Preliminary Report

The *Sarcocystis*-Cyst Containing Beef and Pork as the Sources of Natural Intestinal Sarcocystosis in Thai People

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Background: *Human intestinal sarcocystosis is a zoonotic disease caused by two coccidians, i.e. Sarcocystis fusiformis (syn. S. bovihominis, S. hominis) due to consumption of raw infected beef, and Sarcocystis meischeriana (syn. S. suihominis) due to consumption of infected raw pork. In 1987, survey of the macroscopic S.fusiformis cysts in market beef mainly from old water buffalos aged more than 15 years were commonly observed in Bangkok. In 2005, the macroscopic cyst was no longer seen in beef of cattle and water buffalo aged less than three years.*
Objective: *The epidemiological investigation of Sarcocystis spp. infected meat in Bangkok and Lampang.*
Material and method: *Samples for each of the tongue and beef of cattle and water buffalo, pork from Bangkok markets and pork of domestic swine from some remote villages in various subprovinces (Ampurs) in Lampang were obtained for microscopic examination by H and E and selectively by PAS staining.*
Results: *The microscopic S. fusiformis cysts were seen in all five specimens of tongues and ten specimens of muscles of cattle and water buffalo obtained from fresh-food markets in Bangkok. Ten samples of pork from Bangkok markets revealed no coccidian infection. The microscopic S. meischeriana cysts were seen in three specimens of swine muscles collected from two subprovinces in Lampang.*
Conclusion: *The present merozoites in coccidian cysts retrieved from beef and pork are similar to those previously observed in human intestine. This may histologically indicate an invasive sarcocystosis by both species leading to a condition presently known as chronic inflammation of undetermined etiology in man.*

Keywords: *Sarcocystis fusiformis, Sarcocystis bovihominis, Sarcocystis meischeriana, Sarcocystis suihominis*

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Intestinal sarcocystosis in animals and man is a coccidian infection caused by *Sarcocystis* spp. each having an obligatory heteroxenous life cycle concerning the prey-predator (intermediate host-definitive host) cycle[1-3]. The gametogony, syngamy in sexual phase and sporulation of the coccidian occurred in the intestine of the definitive host usually a predator. The asexual phase and the early sexual phase developed in the extraintestinal tissues with formation of the skeletal-muscle cysts in the intermediate host usually a prey[1-3]. Human intestinal sarcocystosis is caused by two species of coccidian parasites namely *Sarcocystis fusiformis* (syn. *Sarcocystis bovihominis, S. hominis*) by consumption of the raw infected beef and *Sarcocystis meischeriana* (syn. *Sarcocystis suihominis*) by the raw infected pork[1-3].

Asymptomatic intestinal sarcocystosis in man under the experimental and natural situations was recognized by the shedding of oval sporocysts in stools[4-7]. In the raw-beef-consuming Thai patients, the sexual stage and sporulation including asexual forms of *Sarcocystis fusiformis* in combination with *Clostridium* spp. infection or actinomycosis were observed histologically in the small and large bowels resected under segmental eosinophilic necrotizing enterocolitis and eosinophilic granuloma of the intestine[8,9]. This is due to the local dietary habit of some

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groups of Thai people in consuming raw meat in the chilli-hot dish of "Larb dib" together with vegetables contaminated by some certain bacteria. The necrotizing inflammatory bowel condition when found needs a prompt surgical resection due to pending or actual peritonitis similar to pig-bel disease caused by *Clostridium perfringens* type C infection after a heavy pork meals in the highlands of Papua New Guinea[10].

Segmental eosinophilic or necrotizing enterocolitis also occurred in the patients after consumption of raw pork as observed by one of the authors (PU). These inflammatory bowel diseases happened in the patients without any raw meat consumption possibly due to an unclear asexual pathway leading to chronic infection by the coccidian[8,11].

The epidemiological investigation by surveying the *Sarcocystis* spp.-infected meat in Bangkok and Lampang has indicated that the raw infected beef and swine pork were the natural sources of human infection in Thailand. The histological and electron microscopic morphologies of the cyst-containing skeletal muscle cysts in beef and pork were presented.

Material and Method

In 1987, the *Sarcocystis* spp. cysts in sliced beef and pork were searched for in the fresh-food markets in Bangkok and an abattoir in Bangkok vicinity. In 2005, a second survey was done in the same areas in Bangkok and extended to some villages in a northern province, Lampang, where the habitants commonly have a dietary habit of eating raw meat.

The original binomials were employed i.e. *Sarcocystis fusiformis* (Railliet, 1897) Bernard and Bauche, 1912 with water buffalo-man cycle (syn. *Sarcocystis fusiformis* Railliet, 1897 Babudieri, 1932 with ox-man cycle)[12] and *Sarcocystis meischeriana* (Kuhn, 1865) Labbe, 1899 with pig-or swine-man cycle[12].

The coccidian forms inside the skeletal-muscle cysts in man or animals have been described as bradyzoites, cystozoites, cyst merozoites or just zoites[13-15]. The term of cystozoite was here employed and additionally described in the present study as a prokaryotic or eukaryotic form.

In 1987, the sliced beef were collected from the fresh-food markets in Bangkok. There were the appearances of gray-white spindle-shaped cysts, largest 3.5 x 22 mm (Fig. 1A) and as small as 0.5 x 3 mm. These macroscopic cysts were sparsely distributed, approximately 1 cyst per 0.5-1 kg of beef. They were diagnosed as *S. fusiformis* cysts. The cysts were found in beef having water buffalo (*Bubalus bubalis*) in origin rather than from cattle in origin. No *S.meischeriana* cyst could be seen in pork of pigs (*Sus scrofa*). In an abattoir located in Bangkok vicinity, numerous skeletal-muscle cysts were found in the esophageal muscle of water buffalos over 15 years of age. The cysts were 2-3 x 8-10 mm in average (Fig. 1B). The infected esophagus was routinely discarded by the abattoir veterinarians. The skeletal-muscle cyst was not found in the esophageal walls of cattle and water buffalos younger than three years. A part of the infected esophageal wall was prepared for the histological study by hematoxylin and eosin (H and E) and periodic-acid-Schiff (PAS) for staining of carbohydrates. A part of the cyst was prepared for the study under a transmission electron microscope (TEM).

In 2005, the authors' epidemiological survey revealed no macroscopic coccidian cyst in beef collected from the markets. Meanwhile, there was no old water buffalo brought to the abattoirs in Bangkok vicinity. The skeletal-muscle cyst was not seen in the esophageal muscle walls of young cattle or buffalos averaging younger than three years. Some samples without grossly visible cyst for each of the tongue, beef of cattle and buffalo, pig pork from Bangkok markets and pork of domestic swine (*Sus scrofa scrofa*) from some remote villages in various subprovinces (Ampurs) in Lampang were obtained for microscopic examination by H and E and selectively by PAS staining.

Results

All macroscopic skeletal-muscle cysts obtained in 1987 could be squeezed from the sliced beef or esophageal wall. They contained colorless glue-like matter. These cysts were found between the striated-muscle fibers and encased by a covering consisting of granular eosinophilic PAS-negative material, 2-8 μm in thickness (Fig. 1C). Internally, the covering material was blended with interconnecting septal meshwork of 2-8 μm in thickness and encasing a single or a cluster of 2-16 round coccidian forms, 10-12 μm in diameter known as metrocytes[15].

A central basophilic body of 2-3 μm in diameter was seen inside the individual metrocyte (Fig. 1C). These metrocytes could undergo binary division by endodyogeny (Fig. 1C, inset)[16]. In tracing the cyst inward, the septa became thinner and encased enlarging compartments, each of which contained up to 100 large elongated cystozoites averaging 2-3.5 x 8-13 μm. Each elongated cystozoite had a posterior placed nucleus in the H and E section (Fig. 1J). The central part of the grossly visible muscle cyst of 3 μm up in

length showed the degeneration and necroses of the zoites leaving only compartments containing cellular debris and precipitate of the parasitic fluid. Some microscopic cysts within the muscle monofibers were observed in the muscle tissue.

All five samples of the tongues and ten specimens of the market beef collected in 2005 microscopically revealed intracellular cysts by presenting with 1-3 cysts in 4 H and E sections (each of 2 x 3 cm) and averaging 200-800 microscopic cysts per 100 grams of meat. None of the ten samples of pork from pigs reared in the commercial farms under a hygienic condition revealed *S. meischeriana* infection. Three from five samples of swine pork from four Ampurs in Lampang contained *S. meischeriana* cysts in the same concentration as above. The positive samples were obtained from Ampur Gnao and Ampur Wangnua.

The microscopic cysts were 40-90 μm in width and confined within the monofibers. The full length of the cysts could not be measured since only the oblique sections of the cysts were obtained with the longest oblique length being 360 μm. The cysts were bound by a smooth cyst membrane (Fig. 1F), membrane with short villar projections (Fig. 1G and H) or long projections of 0.1 x 6 μm (Fig. 1D), or absence of the covering membrane allowing the zoites directly contact to the myofibrils and cytoplasm of the striated-muscle fiber (Fig. 1I). The young cysts contained myriad of round or oval pale-blue pro- or eukaryotic merozoites of 1-4 μm in diameter (Fig. 1F)[8]. The intermediate prokaryotic cystozoites with dark-blue staining and elongated-ovoid contour were 0.9-1.7 x 2-4 μm (Fig. 1G). The large prokaryotic cystozoites with similar staining and contour were 1.5-2.5 x 4-9 μm (Fig. 1H). There were the large crescentic cystozoites with centrally placed nuclei (Fig. 1I) and some cysts contained large elongated cystozoites similar to those in Fig. 1J. The young cyst contained several small metrocytes (4-6 μm in diameter) with large prokaryotic cystozoites in the center (Fig. 1D). The septal meshwork and regular metrocyte were not seen in these monofiber cysts. The cyst membrane, villar projections, merozoites, metrocytes, pro- and eukaryotic cystozoites were positively stained with PAS and resistant to diastase.

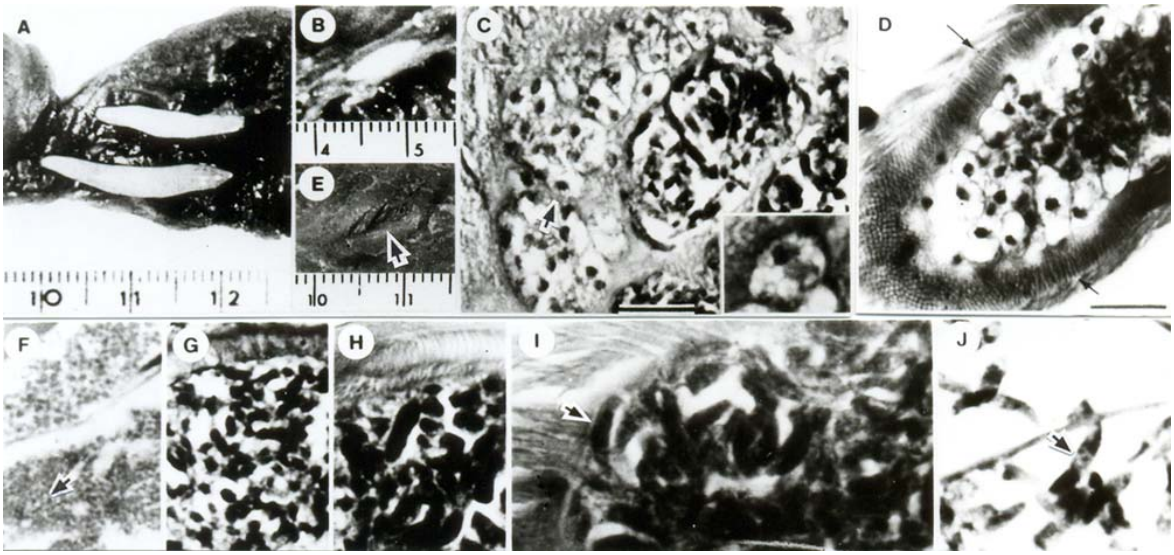

Fig. 1 A: a macroscopic cyst in beef, B: a macroscopic cyst in the esophageal wall of an old buffalo, C: a part of cyst periphery with round metrocytes in the left half, large developing and elongated cystozoites in the right, the cytoplasm of adjacent muscle fiber in the left upper corner and the septum indicated by the arrow, D: villar projections (arrows), E: a translucent cyst (arrow), F: two adjacent young cysts, the upper one containing prokaryotic merozoites and the lower one containing eukaryotic merozoites (arrow), G: intermediate prokaryotic cystozoites with elongated-oval contour seen in full length in the right upper quadrant, H: large prokaryotic cystozoites, I: a large crescentic cystozoite (arrow) and large prokaryotic cystozoites, J: a large elongated cystozoite seen in full length (arrow), A C and J: derived from meat of water buffalo, B and E: cysts in the esophageal wall of water buffalo, D G and I: ox's lingual muscle, F and H: pork of swine, C, D-J: Hematoxylin and eosin, C: bar = 20 μm, C inset and D-J: bar = 10 μm

The gray-white color of the macroscopic cysts in the esophageal wall of water buffalo preserved in 10% phosphate-buffered formalin solution for four years became colorless and translucent (Fig. 1E). Histological study revealed the decrease in intensity of eosin staining on the outer covering and septal meshwork.

TEM study of the macroscopic *S. fusiformis* cyst from the esophageal wall of water buffalo collected in 1987 revealed the fine and coarse dense granules forming the cyst covering and septal meshwork without limiting membrane (Fig. 2A). The large elongated cystozoites had a nucleus in the posterior portion and numerous polysaccharide granules[17] with the diameter of 0.2 μm in the middle portion (Fig. 2B). The anterior part of the cystozoites was rich in micronemes and rhobtries. A conoid structure was present at the anterior end (Fig. 2C). The developing cystozoites were seen with the internal structures attaching directly to the septal meshwork (Fig. 2C). Numerous free ribosomes were observed adjacent to the zoites, where the membrane boundary was absent (Fig. 2D).

The wet mount preparation of fresh content from *S. fusiformis* cyst revealed motility of the elongated large cystozoites about □ hour after the rupture of the cyst as observed under a dissecting microscope. There was a slow spiral rotation of the anterior part leading to a staggering forward movement of the elongate zoites. The developing cystozoites, crescentic zoites and metrocytes remained immotile.

Discussion

The nomenclature for taxonomic names denoting *Sarcocystis* spp. remains inconclusive. The original binomials such as *S. fusiformis* are employed in reference to the newly proposed binomials specifically indicating the pairs of prey-predator cycles i.e. *S. bovihominis* with ox-or water buffalo-man cycle. Similarly, *S. meischeriana* can be referred to as *S. suihominis* with pig-or swine-man life cycle[12,18-20].

The practice of man as the predator by consuming raw infected meat and of some animals acting as appropriate definitive hosts allows the passage of infective sporocysts into the environment. This leads to transmission of the coccidian to the appropriate intermediate host and completion of the life cycle of *Sarcocystis* spp.[1-3].

The sporocysts (9.3 x 12.6-14.7 μm in wet mount) are excysted in the intermediate host's intestine by the host's bile and trypsin and each cyst eventually releases four internal sporozoites[1-4,21,22]. The liberated sporozoites invade the intestinal mucosa of appropriate intermediate host, migrate and pass into various developmental stages of asexual phase in the endothelial cells. These coccidian forms of asexual phase form young cysts in large cells of the myocardium and skeletal muscle with eventual formation of the skeletal-muscle cysts containing large elongated cystozoites as soon as day 54 after the infection[23-25].

The young microscopic cysts within monofibers in Fig. 1D-I represent various developmental stages of the coccidian cysts in the aspects of internal structures and cyst coverings[23]. The cyst membrane may not be formed or becomes ruptured within the monofiber (Fig. 1I). The pro- and eukaryotic merozoites (Fig. 1F), intermediate and large prokaryotic cystozoites (Fig. 1G and H), and small metrocytes (Fig. 1D) seem to represent developmentally younger stages of large

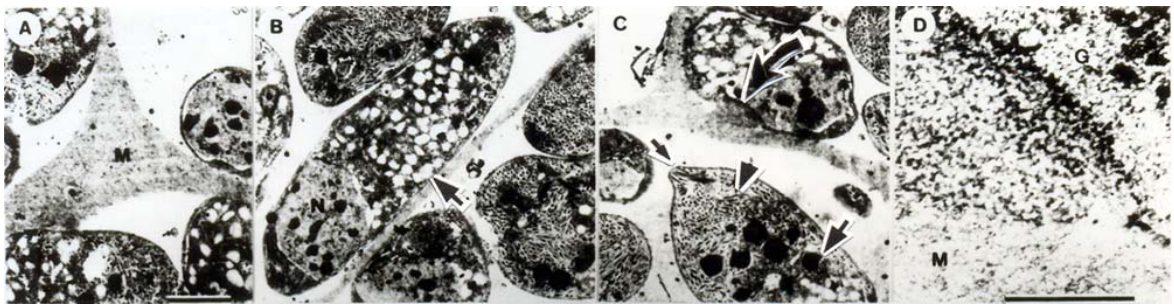

Fig. 2 A: the septal meshwork (M), B: a nucleus (N), polysaccharide granules (arrow), C: a conoid (small arrow), micronemes (arrowhead), a rhobtry (large arrow), attachment of a developing large cystozoite to the septal material (large curved arrow), D: higher magnification at the zone of attachment showing a cluster of free ribosomes between a developing large cystozoite or gametocyte (G) and the meshwork (M), Transmission electron micrographs, A-C, bar = 2 μm, D bar = 0.5 μm

crescentic cystozoites (Fig. I). Eventually, the large crescentic cystozoites transform to large elongated cystozoites (Fig. 1J) acting as the gametocytes in the early sexual phase within the skeletal-muscle cysts in meat (Fig. 1J)[13]. By the prey-predator action, the large cystozoites are transmitted to the definitive host's intestine, where the large elongate cystozoites become motile, invade the intestinal epithelium and develop to micro- and macrogametes. There are syngamy and sporulation with formation of the sporocysts in the mucosa followed by intraluminal release of the infective sporocysts[8,13]. The immotile developing cystozoites, crecencentic zoites, and metrocytes may be infective by locating and developing within the intestinal crypts.

Within the intermediate host, the microscopic skeletal-muscle cysts are prone to become ruptured due to the developmental enlargement. After rupturing of the cysts, some liberated large zoites may become immobilized after binding with the apical complex-associated antibody in the immunized host and change to round form of metrocytes[26]. Binding of the surface membrane and cytoplasmic granules to the corresponding antibody possibly leads to attacks by the eosinophils and lymphocytes on the exposing aspect with somes zoites escaping the killing effect[26-28]. Further binding with this antibody intermixed with inflammatory cell debris allows the deposit of Splendor-Hoeppli (SH) material around the survived metrocytes[29]. This SH material entraps these metrocytes and serves as a shield against further damage by the host's cellular defensive mechanisms.

Multiplication by endodyogeny of the entrapped metrocytes leads to an increasing number of daughter metrocytes. These may eventually transform to large crescentic and elongated cystozoites acting as the gametocytes within the enlarging compartments of endodyogenic packets encased by the cyst covering and septal meshwork of SH material in the extracellular cyst after decades[13]. In the cyst periphery, the partially entrapped metrocytes may undergo endodyogeny and divide to two free metrocyte seen as SH-encased single metrocytes.

Possibly due to a developmental disorder of the cyst, some intermediate prokaryotic cystozoites transform to small metrocytes (Fig. 1D) and likely progress further to regular metrocytes. The large elongated cystozoites from the late development serve as the natural reserve as long as the host's life. The new zoites from the cyst periphery dynamically replaced the degenerated zoites in the cyst center. The SH material seems to give gray-white color to the grossly visible cysts (Fig. 1A and B) and become eluted in formalin solution after four years leaving the cyst appear translucent (Fig. 1E). The absence of macroscopic cyst during the authors' second survey in 2005 was due to the young ages of the cattle and buffalos.

By TEM, the present large cystozoites within the extracellular cyst in water buffalo consist of basic structures i.e. one conoid, micronemes, rhobtries, polysaccharide granules (probably amylopectin in nature for energy reserve) and a nucleus. These basic structures are similar to the zoites of *S. fusiformis* (syn. *S. hominis*, *S. buffalonis* Huong et al, 1997) in water buffalo described from Singapore and Vietnam as well as to the smaller zoites of *Sarcocystis* sp. in skeletal-muscle cyst in man reported form Malaysia[18,30-32]. In addition, the basic structures are also similar to those in the zoites of *Cyclospora cayetanensis* and *Isospora belli* in man described from Western countries, as well as to *Toxoplasma gondii*[16,17,33]. The rhobtries have the function of excreting a lytic enzyme suggested to be cathepsin L-like protease when the zoite penetrates a host cell[34]. The micronemes are known to contain lectin[35]. The direct contact between the internal structures of developing cystozoites and septal material (Fig. 2D) has represented a distinct mean of nutrient transport from the septal meshwork to the zoite under the absence of alimentary tract. The free ribosomes may synthesize the required proteins before being transported into the coccidian body.

Geographically, the *Sarcocystis* spp. skeletal-muscle cysts in cattle (*Bos indicus and Bos taurus*) water buffalo, pig and swine are distributed worldwide[4-7,36-40]. In Thailand the infected tongues and muscles derived from cattle, buffalo and swine are the sources of natural intestinal sarcocystosis in man. For the preventive measure, meat cooked at 100□C for 4 minutes or frozen at -4□C for 48 hours is recommended to ensure disinfection[23].

Intestinal sarcocystosis mainly produces no symptom in repeated exposure ot humans in accordance with dietary habits in Tibet and Thailand, possibly due to the immunities of habitants particularly in Southeast Asia with known high prevalence of skeletal-muscle sarcocystosis[4,5,7,41-43]. However, in the previously unexposed persons living outside Southeast Asia, the large amount of *Sarcocystis* spp. intake can lead to symptoms of diarrhea, generalized myalgias, abdominal pain and distension, eosinophilic myositis and peripheral blood eosinophilia from 1 week to 6 months after the periods of exposure[6,44-47]. For the treatment, cotrimoxazole (trimethoprim 160 mg and

sulfamethoxazole 800 mg) three times a day for 12 days, or albendazole 600 mg twice a day for 20 days were prescribed[46,47].

In human skeletal-muscle sarcocystosis, the predator of man in supporting the prey-predator-cycle concept could not be identified in all reports as reviewed recently[25]. It is of immense interest that the merozoites seen in the animal's skeletal muscle (Fig. 1F) are histologically similar to the merozoites in the intestinal lamina propria and submucosa of man with sarcocystosis and to the merozoites in the endothelial cells of calves[8,23]. This may indicate an unclear complex life cycle of *Sarcocystis* spp. with the development of asexual phase occurring in the definitive host by a complicated alternative pathway detected only under the histological study[8]. In addition, in an experimental animal model of feeding *S. fusiformis* infected bovine heart to dogs the sporulation occurs in the intestinal lamina propria[36]. This indicates the invasive nature of sarcocystosis in the definitive hosts and may lead to the asexual phase and chronicity of the infection.

In the inappropriate intermediate host, the occurrence of developmental disorder of the skeletal-muscle cyst may be responsible for the transformation of intermediate prokaryotic cystozoites (Fig. 1G) to intermediate elongate cystozoites, smaller in size than those of the present large elongate cystozoites and observed in human striated muscle diagnosed only as *Sarcocystis* spp. infection[14]. This phenomenon of small zoite development had occurred earlier in guinea pigs acting as an inappropriate intermediate host in the natural rat-cat cycle for *S. muris*[48]. Possibly, the small zoite formation in the inappropriate intermediate host may represents a coccidian developmental stage in the complicated alternative pathway mentioned prior.

In relevance to the discussion described above, it can be summarized that the *Sarcocystis* spp. life cycles remain unclear and seem to be far more complex than what now known only as the noninvasive infections in the definitive hosts[1-3,19]. Human intestinal sarcocystosis by *S. fusiformis* or *S. meischeriana* from the raw infected beef or pork appears to be an invasive infection in parallel to the known noninvasive mean. The invasive sarcocystosis is capable of leading to chronicity, autoimmunity, and possibly chronic inflammation in the intestinal mucosa of Thai people[8,11,49]. In addition, these two species may be responsible in causing skeletal-muscle sarcocystosis in man since the asexual forms i.e. merozoites were identified in both of the animals' meat and human intestine. Furthermore, the transmission of the *Sarcocystis* spp.-infective forms by untreated or poorly treated drinking water may lead to sarcocystosis in the patient group without raw meat consumption[11, 25].

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เนื้อโคกระบือ และหมูดำที่มีถุงพยาธิของซาร์โคซิสติสเป็นแหล่งที่มาของ โรคซาร์โคซิสโตซิส โดยธรรมชาติในลำไส้คนไทย

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โรคซาร์โคซิสติสของลำไส้ในคนเป็นโรคติดจากสัตว์โดยการกินเนื้อดิบ คือเนื้อโค (Bos indicus, Bos taurus) และกระบือ (Bubalus bubalis) ที่ติดเชื้อ Sarcocystis fusiformis เช่นเดียวกับเนื้อหมู (Sus scrofa) และหมูดำ (Sus scrofa scrofa) ที่มีถุงพยาธิของ Sarcocystis meischeriana จากการสำรวจเชิงระบาดวิทยาพบถุงพยาธิเห็นได้ด้วยตาเปล่าในเนื้อกระบือได้บ่อยมากตามตลาดสดในกรุงเทพฯในปี พ.ศ. 2530 ในปี พ.ศ. 2548 ไม่พบถุงพยาธิที่เห็นได้ด้วยตาเปล่าในเนื้อโคกระบือ ซึ่งมีอายุต่ำกว่าสามปี อย่างไรก็ตามถุงพยาธิที่เห็นได้โดยใช้กล้องจุลทรรศน์ สามารถพบได้จากจำนวนทั้งหมดห้าตัวอย่างของลิ้นโค และสิบตัวอย่างของเนื้อโคกระบือจากตลาดสดในกรุงเทพฯ แต่สิบตัวอย่างของเนื้อหมูจากตลาดสดในกรุงเทพฯไม่แสดงการติดเชื้อ ถุงพยาธิ S. meischeriana พบได้ในสามจากห้าตัวอย่างของเนื้อหมูดำได้มาจากสองอำเภอในจังหวัดลำปางซึ่งอยู่ในภาคเหนือ การกินเนื้อสัตว์ดิบโดยคนนำไปสู่การติดเชื้อซาร์โคซิสติสโดยธรรมชาติในลำไส้คนไทย ลักษณะรูปร่างของถุงพยาธิในเนื้อโคกระบือและเนื้อหมูดำได้ถูกตรวจ เพื่อการศึกษาในระดับจุลพยาธิวิทยาและจุลทรรศน์อิเล็กตรอน ในรายงานนี้ merozoites ที่พบในถุงพยาธิในเนื้อสัตว์ มีลักษณะเช่นเดียวกับ merozoites ที่เคยพบในลำไส้คน ข้อมูลจากการศึกษาด้วยวิธีจุลพยาธิวิทยานี้อาจระบุถึง invasive sarcocystosis ในคนโดยเชื้อทั้งสองชนิดและนำไปสู่การอักเสบเรื้อรัง โดยทราบสาเหตุ