Journal of Helminthology (2001) **75,** 51–56 DOI: 10.1079/JOH200031

Experimental *Oesophagostomum bifurcum* in monkeys

M.L. Eberhard[1]*, E. Kovacs-Nace[1], J. Blotkamp[2], J.J. Verwij[2], V.A.A. Asigri[3] and A.M. Polderman[2]

[1]Division of Parasitic Diseases, CDC, US Public Health Service, Department of Health and Human Services, 4770 Buford Highway NE, Atlanta, Georgia 30341-3724, USA: [2]Department of Parasitology, University of Leiden, The Netherlands: [3]Parasitic Diseases Research Centre, Ministry of Health, Tamale, Ghana

Abstract

Oesophagostomum bifurcum larvae, cultured from human stools collected in northern Ghana, were used to establish experimental infections in monkeys. A patent infection was established in a rhesus monkey (*Macaca mulatta*) and this infection was used to generate larvae to inoculate additional monkeys. In all, 17 animals were inoculated. Thirteen of 15 animals developed antibodies to the infection between 19 and 62 days post inoculation (PI); two animals had a positive response before inoculation. Four of ten animals developed patent infections between 88 and 134 days and passed eggs in the faeces. Egg shedding was consistent in only one animal, but at low levels of one or two eggs per 2 mg direct smear, and extended over a 400 day period. In the other three animals, egg shedding was sporadic and of only 2–4 weeks duration. In seven animals necropsied between 19 and 22 days PI, one to 17 early fourth-stage larvae were recovered from nodules in the bowel wall; in an eighth animal examined at 314 days, six immature adult worms (early fifth stage) were recovered from nodules in the bowel wall. The morphological features and growth of these recovered larvae are described. Three animals were inoculated with larvae that had been dried for one week at 28°C; two animals began shedding eggs at 128 and 134 days PI, respectively. The present results suggest that the parasite obtained from humans is poorly adapted to lower primate hosts, and supports the concept that *Oesophagostomum bifurcum* found in humans and monkeys in the same geographical region of northern Ghana and Togo are distinct and that the infections in humans are not likely to represent zoonotic infections acquired from monkeys.

Introduction

Infections caused by strongyle nematodes in the genus *Oesophagostomum*, commonly called nodular worms, are frequent parasites in ruminants and swine. They also have been reported to be common infections in monkeys and apes, where they cause significant morbidity and mortality (Orihel, 1970; Orihel & Siebold, 1972). For many years, occasional reports of human infections with *Oesophagostomum* suggested that infection with this parasite was sporadic and rare, with most cases documented in Africa, but a few reports from South America and south-east Asia (Beaver *et al.*, 1984). However, because the eggs of *Oesophagostomum* are indistinguishable from other strongyle eggs, particularly those of human hookworms, the true occurrence of the infection in humans is not known. Human cases have, until recently, been viewed as zoonotic in origin and most likely acquired from primates (Eberhard, 1997).

The recent studies of Polderman and colleagues in northern Togo and Ghana have clearly shown that in

*Fax: (770) 488-4253
E-mail: mle1@cdc.gov

Table 1. Summary of animal inoculations, results of serological assays, stool examinations, and necropsies of monkeys experimentally-inoculated with *Oesophagostomum bifurcum*.

Animal no.	L3	Serology	Egg shedding	Necropsy results
1	50§	+day 62	+day 105	14 adult worms @ day 400
2	80§	+day 20	−day 161	NPF @ day 161
3	150§	+day 20	+day 88	NPF @ day 159
4*	50	+day 35	−day 199	NPF @ day 199
5*	50	+day 37	−day 199	NPF @ day 199
6	50	+day 58	−day 420	No necropsy
7	50	+day 32	−day 420	No necropsy
8	50	+day 19	NA	3 larvae @ day 19
9	50	+day 19	NA	3 larvae @ day 19
10	50	+day 20	NA	17 larvae @ day 20
11	50	+day 20	NA	9 larvae @ day 20
12*	50	+day 22	NA	4 larvae @ day 22
13	50	−day 19	NA	1 larva @ day 19
14	50	−day 20	NA	11 larvae @ day 20
15*	100‡	+day 20	+day 128	6 young adult worms in nodules @ day 314
16*	100‡	¶	+day 134	NPF @ day 314
17*	100‡	¶	−day 274	NPF @ day 274

NPF No parasites found.
§ Larvae cultured from human stool.
‡ Larvae had been dried for 7 days.
¶ Positive serologic response at time 0 (preinoculation).
* Cynomolgus monkeys.

some foci, *Oesophagostomum* infections can be extremely common in humans (Polderman *et al.*, 1991; Krepel *et al.*, 1992; Blotkamp *et al.*, 1993; Polderman & Blotkamp, 1995). In some villages, over 50% of the inhabitants were infected, based on stool culture and examination of infective larvae to distinguish *Oesophagostomum* from hookworm infections. Because monkeys are not very common around many of the infected villages, transmission from a simian reservoir would not seem very likely. Moreover, the probable species involved, *O. bifurcum*, is not found in ruminants or pigs. The high prevalence in humans in the area is, therefore, considered to be the result of human to human transmission. Whether or not monkeys play an additional role in transmission is not known.

To better characterize the prepatent period, level of egg shedding and development of an immune response, we undertook experimental infections of laboratory-housed monkeys. This paper describes our observations on experimental *Oesophagostomum* infections in monkeys.

Materials and methods

In February, 1994, several small vials containing the sediment from stool cultures collected a month earlier from infected humans in Ghana were received at the CDC laboratory. Initially, the cultures contained both hookworm (*Necator americanus*) and *Oesophagostomum* larvae, but upon receipt, only the *Oesophagostomum* larvae were alive. The *Oesophagostomum* infective larvae were easily recognized by their long, whip-like tail. Larvae were separated and washed in fresh PBS, counted, and gravity sedimented to consolidate the larvae into small volumes of fluid.

Four rhesus monkeys (*Macaca mulatta*), all laboratory-bred, were available for study initially. Two blood and stool samples were collected one week apart from each animal prior to being inoculated. Serum and PBMCs were separated and frozen and a standard formalin-ethyl acetate (FEA) concentrate stool examination for intestinal parasites was conducted.

Although the number of viable *Oesophagostomum* larvae was limited, three animals (nos. 1–3) were inoculated; one animal received 50 larvae, one animal received 80 larvae, and one animal received 150 larvae (table 1). The fourth animal served as an uninfected control. Larvae were administered to monkeys *via* gavage using a naso-gastric infant feeding tube placed into the stomach.

Beginning one month after administration of larvae, weekly stool samples were collected from each of the three inoculated monkeys and a 1 g portion was processed by formalin-ethyl acetate concentration procedure. An aliquot of the sediment was examined for strongyle eggs. When strongyle eggs were observed, the number of eggs was counted and recorded. In animal no. 1, direct 2 mg smears of stool also were prepared and the number of eggs counted and recorded. A fresh stool sample was collected from monkey no. 1 which developed a patent infection and consistently passed eggs. Using the Haradi-Mori culture technique, the stool was cultured at room temperature (~22°C) or in an incubator at 27°C. Larvae were harvested 5–7 days later.

An additional 14 monkeys, eight laboratory-born rhesus and six cynomolgus (*Macaca fascicularis*) (not laboratory-bred but housed in captivity for the previous 4–5 years), were inoculated with larvae cultured from the original experimentally infected monkey (table 1). These animals were all inoculated *via* gavage in the same manner as the first three animals. Three of the cynomolgus monkeys (see below) were inoculated with 100 third-stage larvae (L3), and all subsequent monkeys were inoculated with 50 L3.

https://doi.org/10.1079/JOH200031 Published online by Cambridge University Press

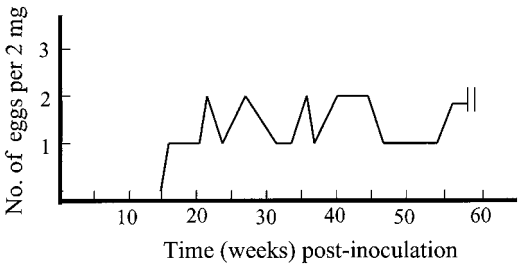

Fig. 1. The number of *Oesophagostomum* eggs detected in 2 mg direct smears during 42 weeks of patency in monkey no. 1.

Experiments to study the effects of drying on viability of larvae are the subject of another report (Pit *et al.*, 2000). However, one batch of larvae dried for 7 days at 28°C and then resuspended in spring water regained a high rate of motility. Aliquots of 100 larvae were administered to each of three cynomolgus monkeys (nos. 15–17; table 1).

The serological assay used to monitor immunological responses of the experimentally infected monkeys was very similar to the assay developed to detect *Oesophagostomum* infections in humans (Polderman *et al.*, 1993). It was modified for use with primate sera where determination of total IgG levels worked much better than testing for specific IgG4 levels.

In those animals which were examined at necropsy,

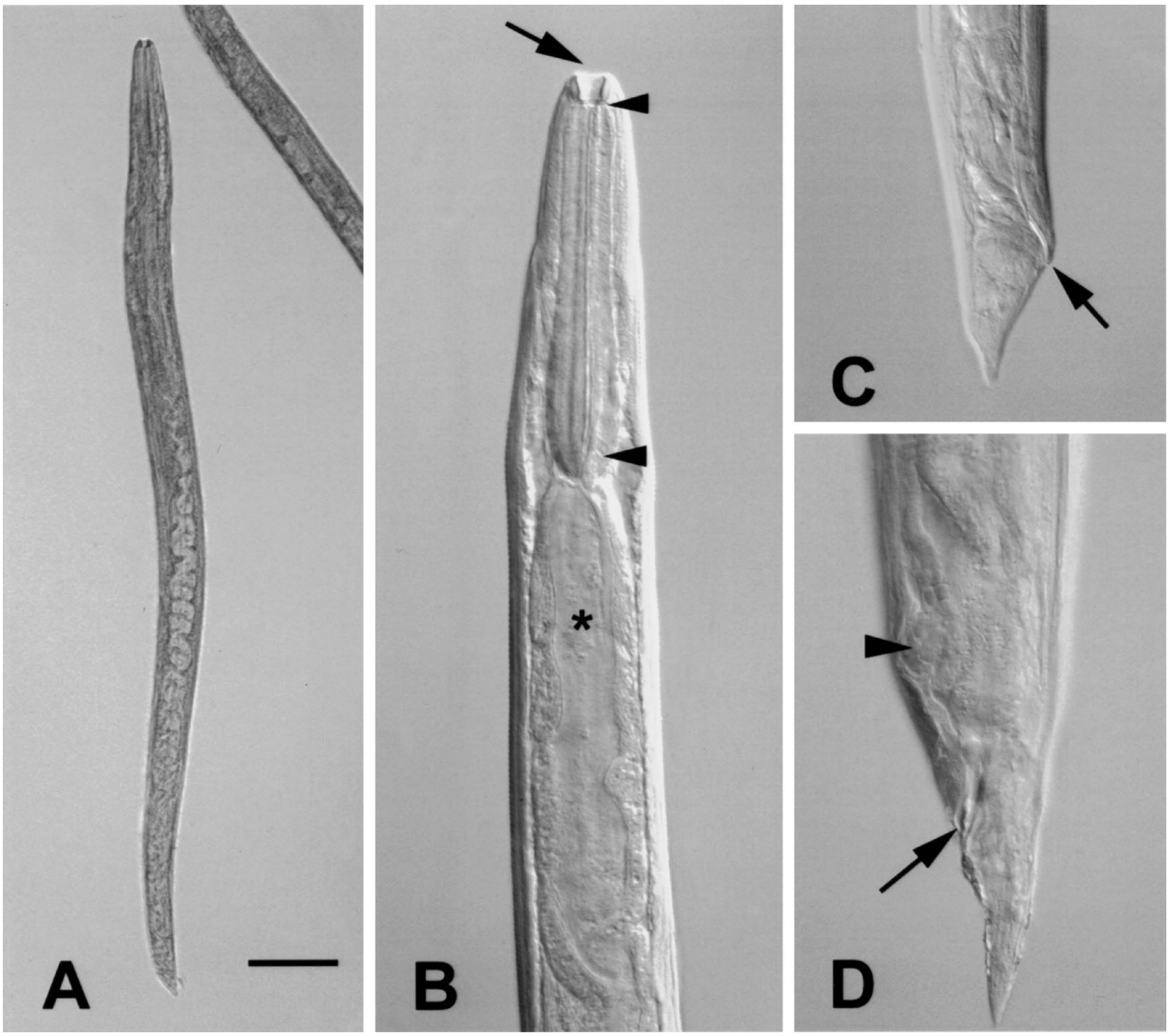

Fig. 2. Early fourth-stage larvae recovered 20 days post-inoculation from bowel nodules. A. Complete larva with rudimentary buccal capsule and the loss of the long whip-like tail. Scale bar = 200 μm. B. Anterior end with the developing buccal capsule (arrow); the oesophagus (darts) is clearly evident as is the intestine (asterisk). Scale bar = 50 μm. C. Tail of a male larva showing the cloaca opening (arrow). There is no marked development of spicules. Scale bar = 50 μm. D. Posterior end of a female larva with anal opening (arrow) and developing ovejector (dart). Scale bar = 50 μm.

https://doi.org/10.1079/JOH200031 Published online by Cambridge University Press

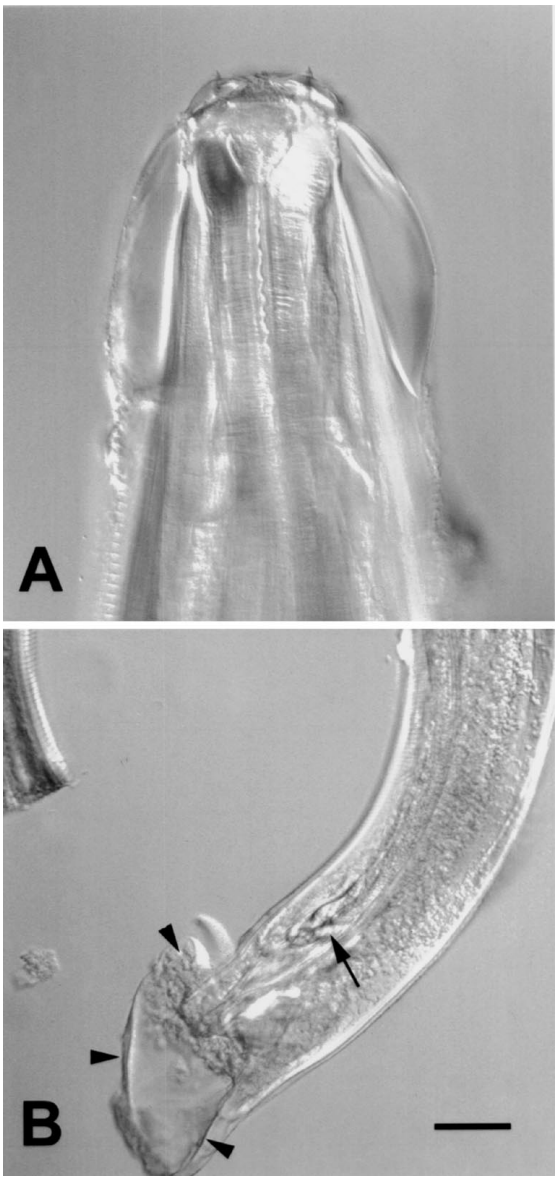

Fig. 3. *Oesophagostomum* recovered from nodules in the bowel of monkey no. 15 at 314 days. A. Anterior end with well developed buccal capsule and cuticular ornamentation. Scale bar = 50 μm. B. Posterior end of a male worm, with a well developed bursa (darts) and cuticularized spicules (arrow). Scale bar = 100 μm.

all portions of the small and large bowel were searched for evidence of infection, either worms, or nodules, although the greatest time was spent examining the region of the ileo-caecal junction.

Results

Eggs of *Oesophagostomum* were first detected in the stools of two animals (nos. 1 and 3) at 88 and 105 days post infection (PI), and at 128 and 134 days in two of the animals (nos. 15 and 16) which had received desiccated larvae (table 1). Eggs were not detected in the stool of six other animals that were followed over these longer time periods. Of the four animals that developed patent infections, eggs were detected sporadically in three animals (nos. 3, 15 and 16) over a 2–4 week period, and then no further stool samples were positive.

In monkey no. 1, which shed eggs consistently, the numbers of eggs detected at any time was low, never more than eight or ten per coverslip. In direct wet examination of 2 mg samples, one or two eggs were counted. However, this animal shed eggs consistently for 42 weeks (fig. 1). Because of health conditions unrelated to the *Oesophagostomum* infection, this animal was euthanized 400 days after being inoculated. At necropsy, four adult male and ten adult female worms were recovered from the lumen of the ileo-caecal region of the bowel.

Most of the animals were killed and necropsied. The findings at necropsy are summarized in table 1. Of 15 experimentally inoculated animals, worms were recovered from nodules in eight and adult worms from the gut lumen in one. In all instances, the worms were alive. All larvae and immature worms were contained in a nodule, and each nodule contained only a single worm. All nodules, regardless of the time recovered, were small, measuring 3–6 mm in diameter, and were located in the submucosal layer. Seven of the 15 animals (nos. 8–14) were necropsied around day 20 PI, and the larvae recovered at this time were fourth-stage larvae (L4), measuring 2.1–3.5 mm in length by 0.10–0.17 mm in diameter (fig. 2A–D). There was no difference in the size of male and female larvae. In one animal (no. 15) examined at 314 days PI, six young adult worms were recovered from nodules in the bowel wall (fig. 3A,B). Two of the nodules were fixed for sectioning, and four were dissected to recover worms. Three of the recovered worms were male, and measured 7–9 mm in length by 0.34–0.40 mm in diameter. One broken female worm, missing the tail, measured 9.5 mm in length by 0.38 mm diameter.

The results of serological assays, summarized in table 1, demonstrate that 13 of 15 monkeys developed a detectable antibody response. In seven of the animals, antibodies were detected as early as 19–20 days PI. The two animals which did not seroconvert were necropsied at day 19–20 PI, and it is likely that they would have developed antibodies as larvae were recovered from the host tissues. In two animals (nos. 16 and 17), antibodies were detected at time 0 (preinoculation). Both of these animals, although housed indoors for a number of years, very likely had exposure to natural infection prior to captivity.

The three cynomolgus monkeys (nos. 15–17) inoculated with dried larvae all demonstrated heightened serological responses, two animals shed eggs at 128 and 134 days, respectively, and, from one animal (no. 15), six immature adult worms (fig. 3A,B) were recovered from bowel nodules (table 1). No lumen-dwelling worms were found in this animal.

Although grossly visible and palpable at necropsy, nodules were not detected by ultrasound due to the small size of the nodules (2–3 mm) (fig. 4A,B). Using an endoscope, we also were unable to visualize nodules nor

https://doi.org/10.1079/JOH200031 Published online by Cambridge University Press

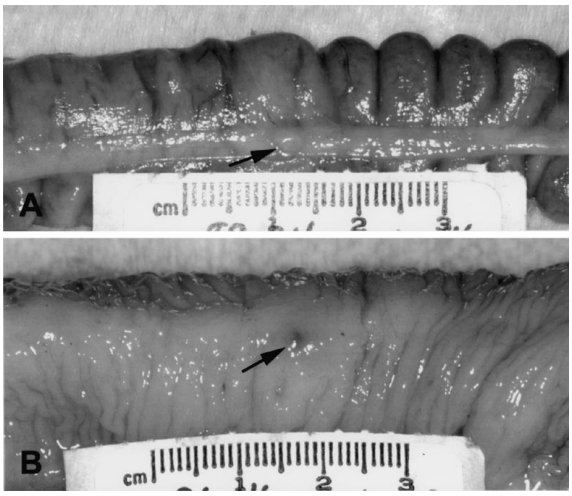

Fig. 4. Bowel of an infected monkey showing the nature of the nodules produced by *Oesophagostomum* larvae. These nodules, at 314 days PI, contain immature adult worms (as illustrated in fig. 3). A. Serosal surface with a small nodule (arrow) in the tinea. B. Mucosal surface with a small haemorrhagic nodule (arrow) that communicates with the lumen.

mucosal ulcers (fig. 4B) which occasionally communicate between the nodule and lumen. However, in two animals (nos. 1 and 3) which were passing eggs, we were able to observe adult worms in the bowel by endoscopy.

Discussion

We were able to infect monkeys with *Oesophagostomum* larvae cultured from human stools collected in northern Ghana. The combined results of seroconversion, worm recovery or detection of eggs in stools indicated that 16 of 17 animals harboured some stage of the parasite. Thirteen of 15 animals showed evidence of seroconversion and nine of 15 animals were positive for developing larvae or adult worms. Egg shedding was detected in four of ten animals followed for sufficient time, and, in only one animal was egg shedding consistent over time. The number of eggs produced by the infection in this one monkey is similar to that calculated for human infections with *Oesophagostomum*. Based on the recovery of ten female worms and a count of one egg per mg of stool, it can be estimated that each female worm produced approximately 50 eggs per g of stool. This is within the range of 16–68 eggs per g stool (median 34) that has been calculated for human *Oesophagostomum* infections (Krepel & Polderman, 1992). However, the fact that three of four animals which developed patent infections only had sporadic egg production for brief periods of time suggest that monkeys are not an optimum host for this parasite. Orihel (1971), working with experimental *Necator americanus* infections in patas monkeys, made the same observation and drew the same conclusion.

We were able to establish that larvae which had been dried not only regained motility, but also retained their ability to infect the vertebrate host. In two of three animals inoculated with dried larvae, eggs were detected in the stools between 128 and 134 days PI. Additionally, in one of these animals six immature adult worms were recovered from nodules in the bowel at necropsy. This observation raises the issue of delayed or asynchronous development. In this one animal (no. 15), some of the developing worms migrated out of the nodules and back into the lumen, completed development, mated, and produced eggs. However, not all worms behaved the same, as some remained in the tissues. Although it is well recognized that such delayed development occurs in this group of parasites, the factors which control or influence this behaviour are not well understood.

Experimental infections in monkeys resulted in seroconversion in 13 of 15 animals. Eight of ten animals seroconverted as early as day 19 or 20 PI, while an additional five animals all became seropositive between 32 and 62 days PI. It would appear that this assay is a sensitive indicator of infection. We have no data to indicate how long the antibodies might persist in these experimentally-infected animals.

Although very limited, the observations obtained in this study indicate that larvae which successfully penetrate the bowel wall and become encased in a typical nodule develop rapidly. By day 20 PI, larvae had undergone substantial development. It is particularly noteworthy that in one instance, immature adult worms were recovered from nodules as long as 314 days PI. This suggests that some mechanism was operative which retarded or arrested the movement of worms back into the lumen. A full description of larval development cannot be provided as larvae were recovered only at two widely spaced intervals, but the state of development can be interpreted from the observations. There is rapid differentiation in the first 20 days after the infective larvae enter the bowel wall and encyst. The developing larvae lose the long whip-like tail characteristic of third-stage larvae, moult for a third time, and increase in size. The buccal capsule begins to form, and developing male and female genital structures are clearly present. Based on morphology, it seems certain that the third larval moult, although not observed, had occurred prior to this time. The larvae found 19–22 days PI are therefore all considered to be L4. In those worms recovered at 314 days PI, but still contained in nodules, the ornamentation and buccal capsule of the anterior end are fully formed as are the spicules and cloaca in the male. It seems probable, based on the morphological features observed, that these worms have undergone the fourth and final moult, and are young adult worms.

In conclusion, we have shown that the human parasite can infect a high percentage of monkeys, that adult worms matured in very few instances, that egg production was of short duration in the majority of cases, and that in some instances immature worms were arrested in the tissues. The last three of these observations would seem to suggest a parasite that is poorly adapted to the primate host. This has important epidemiological implications. Initially, it was assumed that human infections, although very common, were acquired from primates as zoonotic infections. However, it is now clear that in some areas of Ghana, where monkeys are frequently infected, there is intimate contact

between people and monkeys, yet people remain uninfected. In other areas, people are commonly infected, but monkeys are very rare. This would imply that the infection in humans is a result of person to person transmission. The results of the present study confirm that the human parasite does not effectively infect monkeys. This further substantiates epidemiological data which suggests that human and monkey *Oesophagostomum* are not commonly shared between these two hosts.

References

Beaver, P.C., Jung, R.C. & Cupp, E.W. (1984) The Strongylida: hookworms and other bursate nematodes. pp. 269–301 in *Clinical parasitology*, 9th edn. Philadelphia, Lea & Febiger.

Blotkamp, J., Krepel, H.P., Kumar, V., Baeta, S., Van't Noordende, J.M. & Polderman, A.M. (1993) Observations on the morphology of adults and larval stages of *Oesophagostomum* sp. isolated from man in northern Togo and Ghana. *Journal of Helminthology* **67**, 49–61.

Eberhard, M.L. (1997) Oesophagostomiasis. pp. 1499–1503 *in* Connor, D.H. *et al.* (*Eds.*) *Pathology of infectious diseases*, vol. II. Stamford, Appleton & Lange.

Krepel, H.P & Polderman, A.M. (1992) Egg production of *Oesophagostomum bifurcum*, a locally common parasite of humans in Togo. *American Journal of Tropical Medicine and Hygiene* **46**, 469–472.

Krepel, H.P., Baeta, S. & Polderman, A.M. (1992) Human *Oesophagostomum* infection in northern Togo and Ghana: epidemiological aspects. *Annals of Tropical Medicine and Parasitology* **86**, 289–300.

Orihel, T.C. (1970) The helminth parasites of nonhuman primates and man. *Laboratory Animal Care* **20**, 395–401.

Orihel, T.C. (1971) Primates as models for parasitological research. pp. 772–782 *in* Goldsmith, E. & Moor-Jankowski, J. (*Eds*) *Medical primatology.* Proceedings of the Second Conference on Experimental Medicine and Surgery in Primates, New York, 1969, Basel, Switzerland, S. Karger.

Orihel, T.C. & Siebold, H.R. (1972) Nematodes of the bowel and tissue. pp. 76–103 *in* Fiennes, R.N.T.W. (*Ed.*) *Pathology of simian primates, Part II*. Basel, Switzerland, S. Karger.

Pit, D.S.S., Blotkamp, J., Polderman, A.M., Baeta, S. & Eberhard, M.L. (2000) The capacity of L3 larvae of *Oesophagostomum bifurcum* to survive adverse conditions. *Annals of Tropical Medicine and Parasitology* **94**, 165–171.

Polderman, A.M. & Blotkamp, J. (1995) *Oesophagostomum* infections in humans. *Parasitology Today* **11**, 441–481.

Polderman, A.M., Krepel, H.P., 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**, 336–344.

Polderman, A.M., Krepel, H.P., Verweij, J.J., Baeta, S. & Rotman, J.P. (1993) Serological diagnosis of *Oesophagostomum* infections. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **87**, 433–435.

(Accepted 12 May 2000)