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

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## **Chapter 9**

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### **General conclusions and discussion**

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## GENERAL CONCLUSIONS

In the past century incidental clinical cases of human *Oesophagostomum bifurcum* infections have been described from all over the world, therefore, the disease was considered as a rare zoonosis. After the report from Gigase *et al* (1987) of 54 cases of human oesophagostomiasis in Dapaong (northern Togo), field research was carried out for several years. Coprocultures allowed parasite specific diagnosis of *O. bifurcum* in an hookworm endemic area. Human infections with both parasites appeared to be very common in northern Togo (Krepel, 1994). The observations described in the present thesis lead to the following conclusions on human infections with *O. bifurcum*:

- In northern Togo more than 100 000 people (30% of the population) are infected with *O. bifurcum* and 230 000 (70%) are infected with hookworm. Women are more often infected with *O. bifurcum* than men (chapter 3).
- Collection of stool samples on subsequent days does not give better estimates of the individual infection status with *O. bifurcum* and hookworm than multiple coprocultures from one stool sample (chapter 2).
- Treatment of an *O. bifurcum* infected population before the rainy season results in rapid reinfection during the rains. When treatment was given after the rains, larval counts remain low until the following rainy season (chapter 4).
- *O. bifurcum* larvae cultured from human stools, unlike the larvae of *N. americanus*, are shown to survive desiccation for a prolonged period of time. Desiccated larvae can be rehydrated in gastric juices suggesting the possibility of dust-born infections (chapter 5).
- Anti-*O. bifurcum* IgG4 antibodies can be measured in sera from northern Togo as well as from central Togo (where *O. bifurcum* larvae are never found in stool cultures). Absorption experiments show that *N. americanus* specific IgG4 antibodies may cross-react with *O. bifurcum* antigens. In contrast to IgG<sub>4</sub>, *O. bifurcum* specific IgE antibodies are detected in sera from individuals from northern Togo (*O. bifurcum* endemic), but not in those from central Togo (chapter 6).
- Co-infection with *O. bifurcum* and *N. americanus* suppresses parasite-specific cellular respon-

siveness but will not direct cytokine production towards dominant Th1 or Th2 type responses (chapter 7).

- The use of PCR to amplify *O. bifurcum* and hookworm specific DNA in faecal samples is a highly specific and sensitive method for the differential diagnosis of the two infections (chapter 8).

During the field research on human *O. bifurcum* infections in northern Togo, additional observations were made which are important for a better understanding of the different aspect of the diagnosis and transmission of *O. bifurcum*. These additional observations are presented in a different letter type. The significance and implications elicited by some of these additional findings are discussed below.

## DIAGNOSIS

In general, the ideal diagnostic tool should be highly sensitive and highly specific. For most tests, sensitivity depends on the prevalence and intensity of infection within the study population. In general, the preferences for a specific diagnostic tool will depend on the number of people to be examined, the prevalence and intensity of infection in the study

population, the available laboratory equipment, the professional skills of the technical staff and the financial possibilities.

*O. bifurcum* infections can be diagnosed with different methods, depending on the question that needs to be answered. Diagnosis based on the results of coproculture, serology or the use of PCR for parasite-specific DNA detection, may be used for transmission studies in a community, as described in this thesis. Individual pathology is clinically diagnosed, if necessary assisted by ultrasound (Storey *et al.*, 2000). In such cases, however, coproculture cannot be used as a diagnostic tool, because pathology is caused by the encapsulated larvae and not by the egg producing adult worms in the intestinal lumen. The diagnostic value of parasite-specific serology and DNA-amplification for individual diagnosis has to be evaluated further.

### **The coproculture method**

The coproculture allows a parasite-specific diagnosis in a region where both *O. bifurcum* and hookworm are endemic. The development from egg to larvae in the coproculture was investigated in additional experiments.

Eight (8) parallel coprocultures made from 5 individual stools,

showed that the maximum number of larvae can be found after 11 days of culture, but 42% of those larvae can already be found after 5 days. After 7 days of culture, 92% of the total number of larvae can be detected, allowing a practical working schedule of one week.

The use of a large quantity of stools (3 g) in the coproculture should permit a close estimation of the worm burden. Larval counts, as well as egg-counts, showed a good correlation with the observed worm burden, when the Kato-Katz methodology and coprocultures were compared (Krepel *et al.*, 1992b), and therefore, allowed a semi-quantitative estimation of the intensity of infection (Krepel *et al.*, 1995a). In several other helminth infections, such as hookworm and *Schistosoma mansoni* infections, significant day-to-day variation of egg-output and heterogeneous distribution of the eggs in stools have been documented (Hall, 1981; Anderson & Schad, 1985; Polderman *et al.*, 1985; Engels *et al.*, 1996, 1997; Yu *et al.*, 1998). In our study, within-specimen variation did not differ significantly from day-to-

day variation, probably because of the variation created by the coproculture technique (chapter 2). Differences in efficacy of eggs to develop into infective larvae will be a first source of variation in the number of larvae found. In a previous study conducted by Krepel *et al.* (1992b), larval counts after coproculture were relatively low and never exceeded 10% of the eggs found in the Kato-Katz smear.

In an additional unpublished study (table 1), however, modified methodology and repeated examination of culture fluid improved larval recovery (median 22%). But larval hatching in coproculture showed extreme variability: sometimes only a few larvae were found in the coproculture from a patients' stool, even though the corresponding Kato-Katz smear contained many eggs. In several other samples, coproculture revealed much higher larval counts (up to 458%) than the initial egg-counts in stool samples would have suggested. The observed hatching index of 22% corresponds well with that of 24% found for *N. americanus* egg viability and larval recovery (Udonsi, 1988).

*Table 1: Hatching of O. bifurcum and hookworm larvae in coproculture. One 25 mg Kato-Katz-smear was prepared from stools from 34 infected individuals, and examined within 30 minutes of preparation. The result is given as number of eggs per 3g faeces. In addition, a duplicate coproculture (3 g) was made from each stool.*

Patients		Kato-Katz	Coproculture	larval hatching in
		Number eggs/3g stools	number L3/ 3g stools	%
n = 34	Median	1500	322	22%
	(range)	(120-61560)	(1-2396)	(0-458)

A second source of variation in larval counts is a consequence of the method used to recover larvae from the culture fluid; i.e. a variable number of larvae stays in the petri-dish after pouring the culture fluid into the tube, and a variable percentage of the total number of larvae is taken up in the 100 µl of sediment to be examined.

To evaluate these possible variations of the coproculture method, coprocultures were made from stools from 31 infected individuals, and processed as described in chapter 2. Each petri-dish was washed 5 additional times, and each wash fluid collected in a separate conical tube, i.e. 6 collection tubes were examined per sample.

The total number of larvae in each tube was determined by taking up 100 µl of sediment every two hours until no larvae were found in two successive ex-

aminations. The sum of larvae found in tube 1 to 6 was considered as the total number of larvae present in the coproculture (or petri-dish).

The total number of *O. bifurcum* and hookworm larvae found per coproculture ranged from 2-199 and 4-475, respectively (Table 2, column a). For both parasites, at least 90% of the total number of larvae present in the entire coproculture (a) was found in the first tube (d). In the first sediment of the culture fluid, 45% of total *O. bifurcum* and 46% of hookworm larvae were found (b), and recovery increased further with multiple sampling of the sediment (c). However, the recovery rates of larvae from culture fluids at the first examination varied greatly (0%-90%), and in some cases, even duplicate readings would not approach the total number of larvae present in the tube.

Table 2: Quantitative recovery of *O. bifurcum* and hookworm larvae from coproculture according to the number of culture fluids considered and the number of examination of the first culture fluid. Percentages are calculated as compared to the total number of larvae found in the coproculture.

		a	b	c	d
<i>O. bifurcum</i>	Median	20	9	13	18
	Range	2-199	0-122	1-175	2-197
	Median %	--	45%	65%	90%
	Range %	--	0-74%	15-100%	60-100%
Hookworm	Median	65	30	52	64
	Range	4-475	1-188	4-306	4-471
	Median %	--	46%	80%	98%
	Range %	--	16-90%	51-100%	68-100%

a= total number of larvae found per coproculture; b = number of larvae found in the first 100  $\mu$ l examined of the first tube; c= number of larvae found in the first 200  $\mu$ l (2 x 100  $\mu$ l) examined of the first tube; d= total number of larvae found in the first tube

Consequently, for correct determination of larval recovery, the culture fluid sediment should be examined several times, allowing the larvae to settle on the bottom of the tube for at least two hours after each examination. This time-period cannot be shortened by centrifugation of the tubes, probably because this makes the larvae whirl in the tube. The larvae are best immobilised with a drop of 1/15 diluted Lugol on a microscope-slide. Heating the tubes or adding formalin to the culture fluid reduces visibility for the characteristic features of both *O. bifurcum* and hookworm larvae.

In conclusion, the variation in the number of larvae found is due to

- The variation in the day-to-day excretion of the eggs by the female worm.
- The variation in the distribution of the eggs in the feces.
- The variation in the hatching capacity of the eggs.
- The variation in the number of larvae recovered from the petri-dish.
- The variation in the number of larvae picked up from the sediment

Despite the high variability, coproculture remains obligatory for parasite-specific diagnosis in concurrent *O. bifurcum* and hookworm infections. However, for each specific application a compromise between



accuracy and operationality has to be established.

### **Other diagnostic tools**

Apart from high variability which may lead to an underestimation of the prevalence of infection in low endemic regions, the coproculture method has some additional shortcomings, such as the need to process the stools immediately and the time lag of a week before the results can be obtained. In addition, pathology of *O. bifurcum* infections is not caused by the lumen-dwelling, egg laying worm but by the migrating and highly immunogenic larvae encapsulated in the intestinal wall. An alternative diagnosis might be based on serology. The detection of parasite-specific IgG4 antibodies in the serum of infected patients gave promising results (Polderman *et al.*, 1993). However, further research showed that serological diagnosis was hampered by cross-reactivity between *N. americanus* specific IgG4-antibodies and *O. bifurcum* antigen. The detection of IgE antibodies against *O. bifurcum* or hookworm was more specific, but not sensitive enough to detect all infections (Chapter 6). Therefore, IgE specific serology would need further improvement. Other shortcomings of the ELISA technique are the diffi-

culty to collect antigen, and blood from patients, the costs and the necessity of a well-equipped laboratory.

The PCR used to detect parasite specific DNA in frozen stool samples was 100% specific, and highly sensitive (93%) as compared to coproculture (Chapter 8). This new method should allow to screen people, when diagnosis in the field, based on coproculture is not feasible. However, the need for high-standard laboratory facilities may restrict the use of PCR in developing countries.

## **TRANSMISSION**

### ***Distribution of human Oesophagostomiasis***

The prevalence of infection with *O. bifurcum* and hookworm was determined in 65 villages of northern Togo and was found to be 30% and 70 %, respectively (Chapter 3).

Additional surveys in villages south of the study area (Mogou, Sagbadai, Sokode, Lama Tessi) (Figure 1) showed that infections with *O. bifurcum* in humans were definitely confined to the most northern region of Togo. In addition, surgeons from central Togo claimed to have never seen patients with a typical "tumeur de Dapaong". Dr Baeta, who recorded 54 cases of human oesophagostomiasis during his

four years' stay in Dapaong, encountered only one case during his years of practice in Lomé. This case concerned a 10 year old boy that came originally from northern Togo (Krepel, 1994).

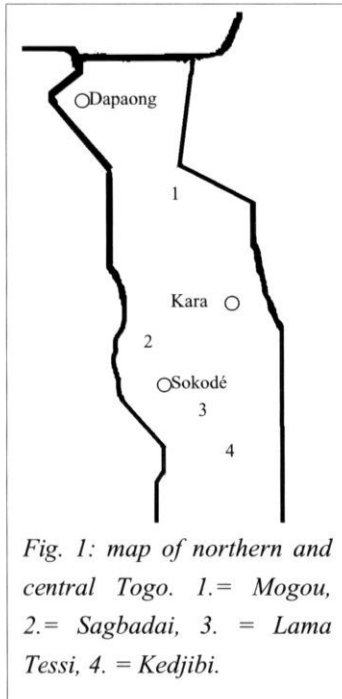


Fig. 1: map of northern and central Togo. 1.= Mogou, 2.= Sagbadai, 3. = Lama Tessi, 4. = Kedjibi.

Moreover, stools were collected from 90 emigrants from northern Togo, now living since 10 years or less in a village (Kedjibi) (Figure 1) in the central region of Togo, approximately 50 km south of Sokodé. The village is quite remote and difficult to access from the main road. The whole populations of Kedjibi originated from villages in northern Togo, in which prevalences of 78% had been measured (Chapter 3). At

first sight the living conditions of the people seemed similar to those in northern Togo, i.e the village and the houses were set up in a characteristic style different from the villages in central Togo. From each individual the village of origin and the duration of stay in Kedjibi were registered, as well as their infection status (based on coproculture). The average duration of stay was 5 years. Fifty eight percent (58%) of the population of Kedjibi was infected with hookworm. But, remarkably, only 3 individuals were infected with *O. bifurcum*. They had left northern Togo less than 3 years ago.

Apparently the conditions necessary for transmission of *O. bifurcum* were absent in central Togo, even though transmission with hookworm was not hampered and the moist environment of central Togo would seem to facilitate larval development.

Different experiments described in Chapter 5 show that the larvae of *O. bifurcum* are extremely sturdy, and will survive adverse environmental condition such as desiccation for prolonged periods of time, high acidity or even freezing.

The precise mode of transmission of *O. bifurcum* infections in humans is not yet known, but analogy to *Oesophagostomum spp.* infections in

animals suggest that transmission is direct and oral (Polderman & Blotkamp, 1995).

The population of northern Togo lives in small gatherings of clay huts called "soukoula's". Pigs, goats, sheep and cattle are raised close to the house. Few houses have latrines, and most people defaecate in the fields surrounding the compounds. Transmission could be the result of ingestion of contaminated soil, in which case the larvae should be found in the soil.

A hundred soil samples were collected at these defaecation sites in highly infected villages, between May and October 1996. The samples were analysed with the Baermann method described in Chapter 5. All samples contained different plant-nematodes and in 7 samples, *Oesophagostomum* larvae were found. These larvae have been collected and will be analysed by PCR. In addition 20 villagers were asked to wash their hands in a bucket with clean water. The washing water was collected and the sediment was examined microscopically. It contained plant-nematodes only, but potentially such close contact with soil and low hygiene standards could easily cause infection with *O. bifurcum*.

Transmission is mainly confined to the rainy season (Krepel *et al*, 1995b; Chapter 4), when the larvae have a better chance to develop in the moist environment. In that period the fields are being laboured and the population is in close contact with the (contaminated) soil.

The population of northern Togo lives in close contacts with pigs. Many authors have commented on the role of pigs in the spread of intestinal helminths (Ackert & Payne, 1922; Jones, 1976). By eating contaminated human faeces pigs can either inactivate the eggs or disseminate *O. bifurcum* and hookworm infections by transporting the eggs from the defaecation site to the house.

In northern Ghana, four parasite-free pigs were fed fresh faeces, from patients heavily infected with both helminths. Four to five percent of the viable *O. bifurcum* and *N. americanus* eggs were retrieved as third-stage larvae after coproculture of the pigs' faeces (Steenhard *et al.*, 2000). Pigs are free to scavenge during the dry season, and it is reasonable to postulate that they may act as transport hosts, relocating eggs from the defaecation sites to the human housing area, creating a reservoir of infection, and thereby intensifying

the contact between the infective larvae and their hosts. It is also possible that preventing pigs from consuming human faeces during the rainy season (when they are tied up to keep them from destroying the growing crops), is a factor that contributes to the intense transmission of *O. bifurcum* and hookworm during this season, because 95% of the *O. bifurcum* eggs are inactivated by the pig. (Steenhard *et al.*, 2000).

#### ***The impact of treatment on O. bifurcum infections***

The goal of treatment may be twofold: control of morbidity and control of transmission. *O. bifurcum* infection-related morbidity is very difficult to quantify because most individuals in northern Togo are simultaneously infected with many other parasites (hookworm, *Schistosoma spp.*, *Plasmodium spp.*, and intestinal protozoa) and therefore may experience multiple disease processes such as abdominal discomfort, diarrhea or obstruction, anaemia and hepatomegaly. Only some *O. bifurcum* infected individuals suffer from the infection by presenting a “tumeur de Dapaong”. The success of treatment of these cases can be monitored by sonography (Storey *et al.*, 2000)

The use of anthelmintic drugs in mass treatment campaigns with the objective to reduce transmission has been extensively described for many parasites (Anderson & Medley, 1985; Anderson & May, 1985; McCallum, 1990; Chan *et al.*, 1994). Despite efficient chemotherapy, control programs are often not effective due to the rapidity by which average intensity of infection in treated individuals reaches pre-control levels following cessation of treatment. In general terms, it has been described that successful control of transmission depends on various factors such as the reproductive life expectancy and the egg production of the worm, the parasite load and its aggregation in the population, the effect of the drug and the most effective treatment schemes (Anderson & May, 1982; Anderson & Medley, 1985; Anderson, 1986).

Earlier observations suggested that transmission of *O. bifurcum* is mainly confined to the rainy season (Krepel *et al.*, 1995a) and that Albendazole kills adult *O. bifurcum* as well as hookworms in the intestinal lumen (Krepel *et al.*, 1993). Chapter 4 describes the reinfection patterns with *O. bifurcum* and hookworm after treatment with Albendazole at different seasons of the year. Reinfection is confined to the rainy sea-

son i.e. following treatment after the rains, larval counts remain low until the next rainy season.

The effect of treatment on different age categories of the study population described in chapter 4 is analysed further.

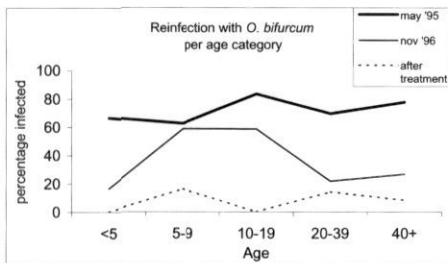


Fig. 2: Prevalence of infection with *O. bifurcum* per age category, at the onset (—, May '95) and end (—, November '96) of the study and right after treatment (---) with Albendazole

Figure 2 shows the prevalence of infection with *O. bifurcum*, per age category at the onset (May '95) and end (November '96) of the study and right after treatment. Treatment of *O. bifurcum* infection is not age dependent, all age categories showed low prevalences of infection directly after treatment. However, reinfection with *O. bifurcum* is mainly confined to individuals between 5 and 19 years. The prevalence of infection with *O. bifurcum* in individuals younger than 5 years or older than 20

years remains low after treatment and possible re-exposure.

Repeated selective treatment of heavily infected individuals as well as the most infected age-groups could be a practical approach to the control of transmission within a community, but this strategy needs further research and evaluation in the endemic area.

The study described in chapter 4 shows that treatment is most effective when given just after the rains, but transmission may still continue, because only randomly selected groups of individuals were treated. For ethical reason all participants were treated at the end of our study. The rapidity of reinfection, when all individuals from a well-defined section of the village were treated in the dry season, was determined in two additional surveys.

After the last survey in November 1996, all participants (n= 197) of the study described in chapter 4 were treated with Albendazole in the following doses: 200 mg for a body weight of less than 20 kg, 400 mg for 20 to 40 kg body weight, 600 mg for 40 to 60 kg body weight and 800 mg for individuals over 60 kg, given as a single oral doses. The tablets were swallowed under direct su-

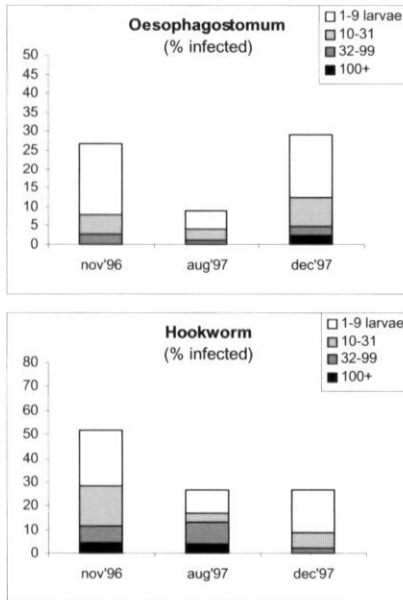


Fig. 3: Prevalence and intensity of infection and reinfection after treatment in November 1996 of the whole study population described in chapter 4.

pervision. Follow-up surveys were conducted in August and December 1997. Stool samples were processed as described in chapter 4. Nine months post treatment (p.t.) only 9% of the population was infected with *O. bifurcum*, and 27% with hookworm (figure 3). Thirteen months p.t., though, prevalence and intensity of infection with *O. bifurcum* had almost reached pretreatment level, while prevalence of infection with hookworm was still lower than before treatment.

Since Albendazole is not 100% efficient, a single mass treatment cam-

paign will never eradicate the parasite from the endemic community; but treatment of a whole population, once a year, at the end of the rainy season, may keep transmission low.

## FURTHER PROJECTS

The research described in this thesis has shown that there are good diagnostic methods i.e. coproculture, parasite-specific ELISA and PCR, which may be useful measures for monitoring and surveillance in disease control. In addition, longitudinal studies on the efficacy of treatment have shown that it is likely that both hookworm and *O. bifurcum* can be controlled by periodical treatment. Such an approach might not result in complete eradication of both parasites but will probably have beneficial effect on the reinfection pattern and morbidity, which, however, requires further evaluation.

In addition, and for purposes to investigate co-infections with *O. bifurcum* in hookworm-endemic areas, the IgE-based serology, as well as the DNA-based diagnosis need further refinement before it can be used on a large and systematic scale. Also, genome analyses of *O. bifurcum* should allow better classification of the parasite by comparing

*Oesophagostomum* spp. from monkey, pigs and those found in humans.

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