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# Studies on *Sarcocystis* in Malaysia

## I. *Sarcocystis levinei* n. sp. From the Water Buffalo *Bubalus bubalis*

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**Summary.** Light and electron microscopic studies and feeding experiments have confirmed the presence of two species of *Sarcocystis* in the water buffalo *Bubalus bubalis*. One is the already known species with large macroscopic sarcocysts, *Sarcocystis fusiformis* (Railliet, 1897) Bernard and Bauche, 1912 and the other is *S. levinei* n. sp. which is being described in detail. The sarcocysts of *S. levinei* are 0.9 × 0.1 mm and the zoites in them 17.8 × 4.2 μm. Ultrastructurally, the primary cyst wall shows sloping villi with irregular wavy outlines. Within the villi are coarse granules and annulated fibrils. Trabeculae are present. The sexual stages of *S. levinei* occur in the subepithelial tissue of the small intestine of the dog and sporocysts shed by this definitive host are 15–16 by 10 μm.

### Introduction

The occurrence of *Sarcocystis* in the water buffalo has been known since the first report by Railliet (1897) who briefly described the large "balbianids" and named the parasite *Balbiania fusiformis*. As pointed out by us (Dissanaike et al., 1977) this species name had been indiscriminately used for the parasites of cattle until very recently. Levine (1977) has clearly shown that the name *S. fusiformis* should be confined to the species forming large sarcocysts in the buffalo and, as earlier indicated by Bernard and Bauche (1912), it should be correctly named *Sarcocystis fusiformis* (Railliet, 1897) Bernard and Bauche, 1912.

During our studies on this species we came across another *Sarcocystis* species in the water buffalo. This produces much smaller, microscopic sarcocysts than *S. fusiformis*. We have already reported that the sexual stages of the two species in the buffalo (*S. fusiformis* and the new species) occur in the lamina propria of the small intestines of cats and dogs respectively (Dissanaike et al., 1977). The smaller sarcocyst-forming species is now described in detail as a new species *Sarcocystis levinei*, in honour of Professor Norman D. Levine, who has helped to clarify the nomenclature of the species of *Sarcocystis* in buffalo and cattle (personal communication and Levine, 1977).

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## Materials and Methods

Only specimens of oesophageal muscles were collected from the abattoir at Shah Alam, Selangor, Malaysia, and they were found to be invariably infected with the macroscopic sarcocysts of *S. fusiformis* and the microscopic sarcocysts of the new species. Measurements of the small cysts were made by examining thin strips of infected muscle under a low-power microscope (×30). Zoites (merozoites) were measured in saline smears under the phase contrast microscope after rupturing the cysts. Zoites were also examined in dry-fixed smears stained with Giemsa stain. Infected muscle was fixed in alcoholic Bouin's fluid and Carnoy's fluid for light microscopic studies. Paraffin sections were cut at 5 μm and stained with haematoxylin and eosin. Infected muscles were also processed in the standard way for electron microscopy (Kan and Dissanaike, 1976). Thin sections were viewed with a Hitachi HS-8 electron microscope at 50 kV.

In the first series of experiments, oesophageal muscle containing both the large and the microscopic sarcocysts were fed to three dogs. In subsequent experiments the large sarcocysts of *S. fusiformis* were dissected out and fed to one dog and four cats while the muscles (containing the small sarcocysts) were fed to two dogs and two monkeys (see Table 1). Stools of all animals were examined, after sugar flotation, 1–2 weeks before feeding and for about 2 months' post-feeding.

As facilities for rearing dogs and cats in the conventional method were not available, the dogs and cats used in these experiments in the Central Animal Facility of the Faculty of Medicine were strays of mixed breed that had been fed only on boiled fish and rice from the day they were acquired. The dogs varied from over 6 months to 105 months in approximate age and were fed between 3 weeks to about $7\frac{1}{2}$ years after being on this diet (Table 1). The cats were 6–11 months in approximate age and were fed between 16–104 days after being on this diet. The monkeys were acquired from a dealer 16–17 months before being fed. Their diet has always been papaya and food pellets (from Zuellig Feedmills, Malaysia) supplemented with Vitamin C. All animals were examined daily for 7–10 days and found free of sporocysts of *Sarcocystis* before being used for the experiments, although some of them (dogs and cats) shed *Toxocara*, hookworm and *Trichuris* eggs and oocysts (unsporulated) of *Isospora* species from time to time during the experiments.

The small intestines of three of the infected dogs were biopsied on days 10, 20 and 23 respectively post-feeding to examine for sexual stages, while scrapings of the mucosa were examined for oocyst stages. The same was done with three cats biopsied on days 5, 15 and 17 respectively (Table 1). Paraffin sections were prepared from small intestinal tissue for examination of the various stages.

Four control dogs and two control cats were negative during the period of the experiments. All animals were kept in individual cages, the cats in galvanized iron cages, dogs in individual cement-floored rooms with tiled sides and iron bars in front; and the monkeys in iron cages or anodized aluminium cages.

## Results

### a. *Light Microscopic Appearance of Sarcocysts and Zoites of S. levinei*

The sarcocysts are thin and spindle-shaped, averaging 0.9 × 0.1 mm (range: 0.8–1.15 × 0.09–0.14 mm, $N = 12$) (Figs. 1, 2). The cyst wall appears thin and the tightly packed zoites are divided into a few irregularly shaped compartments by thin septa (Fig. 2). The zoites, measuring 17.8 × 4.2 μm (range: 17.0–18.2 × 3.8–4.6 μm, $N = 10$) are banana-shaped with the anterior end slightly more pointed (Fig. 3). When stained with Giemsa, the zoites show an anterior pink staining zone followed by a zone of dark-staining granules behind which is the nuclear region followed by another granular region (Fig. 3).

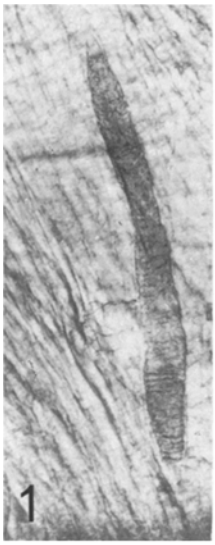
### b. *Light Microscopic Appearance of Sexual Stages of S. levinei*

The results of feeding experiments are summarized in Table 1. The only sexual stages seen were in experiments where the intestines of three dogs were biopsied on days 10,

**Table 1.** Summary of feeding experiments

| Animal | No./sex | Date acquired | Approx. age (mths.) | Date fed | Material fed | Infection | Sporocysts shed | Pre-patent period (days) | Patent period (days) | Day of biopsy/ killing (post-feeding) | Stages seen in lamina propria on biopsy/death |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Dog | P1 | 20.6.69 | 105 | i. 30.11.76 | Sf + om | + | *S. levinei* | 34 | 4 | 36 | Sporulated oocysts |
| | F | | | ii. 25.2.77[a] | om only | + | *S. levinei* | 12 | a. 13<br>b. -ve–33<br>c. 30+ | — | |
| | 2113 F | 1.11.76 | 6 | 30.11.76 | Sf + om | + | *S. levinei* | 21 | 31 | 23 | Sporulated oocysts |
| | 2123 F | 5.11.76 | 6 | 30.11.76 | Sf + om | +[b] | *S. levinei* | 18 | 47 | 20 | Sporulated oocysts |
| | 2107 F | 9.10.76 | 6 | 2.2.77 | om only | + | *S. levinei* | 19 | 8 | 10 | Early oocysts |
| | 2117 M | 2.11.76 | 10–12 | 2.2.77 | om only | + | *S. levinei* | 13 | 14 | — | — |
| | 2154 M | 28.12.76 | 6 | 14.1.77 | Sf only (35) | –ve | — | — | — | 6 | — |
| Cat | 1900 M | 11.10.76 | 11 | 14.1.77 | Sf only (15) | + | *S. fusiformis* | 14 | 26 | 17 | Sporulated oocysts |
| | 1903 F | 11.10.76 | 6 | 2.2.77 | Sf only (30) | + | *S. fusiformis* | 12 | 3 | 15 (killed) | Sporulated oocysts |
| | 1919 M | 17.1.77 | 8 | 2.2.77 | Sf only (30) | + | *S. fusiformis* | 8 | 6 | 5 | G'cytes, early oocysts |
| | 1920 M | 17.1.77 | 8 | 2.2.77 | Sf only (30) | + | *S. fusiformis* | 12 | 15 | — | — |
| Monkey | M7 | 19.8.75 | 48 | 2–4.2.77 | om | –ve | — | — | — | — | — |
| | M8 | 22.9.75 | 48 | 25.2.77 | om | –ve | — | — | — | — | — |

Sf = *S. fusiformis* sarcocysts. Number of cysts fed in parentheses
om = Oesophageal muscle containing *S. levinei* sarcocysts
[a] = Second feeding 87 days after first or 50 days after stopped shedding sporocysts from first feed. a = first patent phase, b = negative phase, c = second patent phase
[b] = Passed *I. heydorni* type oocysts 10 days post-feeding for 2 days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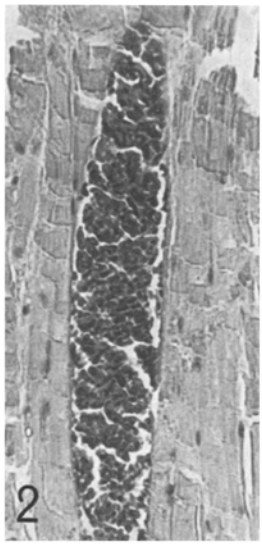
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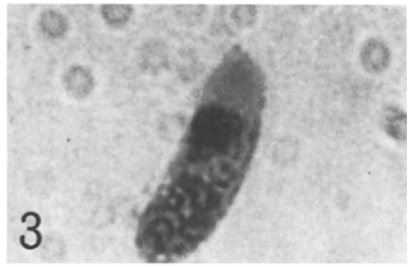
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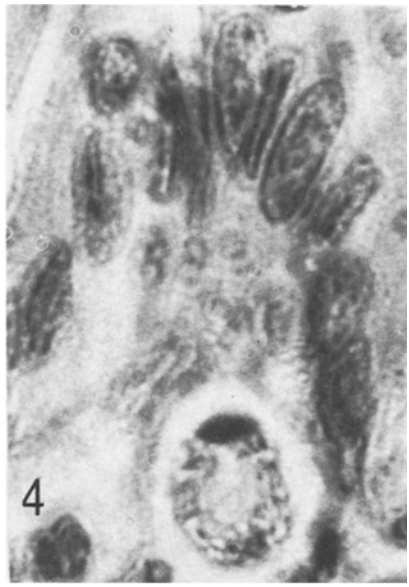
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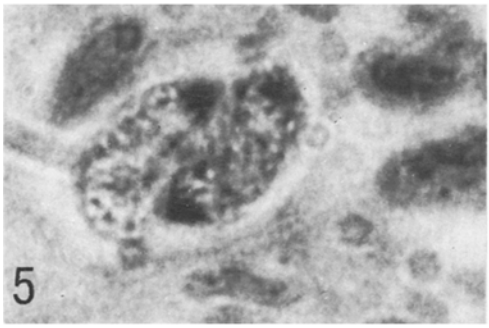
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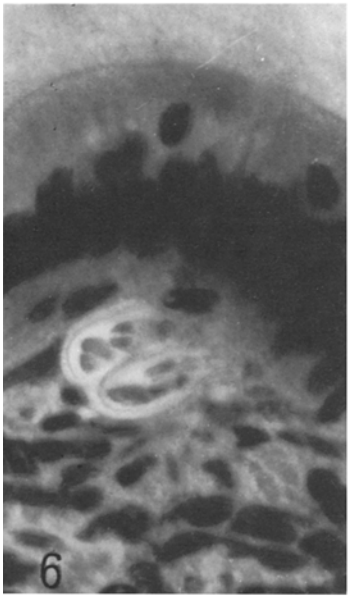
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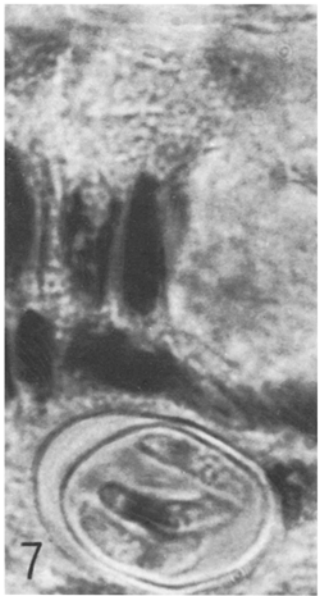
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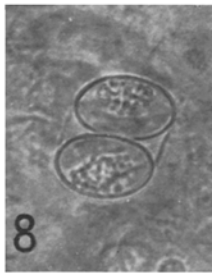
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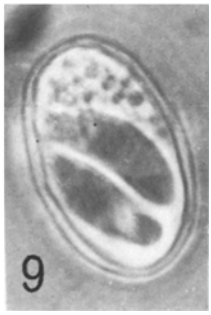
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20 and 23. Binuclear stages of the oocysts (Fig. 4) and sporulated oocysts (Figs. 5–8) were seen in the lamina propria of the small intestine just beneath the epithelium. The parasite in the binuclear stage is 11.5–12.7 μm by 8.5 × 9.2 μm (Fig. 4) and the oocysts as seen in sections are 18 × 14 μm (Figs. 6, 7). In whole mounts of the villi from scraping of the intestine, the oocysts are 21 by 15 μm (Fig. 8) and sporocysts in stools passed by the dogs (Fig. 9) measure 15–16 by 9–10 μm. Each sporocyst contains four sporozoites and the residual granules are either condensed at one pole or scattered. The sporozoites seen in sections (Fig. 8) are 8.3 × 1.5 μm.

### c. *Electron Microscopic Appearance of Sarcocysts and Zoites of S. levinei*

The features of the cyst wall and zoites of *S. levinei* are summarized in Table 2.

*Cyst Wall.* The primary cyst wall (PCW) is a thin, electron-dense layer, about 39 nm thick, with invaginations of about 26 nm deep and regularly spaced at intervals of about 68 nm (Fig. 10). Projecting from the PCW are long, sloping, villi-like cytophaneres about 7.4 μm in height (Fig. 11). Each projection has an irregular, wavy, electron-dense wall which is continuous with the PCW (Figs. 10–11). Within the projections are hollow, annulated fibrils which run along the longitudinal axis of the projections. Scattered between these fibrils are numerous coarse, electron-dense granules (Figs. 10–12).

The ground substance beneath the PCW is about 1.9 μm thick. This layer is filled with fine fibrillar elements and coarse, electron-dense granules (Figs 10–12). The ground substance extends into the sarcocyst to divide the zoites into compartments (Fig. 12).

*Zoites* (*merozoites*). The zoites are banana-shaped and show the characteristic features of *Sarcocystis* zoites (Fig. 13). Surrounding each zoite is a double-layered membrane. Like other *Sarcocystis* spp. each zoite has an anterior conoid and 22 subpellicular microtubules. At the anterior half of the zoite are the micronemes (about 200–300 in number) and 8 rhoptries. The nucleus containing electron-dense

**Fig. 1.** Cyst of *S. levinei* in fresh oesophageal muscle preparation. ×60

**Fig. 2.** Longitudinal section of *S. levinei* cyst. ×215 H & E

**Fig. 3.** Zoite of *S. levinei* from smear stained in Giemsa. ×2,125

**Fig. 4.** Binuclear stage oocyst of *S. levinei* in intestine of dog 10 days after feeding sarcocysts. ×2,125 H & E

**Fig. 5.** Sporulated oocyst of *S. levinei* showing 2 sporocysts, one in binuclear stage. ×2,220 H & E

**Fig. 6.** Section of small intestine of dog 20 days after feeding sarcocysts of *S. levinei* (showing sporulated oocysts in lamina propria). ×990 H & E

**Fig. 7.** Same as above showing section cut through only one sporocyst of *S. levinei.* ×2,200 H & E

**Fig. 8.** Sporulated oocyst of *S. levinei* from scraping of intestine villi of experimentally infected dog, 23 days after feeding sarcocysts. ×875.

**Fig. 9.** Sporocyst from faeces of dog infected with *S. levinei.* ×2,125

**Table 2.** Measurements and other features of the cyst wall and zoites of *Sarcocystis levinei* from the water buffalo, *Bubalus bubalis*

| | |
|---|---|
| I. | Cyst wall |
| (a) | Primary cyst wall |
| (i) | Thickness of PCW: 39 nm (33–44) |
| (ii) | Depth of invaginations of PCW: 26 nm (18–33) |
| (iii) | Distance between invaginations: 78 nm (56–89) |
| (b) | Cytophaneres |
| (i) | Appearance: sloping, with irregular, highly-folded wavy walls |
| (ii) | Height: 7.4 μm (6.4–10.0) |
| (iii) | Outer diameter of fibrils within cytophaneres: 25 nm (22–27) |
| (iv) | Inner diameter of fibrils within cytophaneres: 12 nm (9–16) |
| (c) | Ground substance |
| (i) | Thickness of ground substance: 1.90 μm (1.57–2.07) |
| (ii) | Trabeculae present with no limiting membranes |
| II. | Zoites (merozoites) |
| (i) | Number of subpellicular microtubules: 22 |
| (ii) | Number of micronemes: 200–300 |
| (iii) | Number of rhoptries: 8 |
| (iv) | Structure of rhoptries: uniformly electron-dense with no limiting membrane |
| (v) | Nucleus: posterior half of zoite |
| (vi) | Micropores: present at anterior half of zoite |
| III. | Metrocytes |
| (i) | Present but few |
| (ii) | Position: peripheral |
| (iii) | Micropores: several |

masses of chromatin is situated at the posterior half of the zoites. Anterior to the nucleus is the mitochondrion which extends a branch backwards alongside the nucleus. Masses of lipid droplets are found behind the nucleus at the posterior end. A micropore is seen at the region of the micronemes (Fig. 14).

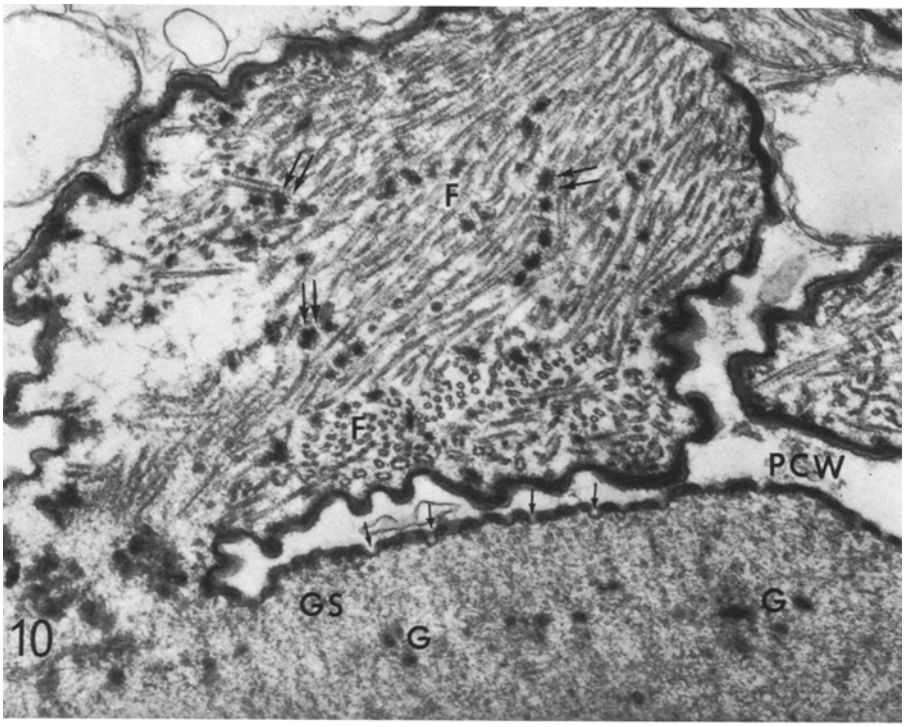
*Metrocytes.* These are large and irregularly shaped with a large nucleus and several micropores along the double-layered membrane (Fig. 12). The metrocytes are relatively few in number and are peripheral in position.

## Discussion

Soon after the original description of *S. fusiformis* from the water buffalo by Railliet (1897) there were several reports of this parasite. Shipley (1903) reported the parasite from Ceylon (Sri Lanka) but labelled it *S. tenella* after getting it identified by Railliet. Von Linstow (1903), describing the same parasite from the water buffalo

**Fig. 10.** Electron micrograph of *S. levinei* showing the primary cyst wall (*PCW*) with regularly spaced invaginations (*arrowed*). Note longitudinal and cross-sections of hollow annulated fibrils (*F*) and coarse granules (*double arrows*) within the villi-like projection of the PCW. Coarse electron-dense granules (*G*) are also seen within the ground substance (*GS*) beneath the PCW. ×36,000

**Fig. 11.** Electron micrograph showing irregular, wavy outlines of long, sloping, villi-like projections of PCW. Note also the annulated fibrils and coarse granules within these projections. ×11,200

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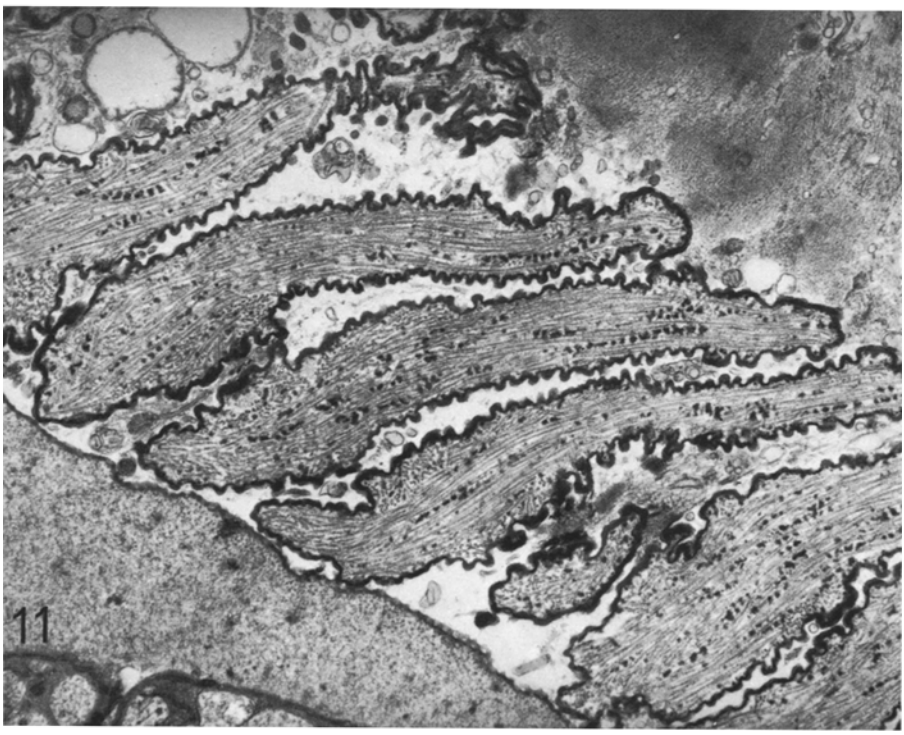

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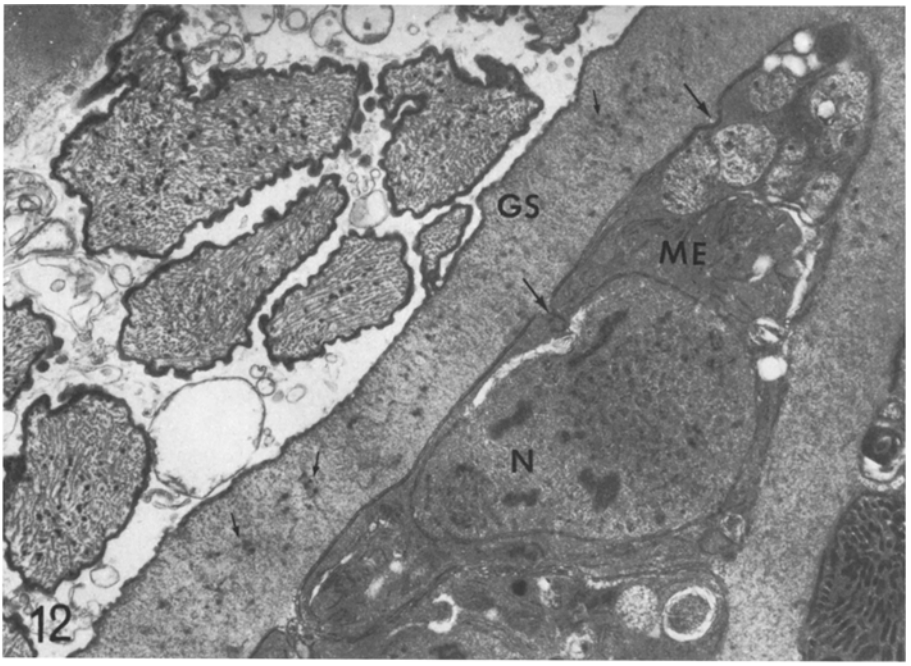
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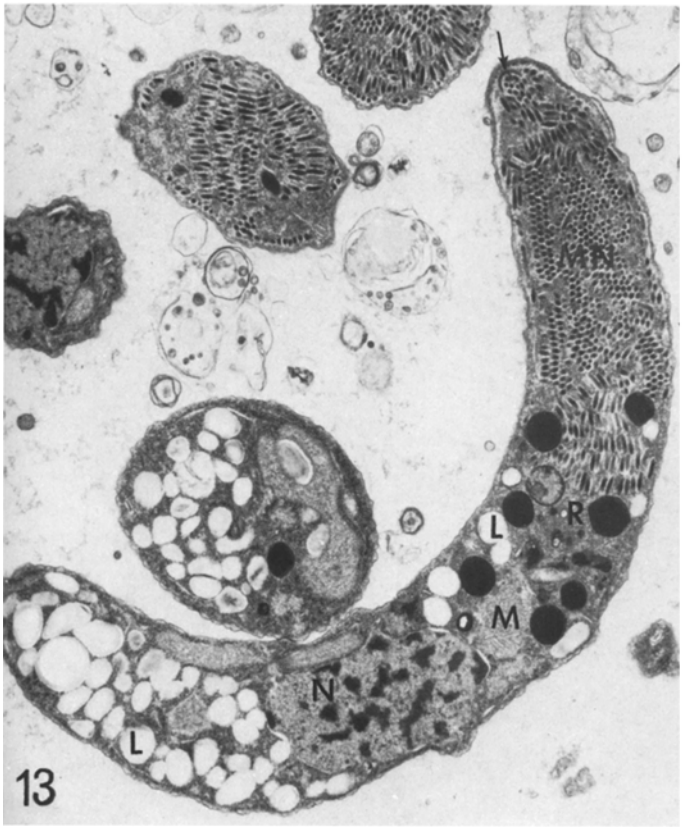
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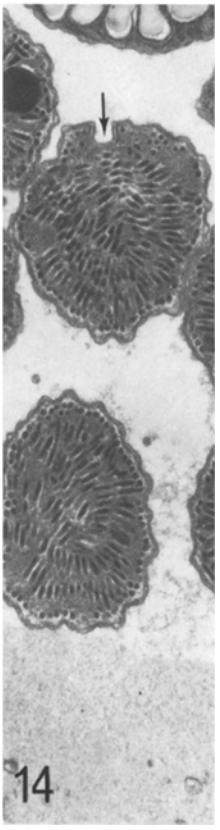
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in Siam as *Balbiania siamensis*, said that possibly the same species was found earlier by de Jongh in the buffalo in Java and Sumatra. Willey et al. (1904), reporting on the same parasite from Ceylon, called it *S. bubali*. It is interesting to note that they instituted feeding experiments in a dog but, apart from stating that "after the lapse of several months the dog may be examined, but it is not unlikely that it will be found that the dog is not a facultative host of *S. bubali*," they gave no further results. It is also no doubt the same species that Bhatia (1938) records from the buffalo in Ceylon as *S. blanchardi*. All these reports are of the large sarcocyst-producing species and it is clear that the small sarcocyst-producing species has not hitherto been recognized.

The demonstration that one intermediate host can harbour more than one species of *Sarcocystis* (Rommel et al., 1972; Heydorn and Rommel, 1972; Rommel and Heydorn, 1972) initiated the search for other species from the same host. In fact at the beginning of our studies we wrongly assumed that there was only one species in the water buffalo, namely, that producing large sarcocysts. Light microscopic and subsequent electron microscopic studies of oesophageal muscle soon revealed that there was another species producing small sarcocysts in which the wall had sloping villi quite unlike the cauliflower-like villi of *S. fusiformis*. The presence of the new species was finally confirmed by feeding experiments briefly reported by us earlier (Dissanaike et al., 1977) and described in more detail in the present paper. The obvious difference in the sarcocyst sizes, the cyst wall and the zoites both in light and electron microscopic sections, the different definitive hosts and the sporocyst sizes clearly distinguish the two species in the water buffalo. The more detailed ultrastructural features of the new parasite as compared to *S. fusiformis* will be discussed in a subsequent paper (Kan and Dissanaike, in preparation).

If we follow the nomenclature suggested by Heydorn et al. (1975) it would be necessary to name our new parasite *S. bubalicanis*. Although this is a very convenient name reflecting the life cycle of the parasite, we are of the opinion that it is unwise, particularly in view of the recent demonstration by Dubey et al. (1977), that two species of *Sarcocystis* can share the same definitive and intermediate hosts (as in *S. bertrami* and *S. fayeri* of the horse). We are therefore naming our parasite *S. levinei*.

It is necessary to distinguish *S. levinei* from the similar microscopic cyst-producing species from cattle. *S. hirsuta* (syn. *S. bovifelis*) has the cat as definitive host, produces sporocysts that are smaller (12.5 by 7.8 $\mu$m) than the present species and has villi in its cyst wall which are ultrastructurally similar to those of *S. levinei*, but these villi do not contain the coarse granules or the wavy wall so characteristic of *S. levinei*. On the other hand, *S. cruzi* (syn. *S. bovicanis*) like *S. levinei*, uses the dog as definitive host which sheds sporocysts of similar size (16.3 by 10.8 $\mu$m). It differs however in that the villi of the cyst wall are small flattened protrusions without fibrils and are irregular in size and arrangement.

**Fig. 12.** Electron micrograph showing a metrocyte (*ME*) just beneath the ground substance (*GS*) which contains coarse, electron-dense granules (*small arrows*). Note large nucleus (*N*) and micropores (*large arrows*) along cell wall of metrocyte. ×11,200

**Fig. 13.** Electron micrograph showing a zoite of *S. levinei*. Note anterior conoid (*arrowed*), micronemes (*MN*), rhoptries (*R*), mitochondrion (*M*), nucleus (*N*) and lipid droplets (*L*) within zoite. ×8,000

**Fig. 14.** Electron micrograph showing micropore (*arrowed*) in region of micronemes. ×11,200

One of our experimental dogs (P1) which was re-fed 87 days after the first feed (with oesophageal muscle) and 50 days after it had stopped shedding sporocysts from the first infection (Table 1), shed sporocysts again after a prepatent period of 12 days and continued to shed them for 13 days. It then became negative for 33 days and began reshedding sporocysts against for 30+ days. While this confirms the observations of other workers that there is no immunity to reinfection in the definitive host, it is difficult to explain the reshedding of sporocysts after a negative phase of 33 days. Dubey's (1976) report of the reshedding of *Toxoplasma gondii* oocysts by chronically infected cats in the absence of exogenous reinfection is worth considering in this connection. It is possible that an hitherto undetected extra-intestinal or tissue stage gave rise to the second lot of sporocysts, but this possibility has been ruled out by Fayer (1974). On the other hand, the dog may have swallowed its own sporocysts from its faeces although reinfection by this method is not supported by the work of others (Fischle, 1973; Rommel et al., 1974; Fayer, 1974), nor is this possibility supported by the findings of Fayer and Leek (1973) in their in vitro studies. It is interesting to note that this same dog (P1) that reshed sporocysts was also the oldest dog used in the experiments (8 years) whereas all other dogs were around one year old. It was also the dog that was kept longest on a meat-free diet. The above observations need further investigation with this and other species of *Sarcocystis*.

Another observation that needs further investigation is the origin of the *I. heydorni*-like oocysts that were shed by dog 2123, 10 days after feeding buffalo oesophageal muscle (Table 1).

In comparing the sexual stages of *S. levinei* with those of *S. cruzi*, described in detail by Fayer (1974) under the incorrect name *S. fusiformis*, it is clear that there are similarities. Only a more detailed study of these stages in *S. levinei* will reveal distinguishing features. In this context it is not possible to decide which parasite was seen by Wenyon and Sheather (1925) andWenyon (1926) in sections of the small intestines of dogs in Ceylon, where mature oocysts "occurred in the subepithelial tissues" and sporocysts were 13.5–15.5 $\mu$m in length. While Dissanaike and Jayesuriya (1959) referred to microscopic sarcocysts in cattle in Sri Lanka and labelled them *S. hirsuta* it is not certain what species they saw. If it was *S. cruzi* that they referred to and if *S. levinei* occurs in buffaloes in Sri Lanka in addition to *S. fusiformis*, then Wenyon and Sheather's parasite could have been either *S. cruzi* or *S. levinei*.

We have found that both *S. cruzi* and *S. hirsuta* occur in cattle in Malaysia (confirmed by electronmicroscopic studies and feeding experiments) and yet neither of them appears to infect the buffalo and vice versa. Thus there appears to be a host restriction on the part of the parasites even among closely related bovine intermediate hosts.

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Species diagnosis of *Sarcocystis levinei* n. sp:

| | |
|---|---|
| Type host: | *Bubalus bubalis.* |
| Definitive Host: | Dog. |
| Locality: | Peninsular Malaysia. |
| Cysts: | Thin and spindle-shaped, 0.9 mm by 0.1 mm.<br>In oesophageal muscle.<br>Cyst wall thin with sloping, cytophaneres (height 7.4 $\mu$m). Wall of cytophaneres wavy and irregular. Within cytophaneres are hollow, annulated fibrils and coarse, electron-dense granules.<br>Trabeculae present – relatively few and thin, with no limiting membrane. |
| Zoites: | Banana-shaped, 17.8 × 4.2 $\mu$m (in fresh preparations).<br>Micronemes: 200–300. Rhoptries: 8.<br>Micropores: present. Nucleus: posterior to midline. |
| Sexual stages: | In lamina propria of villi of small intestine. Sporocysts (15–16 by 9–10 $\mu$m). |
| Syntypes: | In Department of Parasitology, Faculty of Medicine, University of Malaya. |

## Abbreviations

| | | | |
|---|---|---|---|
| F | fibrils | ME | metrocyte |
| G | granules | MN | micronemes |
| GS | ground substance | N | nucleus |
| L | lipid droplets | PCW | primary cyst wall |
| M | mitochondrion | R | rhoptries |

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