

Observations on mass production of *Calicophoron microbothrium* metacercariae from experimentally and naturally infected *Bulinus tropicus*

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ABSTRACT

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In an attempt to establish an ideal method for mass production of *Calicophoron microbothrium* metacercariae, a study was carried out to compare the shedding capacities of *Bulinus tropicus* naturally and experimentally infected with *C. microbothrium*. A total of 906 F1 *B. tropicus* between 4 and 5 weeks old were each experimentally infected with two *C. microbothrium* miracidia and monitored for 12 weeks. The infected snails were fed on dried lettuce and fish flakes and were kept in 1 ℓ plastic aquaria housed in a snail room where temperature, light and humidity were controlled. Seventy-four percent of the experimentally infected snails died during the prepatent period and of the remaining, only 13.2% developed patent infection, while 12.5% were refractory. Snail growth rate was poor and the average shedding rate was 20 cercariae per snail per day.

Compared to the experimentally infected snails, 2200 adult *B. tropicus*, collected from the field and naturally infected with *C. microbothrium*, yielded high numbers of metacercariae. Eighty-four percent of the snails died within 7 weeks of the study with peak mortality occurring from the 2nd to the 4th week of infection and coinciding with an overall decrease in the number of cercariae shed.

Keywords: *Bulinus tropicus*, *Calicophoron microbothrium*, mass production, metacercariae, snails

INTRODUCTION

Ruminant amphistomosis is a parasitic infection of cattle, sheep, goats and wild ruminants caused by pathogenic and immature amphistomes in the small intestines of susceptible hosts (Horak 1967; Dutt 1980). The disease is characterized by sporadic epizootics of acute parasitic gastroenteritis and subsequent loss of production associated with high morbidity and mortality particularly in young ruminants (Butler & Yeoman 1962; Boray 1969; Horak 1971;

Dutt 1980). The disease is confined mainly to tropical and subtropical countries in Africa, Asia, Australia and some European countries (Horak 1971; Kelly & Henderson 1973).

In Zimbabwe and the rest of southern Africa, *Calicophoron microbothrium*, with *Bulinus tropicus* as its intermediate host (Swart & Reinecke 1962a; Over 1982; Chingwena, Mukaratirwa, Kristensen & Chimbari 2002), has a wide distribution and has been incriminated in the majority of outbreaks of amphistomosis in ruminants (Dinnik & Dinnik 1962; Dinnik 1965; Horak 1971).

The epidemiology of amphistomosis has been reported mostly in India and other tropical countries (Okafor, Mbata & Anosike 1988; Rolfe, Boray, Nichols & Collins 1991; Prasad & Varma 1999; Bedarkar, Narladkar & Deshpande 2000), and knowledge on

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other aspects of the disease such as its pathogenesis, perceived mechanisms of resistance (Butler & Yeoman 1962; Horak 1967, 1971) and clinical pathology (Horak 1967) is limited, available related literature having been assembled from field observations.

In order to acquire full knowledge of the pathogenesis of the disease, the mechanisms of resistance and the clinical pathology, systematic experimental infections using susceptible hosts are required, followed by time series observations and sampling strategies. For such studies to succeed large stocks of viable amphistome metacercariae are required (Swart & Reinecke 1962a) and the first step is to develop a routine technique for establishing and maintaining infected snails for harvesting metacercariae. Swart & Reinecke (1962b) successfully infected large numbers of first generation (F1) *B. tropicus* under laboratory conditions, while Rolfe, Boray & Collins (1994) used naturally infected *B. tropicus* for obtaining infective metacercariae for experimental infection in sheep.

The objective of this study was to establish a method of harvesting amphistome metacercariae in large quantities for experimental studies.

MATERIALS AND METHODS

Experimental infection of *B. tropicus* with *C. microbothrium* miracidia

Collection and culture of C. microbothrium eggs

Live, sexually matured *C. microbothrium* collected from the rumens of cattle slaughtered at a local abattoir, were preserved in physiological saline until they were confirmed to be *C. microbothrium* according to Eduardo (1982a, b, c). To obtain the eggs, the flukes were crushed with a pestle in a mortar before mixing with distilled water. The contents were then serially sieved through sieves of 425 and 150 μm and finally a 63 μm sieve from which the eggs were collected. The eggs were then washed into a conical flask and left to stand for 10 min before the excess water was decanted and the sediment transferred into petri dishes for incubation. The eggs were incubated at 25 °C until Day 12 when hatching occurred upon exposure to a 40 watt light bulb.

Propagation and maintenance of B. tropicus juveniles

First generation *B. tropicus* juveniles used in this study were bred from adult *B. tropicus* collected from natural habitats and maintained in the laboratory.

After hatching, the juvenile snails were kept in 1 ℓ plastic aquaria containing de-chlorinated water from an artificial pond located outside the snail room and fed on dried lettuce and commercial fish flakes. The same source of water was used for the maintenance of the naturally infected snails in the laboratory.

Experimental infection of B. tropicus with C. microbothrium miracidia

A total of 906 F1 *B. tropicus*, between 3 and 4 weeks old, were individually exposed for 4 h to 2 newly hatched miracidia of *C. microbothrium* in 10 ml plastic cups, each containing 3 ml of de-chlorinated pond water, before being pooled into 1 ℓ plastic aquaria holding a maximum of 10 snails each. At the time of the transfer, stereoscopic examination failed to reveal any miracidia remaining in the plastic cups.

The infected snails were housed in a snail room at 30 °C and observed once per day for 12 weeks to record the mortality pattern, the growth rate and any abnormalities that could occur during the study period. Florescent bulbs supplied light during the day. De-chlorinated pond water (pH 6.9) was used in the aquaria and the water was replaced every 2 days. The snails were fed on dried lettuce and commercial fish flakes. At 4 weeks post-infection each snail was exposed to light for 1 h every 2 days to stimulate cercariae shedding. Snails shedding cercariae were placed in separate aquaria and kept in the dark for mass production of cercariae.

Mass production of *C. microbothrium* metacercariae from experimentally infected *B. tropicus*

Durie's (1955) technique for harvesting large numbers of amphistome metacercariae was used with some modifications. Inside a 500 ml glass beaker painted black on the outside, and midway to the capacity graduation mark, a cellulose acetate strip 10 mm wide was pasted right around the wall. De-chlorinated water from an artificial pond outside the snail room was added into the beaker to midway the width of the cellulose acetate strip. *Bulinus tropicus* shedding cercariae were then placed in nylon gauze bags, dropped into the beaker and placed in a covered wooden box fitted with a light source, making sure the light source was directly above the beaker and high enough to avoid overheating the water. These snails were exposed in this way every 2 days for 3 h before they were returned to the dark aquaria. Metacercariae harvested at each exposure were counted and kept moist at 25 °C.

Mass production of *C. microbothrium* metacercariae from naturally infected *B. tropicus*

A total of 2 200 adult *B. tropicus*, naturally infected and shedding *C. microbothrium* metacercariae, were collected from a single natural habitat and maintained in the laboratory in dark 5 ℓ plastic aquaria filled with de-chlorinated pond water, with each aquarium holding approximately 80 snails. Immediately after collection, the snails were individually screened and each one that either failed to shed cercariae or shed other than amphistome cercariae was discarded after several daily exposures to direct sunlight. The identity of the cercariae shed by the *B. tropicus* was confirmed by feeding 15 000 metacercariae to a sheep, which was sacrificed at Day 56 post-infection. The mature amphistomes recovered from the rumen of the sheep were identified as *C. microbothrium*, according to Eduardo (1983).

The management regimen and observation of the naturally infected *B. tropicus* was similar to that described for the experimentally infected group excepting that lettuce and fish flakes formed the basal diet and that mud collected from the natural habitat was added into the aquaria in an attempt to emulate the natural habitat conditions.

For mass production of cercariae, nylon gauze bags (2 mm) each holding up to 20 snails were used to confine snails during shedding to minimize ingestion of metacercariae by the snails. The open-end of each snail bag was stapled, individually immersed in a plastic container holding 500 mℓ of de-chlorinated pond water and exposed to sunlight for 3 h before being returned to the dark aquaria. The harvested metacercariae were then stored under moist conditions at 25 °C until required for use. The harvesting procedure was repeated on alternate days until few or no cercariae were released from the snails. The growth and mortality rates and any abnormalities observed during the study period were recorded.

RESULTS

Experimental infection of *B. tropicus* with *C. microbothrium*

Eggs and embryo development

A large number of eggs were obtained by crushing mature *C. microbothrium* flukes. The eggs were oval in shape, colourless and filled with vitelline cells. The anterior end was tapered and operculated while the posterior end was broad. On the 8th day of incuba-

tion there was a decrease in the number of vitelline cells and an increase in the size of the embryo, which in many eggs had assumed a concave position. By Day 12 of incubation the embryo had taken up most of the shell space and some embryos could be seen wriggling inside the shell, signifying maturity and readiness to hatch. Exposure of eggs to an artificial light source on this day stimulated hatching spontaneously.

Experimental infection of B. tropicus and mass production of metacercariae

Confinement of individual snails in small plastic cups with small volumes of de-chlorinated pond water facilitated the infection as it promoted close contact between the snail and the miracidia. Upon exposure to the snails, the miracidia became very active and made numerous oscillatory movements around the snail and/or the snail faecal material. The majority of the snails retreated from the miracidia by migrating out of the water. After vigorous movement during the first 30 min of contact the snails became relatively inactive apparently indicating successful miracidial penetrations as was supported by failing to observe miracidia microscopically in the cups after the snails had been removed.

The attempt at mass production of *C. microbothrium* using experimentally infected *B. tropicus* was largely unsuccessful as low numbers of cercariae were recovered and snail mortality was high. Of the 906 *B. tropicus* infected 673 (74.3%) died during the 13 weeks of the study, 120 (13.2%) shed cercariae and 113 (12.5%) survived the infection but remained refractory. The first cercariae were released on Day 42 post-infection, at an average rate of 20 cercariae per snail per day. As illustrated in Fig. 1, snail losses occurred throughout the study period. After a high proportion (34%) of snails had been lost in the first 4 weeks of infection, a steady loss occurred from Weeks 5–12, when only 25.7% of the infected snails remained.

The growth rate of the snails was poor and the majority remained stunted. Only 8% of the infected snails had marginal increases in shell length, ranging from about 2 to 3 mm over the study period. One percent of snails survived for 20 weeks outside the study period before they were discarded.

Mass production of C. microbothrium metacercariae from naturally infected B. tropicus

The shedding rate of naturally infected snails could not be determined because of the large numbers of

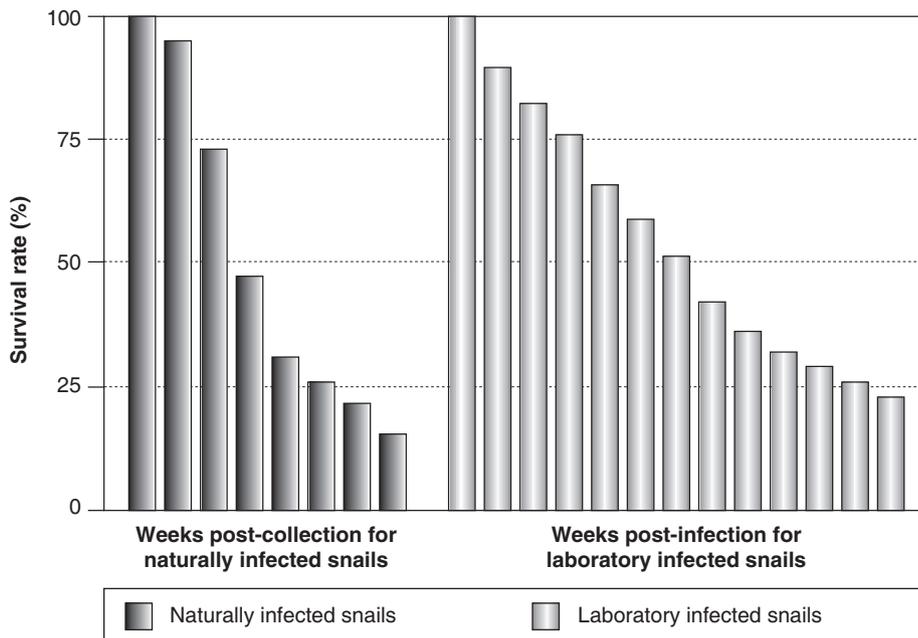


FIG. 1
Weekly survival rates (%) of *Bulinus tropicus*, either experimentally or naturally infected with *Calicophoron microbothrium* miracidia

cercariae that were released per exposure. Cercariae emergence commenced soon after exposure to direct sunlight, initially in small numbers but peaking within an hour by which time they were so many swimming cercariae. The cercariae encysted approximately 10 min after release, at the level of the water meniscus. However, perhaps because of a shortage of space for cercariae released, some cercariae encysted below the meniscus, some on the water surface and some on the bottoms of the containers. Shedding had almost ceased by the end of 2 h, while cercariae encystment was completed after 3 h. After recovery of high numbers of metacercariae during the first 3 weeks, the numbers started to diminish gradually, in concert with the death of high yielding snails (Fig. 1).

Only 15% of the 2 200 *B. tropicus* naturally infected with *C. microbothrium* survived infection till the end of the study. Snail mortalities occurred throughout the study period, with peak mortality from the 2nd to the 4th week of collection from the field (Fig. 1). After a 95% survival rate of the snails at the end of the first week post-collection, at the end of Week 3 post-collection only 46% remained. Snail mortality decreased from the 5th week until the end of the study and during this period the shedding rate reduced drastically.

DISCUSSION

The crushing of adult flukes to harvest *C. microbothrium* eggs as done in this study, proved simpler,

faster, cheaper and less labour intensive than where mature flukes are left to deposit the eggs after collection (Tripathi & Srivastava 1987; Prasad, Malviya, Varma & Dwivedi 1994). Although detailed parasite systematics and biology studies were not done in this study, the observed development pattern and behavioural characteristics of *C. microbothrium* larval stages are similar to those described in other amphistome species (Dutt 1980; Sey 1991; Gupta 1993). Since the miracidia were observed to become less active within 2 h of hatching, exposure of miracidia to snails should be fairly rapid to achieve infection while the miracidia are still energetic. To counter this problem it is advisable to serially expose incubated eggs to a light source in batches 1 h apart, so that active and energetic miracidia are always available for each batch of snails to be infected.

This study confirmed that, as reported previously (Swart & Reinecke 1962a; Dinnik 1962; Over 1982; Chingwena *et al.* 2002) *B. tropicus* is the intermediate host of *C. microbothrium* in southern Africa including Zimbabwe. While *C. microbothrium* did infect *B. tropicus* in the laboratory, with a minimum prepatent period of 42 days, the experimentally infected snails did not yield large numbers of metacercariae due to high mortality, poor growth rate and low per capita shedding rate.

Most experimentally infected snails were lost during the first 4 weeks of the study, most likely from the stress of infection. The infection dose used in this study was adequate considering that in the field, the dose might be multi-miracidial unless susceptible

snails invoke retraction strategies as observed in this study to avoid multiple infections. Swart & Reinecke (1962b) massively infected a large number of *B. tropicus* with *C. microbothrium* under laboratory conditions. In this study, massive infection as described by Swart & Reinecke (1962b), was attempted as a sideline but caused 100% mortality a few days after exposure and was consequently abandoned.

Apparently only 13.2% of the experimentally infected snails became infected. No dissections were done to determine the actual number of snails that had become infected, but this result was similar to that obtained by Prasad *et al.* (1994) who confirmed through dissection that only 15.2% of 550 *Indoplanorbis exustus* experimentally infected with *Paraphistomum epiclitum* became infected.

The individual snail infection method used in this study could be expected to succeed but it appears that the study failed to reproduce the critical natural parameters an infected snail would require to survive the infection in the field. Swart & Reinecke (1962a) reported that water pH, temperature, water maturity, aeration and light source were among the parameters that had to be adjusted to sustain the infected snails, but they did not report survival rates.

Unlike the experimentally infected *B. tropicus*, naturally infected *B. tropicus* produced large numbers of *C. microbothrium* cercariae and the production was sustained for 3 weeks after which it started to decline. However, snail mortality was comparatively greater than the experimentally infected group, peaking at 3 weeks and coinciding with a fall in total weekly cercariae production. The death of the snails was possibly related to the stress of stimulation and massive release of cercariae. Cercariae are released through rupturing of snail tissues and considering the production capacity displayed by the naturally infected snails in the present study, both the damage to the snail tissues and the massive physiological/nutritional strain of the maturing cercariae are likely to have overwhelmed these intermediate hosts.

The cercariae production rate decreased and coincided with the peak in snail mortality at 3 weeks. It is possible and logical to conclude that the peak in mortality at 3 weeks was due to the death of high yielding snails under severe shedding stress and therefore died quicker leaving less productive snails. This is contrary to the report by Swart & Reinecke (1962b) where heavily infected snails were sustained for up to 10 months despite regular exposures to a 40 watt yellow light. From this study, cercariae shed-

ding peaked at 3 h after exposure to sunlight and confirms Swart & Reinecke's (1962b) findings where cercariae emergence peaked in the first 2–3 h, and it would appear any exposure exceeding 3 h at a given time can lead to snail fatigue and high mortalities. In nature the infected snails may conceivably have many ways of avoiding excessive shedding, which include aestivation and residing in the shade of aquatic plants to avoid light exposure (Mavenyengwa, personal observation 2003). Under such conditions shedding can be expected to be regulated, thus prolonging snail survival.

The possibility that addition of mud from the natural habitat to the aquaria may have favoured development and survival of the naturally infected snails, should be investigated. No growth retardation tendencies or signs of calcium deficiencies were found among the naturally infected snails. This is possibly related to the mineral content of the habitat soil and subsequent adjustments of the water pH to simulate field conditions.

Induction of experimental amphistomosis in the final host requires large numbers of viable metacercariae for infection (Swart & Reinecke 1962a; Horak 1967) and observations made in this study highlight some of the difficulties that can be encountered in an attempt to raise such numbers of *C. microbothrium*-infected *B. tropicus* under laboratory conditions. Swart & Reinecke (1962b) using the mass infection technique succeeded in infecting a large number of young snails confined in a container although they neither mention the number of snails infected nor the quality of the water used. In the present study, raising such snails proved laborious, time consuming and produced insufficient quantities of metacercariae for experimental purposes. The use of naturally infected *B. tropicus* for mass production of cercariae proved relatively easy but had disadvantages including working with an unknown parasite species, which can only be determined at the end of the study. This constraint can, however, be overcome by conducting primary infections in a single ruminant host to obtain the adult amphistomes for confirmation of parasite identity before embarking on any major work on experimental amphistomosis. The other disadvantage is the probability of getting mixed infections from a single habitat. However, despite these disadvantages, it was found that the use of naturally infected snails for harvesting metacercariae of *C. microbothrium* was relatively easier, more efficient, and time conserving provided that stable habitats with naturally infected *B. tropicus* (common in Zimbabwe soon after the rainy season)

are identified and the seasonal occurrence of amphistomosis is considered.

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