood of detecting an infection

depends on the number and size of faecal samples examined, the intensity of infection and the daily egg-output of the worm (Hall, 1982).

Larval counts (as well as egg-counts) of *O. bifurcum* showed a good correlation with observed worm burdens (Krepel *et al.*, 1992), and therefore allow a semi-quantitative estimation of the intensity of infection (Krepel *et al.*, 1995).

In several other helminth infections, such as hookworm and *Schistosoma mansoni* infections, significant day-to-day variation of egg output have been carefully documented (Hall, 1981; Anderson & Schad, 1985; Polderman *et al.*, 1985; Engels, Sinzinkayo & Gryseels, 1996). In addition, the distribution of eggs in stools may be heterogeneous, such that different specimens taken from the same stool sample result in different estimates of the intensity of infection (Hall, 1981; Anderson & Schad, 1985; Engels *et al.*, 1997; Yu *et al.*, 1998). Low level infections may even remain undetected if only one stool examination is done (Barreto *et al.*, 1978; Polderman, 1979; Gryseels, Nkylikyinka & Engels, 1991). Anderson & Schad (1985) pointed out that as a result of this within-specimen variability and day-to-day fluctuation, egg-output should be interpreted in a qualitative way, and does not reliably reflect worm burden.

The aim of this study was to determine the sensitivity of the coproculture method for specific *O. bifurcum* and hookworm diagnosis and to assess to what extent the within-specimen and day-to-day variation influence reliability of diagnosis and determination of infection intensity.

MATERIAL AND METHODS

Study population

To determine day-to-day fluctuation, 25 randomly chosen school children (10-18 years) from Mire (Northern Togo) were asked to give stools for seven consecutive days, of which 3 g of stools were cultured each day. In a preliminary study in the same village, the prevalence of infection with *O. bifurcum* and hookworm was 57% and 79%, respectively (n = 112, age range: 4-60 years).

To determine within-specimen variation, 10 coprocultures were made from a single stool specimen taken from 41 individuals coming from two endemic villages: Pana and Lotogou. In a previous study, prevalences of infection with *O. bifurcum* and hookworm, were 37% and 78% (Pana), and 54% and 80% (Lotogou), respectively.

Adults gave informed consent; parents and the school director gave consent for the children. All participants found infected were treated with Albendazole.

Coproculture

From each stool sample a modified duplicate coproculture was made as previously described by Polderman *et al.*, (1991). Briefly, 3 g of faeces, weighed on a scale, were mixed with an equal quantity of "vermiculite", divided in two and placed on moist filterpaper in two petri-dishes. Stools were cultured for a week and stirred every day to reduce the growth of maggots and fungi. Larvae migrated from the faeces to the clean water surrounding the filterpaper. On day 7, the culture fluid was poured off into a conical tube, the petri-dish was rinsed and the water added to the conical tube. After two hours of sedimentation, 100 µl of sediment was taken up with a micropipette and examined microscopically at low magnification (4x10), larvae were identified and counted by species. To minimize between-observer variation, all cultures were made (H.S.) and read (W.dG.) by the same person.

Statistical analysis

For the calculation of the sensitivity, the prevalence after 10 coprocultures is used as gold standard (i.e. if at least one coproculture was positive the person was considered infected). The sensitivity of the coproculture-method within a specimen was calculated by comparing the proportion positive cases after a single or more coprocultures with the gold standard. The overall trend in cumulative proportions of positives has been calculated on the basis of all possible permutations. This means that the chronological order in which the coprocultures were read was not explicitly taken into account. For example, if 10 coprocultures are made from an individual stool, the cumulative prevalence of 2 coprocultures is the average of 45 possible combinations (1 and 2, 1 and 3, ... ,9 and 10).

It was not always possible to make 10 coprocultures, for 4 patients 9 coprocultures could be made, and for one patient 8 cultures. In those cases the prevalence after 9 and 8 coprocultures respectively was considered as gold standard.

The day-to-day and within-specimen variation of each infected person is given by an individual coefficient of variation: CV= (standard deviation /

mean) x 100%. As a first simple comparison, the geometric mean of the coefficients of variation of all individuals was used to compare day-to-day and within-specimen variation.

In order to more adequately compare the day-to-day and within-specimen coefficients of variation, the same amount of faeces examined (3g) had to be considered. Therefore, 20 random combinations of duplicates were made from the 10 single coprocultures of each individual from Pana and Lotogou. Theoretically, $n!/((n/2)!.2^{n/2})= 945$ combinations of 5 duplicate coprocultures can be made out of n=10 coprocultures. The standard deviation per person is the average of the 20 standard deviations found.

The Kolmogorov-Smirnov two samples test was used to compare the complete frequency distributions of the day-to-day coefficient of variation and the within-specimen coefficient of variation, for both *O. bifurcum* and hookworm.

RESULTS

Due to day-to-day fluctuation and within-specimen variation, the percentage of people found to be infected increases with the number of cultures made (Figure 1). After collecting stool-specimens for 7 days, all of the 25 children from Mire appeared to be infected with *O. bifurcum* and hookworm. Four children (16%) had at least one negative stool and thus infection with *O. bifurcum* would not necessarily have been detected with only one sample. If stools are collected on one day only, the observed prevalence of infection with *O. bifurcum* is 92.5%, increasing to 97.3% after 2 stool collections. The coprocultures made from each stool of these 25 children over 7 days contained all hookworm larvae.

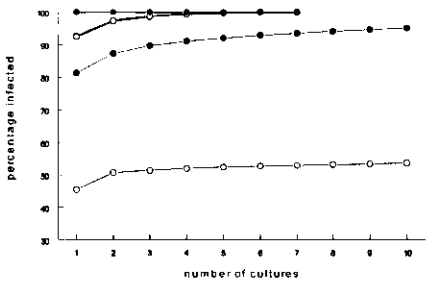

Fig. 1. Cumulative percentage of O. bifurcum (o) and hookworm (•) infection after one or more coprocultures made from a single stool sample of 41 individuals from Pana and Lotogou (—); and after 1-7 days of stool examinations among 25 children from Mire (__).

Table 1. Sensitivity of the coproculture technique at different intensities of Oesophagostomum bifurcum or hookworm infection.

Mean no of larvae/10 coprocultures	*O. bifurcum*				Hookworm			
	n	sens 1	sens 2	sens 3	n	sens 1	sens 2	sens 3
0.1- 9.9	6	65.7	80.3	85.6	12	59.5	74.4	81.5
10.0-32.9	7	84.5	96.7	99	15	95.3	99	99.8
33.0+	9	98.8	100	100	12	99.1	100	100
Total	22	84.5	92.9	95.3	39	84.9	91.3	93.9

(n = number of patients; sens 1= sensitivity for 1 coproculture/stool; sens 2 = sensitivity for duplicate coprocultures/stool, sens 3 = sensitivity for 3 coprocultures/stool)

In Pana and Lotogou, 184 out of 404 (45.5%) cultures made from 41 individuals contained *O. bifurcum* larvae. In 53.7% of these individuals at least one culture contained *O. bifurcum* larvae, if 10 cultures were conducted. With a duplicate culture 94% of the *O. bifurcum* infected patients were already detected, corresponding to a prevalence of infection of 50.7%. For the diagnosis of hookworm infections in Pana and Lotogou, the percentages of infected people increased with increasing numbers of cultures made from a single stool specimen. Only 81.3 % of the 41 individuals were found infected with hookworm if one coproculture was conducted, whereas 95.1% of these individuals appeared to be infected if 10 coprocultures were made.

The percentage of people detected as being infected depends not only on the number of coprocultures made per person, but also on the intensity of infection in that population. The Table shows that, in this study-population, 80% of the light *O. bifurcum* infections (mean number of larvae 0.1-9.9) are correctly diagnosed with a duplicate culture, but 97% sensitivity was achieved if more than 10 larvae were found in a coproculture. The sensitivity of the coproculture was similar for hookworm detection, 74% of the light infections were detected with duplicate cultures, and the sensitivity was 99% if more than 10 larvae were recovered (Table 1).

The intensity of infection with *O. bifurcum* varied from very light (2 larvae in 21g of stools) to extremely

high (7193 larvae in 18g of stools) in the 25 randomly chosen children from Mire. There was a considerable variation in the number of larvae found in individual stools on different days, with e.g. 4 larvae on day one and 206 larvae the next day in the same person.

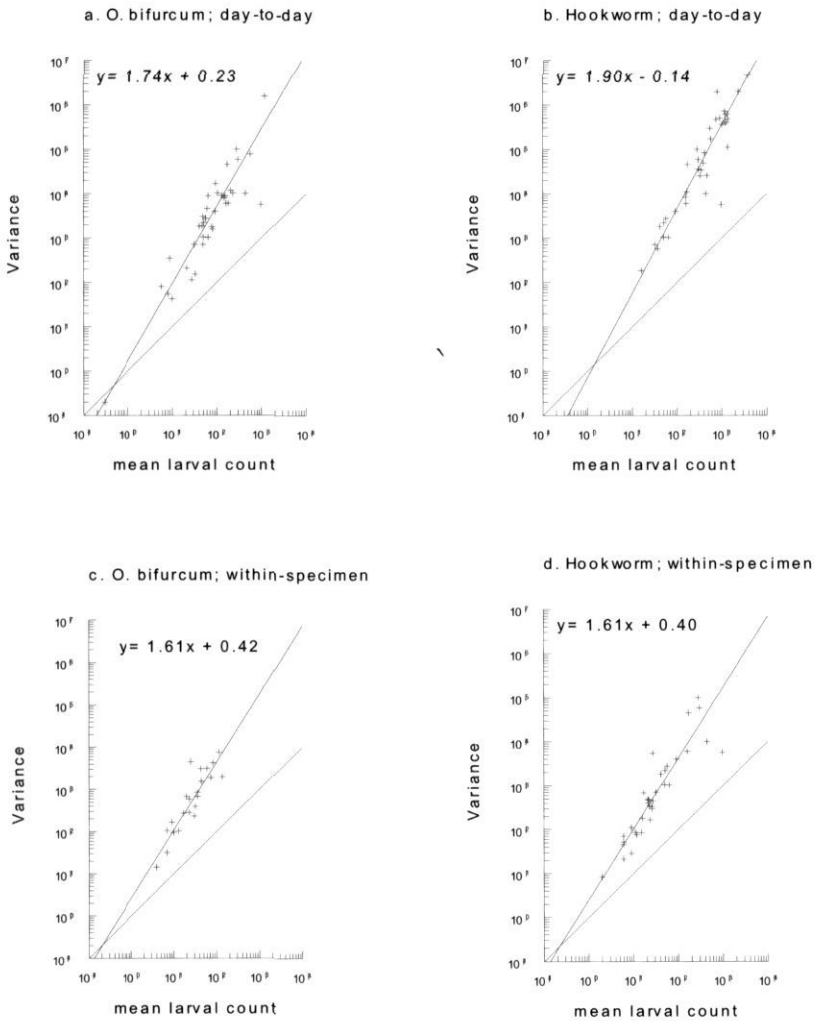

Fig. 2. Scattergrams of the variances and mean larval count in coprocultures made from 7 different stools from each individual with O. bifurcum (a) and hookworm (b), and in 10 coprocultures from one stool specimen (c and d). (----) variance = mean, which would represent a homogeneous distribution of larval-counts; (—) best fitting linear regression line (log variance = b × log mean + a).

The geometric means of the coefficients of variation were 77% and 62% for *O. bifurcum* and hookworm, respectively.

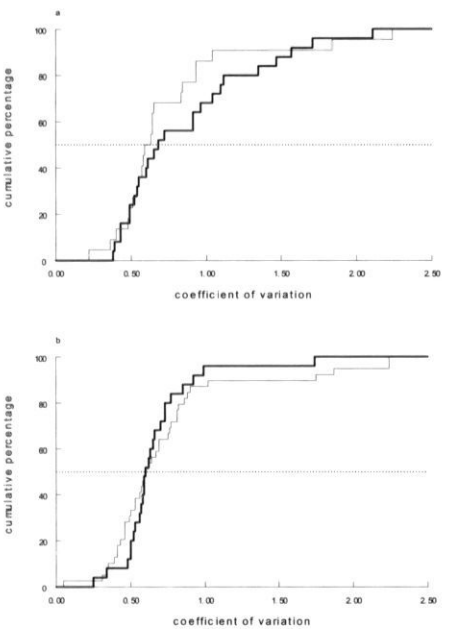

Fig. 3. Cumulative frequency of the day-to-day coefficient of variation (thick line) and the within-specimen coefficient of variation (thin line) for O. bifurcum (a) and hookworm (b). According to Kolmogorov-Smirnov's two samples test there is no significant difference between the frequency distribution of both coefficients of variation, p= 0.602 for O. bifurcum and p= 0.563 for hookworm.

The variance increases with increasing intensity of infection (Figure 2a,b). Variance exceeding the mean, as in this case, is indicative of a heterogeneous distribution of the number of eggs daily found in the stools.

If 10 coprocultures were made from one stool sample, each coproculture contained a variable number of larvae. For example one coproculture contained 6 larvae and another coproculture, of the same stool, contained 172 larvae. The average coefficient of variation of the number of *O. bifurcum* larvae found was 66%, with a mean of 18 larvae. Similarly, for hookworm infections, the coefficient of variation was 64%, with a mean of 18 larvae found. Again the variance increases with increasing intensity of infection and the variance exceeding the mean indicate a heterogeneous distribution of the eggs in the faeces (Figure 2c,d).

Cumulative frequencies of the day-to-day coefficient of variation and the within-specimen coefficient of variation are both plotted in one graph for each nematode (Figure 3). For a more precise comparison of the coefficients of variation, the same amount of faeces (3g) has been considered. There was no significant difference between the cumulative frequency of the day-to-day coefficient of variation and the within-specimen coefficient of variation, for either of

the species (Kolmogorov-Smirnov: p= 0.602 for *O. bifurcum*, p= 0.563 for hookworm).

DISCUSSION

Where hookworm and *O. bifurcum* infections co-exist, parasitological diagnosis can not be based on egg differentiation, other stages of the worm have to be identified (Blotkamp *et al.*, 1993). The third-stage larvae (L3) cultured from fresh stools of *O. bifurcum* infected subjects are morphologically different from those of other helminths. Therefore, in our research, diagnosis is based on the detection of third-stage larvae.

If using coprocultures as the basic tool for diagnosis, differences in efficacy of the eggs and larvae to grow into infective larvae will be a source of variation in the number of larvae found. In addition, day-to-day variation in faeces composition and egg production, clustering of eggs in the stool sample, and differences in volume examined, will obscure a proper estimation of the prevalence and intensity of infection (Anderson & Schad, 1985; Hall, 1981; Engels *et al.*, 1996).

To achieve a better understanding of the transmission dynamics of *Oesophagostomum* infections in man, it is necessary to quantify worm loads of infected individuals over time. For this the sensitivity of stool-cultures as a diagnostic tool, the validity of counting L3 larvae, the variation of the larval counts in infected individuals, and the variation of counts over time need to be established and the causes for variation need to be analysed. The prepatent periods of *Oesophagostomum* and hookworm are measured in weeks, while their reproductive life span may be measured in years (Hoagland *et al.*, 1978). Therefore, we considered the worm burden to be constant over a short period of seven days, and this time-span suitable to determine day-to-day variation of egg production.

Both in *Oesophagostomum* and hookworm infections, the sensitivity of a single stool duplicate coproculture was of above 90% in comparison to a gold standard of 10 coprocultures made from a single stool. Obviously, sensitivity depends on intensity of infection. Thus, we can only conclude that prevalence of infection can reliably be determined with a duplicate coproculture in a village with comparable prevalences and intensity of infection as our study villages.

Determination of the worm burden in intestinal helminths is also obscured

by day-to-day and within-specimen variation (Hall, 1981; Anderson & Schad, 1985). Since hookworms live in the small intestine, and caecum and colon are the principal blenders, a better mixture of egg and faeces could be expected as the faeces stay longer in the intestine (Hoagland *et al.*, 1978; Hall, 1982). Yet, poorly mixed hookworm eggs have been detected by Hall (1981) if 25 mg sample of faeces were examined. In our study, a much higher quantity of faeces was examined, and less within-specimen variation would be expected. Still, there was a considerable variation in the number of larvae found in different samples out of one stool specimen. The intensity of infection per individual varied also considerably from day-to-day: the coefficient of variation of 77% was within the range found previously for *S. mansoni* egg-output (Barreto *et al.*, 1978; Engels *et al.*, 1996) and for hookworm egg-output (Hall, 1981). The daily variation in the faecal bulk (Hall, 1981) can change the concentration of parasite eggs in the stools (Scott, 1938) and thus might influence the number of eggs found (Hall, 1982). Our observations indicate, however, that within-specimen variation did not differ significantly from day-to-day variation. This means that either there is no specific day-to-day component or variation created by the coproculture technique is so high that within-specimen and day-to-day variation can not be distinguished. Where diagnosis was based on egg count techniques, day-to-day variation and within-specimen variation could clearly be differentiated (Hall, 1981; Anderson & Schad, 1985). Therefore, the absence of a particular day-to-day fluctuation component is quite unlikely, and the high variation should be attributed to the coproculture method. Indeed, new experiments show a considerable variation of the coproculture technique on the larval recovery (Pit *et al.*, in prep.).

In conclusion, prevalences of infections in an *O. bifurcum* and hookworm endemic population tend to be only slightly underestimated by means of a single coproculture. The commonly applied duplicate coproculture seems to provide an adequate estimate of the prevalence. The determination of intensity of infection is obscured by day-to-day, within-specimen and also methodological variation. For a proper assessment of infection intensity, repeated stool examination can as well be done on the same stool sample. Laborious stool collection of new samples on subse-

quent days does not considerably add to the sensitivity of the coproculture procedure.

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