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STUDIES ON *DICTYOCAULUS FILARIA*
I. MODIFICATIONS OF LABORATORY PROCEDURES

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ABSTRACT

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Techniques used in a study of the life-cycle of *Dictyocaulus filaria* (Rudolphi, 1809) are described. First stage larvae migrate from faecal pellets placed in water. Infective larvae are cultured in clean water at room temperature and aerated overnight for 7 days. Sheep are infested *per os* with infective larvae. At various intervals after infestation they are killed and their organs are cut into small pieces, placed in a modified Baermann apparatus and the parasites allowed to migrate from the tissue into physiological saline at 37°C for 6 to 8 hours. Larvae are collected from lymph drained from the thoracic duct with a catheter. Some of the recovered worms are killed with an iodine solution; others intended for morphological studies are killed by heating to 60°C and preserved in a mixture containing triethanolamine.

INTRODUCTION

"For out of old feldes, as men seith,
Cometh al this newe corn fro yeer to yere;
and out of olde bokes, in good feith,
Cometh al this newe science that men lere".

Chaucer.

Before carrying out studies on *Dictyocaulus filaria* (Rudolphi, 1809) it was essential to investigate the methods of cultivation of infective larvae as well as the recovery and fixation of the parasites from experimentally infested lambs. The procedures used by various workers are often poorly described and usually scattered in the literature. This report describes modifications and improvements of the standard techniques.

HARVESTING OF FIRST-STAGE LARVAE

Various methods have been used for the cultivation of first stage larvae. Romanovitch & Slavine (1914) placed bronchial mucus of infested animals in water while Daubney (1920) dissected eggs from gravid females and allowed them to hatch in tap water. Guberlet (1919) recovered eggs in the same way or allowed the females to lay them; these eggs hatched in 24 to 36 hours at 16° to 20°C when they were placed in water, saline or moist soil. Although he obtained eggs from faeces he does not describe his method.

Larvae were isolated from faeces by a modification of the Baermann technique, which is also used for the extraction of hook worm larvae from the soil (Kauzal, 1933; Shaw, 1934; Goldberg, 1952; Rose, 1955).

Technique

Faecal pellets are placed on a 400 mesh to the linear inch (25,4 mm) sieve (Endecott) to a maximum depth of three layers and the sieve is then lowered into an enamel bucket containing enough tap water to float the pellets within its rim. Air bubbles below the sieve are eliminated by tapping it sharply. The sieve and pellets are removed 30 minutes later, allowing the trapped water to drain into the bucket. The faecal filtrate is poured into widemouthed flasks to a depth of 5 cm and an equal amount of distilled water added. Within 30 minutes most of the harvested larvae settle to the bottom of the flask. The supernatant is then decanted, the flasks refilled to a level of 10 cm with distilled water and allowed to stand for a further 30 minutes. This process is repeated until the supernatant fluid is clear and suitable for cultivation of the harvested larvae.

The advantage of this apparatus over the standard Baermann funnel is that less space is required for processing large amounts of lightly infested faeces.

CULTIVATION OF INFECTIVE LARVAE

Romanovitch & Slavine (1914) and Guberlet (1919) cultured larvae of *D. filaria* in water but do not describe their methods. Guberlet also cultured them in moist soil and faeces while Daubney (1920) used boiled sheep faeces and charcoal as a culture medium. Better results were obtained when larvae were cultured in clean tap water than in various media containing faeces (Kauzal, 1933). The larvae used by Lucker, Vegors & Douvres (1964) were cultured in water a few millimetres deep. Infective larvae may also be cultured on moist granular bone charcoal (Goldberg, 1952).

It was shown by Soliman (1953a) that oxygen is essential for the development of both *D. filaria* and *Dictyocaulus viviparus* (Bloch, 1782) and that bacterial contamination or putrefaction of organic material hastened larval death. He provided optimum conditions for larval development by continuous aeration of the water and replacing it every day.

Porter & Cauthen (1942) cultured larvae of *D. viviparus* on moist filter paper in stender dishes at 22 to 28°C. Larvae of this species were cultivated by Michel (1954), who washed and resuspended them in water which was mechanically aerated and kept at room temperature for 7 days. Jarrett, McIntyre & Urquhart (1954) cultivated larvae by the latter method but found that these larvae did not give satisfactory results when used to infest calves. They obtained better results with larvae cultured as follows: beakers containing infested faeces to a depth of 25 to 50 mm (1 to 2 in) are set in a glass tank with a glass lid. Water in the tank kept the atmosphere moist. After 7 to 10 days the larvae are harvested by washing the faeces and the sides of the beakers with a little water. Lucker & Vegors (1964) found that infective larvae of cattle lungworm may be cultivated in animal-charcoal cultures kept at room

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temperature for 7 days or in water 0,5 cm deep in Syracuse watch glasses.

The pre-infective larvae of *D. filaria* are more sensitive to desiccation than the infective stages (Guberlet, 1919). Infective larvae are resistant to low temperatures and viability is not affected by freezing for 10 to 12 days, nor does repeated freezing and thawing affect them adversely (Guberlet, 1919). The resistance to low temperatures of the pre-infective and infective larvae of both *D. filaria* and *D. viviparus* was confirmed by Daubney (1920), who exposed them to temperatures below 0°C for limited periods. It was shown by Rose (1955) that infective larvae may survive continuous freezing for 19 days but that first and second stage larvae did not live for more than 12 days. He also found that development to the infective stage continues at 5°C but under these conditions takes 20 days; the same stage is reached in 5 days at 20° or 25°C. Soliman (1953a) stored infective larvae of *D. filaria* and *D. viviparus* at 8°C; the latter species was stored by Goldberg (1952) at 4°C and by Lucker *et al.* (1964) at 6,7°C (44°F).

Technique

Larvae are cultivated to the infective stage as described by Michel (1954) but the water is mechanically aerated overnight, not continuously.

As Jarrett *et al.* (1954) had obtained satisfactory development of *D. viviparus* in cultures containing faeces, a series of experiments was carried out to determine whether clean water is essential for the cultivation of *D. filaria* larvae. First stage larvae leave the faecal pellet immediately it comes into contact with water and thereafter actively avoid soluble faecal material. The larvae can be diverted in any desired direction during their rapid migration from faecal pellets by the judicious placing of such material. Furthermore, larvae collected in clean water are immobilized by the addition of soluble faecal material taken from the faeces from which they have recently been harvested. If the concentration of these faecal solutes is increased it kills them.

Infective larvae are kept at room temperature with overnight aeration for periods of up to 4 months and such larvae give satisfactory results when used for the infestation of "donor" sheep. In these studies, however, the larval cultures used for infestation are not older than 3 weeks.

Occasional fungal growth in a culture is reduced by pouring it into a measuring cylinder and allowing the heavier fungal growth to settle. The majority of larvae remain in suspension and are then decanted into clean flasks. Usually these fungal growths have no apparent adverse effect on the larvae but on one occasion predacious fungi eliminated thousands of larvae within 24 hours. These predacious fungi, which appear to be contaminants in the faeces of a single donor sheep, died out spontaneously during attempts to control them with serial dilutions of copper sulphate.

The findings of Panasyuk (1959) are of interest in regard to the purification of cultures. He reported that pyraldine in concentrations of 1:500 and 1:1 000 did not interfere with the normal development of *D. filaria* larvae but killed other nematode larvae and protozoans.

Infestation of the host

Lambs may be infested with *D. filaria* by dosing the infective larvae in gelatine capsules (Guberlet, 1919; Shaw, 1934) or by drenching the larvae suspended in water (Kauzal, 1933; Shaw, 1934). The latter method was used to infest calves with infective larvae of *D. viviparus* (Jarrett *et al.*, 1954; Lucker & Vegors, 1964).

It was reported by Panasyuk (1955) that 4 out of 5 lambs were successfully infested percutaneously with infective larvae of *D. filaria*. This was not confirmed by Borodina (1957). Prior to this, Soliman (1953a) showed that neither sheathed nor exsheathed larvae of *D. filaria* and *D. viviparus* were able to penetrate the skin.

Sheep and mice were successfully infested intravenously with exsheathed larvae of *D. filaria* by Michel (1956). He suspended larvae in 7 per cent Milton* in a shallow dish and added silver sand until there was no free fluid showing above the sand. After 6 hours at room temperature the exsheathed larvae were washed out of the sand, thoroughly washed through a 400 mesh to the linear inch (25,4 mm) sieve and then suspended in normal saline.

Calves were infested with *D. viviparus* by subcutaneous injection of first and third stage larvae (Wade & Swanson, 1958).

Technique

After the number of live larvae in a culture has been determined, the number per unit volume is either reduced by dilution or concentrated by sedimentation until the required number is present in 20 ml water. The larval suspension is drawn into a 50 ml syringe fitted with a blunt 14 gauge needle and is then squirted into the back of the sheep's throat.

Recovery of parasites from host tissues

Hobmaier & Hobmaier (1929) recovered larvae from the liver, mesenteric lymph nodes and lungs of infested sheep but unfortunately do not describe their techniques.

Large numbers of larvae were found in press preparations of the mesenteric lymph nodes of lambs 1 to 3 days after infestation by Kauzal (1933). He extracted larvae from the lungs of lambs and various organs of guinea pigs by mincing them in saline and incubating them at 37°C.

Michel (1956) extracted larvae of *D. filaria* from the lungs and lymph nodes of sheep, rabbits and mice in a Baermann apparatus placed at 37°C overnight. In the case of sheep the minced tissue remaining after this process was discarded by that of the rabbits and mice was subjected to peptic digestion.

Soliman (1953b) investigated the life-cycle of *D. filaria* in experimentally infested rabbits, guinea pigs and mice. He examined press preparations of parts of the livers and the mesenteric lymph nodes; the remaining parts of these organs as well as the lungs were placed in a Baermann apparatus. He removed *D. viviparus* from the bronchial tree after it was lavaged with saline. Thereafter the whole lung plus the mesenteric lymph nodes and selected parts of the liver were minced and extracted with saline in a Baermann apparatus at 37°C for 8 hours. A similar method for the recovery of *D. viviparus* from the lungs of cattle was used by Michel (1954), who trapped the worms by passing lung washings through a 300 mesh sieve. Jarrett & Sharp (1963), who describe their methods in detail, found that cooling of the tissues during their preparation affects the activity of the larvae. They overcome this by warming the work bench and main-

*Milton (The chemical composition and manufacturers are not given by Michel but it was probably a $NaHCl_3$ solution).

taining it near body temperature by flooding it with hot water.

Technique

The sheep are killed by electrocution by attaching one terminal to the lips, the other to the anus, and 110 volts of alternating current are applied twice for 10 seconds with a 5-second interval.

Great care is taken to clean the instruments and hands before each organ is removed from the carcase and cut into small pieces.

The lungs are removed with the trachea intact. The bronchial tree is opened with scissors, the visible worms removed and the whole lung washed in saline (0,85 per cent NaCl solution). Each lobe is separated from the lung, cut into pieces 1,5 cm square and placed in saline at 37°C in a separate widemouthed vessel. Each vessel is labelled and is hereafter referred to as vessel A. Similar vessels are used to collect the tissues of the other organs examined, which are cut into pieces 0,5 to 1,5 cm square (depending upon the size of the organ) and immersed in saline in a separate vessel A. A single layer of tissue is placed in each vessel A.

For each vessel A an identically labelled vessel B is provided. The inner diameter of its mouth is a few millimetres more than the outer diameter of the mouth of vessel A. Both vessels are filled to the brim with saline.

Nylon mesh with an aperture of 250μ is moistened in saline and held in position over the mouth of vessel A with an elastic band. This procedure forces the floating lung tissue below the level of the saline. Vessel A is then inverted and the gauze covered mouth and neck allowed to slide into the mouth of vessel B, so that the shoulders of vessel A rest on the rim of the mouth of vessel B. The nylon mesh is kept taut as vessel A is forced vertically downwards into vessel B to prevent air bubbles from forming under the gauze.

The apparatus is placed in an incubator at 37°C for 6 to 8 hours. Vessel A is then removed and vessel B set aside for an hour to ensure that recovered worms gravitate to the bottom. The supernatant is decanted.

Incubation for longer periods results in the death of large numbers of worms, which cannot then be satisfactorily prepared for morphological studies. Organ digestion with pepsin/HCl is unsatisfactory for the same reason.

Examination of the contents of vessel A showed that less than one per cent of the worms failed to migrate into vessel B.

The large number of specimens made it essential to use something less bulky than the classical Baermann apparatus.

Recovery of parasites from body fluid

Kauzal (1933) was unable to demonstrate larvae of *D. filaria* either in blood from the portal vein, the vena cava, or the heart, or in lymph from the thoracic duct of experimentally infested lambs. Fifth stage *D. viviparus* were, however, recovered from the heart of guinea pigs 9 days after infestation (Poynter, Jones, Nelson, Peacock, Robinson, Silverman & Terry, 1960).

Technique

Cerebro-spinal fluid is withdrawn from the cisterna magna with a syringe and 19 gauge needle immediately after electrocution. The puncture site is in the lowest point of a depression palpable between the occipital crest and the arch of the first vertebra. The needle is inserted parallel to the longitudinal axis of the head and sudden cessation of tissue resistance indicates entry into the subarachnoid space (Innes & Saunders, 1962, p. 36).

Fluid from the abdominal and thoracic cavities is collected immediately after opening the carcase.

Blood removed from the portal vein with a syringe and 14 gauge needle is diluted with water to haemolyse it. Thereafter it is repeatedly sedimented and decanted until a clear sediment is obtained.

Lymph was originally collected from the thoracic duct either with a syringe and 24 gauge needle during massage of the mesentery or by ligaturing both ends of the thoracic duct and removing it entirely by blunt dissection. Since neither of these methods proved satisfactory for the recovery of larvae from the thoracic duct a further method was investigated.

A plastic tube is inserted into the thoracic duct just caudal to its entry into the external jugular vein and another is inserted into the vein itself. These tubes are led to the exterior and joined to allow normal lymph flow.

Lymph is collected in containers which are replaced every 30 minutes and during collection the tubes are disconnected and the tube in the jugular clamped. Preliminary observations showed that this method is satisfactory for lymph collection over a period of 4 hours on 2 successive days.

Killing and preservation of parasites

As morphology was not important in their investigations the workers referred to above do not describe the methods used to kill and preserve the recovered worms.

Sheathed larvae were killed by Guberlet (1919) by dropping them in boiling 70 per cent alcohol containing 10 per cent glycerine. Daubney (1920), in his excellent account of the preparasitic larvae of both *D. filaria* and *D. viviparus,* described live specimens as well as others which were killed with alcohol vapour and stained with acid carmine.

Technique

Worms are killed by one of two methods. For the determination of total number of worms recovered the parasites are killed by the addition of an iodine solution. A 45 per cent stock solution of iodine is prepared by dissolving 7,3 kg potassium iodide in 5 litres of warm distilled water; 4,5 kg iodine crystals are added and the solution made up to 10 litres with distilled water. This stock solution is diluted to 10 per cent and added to the flask containing the worms in physiological saline, until the worms are pigmented a light brown.

For the preservation of worms for later morphological studies the live parasites, collected in physiological saline, are rapidly heated to 60°C in a waterbath. The suspension is stirred continually during heating. The adult worms are then placed in 10 per cent formalin.

Immature worms are killed in the same way. Thereafter they are rapidly cooled to room temperature. An equal volume of 10 per cent formalin is added and after an hour the supernatant is decanted. Subsequently an equal volume of the following mixture is added:

40% formaldehyde	350 ml
triethanolamine	100 ml
50% picric acid	10 g
Distilled water	4 540 ml

Larvae collected in lymph are killed and fixed as described above but the specimen is diluted ten fold with 5 per cent formalin before the addition of the

mixture containing triethanolamine. The larvae are allowed to settle for 12 hours before the supernatant is decanted.

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