Ultrastructural and Transmission Evidence of *Sarcocystis cruzi* Associated with Eosinophilic Myositis in Cattle

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ABSTRACT

Skeletal muscle, diaphragm, tongue, esophagus and heart of beef carcasses that were condemned for eosinophilic myositis and those that were unaffected were collected at an abattoir in Colorado and studied to determine the involvement of *Sarcocystis* spp. All affected carcasses contained similar granulomatous lesions with adjacent infiltrations of leukocytes. Intact or fragments of sarcocysts were found within 32 of 363 granulomas, and whole sarcocysts were present in nearby unaffected muscle cells. Light and electron microscopic examinations revealed that sarcocysts, affected or unaffected by cellular response in condemned carcasses, as well as those found in unaffected carcasses, were consistent with those of *S. cruzi*. Transmission experiments confirmed that *S. cruzi* were present in all carcasses, and that dogs, but not cats, were the definitive hosts. The results of pepsin-HCl digestion assays showed that unaffected carcasses that were approved for human consumption generally contained more infective parasites than carcasses that were condemned for eosinophilic myositis. This study provides evidence to support the suggestion that dogs, rather than cats, and unaffected rather than eosinophilic myositis-affected carcasses, have greater potential for contributing to the perpetuation of eosinophilic myositis in the cattle industry.

RÉSUMÉ

Dans un abattoir du Colorado, on a étudié l'implication possible de *Sarcocystis* spp. dans la myosite éosinophilique à partir de muscles squelettiques, de diaphragmes, de langues et de cœurs de bovins de boucherie atteints ou non de cette condition. Les carcasses affectées présentaient des lésions granulomateuses avec une infiltration adjacente de leucocytes. Des sarcocystes entiers ou fragmentés ont été retrouvés dans 32 des 363 granulomes étudiés et des sarcocystes entiers ont été rencontrés dans les cellules musculaires avoisinantes. La microscopie photonique et électronique a permis de classer les sarcocystes observés appartenant à *S. cruzi* et ce indépendamment de la réponse cellulaire observée et/ou de la condamnation de la carcasse. Des études de transmission ont permis de définir que *S. cruzi* était présent dans toutes les carcasses examinées et que le chien et non pas le chat est l'hôte définitif de *S. cruzi*. De plus, à partir de carcasses non-affectées et approuvées pour consommation humaine on a généralement retrouvé plus de parasites infectants que dans les carcasses condamnées pour myosite éosinophilique. Cette étude démontre d'une part que le chien plutôt que le chat et d'autre part que les carcasses non-affectées vs celles affectées de myosite éosinophilique seraient des facteurs importants contribuant à la perpétuation de l'affection chez les bovins. (*Traduit par Dr Pascal Dubreuil*)

INTRODUCTION

For many years, *Sarcocystis* spp. has been suspected as an etiological agent of eosinophilic myositis (EM), which is often recognized as small disseminated lesions that are usually green to greyish-green (1,2). Recent reports have provided evidence that sarcocysts are directly associated with the lesions and contribute to condemnations and down-grading of carcasses at meat-processing plants (3-6). One study examined cattle and sheep and found that up to 5% of carcasses were condemned for EM in the USA (3).

Currently, there are no tests that can predict the presence of EM prior to slaughter. Determination of the species of *Sarcocystis* involved would expedite the development of a reliable serological or nucleic acid hybridization assay. Immunoglobulins that are specific for species of *Sarcocystis* have been found in both affected and nonaffected cattle (7), and studies using experimentally infected calves failed to produce EM (8-10). Consequently, there is confusion about the species identity of sarcocysts that are responsible for the lesions of EM in cattle. However, it is possible to identify species of *Sarcocystis* by examining the ultrastructure of sarcocyst walls (11). Once the location of the parasite has been determined the tissue can be processed for electron microscopy (4).

In the present study, beef carcasses affected and unaffected with EM were sampled and compared for *Sarcocystis* spp. infection using light microscopy and a digestion technique. The cellular immune response in the infections was assessed, and the ultrastructure of selected sarcocysts examined in order to determine species identity. Dogs and cats were used in transmission studies to confirm species identification of associated sarcocysts.

MATERIALS AND METHODS

TISSUE COLLECTION

During 1988 and 1989, unaffected and EM-condemned carcasses were made available from an abattoir

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(Monfort of Colorado Inc., Greeley, Colorado). The animals were 1.5–2 year old steers and heifers that were obtained from feedlots in the mid-western USA, but primarily from Colorado. Portions of unaffected hearts and esophagi were obtained from carcasses that were approved for human consumption by US Department of Agriculture meat inspectors. Carcasses with disseminated eosinophilic granulomatous lesions were condemned by the inspectors and made available for this study. Portions of skeletal muscles, diaphragm, tongue, esophagus and heart were obtained from affected carcasses. The gross condition of the tissues was recorded and portions processed for histological study, digestion, or transmission experiments.

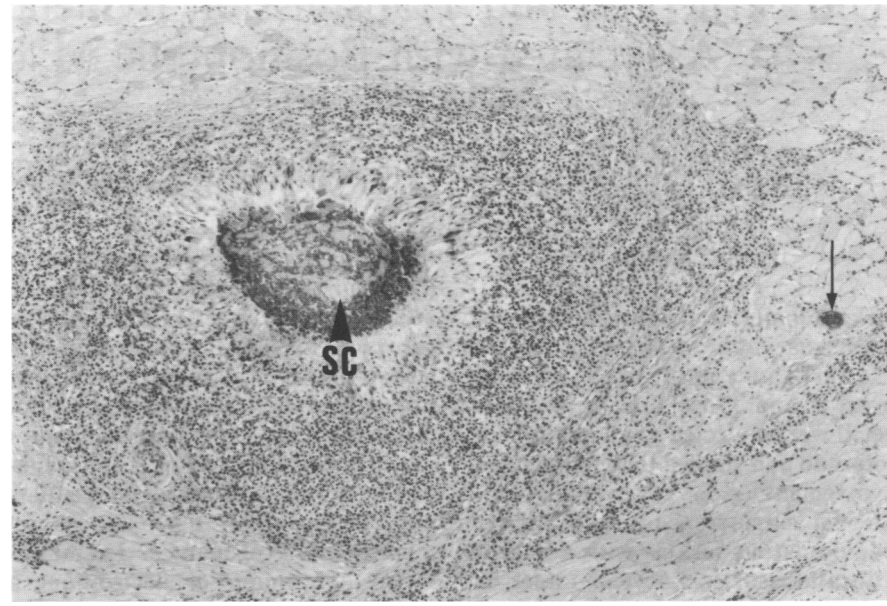

Fig. 1. Light micrograph of a section through a granulomatous lesion in the muscle of a condemned beef carcass. A sarcocyst (SC) is in the center of the granuloma, and another (arrow) appears unaffected outside the lesion. H & E. ×70.

HISTOLOGICAL STUDY

Muscles of the various organs were sliced perpendicular to muscle fasci and, whenever present, lesions and some surrounding tissues were removed for histological study. Depending on the number of foci present, 2–12 sections from each organ were fixed in neutral-buffered 10% formalin, processed by standard histological techniques, sectioned at 6 μm, stained with hematoxylin and eosin (H & E), and examined by light microscopy.

ELECTRON MICROSCOPY

Histological sections of muscles that contained sarcocysts without cellular reaction or sarcocysts within granulomatous reactions were processed for transmission electron microscopy (4,12). The slide was soaked in xylene, the coverslip removed, and unwanted portions of the tissue trimmed. Dehydration of the specimen was performed by flooding the slide with propylene oxide, and the tissue was embedded by inverting a capsule of epon over it. The epon was polymerized by incubation of the capsule, epon, tissue-section and slide for 48 h at 60°C. Drops of ice-cold water were placed at the base of the capsule and a razor blade used to free the embedded tissue and capsule of epon from the glass slide. The block was trimmed and thin sections cut and stained with lead citrate. A Philips EM 410 LS transmission electron microscope was used to examine the sections and take photographs.

DIGESTION OF TISSUES

Unaffected or condemned heart was cut into small cubes and ground in a meat grinder. One hundred grams of ground meat were placed in a flask containing 400 mL of a pepsin-HCl solution (0.6% pepsin, 0.8% NaCl, 0.8% HCl) and incubated with gentle agitation for 1 h at 37°C. Four layers of gauze were used to remove undigested tissue and the filtrate was washed by centrifugation, in phosphate-buffered (pH 7.0) saline (PBS). The bradyzoites were purified further on a Percoll gradient. One volume of bradyzoite-PBS suspension was mixed with two volumes of isotonic Percoll stock solution and centrifuged in 15 mL conical tubes using a swing-out rotor at 1160 × *g* for 20 min at room temperature. Bradyzoites were recovered from the pellet, washed twice in PBS and the number of organisms estimated by examining an aliquot in a hemacytometer.

TRANSMISSION EXPERIMENT

Nine 6 wk old dogs that were born and raised in isolation and nine similarly raised 3–8 month old cats were used in transmission studies. Care and housing of animals were in accordance with the National Institute of Health guidelines. Fecal samples of all animals were collected daily, ten days prior to inoculation, and processed by the Sheather's sugar flotation method for the presence of parasitic forms. One hundred grams of ground EM-condemned heart or esophagus were used to orally inoculate two dogs and two cats. Similarly, 100 g of ground, unaffected heart were used to infect two dogs and two cats. An uninoculated dog and a cat were kept as controls. The feces of dogs were collected daily until 19 or 21 days postinoculation (PI) and preparations of fecal flotations examined for the presence of oocysts and sporocysts. The feces of cats were similarly collected and monitored for the presence of *Sarcocysts* spp. Sporocysts observed in fecal preparations were measured on a light microscope using oil immersion and an ocular micrometer. One cat from each of the four groups was euthanized 22 days PI and the small intestine removed. Mucosal surface scrapings obtained at 5 cm intervals along the small intestine were placed on slides and microscopically examined for evidence of *Sarcocystis* spp.

RESULTS

GROSS PATHOLOGICAL FINDINGS

All samples collected from 19 bovine carcasses that were condemned for EM contained small disseminated lesions of

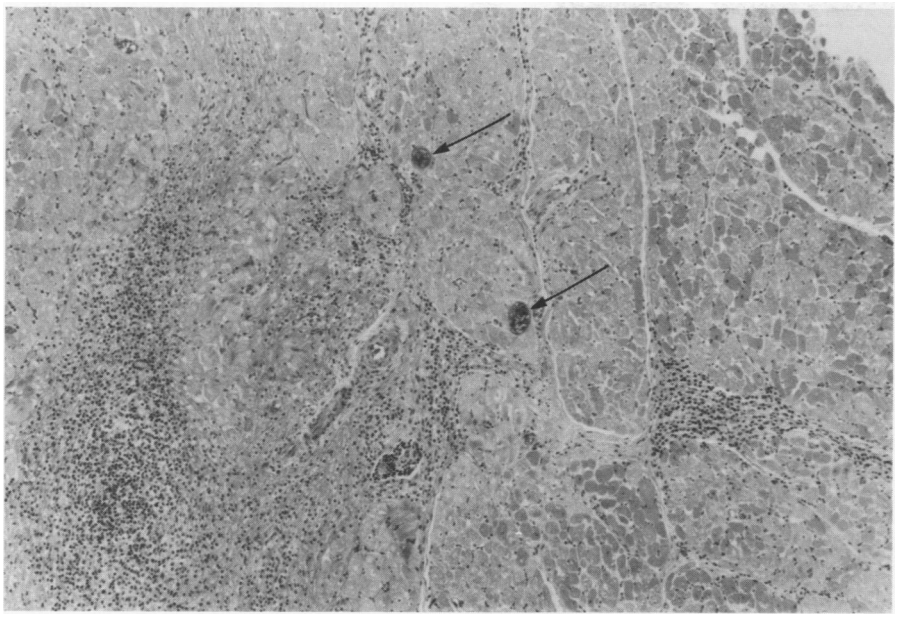

Fig. 2. Light micrograph of a typical section through the muscle of a carcass affected with eosinophilic myositis. Note the diffuse leukocytic infiltration and the *S. cruzi*-like sarcocysts (arrows). H & E. ×70.

variable intensity from one organ to another and from one carcass to another. The intensity and prevalence of cysts were highest in myocardial and esophageal muscles. The lesions were discrete, round to fusiform, greyish-green and measured approximately 0.5–4 × 2 mm. Similar but larger cyst-like structures (5–8 × 2–3 mm) were often found in the esophagus. Occasionally, lesions were present only in the heart or skeletal muscles.

HISTOPATHOLOGICAL FINDINGS

The cyst-like lesions consisted of granulomatous reactions with adjacent intense cellular infiltrations (Fig. 1). The intense reaction often encompassed the entire muscle fasciculus. Beyond the granulomas, there were accumulations of mixed inflammatory cells, mainly eosinophils with some neutrophils, macrophages and plasma cells, as well as erythrocytes. A generalized leukocytic response of mostly eosinophils was present in all 549 sections of 19 condemned carcasses (Fig. 2). Eight hundred and fifty-three granulomas were examined and 46 intact or fragmented sarcocysts were found in the center of 32 granulomas. Eosinophilic myositis-associated sarcosysts appeared similar to each other in structure, shape and size. A cyst wall could not be discerned by light microscopy, but within the sarcocysts' septae, numerous zoites and pale to neutral-staining degranulating eosinophils were observed. Within most granulomas and adjacent to the trapped sarcocysts there were areas of caseation necrosis and degenerated, hyalinized remnants of muscle fibers (Fig. 1). Beyond the central area of the granulomas there were numerous leukocytes, primarily eosinophils, that were occasionally surrounded by a layer of elongated epithelioid cells.

Sixty-six percent of sections contained 363 well organized granulomas, and 46 EM-associated sarcocysts were found in 11 of the 19 affected carcasses. Approximately 50% of all sections and 32 sections of EM-condemned carcasses that contained sarcocysts within granulomas also had typical *S. cruzi*-like sarcocysts that were devoid of cellular response (Fig. 1). The light microscopic features of these parasites were all similar to each other, including an indiscernible cyst wall (Fig. 3), and were consistent with the sarcocysts of *S. cruzi*. Two thick-walled *S. hirsuta*-like sarcocysts were found in two myocardial sections that were obtained from an unaffected carcass.

ULTRASTRUCTURE

Following processing for electron microscopy, ultrastructural information was obtained from five sarcocysts within granulomas, and two that lacked cellular response, one of which

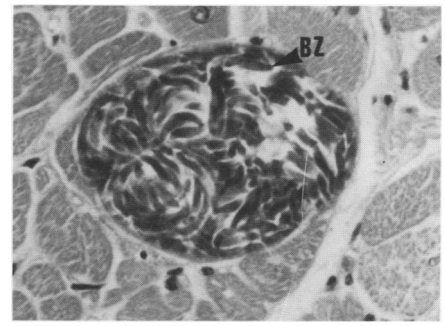

Fig. 3. Light micrograph of a section of an unaffected *Sarcocystis cruzi* sarcocyst. Note the presence of numerous bradyzoites (BZ), and that the thin wall of the sarcocyst is not discernible. H & E. ×800.

was from unaffected beef muscle. The walls of all sarcocysts were similar to each other (Figs. 4 and 5). They were thin (0.44–0.56 μm) and had flattened microvillar protrusions that did not contain fibrils. Although all sarcocysts had prominent septa, the granuloma-associated parasites enclosed an amorphous matrix which contained numerous oval to fusiform zoites (Fig. 4). Sarcocysts that were not associated with cellular response also contained numerous zoites, but lacked the amorphous matrix (Fig. 5). Occasionally, subpellicular microtubules could be discerned as well.

PEPSIN DIGESTS

The zoites that were extracted from 21 of 22 animals were approximately 10–12 μm long. No parasites were found in the esophagus of one unaffected carcass, yet the largest number of parasites (3.7×10^6 per g) was present in an unaffected heart that was passed for human consumption. The mean number of zoites extracted per gram of tissue was 14.5×10^5 for unaffected hearts, 8.2×10^5 for condemned hearts, 3.4×10^5 for unaffected esophagi and 4.4×10^5 for condemned esophagi. Generally, more zoites were recovered from unaffected hearts than hearts that were condemned for EM, and fewer parasites were harvested from EM-condemned carcasses than unaffected tissues. The zoites obtained from normal as well as condemned carcasses appeared to be intact and were often motile.

TRANSMISSION

Oocysts or sporocysts of *Sarcocystis* spp. were not found in the feces of

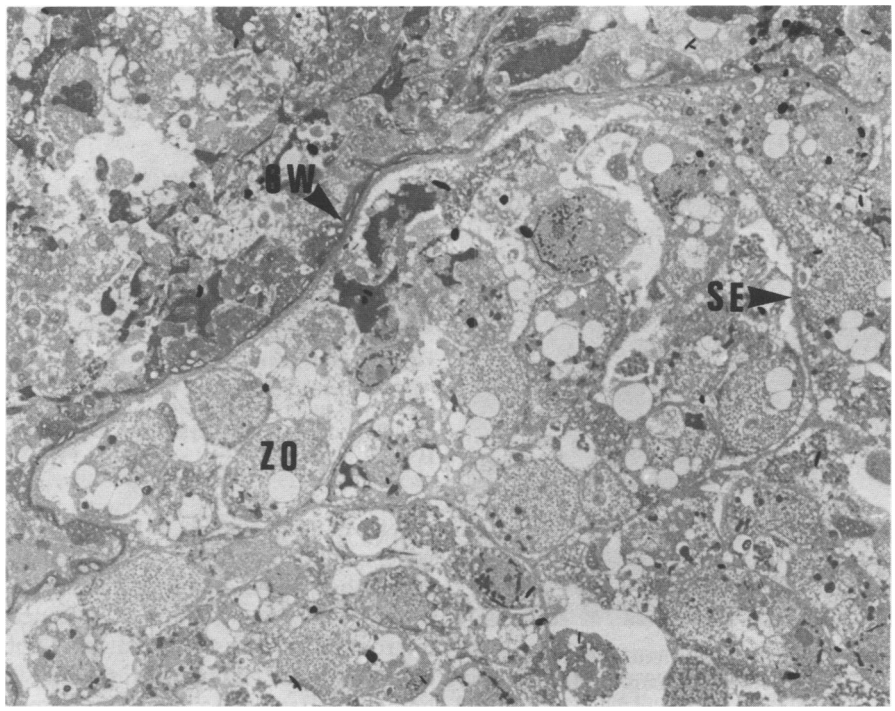

Fig. 4. Electron micrograph of a sarcocyst in a granuloma. The sarcocyst is enclosed by a thin wall (CW), contains septae (SE), and many zoites (ZO). ×3,200.

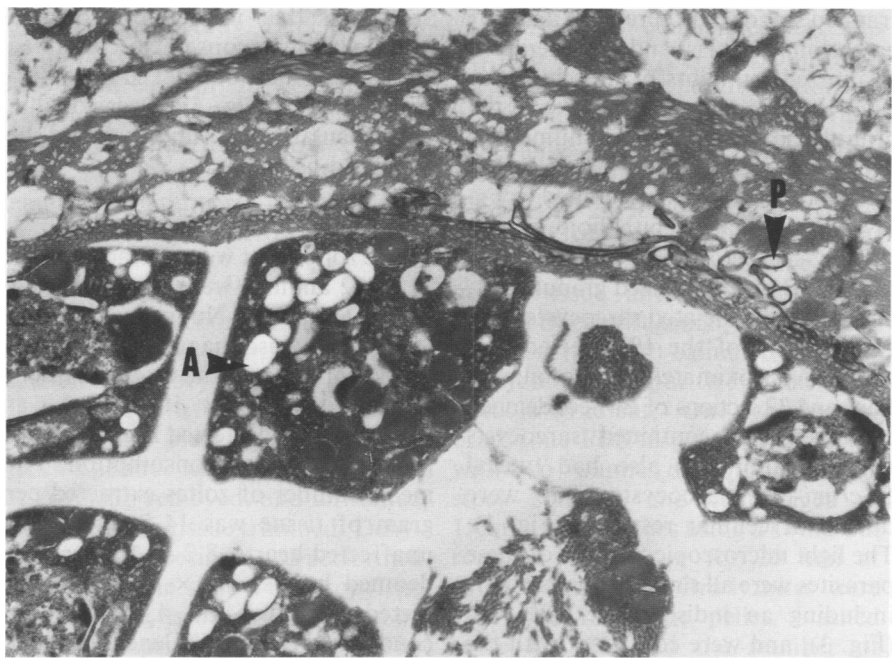

Fig. 5. Electron micrograph of a portion of *S. cruzi* sarcocyst that is without cellular reaction. The bradyzoites contain numerous amylopectin granules (A) and the cyst wall has flattened microvillar projections (P). ×11,800.

dogs and cats prior to inoculation. All cats appeared healthy throughout the experiment and their feces were always well formed. The feces of all dogs were formed or semi-formed before inoculation and the first few days PI. Dogs that were fed unaffected or condemned heart or esophagus became diarrheic two to five days PI and consumed less than the usual amount of food. The uninfected control dog showed none of these signs and sporocysts were never found in its feces. Sporocysts were found in the feces of all inoculated dogs beginning 10–12 days PI and were present daily for at least nine days, regardless of whether esophagus or heart was the source of the inoculum. Most sporocysts were passed between 11–17 days PI by dogs that were fed normal heart muscle. The measurements of the sporocysts passed by these dogs are given in Table I.

DISCUSSION

This study confirms the ubiquity of *Sarcocystis cruzi* in cattle. Ninety-six percent of animals examined by the digestion procedure contained zoites. Although the digestion procedure is more efficient in detecting *Sarcocystis* spp. in muscle than are visual inspection, trichinoscopy, histology or transmission methods (13), the procedure does not detect all infections. Tissues are not digested to completion, and parasites may be trapped and discarded with the unfilterable material. Also, if conditions, such as osmolarity, are inappropriate the zoites may lyse or crenate and become unrecognizable. For these reasons, the digestion method underestimates both the prevalence and intensity of infections. Thus, the statement that virtually 100% of cattle in the USA are infected (14) is probably accurate.

Generally, there were two to three times more parasites per gram of myocardium than per gram of esophageal muscle. This may be due to the predilection of *S. cruzi* for heart muscle. Experimental studies have shown that *S. cruzi* is most commonly found in the heart (9). The reason for finding nearly twice as many zoites in unaffected than EM-condemned hearts is not clear. It is not known whether the zoites extracted from the condemned carcasses included parasites from within granulomas or were only from sarcocysts that were not associated with the lesions of EM. Although all extracted zoites appeared to be similar in size, shape and structure, it was not possible to determine whether one or more species of *Sarcocystis* were harvested. It may be that fewer parasites were harvested from condemned heart because zoites within granulomas were already destroyed or not readily freed by the digestion procedure.

TABLE I. Measurements of sporocysts passed in the feces of dogs that were fed eosinophilic myositis-condemned or unaffected heart or esophagus

	Heart		Esophagus	
	Normal	Condemned	Normal	Condemned
Range (μm)				
n = 30	15.0-15.6 × 8.3-10.0	14.4-16.1 × 8.3-10.0	14.4-15.6 × 8.9-10.0	15.0-16.1 × 9.0-10.6
Mean:	15.4 × 9.7	15.4 × 9.6	15.5 × 9.8	15.3 × 9.7
SD	0.3 0.5	0.4 0.4	0.4 0.4	0.3 0.4

Histological evidence indicated that granuloma-associated sarcocysts were partially destroyed or enclosed by dense, organized accumulations of inflammatory cells. Generalized inflammatory reactions were common in sections of all condemned carcasses, and sections of all lesions contained granulomatous reactions. Most of the granulomas observed in EM-condemned carcasses may have resulted from sarcocysts that served as chronic inflammatory stimuli. It is not surprising that sarcocysts were found in only a small proportion of granulomas. Sarcocysts within lesions were relatively small and a single histological section will not always reveal their presence. Other studies have demonstrated that, when the parasite was present within a granuloma, many serial sections were often required to demonstrate its presence (3-5). Furthermore, because of the destructive nature of granulomatous reactions many sarcocysts were partially or completely destroyed.

There is uncertainty regarding the species identity of sarcocysts that are involved in bovine EM (11). Calves experimentally infected with *S. cruzi* and *S. hirsuta* did not contain EM up to eight months after the inoculation (10) and the lesions were not present in *S. hominis*-inoculated cattle (15,16). Based on light microscopic studies, it has been suggested that *S. cruzi*, *S. hirsuta* and *S. hominis* are involved in these lesions (3). However, identification based solely on light microscopy is not a reliable method of identifying species of *Sarcocystis* and there are no ultrastructural or transmission data to support this claim. Nevertheless, the present study provides evidence which supports the claim that *S. cruzi* is involved in EM (7,17,18). Our histological observations, substantiated by ultrastructural data, indicated that the sarcocyst walls in the lesions were identical to those of *S. cruzi*. The ultrastructural differences seen between the two types of sarcocysts may have been related to age of the parasites, or fixation artifacts. Most often, the zoites in the lesion-associated sarcocysts contained few of the elements of the apical complex, and rhoptries were rarely present. This is considered to be an indication of an immature zoite (9).

The passage of *S. cruzi*-like sporocysts in the feces of dogs that were fed normal or condemned heart or esophagus adds additional support for incriminating canids as the definitive host. However, the patency may have been the result of only sarcocysts that were not associated with cellular reaction.

The general absence of thick-walled sarcocysts of *S. hirsuta* in sections of condemned carcasses is supported by the absence of patent infections in cats that were fed normal or condemned muscle. This information does not preclude the possibility that *S. hirsuta* may also be involved in EM. However, it may be inferred that *S. hirsuta* is not as common as *S. cruzi* in parts of the USA and that the former organism does not contribute significantly to economic losses in the cattle industry.

Despite the lack of experimental evidence, it is possible that all three species of bovine *Sarcocystis* may be involved in EM. Previous ultrastructural studies indicate that other species which may or may not be normal bovine parasites are also involved in EM (4). The present investigation shows that, while some sarcocysts stimulate an overwhelming inflammatory response, sarcocysts with identical walls are entirely free of cellular response. The hypothesis that the walls of some sarcocysts rupture, become leaky, and release toxins (3) may be valid, but it is unlikely that it is age-related. It may be related to a genetic defect in sarcocysts and/or repeated sensitization of the host to a particular species of *Sarcocystis*.

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