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

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Chapter 7

The capacity of L_3 larvae of *Oesophagostomum bifurcum* to survive adverse conditions

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SUMMARY

Human infections with the intestinal nematode *Oesophagostomum bifurcum* are commonly found in the Sudan Savannah of northern Togo and Ghana. Apparently, the long hot, dry season in this region, does not prevent transmission, which is believed to take place through ingestion of the infective third stage larvae (L_3). *Oesophagostomum* L_3 cultured from human stools, unlike the larvae of *Necator americanus*, were shown to survive desiccation for a prolonged period of time. In addition 93% the *O. bifurcum* L_3 frozen for 24 hours at $-15^{\circ}C$ regained motility when brought back into ambient temperatures. The L_3 also larvae survived the acidity of an artificial mixture made to resemble the gastric juices of human. Desiccated larvae could even be rehydrated in this mixture, indicating the possibility of dust-born infections. The sturdiness of the L_3 is likely to contribute to the high transmission in northern Togo and Ghana.

INTRODUCTION

Oesophagostomum bifurcum is a very common nematode parasite of humans in northern Togo and Ghana, with local prevalences of 60% and more (Polderman *et al*, 1991; Pit *et al., 1999*). Elsewhere, the parasite has been encountered only occasionally in humans; incidental cases have been described in a number of African countries (Guinea, Ivory Coast, Nigeria, Uganda, Sudan, Ethiopia, Kenya), in South East Asia (Indonesia, Brunei, Malaysia) and (as a single case) in Brazil. It is likely, however, that such infections may be more common outside Togo and Ghana than believed hitherto, because infection is easily missed.

Oesophagostomum eggs excreted in faeces can not be differentiated microscopically from the eggs of hookworm. The only morphological way to identify which eggs are present is to produce third-stage larvae (L_3) from them, in stool cultures. The L_3 develop through two moults after excretion of eggs by the final host. The development from freshly laid egg to third stage larvae takes 4-7 days, depending on the environmental conditions (Spindler *et al.*, 1936). The *Oesophagostomum*-endemic area in northern Togo and Ghana is characterised by a Sudan type climate with a long hot, dry season (October-April) followed by a rainy season (May-September). The relationship between climatic factors and transmission of parasitic infections is not easily understood.

Oesophagostomum species can infect a range of animals both in tropical and in temperate regions and

observations have been made on the ability of their L_3 to endure periods of low temperature or of drought (Shanker, 1970; Fossing, 1995). As in *Oesophagostomum* infections of pigs and ruminants, humans are thought to be infected through ingestion of the infective third stage larvae. If this is the route of transmission, the L_3 must presumably be able to survive the adverse conditions of the stomach, (i.e. low pH), if they are to establish a patent infection. In ruminants and pigs, infection would seem to follow a logical route: meadows are polluted with droppings containing eggs, which develop into infective larvae and are ingested by grazing animals. Although the route of transmission to humans is less obvious, the factors that influence survival of the infective larvae until they infect humans are likely to be of great importance in transmission.

In northern Togo and Ghana, *Ascaris* and *Trichuris* transmission is very inefficient; few individuals infected with these parasites can be found in the region, even though many infected people enter the area from the south. In contrast, human infections with *Oesophagostomum* are extremely common, indicating that *Oesophagostomum* larvae survive the environmental conditions that kill the eggs of other parasites.

In temperate zones, some nematode species have been shown to survive harsh condition, such as low winter temperatures, remarkably well (Rose & Small, 1980). In sub-Sahelian Ghana and Togo the most prominent adverse climatic factors are those of low humidity and lack of precipitation during the long dry season. During studies on various aspects of the epidemiology and clinical presentation of *Oesophagostomum* infections in the area, we made some unplanned observations on the capacity of the larvae to survive harsh environmental conditions. In the present paper some observations are brought together and some experiments are described to characterise the larvae's capacity to survive such conditions. The behaviour of the L_3 larvae of *Oesophagostomum* is compared with that of *Necator americanus*, the hookworm commonly found in this area.

MATERIALS AND METHODS

Desiccation *and rehydration*

Third stage *O. bifurcum* larvae were cultured from human stools, collected in villages in northern Togo, during the rainy season. The cultures contained *Necator americanus* larvae as well. The natural

exposure of the larvae to desiccation during the dry season was then simulated by putting known numbers of larvae (approximately 100 larvae of each species, suspended in 100 µl of water) on 25 g of soil in a petri-dish or a stool container. The soil had been heated beforehand to kill all free-living nematodes. Sixty-five such dishes were set and left untouched in the laboratory for 1 day -12 months. (Overall, 6809 *O. bifurcum* larvae were used for the desiccation experiments.) At various time- points, the larvae from six petri-dishes were rehydrated by slightly wetting soil. This damp soil was placed in a piece of gauze, which was then knotted to form a bag, hung in water in a glass sedimentation cone and left at room temperature (25-35^{0}C) for 24 hours. The larvae migrating from the soil to the water fell to the bottom of the cone. The sediment from each cone was therefore examined microscopically at low power and any larvae detected were counted. To check the effectiveness of this Baermann method, known numbers of undesiccated larvae from cultures were mixed with the soil and extracted in a similar way. In addition, on several occasions *O. bifurcum* L_3 were desiccated twice, for 6 months each time.

To test the effect of fast dehydration, larvae in a drop of water on the microscope slide were allowed to dry out quickly and were rehydrated 3 to 6 weeks later.

Low temperature

Known numbers of larvae of *O. bifurcum* and *N. americanus* from humans from northern Togo were kept in water at +4^{0}C and at -15^{0}C. At different time intervals the larvae were returned to ambient temperature (approximately 30^{0}C) for at least 2 h and then examined under the microscope. Survival was expressed as the percentage of larvae still alive and moving.

Exposure to Artificial Gastric Juices

Approximately 50 *O. bifurcum* larvae cultured from human stools were kept in artificial gastric juices [1.3 g pepsine (1: 10 000; sigma), 2.5 g NaCl, 3.5 ml HCl (36%), 500 ml H_2O] for 1, 3, or 24 hours. The activiety of the larvae was then observed under the microscope. For practical reasons, larvae were considered to be alive if motile and otherwise to be dead.

To test the possibility that dried *O. bifurcum* larvae were infective after ingestion by the host, larvae were dried in centrifuge tubes, left for 4 months and then resuspended in the artificial gastric juices or natural bile

(recovered from a parasite free patient). They were then categorised as dead or alive, as before.

Animal inoculation

Three monkeys (*Macaca fascicularis*) were each inocultated with 100 L_3 larvae which had been allowed to dry for 7 days at 20^0C. Just prior to inoculation, the dried larvae were resuspended in spring water and administered to the monkeys by stomach gavage.

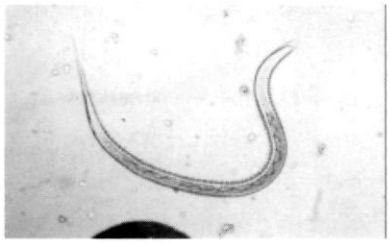
RESULTS

Desiccation

When *O. bifurcum* L_3 were slowly desiccated in a tube, the larvae shrink to almost half their size within the slightly collapsed sheath (Figure 1). This appeared to happen in similar ways in other *Oesophagostomum* species and in hookworm larvae. Only slight or no shrinkage occurred when the larvae were allowed to dry too quickly on a microscope slide.

Many of the *O. bifurcum* L_3 desiccated slowly on soil survived their desiccation for several months (Figure 2). Even after 6 months of dehydration in soil, 20% of the larvae were recovered alive, but no *O. bifurcum* larvae could be recovered after 12 months. The hookworm L_3 appeared dead after just 1 day of desiccation. The *O. bifurcum* L_3 desiccated twice could be revived again.

Since not all viable larvae could be recovered using the Baermann method and the proportion recovered varied (see Table), the exact percentages of larvae surviving desiccation could not be determined.



A

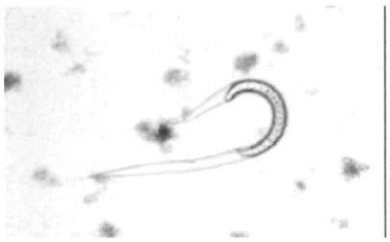


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Fig.1: Normal Oesophagostomum bifurcum third stage larvae (a) and desiccated O. bifurcum larvae (b). The larvae shrink to almost half their size following slow desiccation, with a slightly collapsed sheath.

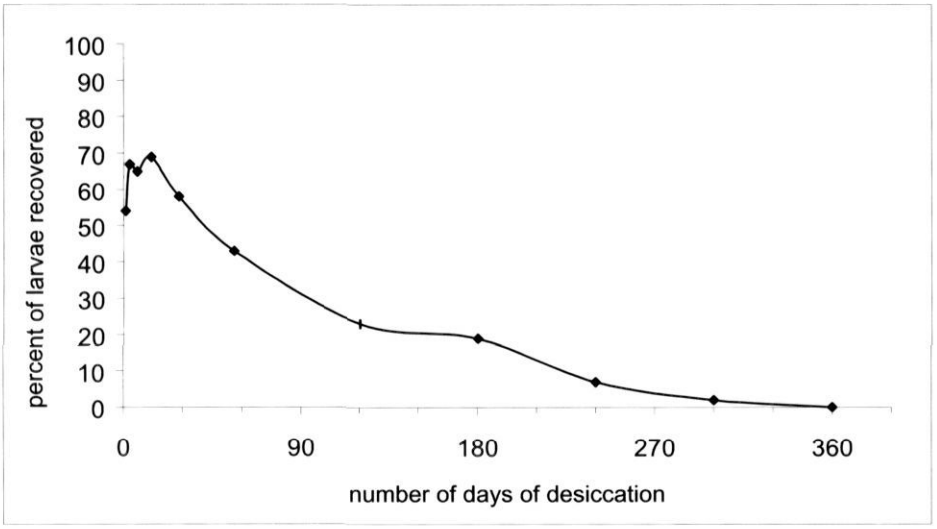

Fig. 2. The effect of desiccation on larvae of O. bifurcum. Percent of larvae recovered after dehydration in soil for a period of 1 day to 12 months. The larvae were allowed to rehydrate in water for 24 hours at room temperature (25-35°C)

Table: Recovery of known numbers of undesiccated Oesophagostomum bifurcum (ob) and Necator americanus (Na) larvae from soil samples

Number of larvae used		recovered		Recovery %*	
Ob	Na	Ob	Na	Ob	Na
20	140	22	28	110	20
20	140	18	42	90	30
20	140	19	43	95	31
25	45	10	29	40	64
26	27	8	4	31	15
21	27	1	5	5	19
20	140	15	14	75	10
20	140	19	20	95	14
20	140	16	51	80	36
39	34	7	12	18	35
39	44	11	18	28	41
36	28	7	12	19	43
31	46	10	10	32	22
40	58	17	12	43	21
41	51	31	21	76	41
27	31	14	27	52	87
25	33	19	19	76	58

**mean (S.D) percentage recovery were 57 (32) for O. bifurcum and 35 (20) for N. americanus*

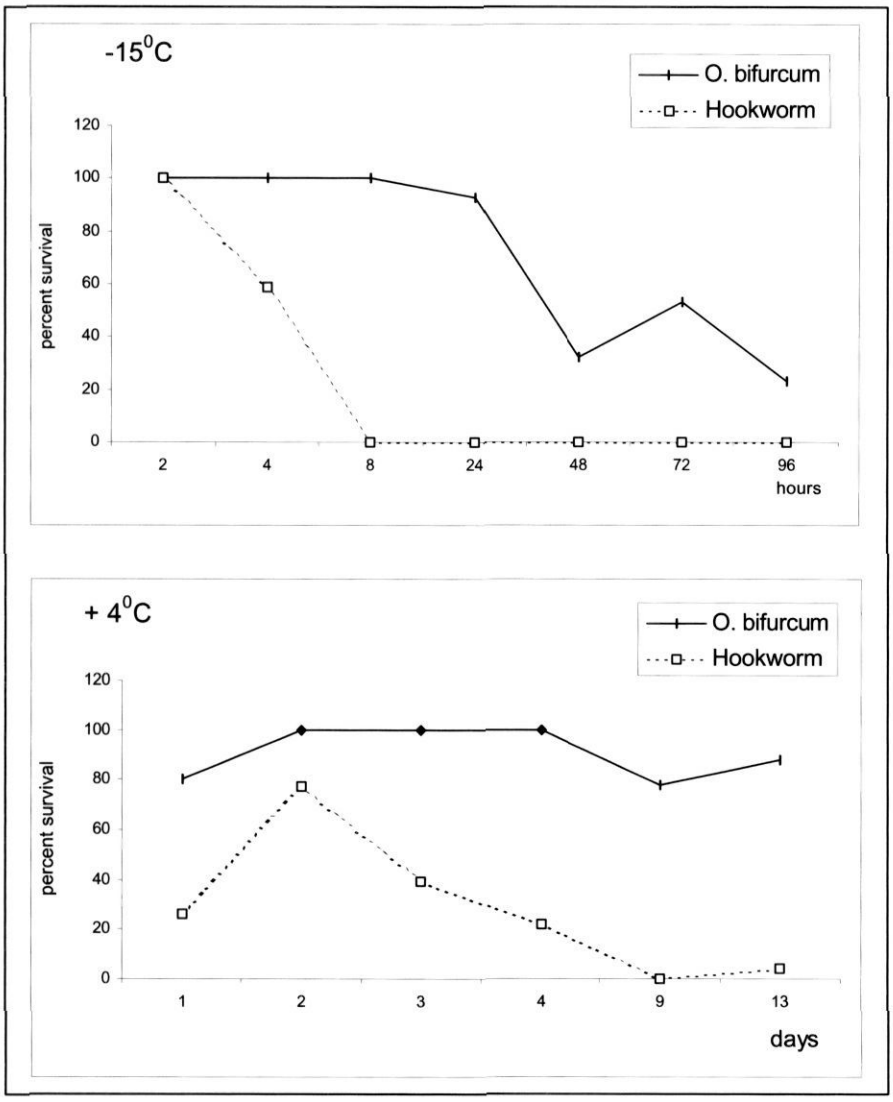

Fig. 3: Survival of Oesophagostomum bifurcum and Necator americanus larvae isolated from human stools and exposed to a temperature of –15°C (a) and 4°C (b). The larvae were allowed at least 2 h to recover at an ambient temperature of approximately 30°C.

Survival at low temperature

The survival of *O. bifurcum* and hookworm L_3 at low temperatures is illustrated in Figure 3. Even after 106 hours at -15°C, 25% of the *O. bifurcum* were moving when thawed. In contrast the hookworm larvae could only survive 4 hours at this temperature.

Survival in gastric juices

All the *O. bifurcum* L_3 larvae cultured from human stools and kept in artificial gastric juices for 1, 3, or 24 hours survived and were active. When gastric juices or natural bile were added to *O. bifurcum* larvae that had been allowed to dry out for 4 months, 10 % of these larvae recovered, and those in the bile were remarkably active. None of the hookworm larvae survived exposure to the artificial gastric juices.

Animal inoculation

All three monkeys developed heightened serological responses to *Oesophagostomum* antigen (data not shown). One began shedding eggs of the parasite after 128 and another after 137 days. Six, immature, adult worms were recovered at necropsy from one of these animals, 314 days after inoculation. No eggs nor worms were observed in the third monkey.

DISCUSSION

In the course of research on various aspects of human infection with *O. bifurcum* in northern Togo and Ghana, some rather astonishing capacities of the free-living larvae of *O. bifurcum* to survive under adverse conditions were observed.

The exceptionally long survival time of desiccated *O. bifurcum* L_3 larvae from human hosts presumably enables the larvae to remain viable during the dry season. Rehydration in itself is not a sigh of survival. In fact most larvae, even the hookworm larvae, regained their original shape, but they did not necessarily move. In general, *O. bifurcum* larvae started moving within a few hours. There have been several previous attempts to test the ability of third stage larvae to withstand desiccation over a period of time. Those of *O. columbianum* survived for a longer period under conditions of high relative humidity but desiccation was deleterious (Shanker, 1970). Goodey (1924) found that the infective-stage juveniles of *O. dentatum* were resistant to desiccation for 1-2 days. Following the present study, the capacity *O. dentatum* L_3 obtained from naturally infected pigs from Guinea and from pigs infected with inbred strains at the veterinary department of the University of Gent (Belgium), to survive desiccation was investigated in a similar way. These did not survive desiccation in soil. On the other hand, 884 *O. bifurcum* L_3 larvae obtained from stools from Mona monkeys in mid Ghana did show the same capacity to survive desiccation. After three months of desiccation in soil, 210 larvae (24%) were recovered.

It seems evident that larvae will only survive within somewhat restricted limits of temperature and humidity. Optimum development and survival of *O. dentatum* larvae harvested in Denmark was in the temperature range from 15°C to 20°C and at humidities from 79.5% to 95.5% (Fossing et al., 1995). Shanker (1970) found that most (72%) *O. columbianum* L_3 survived exposure to a temperature of –18°C for 8 hours, but all were dead after 24 hours. In the present study, 93% of the *O. bifurcum* regained movement when thawed after 24 hours at –15°C. The ability of *O. bifurcum* L_3 to survive freezing at –15°C for several days would not seem of great relevance for a parasite in Togo's northernmost province because the absolute minimum temperatures are around 15°C. However, it is once again proof of the very strong survival capacity of the larvae.

In the oral route of transmission, the L_3 have to survive the acidity of the gastric juices (pH 2.7) before entering the intestine, where in contrast the pH is more alkaline (up to pH 9). When *O. bifurcum* L_3 were exposed to a solution simulating the gastric juices of humans, they remained viable, even after 24 hours. A significant percentage of desiccated larvae rehydrated and regained motility in artificial gastric juice or natural bile. If desiccated larvae can be rehydrated in gastric juices, then Harmattan-wind dispersal of the dried L_3 and inhalation and swallowing could produce infection.

For practical reasons, the larvae which were able to move after desiccation and rehydration were assumed to be viable. A more relevant question, however, is whether they are still able to infect a host. Larvae cultured from human stools, and then dried for 7 days not only regained motility, but also retained their ability to infect monkeys.

The present observations indicate that *O. bifurcum* larvae can survive adverse conditions such as longer periods of drought and can be rehydrated again through ingestion by the host. Dust-born infections are therefore possible. The sturdiness of larvae certainly contributes to the high transmission intensity in the endemic areas of West Africa.

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REFERENCES:

Fossing, E.C., (1995). Development of free-living stages of *Hyostrongylus rubidus* and *Oesophagostomum* spp. at different temperatures and humidities. J. Helminthol, Mar; 69(1): 7-11.

Pit D.S.S., Rijcken F.E.M, Raspoort E.C, Baeta S.M., Polderman A.M. (1999). Geographical distribution and epidemiology of *Oesophagostomum bifurcum* and hookworm infections in humans in Togo. *American Journal of Tropical Medicine and Hygiene,61 (6), 951-955.*

Polderman AM, Krepel HP, Baeta S, Blotkamp J & Gigase P. (1991). Oesophagostomiasis, a common infection of man in northern Togo and Ghana. *American Journal of Tropical Medicine and Hygiene* 44(3): 336-44.

Rose J.H. & Small A.J. (1980). Observations on the development and survival of the free-living stages of *Oesophagostomum dentatum* both in their natural environments out-of-doors and under controlled conditions in the laboratory. *Parasitology, 81, 507-517*

Shanker R. (1970). Effect of temperature and humidity on the survival of infective-stage juveniles of *Oesophagostomum columbianum* (Curtice, 1890) under controlled laboratory conditions. *Indian Veterinary Journal* 47:120-3

Spindler L.A., (1936). Effects of various physical factors on the survival of eggs and infective larvae of the swine nodular worm, *Oesophagostomum dentatum.*