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Exsheathment Characteristics and Infectivity of Revived Anhydrobiotic L_3 of Haemonchus Contortus

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Abstract: This study was conducted to determine the effect of temperature and moisture stress on exsheathment process and infectivity of the infective larvae of the parasitic nematode *Haemonchus contortus.* Laboratory conditions were used in which infective (L_3) of *H. contortus* were either subjected to gradually increasing temperatures, decreasing moisture or both. Post stress viability and exsheatment of L_3 was determined prior to infection experiments. The viability of *H. contortus* after induction of anhydrobiosis declined significantly ($p<0.05$) from 85 to 60% in about 60 days. The results showed significant ($p<0.05$) delay in exsheathment in stressed larvae compared to unstressed larvae leading to low overall establishment (Infection). However, the proportion of the immature in relation to the mature parasites (hypobiosis) was significantly ($p<0.05$) higher in stressed compared to the unstressed L_3. These results indicate that delayed exsheatment in stressed L_3 contributes to low infectivity in ruminants.

Keywords: Exsheathment, haemonchus contortus, infectivity, moisture, stress, temperature

INTRODUCTION

Successful nematode infections in ruminants are the consequences of complex inter relationships between parasite factors and the animal host, husbandry practices and prevailing climatic effects on the parasite (Khan *et al*., 1989; Gruner *et al*., 1989; Berbigier *et al*., 1990; Besier and Dunsmore, 1993; Tembely *et al*., 1997; Wall *et al*., 2004). During hot dry seasons frequently observed during droughts in the tropics, development and survival of trichostrongyles is limited because the eggs and the free living infective larvae (L_3) are subjected to temperature stress and desiccation. Although the L_3 are considerably more resistant to adverse environmental conditions, it has been found out that the longevity is negatively correlated to the stress intensity (Vlassoff *et al*., 2001). This manifests in low parasitic burdens in grazing ruminants attributed to low availability of infective larvae on pasture/herbage. However, recent studies suggest that L3s subjected to repeated short interval cycles of temperature and moisture stress undergo anhydrobiosis and retain significant viability for extended periods (Siamba *et al*., 2009). This means that larvae have the capacity to survive beyond the short interval stress periods and upon revival, L_3s would most likely be available on pastures and may cause the disease unless other mechanisms exist to interfere with the establishment of the parasite upon ingestion by the host. It was therefore hypothesized that delayed exsheathment of the L_3 contributes to the low parasite burden during the dry seasons. The study was therefore conducted to determine the effect of temperature and moisture stress on exsheathment process and infectivity in the infective larvae of the parasitic nematode *H. contortus.*

MATERIALS AND METHODS

Study site: The study was conducted at the National Animal Husbandry Research Centre-Naivasha, Kenya from May 2008 to February 2009. The centre is situated at an elevation of approximately 1700 m above sea level and has a semi-arid climate with strong desiccating winds (upto 13 m/sec) during the dry season. The area is classified as semi arid and seasonal variation in nematode infections has been in described in grazing ruminants (Waruiru *et al*., 1993; Gatongi *et al*., 1998).

Experimental parasites: The study used *Haemonchus contortus* monoculture, established and maintained by regular passage through parasite-free small East African

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Goats (*Capra hircus*) as described by Siamba *et al*. (2009). Infective (L_3) larvae of *H. contortus* for the experiments were obtained by culturing faecal material from donor goats artificially infected with parasite monoculture. Faeces were cultured at 27°C for 10 days. The third stage larvae (L3) was then acquired from the faecal material as described by Hansen and Perry (1990).

Stress conditions and procedures: The L_3 were subjected to artificial (laboratory) stress conditions selected based on the environmental characteristics of the study area. Briefly, 35 disposable weighing dishes (41×41×8) mm^3 (Neolab (r) - Karl-Kolb GmbH and Co.kg, Scientific Technical Supplies, Dreieich, Germany) were evenly filled with 10 g of fine laboratory grade sand, with water field capacity of 39.3% similar to the soils representative of the study site, as a substrate for the larvae. About 5000 *H. contortus* L_3 larvae aliquots in 4 mL of distilled water (dH_2O) were dispensed in each of the 35 dishes ensuring that the distribution in the sand substrate was as even as possible.

The seeded dishes were simultaneously subjected to gradual reduction in moisture and increase in temperature using a programmable Binder cold/heat testing chamber (MK53, Neolab, Karl-Kolb GmbH and Co. kg Scientific, Technical supplies, Dreieich Germany). The incubator was found appropriate because its automatic mechanisms to control the fan speed for required air current over the samples. On attaining the 32°C and moisture content of 2.6%, (attained in 10 days following adjustments of the incubator in line with the weather characteristics of the study site), the dishes were chosen at random for recovery of larvae for viability, exsheathment and infectivity tests.

Recovery from substrate: Infective larvae were extracted from sand using high molarity sucrose solution as described by Freckman *et al*. (1977). Recovered larvae in the test tube were enumerated by serial dilution count (MAFF, 1986)

Viability and exsheathment tests: At the start of the infectivity experiment, percent viability was estimated by the methylene blue exclusion test. This was necessary in order to adjust (the dose) for the dead L_3 as only live larvae were expected to undergo exsheatment and contribute to infection.

In vivo exsheathment was estimated as follows: About 3000 *H. contortus* L_3 of each treatment suspended in 2 mL of dH_2O, were dispensed in 2×4 cm disposable dialysis polyamide bags (Nybold, Switzerland) with pore size of 40 µm. The bags, attached to strings and appropriately labelled, were incubated for various periods between 0 and 360 min in the rumen of 6 sheep surgically fitted with rumen canula and maintained on natural grass. Each sheep carried 5 bags. One bag selected at random from each sheep was removed at hourly interval. The larvae were recovered from the bags and assessed for morphological changes and percentage exsheatment.

Infectivity tests: The infective larvae (L_3) stressed as described above were used to infect experimental animals in order to assess the infectivity of stressed L_3 in the host. Sixteen (16) Small East African (SEA) goats aged between 7-12 months raised worm were purchased from contracted farmers. They were transported to and maintained on slated floor pens at the National Animal husbandry Research Centre-Naivasha. After a 10 day adaptation period, the animals were randomly assigned to 2 treatment groups (1 and 2), with 8 animals in each treatment. Animals in groups 1 and 2 were artificially trickle infected daily for 4 days with 3000 unstressed (controls) and stressed L_3, respectively. The animals were confined and maintained on a diet consisting of a mixture of commercially grown grass and Lucerne hay and offered clean water *Ad libitum*. Faecal egg count was done on day 0 and every after 7 days to monitor progress of infection. The animals were then slaughtered 25 days after the last dose of infection. This was to allow worms to develop to maturity in 21 days.

Differential parasite counts: Experimental animals were slaughtered 25 days post infection and differential parasite counts carried out as described by Hansen and Perry (1990) with modifications. Briefly, the abomasum was ligated and separated from the omasum and duodenum and placed in a tray. The abomasums was opened along the greater curvature to open the contents into the tray. The content was then transferred into a total content jar to allow the opened abomasums to be thoroughly washed paying attention folds of the mucus membranes. The washings were added to the total content jar. The volume of content and washings in the total content jar was made to 2000 mL. The contents were then vigorously stirred to mix the food material, mucus and the water. A total of 200 mL was then washed through a 80 and 40 µm pore sized sieves in aliquots of 40 mL. The second sieve with the parasites was backwashed into a beaker and the volume made to

50 mL. Aliquots of 10 mL of suspension were transferred to 5 Petri dishes and few drops of iodine solution added. After 5 min, the parasites were counted for each Petri dish. Counts for all the dishes were added and multiplied by 10 to estimate the total adult parasite counts.

For inhibited larvae, the opened and washed abomasums was placed in a tray containing warm normal physiological saline with the mucus membrane face down. The abomasums was left to soak for 12 h. After 12 h, the abomasums was rinsed with saline solution and discarded. The saline solution in the tray was then poured through a sieve 40 µm which retained the larvae. The larvae were flushed into a beaker using a wash bottle and the volume made to 200 mL. Using a dissecting microscope, an aliquot of 10 mL in a Petri dish was examined and the larvae counted. Total inhibited larvae were then calculated as follows: Number in 10 mL sub-sample × 20.

RESULTS

As shown in Fig. 1, the viability of *H. contortus* after induction of anhydrobiosis declined significantly ($p<0.05$) from 85 to 60% in about 60 days to a steady state thereafter. The proportion of viable L_3 remained constant after 60 days of storage to the end of the experiment at day 180.

In vivo exsheathment characteristic (percentage) of different larval treatments is presented in Fig. 2. There was a general increase in the exsheathment rates in all treatments with highest increase in the unstressed (T_1) compared to stressed (T_2). Regression analysis indicated that there was significant association of exsheathment with time of exposure. From the trendline equations, it was observed that the rate of exsheathment was not significantly different in unstressed compared to the stressed larvae only that the unstressed larvae exhibited a shorter lag phase than in stressed larvae. These results thus showed significant delay in exsheathment in stressed larvae compared to unstressed larvae (T_1 and T_2). At the end of the 6 h experimental period, the overall exsheathment percentage was significantly ($p<0.05$) higher in unstressed larvae.

As shown in Table 1, it was observed that there was significantly ($p<0.05$) higher establishment of the worms in unstressed compared to the stressed parasites. However, the proportion of the immature in relation to the mature parasites (hypobiosis) was significantly ($p<0.05$) higher in stressed compared to the unstressed.

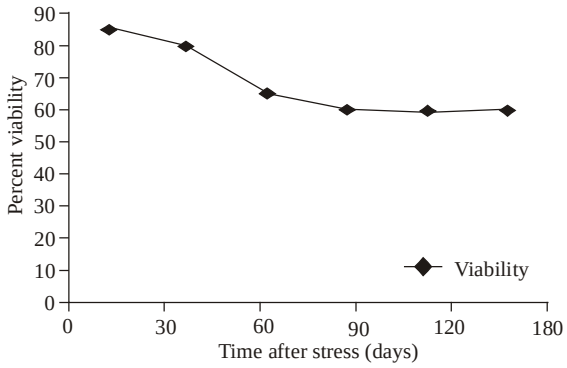

Fig. 1: Viability of anhydrobiotic L_3 of *H. contortus* revived after different periods of storage at 32°C and moisture content of 2.6%

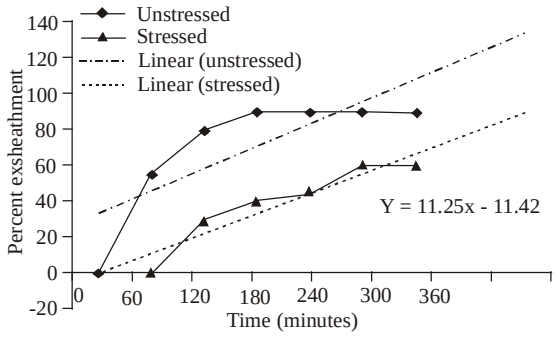

Fig. 2: Rate of In vivo exsheathment of L_3 of *H. contortus* in canulated sheep

DISCUSSION

Low worm burdens during the dry seasons have been consistently observed in grazing ruminants (Khan *et al*., 1989; Gruner *et al*., 1989; Berbigier *et al*., 1990; Besier and Dunsmore, 1993; Tembely *et al*., 1997; Wall *et al*., 2004). Besides the reduced availability of L_3 on pasture, physiological adjustments in the L_3 in response to exposure to stressful environmental factors may ultimately affect the process of infection (from exsheathment to maturity). The current study established that there was significant delay in exsheathment in stressed as compared to the unstressed

Table 1: Exsheathment and establishment characteristics of stressed *H. contortus* in a host following artificial infection

Treatment	Exsheathment % (n = 4)	Mature (n = 5)	Immature (n = 5)	Total established	Hypobiosis* (%)	Established (%)
Unstressed	90.01	3,640[a]	735[a]	4,376[a]	20.21[a]	36.47[a]
Stressed	60.63	460[b]	394[b]	794[b]	85.65[b]	6.60[b]

[abc]: Means with the same superscript in the same column are not significantly different at p = 0.05; *: Derived as the proportion of the immature to the matures (percentage)

parasites. This delayed response may reflect the time required to activate all the mechanisms including enzymes involved in exsheathment. Stress especially desiccation, has been associated with changes in the physico-chemical properties of a number of organelles in nematodes. The changes have been found to include decreased membrane permeability (Ellenby, 1969; Perry, 1977; Wharton and Marshall, 2002). This alteration in membrane property may hinder the release of membrane bound molecules such as enzymes required for resumption and initiation of specific activities such as exsheathment. Since exsheatment marks the mandatory transition from non parasitic to parasitic phases, any interference with the process will affect the worm load. Indeed, the percentage establishment of stressed parasites in the host was significantly lower in stressed compared to the unstressed *H. contortus*. Exsheathment of *H. contortus* occurs in the rumen, delay in exsheatment as recorded in the present study (probably occurring in natural environment under stressful conditions) could result in a significant proportion of the larvae being swept down the gut before they exsheath. As suggested before, this may result from reduced uptake of sufficient water to activate the exsheathment enzymes.

The few parasites that manage to exsheath and end up in the abomasum as required for establishment, face continued increase in temperature (upto 40^{o}C) and other stresses provided by the rumen environment. The response to this challenge may be magnified in already sensitised parasites. Evidence to support this phenomenon has been provided by the high levels of the stress induced sugar trehalose in stressed larvae as well as in immature (arrested larvae) parasites (Siamba *et al*., 2010). This sugar has been associated with protection of organisms against several stresses. The presence of the sugar in arrested larvae may indicate persistent stress stimuli provided by the animal environment. Since elevated levels of trehalose have been associated with retarded development in some species of plants for example tobacco (Vogel *et al*., 1998), it is possible that there is a link between the arrested development (hypobiosis) and trehalose levels. The significantly high levels of hypobiosis in stressed parasites may reflect the persistence (or succession) of stress and spontaneous resumption of development, is likely to be triggered by reduction of trehalose to levels compatible with normal development.

CONCUSION

From this study, it can be concluded that temperature and moisture stress leads to low infectivity as a result of delayed exsheathment in the host. Delayed exsheathment therefore contributes to low parasitic burdens in grazing ruminants during the hot dry seasons in the tropics.

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