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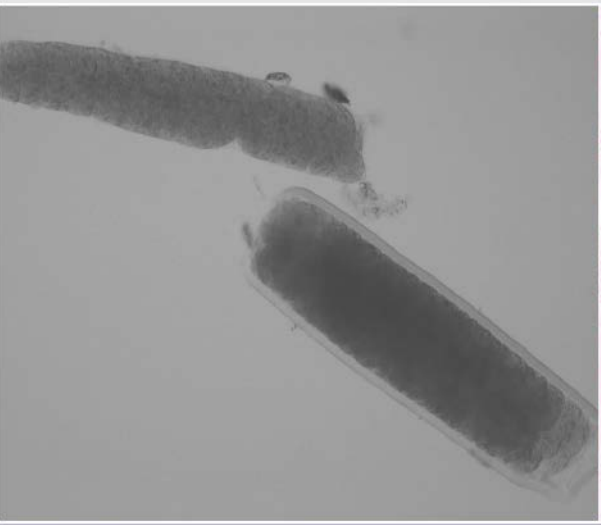

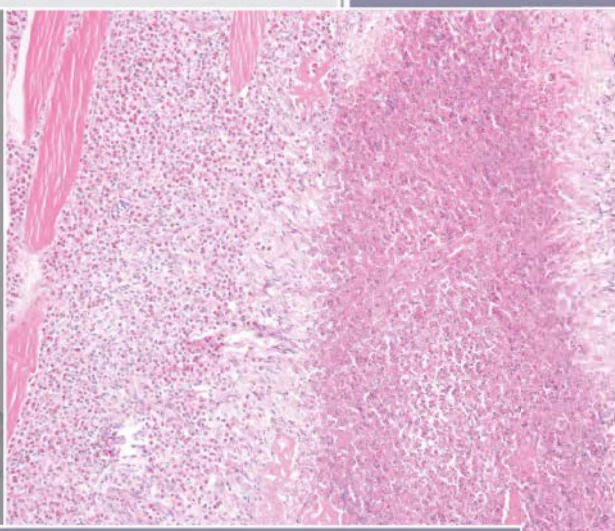

Bovine *Sarcocystis* species and their role in Bovine Eosinophilic Myositis

Lieve Vangeel

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Lieve Vangeel

Thesis submitted in fulfillment of the requirements for the degree of Doctor in Veterinary Science (PhD), Faculty of Veterinary Medicine, Ghent University, 2012

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Jong zijn
betekent
leven met open handen,
kansen grijpen,
groeien,
kopje-onder gaan
en proestend weer boven komen,
roeien tegen de stroom op,
kwistig zijn met je talenten,
geven en blijven geven,
onvermoeibaar zijn.

E. Stynen

Dit werk kwam tot stand in het kader van een specialisatiebeurs van het IWT-Vlaanderen (Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen)

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List of abbreviations

18S rDNA	18S ribosomal DNA
BEM	Bovine Eosinophilic Myositis
CD	Cluster of differentiation
CNS	Central nervous system
COX-2	Cyclooxygenase-2
DPI	Days post infection
DTH	Delayed type hypersensitivity
EC	European Community
ECF-A	Eosinophil Chemotactic Factor of Anaphylaxis
ELISA	Enzyme-linked immunosorbent assay
ESP	Eosinophil Stimulation Promoter
FSIS	Food Safety Inspection Service
HE	haematoxylin and eosin
HLA	Human leukocyte antigen
IFAT	Immunofluorescence antibody test
IHA	Indirect haemagglutination test
LCM	Laser Capture Microdissection
LGMD2A	limb-girdle muscular dystrophy type 2A
MAC387	Calprotectin
MHC II	Major Histocompatibility Complex class II
PAS	Periodic acid Schiff reaction
Pc	polyclonal
PCR	Polymerase Chain Reaction
PV	parasitophorous vacuole
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
rRNA	ribosomal Ribonucleic Acid
spp.	species
TEM	Transmission Electron Microscopy
Th	T helper
USDA	United States Department of Agriculture
VLAREM	Vlaams reglement betreffende de milieuvergunning

LITERATURE REVIEW

Chapter 1

Bovine *Sarcocystis* species: a review of the literature

Partially adapted from:

Vangeel, L., Houf, K., Geldhof P., Vercruysse, J., Ducatelle, R. and Chiers, K., 2012. *Sarcocystis*. In: Dongyou Liu (Ed.), Molecular detection of human parasitic pathogens. CRC Press (Taylor & Francis Group), Boca Raton, pp. 215–223.

1.1 History

Sarcocystis (*S.*) tissue cysts were described for the first time by F. Miescher in 1843 when they were discovered in skeletal tissue of domestic house mice. The cysts, described as "milky white threads", were suspected to have a parasitic origin. They were named after Friedrich Miescher and were termed "Miescher's Tubules". Once Lankester introduced the genus name *Sarcocystis* (*sarx* = flesh and *kystis* = bladder) in 1882, several species were described based on the host species. Confusion occurred, since the life cycle was not known yet and validation of the identity of the species was not possible. Although, investigators named two species in cattle: *S. cruzi* (Hasselmann, 1926) and *S. hirsuta* (Moulé, 1888), any sarcocyst in cattle was considered as one species, i.e. *S. fusiformis* (Dubey et al., 1989a).

In 1972, pioneering research of Rommel and Heydorn (1972) revealed the two host (heteroxenous) life cycle of *Sarcocystis*. Heydorn et al. (1975) also showed that both intermediate and definitive host can be infected by more than one species. Moreover, they were the first providing conclusive evidence of the existence of three structurally different *Sarcocystis* species in cattle, with dogs, cats and man as definitive host. Since the original descriptions were inadequate and it was impossible to confirm that the originally named species were identical to those wherein the life cycle was named, Heydorn et al. (1975) proposed new names (Table 1.1) combining the name of the intermediate and definitive hosts i.e. *S. bovicanis, S. bovifelis* and *S. bovihominis* with dogs, cats and man as definitive hosts, respectively. Nevertheless, Levine (1977) stated that the old names *S. cruzi* (Hasselmann 1926), *S. hirsuta* (Moulé, 1888) and *S. hominis*, first described by Railliet and Lucet in 1891 (Dubey, 1976), were valid following the international code of zoological nomenclature. These two opposite views also caused considerable confusion. Currently, *S. cruzi* (dog), *S. hirsuta* (cat) and *S. hominis* (man) are the only valid names according to the international code of zoological nomenclature (Dubey et al., 1989a).

Table 1.1: important *Sarcocystis* species in livestock

***Sarcocystis* species**	**Synonym (Heydorn et al., 1975)**	**Intermediate host**	**Definitive host**
S. hominis	*S. bovihominis*	cattle	primates
S. hirsuta	*S.bovifelis*	cattle	felids
S. cruzi	*S. bovicanis*	cattle	canids
S. fusiformis	-	water buffalo	cat
S. suihominis	-	pig	primates
S. porcifelis	-	pig	felids
S. miescheriana	*S. suicanis*	pig	canids
S. tenella	*S. ovicanis*	sheep	dog, fox, coyote
S. gigantea	*S. ovifelis*	sheep	cat
S. medusiformis	-	sheep	cat
S. arieticanis	-	sheep	dog
S. capracanis	-	goat	dog, fox, wolf
S. hircicanis	-	goat	dog
S. moulei	*S. caprafelis*	goat	cat

1.2 Taxonomy

Sarcocystis, a genus of tissue cyst-forming coccidia, belongs to the family Sarcocystidae, phylum of Apicomplexa (also known as Sporozoa) and subkingdom of Protozoa (Levine, 1986). Related genera include *Besnoitia*, *Cystoisospora, Frenkelia, Hammondia, Neospora* and *Toxoplasma*. It has been suggested by different authors that *Sarcocystis* and *Frenkelia* belong to the same genus (Odening, 1998, Tenter et al., 2002). Frenkel and Smith (2003) suggest however to keep the generic distinction.

Sarcocystis is the largest genus within this family. Over 200 species have been characterized (Frenkel and Smith, 2003), and more continue to be described, making *Sarcocystis* an ubiquitous parasite of mammals (especially sheep, pigs, and cattle), reptiles, and birds (see Table 1.1).

Much of the confusion concerning the taxonomy of *Sarcocystis* species is due to misinterpretations of original descriptions and inadequate new descriptions (Tenter, 1995). In the late 1980's, isoenzyme analysis was used to examine the relatedness of a range of pathogenic and non-pathogenic *Sarcocystis* species from sheep, goats, cattle and mice (O'Donoghue et al., 1986). However, this method based on the detection of differences in the electrophoretic mobility has not been developed further. Few years later, molecular techniques have been applied to examine the phylogenetic relationships of *Sarcocystis* species to each other, and to other cyst-forming coccidians, such as *Toxoplasma gondii* and *Neospora caninum* (Johnson et al., 1988; Tenter et al., 1992; Ellis et al., 1995). Genomic analysis is beginning to provide more insight and new taxonomic schemes are proposed (Tenter et al., 2002). However, relatively few species have been studied in detail and for now, most descriptions are still based on the morphology (Frenkel and Smith, 2003).

1.3 Life cycle of bovine *Sarcocystis* species

The *Sarcocystis* species that are described in *Bos taurus*, namely *S. cruzi*, *S. hirsuta* and *S. hominis*, require a heteroxenous (obligatory two-host) life cycle (Figure 1.1), alternating between hosts that serve as each other's predator and prey (definitive – intermediate host) (Dubey, 1976). The asexual stages of *S. cruzi*, *S. hirsuta* and *S. hominis* with establishment of tissue cysts (sarcocysts) occur in domestic cattle, the intermediate hosts. The sexual stages, with formation of oocysts, take place in carnivorous definitive hosts, which are canids (dogs, coyotes, wolves, and foxes), felids, and primates (humans, rhesus monkeys, baboons and possibly chimpanzees) for *S. cruzi*, *S. hirsuta* and *S. hominis*, respectively (Heydorn et al., 1975; Dubey, 1976; Dubey, 1983b).

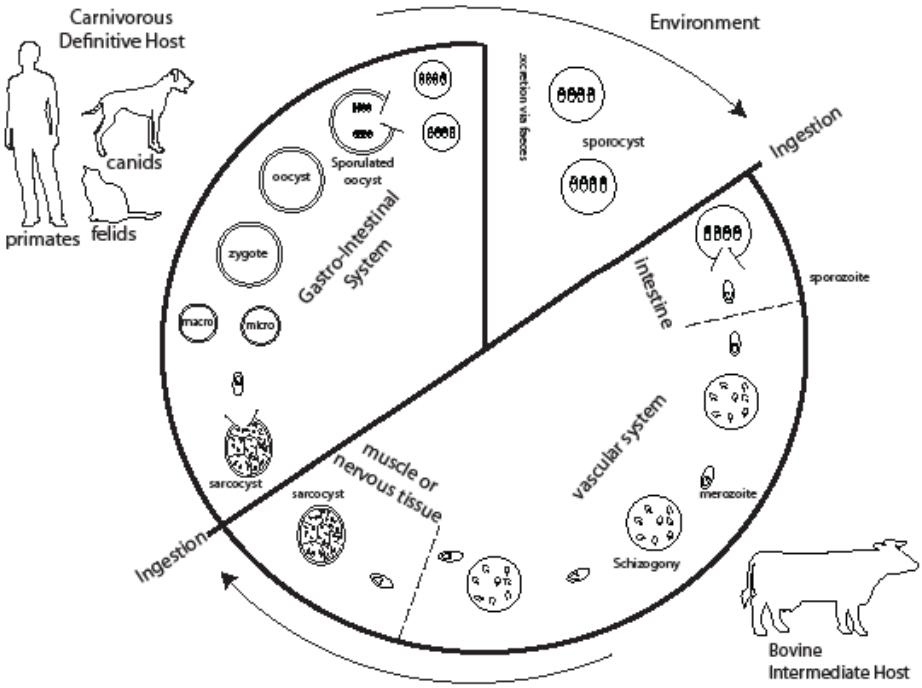


Figure 1.1: schematic presentation of the life cycle of *S. cruzi*, *S. hirsuta* and *S. hominis*. (Adapted from Vangeel et al., 2012)
The asexual stages with establishment of tissue cysts (sarcocysts) occur in cattle, the intermediate host. The sexual stages, with formation of oocysts, take place in the definitive hosts, which are canids, felids and primates.

1.3.1 In the definitive host (canids, felids, primates)

The definitive host gets infected by ingesting raw or undercooked bovine meat, containing mature sarcocysts. After ingestion, the sarcocyst wall is mechanically disrupted or digested in the stomach and intestine and the enclosed bradyzoites are liberated (Dubey and Lindsay, 2006). They penetrate goblet cells on the surface lining of the small intestine. Each intracellular bradyzoite develops in a male or female stage (micro- and macrogamonts) moving into the lamina propria. Fusion of a macrogamont and a microgamont results in a zygote which develops into an oocyst. Spherical oocysts pass into the intestinal lumen and contain two sporo-

cysts, each with four sporozoites and a granular residual body (*Isospora* type of oocyst). As the oocyst wall is very thin, oval sporocysts are frequently liberated before excretion and individual sporocysts are excreted with the faeces. The sporocysts are immediately infective after being shed in the environment. This is a specific characteristic of the genus *Sarcocystis*, because other members of the family Sarcocystidae shed unsporulated oocysts (Mehlhorn and Heydorn, 1978). The shedding is usually low and intermittent, but can sometimes occur in large numbers (Mehlhorn and Heydorn, 1978; Heckeroth and Tenter, 2007). Literature on the number of sporocysts excreted by definitive hosts is however scarce. Fayer (1977) and Latif et al. (1999) reported shedding of more than 2 million sporocysts a day by infected dogs. Dubey et al. (1989a) and Pena et al. (2001) showed however, that the number of sporocysts excreted by cats and humans is low in contrast to dogs. During an experimental infection of six persons with 200 g raw meat a mean sporocyst count between 1.2 and 23.5 sporocyst per gram faeces was reported (Pena et al., 2001). The prepatent period (the interval between infection by a parasitic organism and the first diagnostic stage of the organism detectable in that host) of *S. hirsuta* is 7-9 days and for *S. cruzi* and *S. hominis* it is 9-10 days. The patent period (the period of time in the course of a parasitic disease during which the parasitic organisms can be detected in the body) for bovine *Sarcocystis* species is more than six weeks (Mehlhorn and Heydorn, 1978).

1.3.2 In the intermediate host (cattle)

The bovine host becomes infected by ingestion of sporocysts in faecal contaminated feed and/or water. In the small intestine the sporocyst wall ruptures, releasing the sporozoites (Dubey and Lindsay, 2006). They enter the intestinal wall and migrate to the endothelial lining of arteries, particularly those of the intestines and mesenteric lymph nodes. The sporozoites multiply in close association with the endothelium, resulting in first-generation schizonts containing merozoites (*S. hirsuta* 7 - 23 days post infection (DPI); *S. cruzi* 7 - 26 DPI; *S. hominis* unknown) (Dubey, 1982a, b, c; Dubey et al., 1988). The schizogony in *Sarcocystis* species happens by endopolygeny (a process of asexual reproduction, in which many daughter cells are produced inside a mother cell). Merozoites released from the schizonts enter the bloodstream and develop into second-generation schizonts (*S. hirsuta* 15 - 23 DPI; *S. cruzi* 19 - 46 DPI; *S. hominis* unknown) in the endothelium of capillaries of virtually every organ, predominantly in renal glomeruli (Dubey, 1982b, c; Dubey and Fayer, 1983; Mehl-

horn, 1988; Dubey et al., 1988). However those of *S. hirsuta* are restricted to endothelium of capillaries in muscles (Dubey, 1982c). Both first- and second-generation schizonts are located within the host cytoplasm and not surrounded by a parasitophorous vacuole (PV). Merozoites emerge from the schizonts and circulate extracellularly or may enter mononuclear cells, where they divide (Dubey and Fayer, 1983). Division in blood cells has not been seen for *S. hirsuta* (Dubey et al., 1989a). Merozoites from the terminal schizont generation, leave the blood stream and invade muscle cells (skeletal or cardiac) (Dubey et al., 1989a) or occasionally neural tissue (*S. cruzi*) (Heydorn et al., 1975). Merozoites entering muscle cells round up to form metrocytes and initiate sarcocyst formation. A wall develops around the metrocytes, forming an immature sarcocyst. Consequently the sarcocyst is always located within a PV in the host cell. Multiplication by endodyogeny (a process of asexual reproduction, in which two daughter cells are produced inside a mother cell) in the tissue cyst leads to the accumulation of numerous metrocytes (globular mother cells) and the sarcocyst increases in size. In the centre of the cyst, the small, rounded, non-infectious metrocytes differentiate in crescent- or banana-shaped bodies, called bradyzoites, which are infective for the definitive host. Bradyzoites measure from 3-5 µm up to 18 µm depending on the species. A cyst is mature when it contains more bradyzoites than metrocytes (Mehlhorn and Heydorn, 1978). Maturation varies for each species and takes approximately 62 - 98 days for *S. cruzi* and *S. hominis*; however, for macrocyst-forming species as *S. hirsuta*, it can be up to 1 – 4 years (Heydorn et al., 1975; Rommel et al., 1995). Mature sarcocysts of each species differ in size (from 0.1 mm to 30 mm) and shape. Some always remain microscopic, whereas others (can) become macroscopically visible (e.g. *S. hirsuta*) (Dubey et al., 1990). Whether *S. hominis* produces macroscopic cysts has not been determined (Böttner et al., 1987; Dubey et al., 1988). Sarcocysts can persist for months or years in the intermediate host. In old cysts, bradyzoites that are located centrally degenerate and are replaced by a hollow (Dubey et al., 1989a).
Sarcocysts develop distinct cyst walls that vary in thickness and organization of the villar protrusions (Dubey et al., 1989a; Odening et al., 1995) (described in detail in 1.4.1.1 “Morphological techniques”). Internally the sarcocyst is compartmented by septae, which is a specific characteristic of *Sarcocystis* species (Mehlhorn and Heydorn, 1978).

Although ingestion of sporocysts is the major mode of transmission for cattle, vertical transmission of *S. cruzi* has been documented in experimental studies (Savini et al., 1996a) and sporadically in natural infections (Dubey and Bergeron, 1982; Hong et al., 1982), though rarely (Moré et al., 2009).

To date, the possibility of transmission through blood is not shown in natural infections, but Fayer and Leek (1979) have described that *Sarcocystis* can be successfully transmitted between intermediate hosts by transfusion of blood, containing merozoites.
Since lactogenic transmission was described for *Toxoplasma gondii*, transmission via milk or colostrum was also tested experimentally for *S. cruzi*, but evidence was not found (Fayer et al., 1982).

1.4 Detection and identification

1.4.1 In the intermediate host

A variety of techniques have been used to investigate *Sarcocystis* species in muscles of bovines. Each method has its advantages and disadvantages.

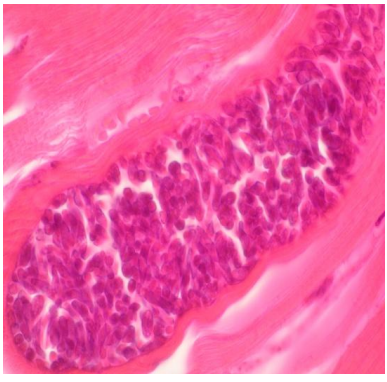
1.4.1.1 Morphological techniques (Gross – Light microscopy - TEM)

Gross inspection of muscle tissue is only possible for macrocyst-forming species (Böttner et al., 1987; Dubey et al., 1990) and this technique is therefore not sensitive enough to be a reliable detection method (Table 1.2).
Sarcocysts can be detected in unstained fresh squash preparations by **trichinoscopy** or **stereoscopy** at magnification of 10-60X (Gut, 1982). Although these techniques are not suitable for identification, relatively more tissue can be examined in contrast to histological examination; hence it is more sensitive. However, sarcocysts may be easily overlooked by untrained observers (van Knapen et al., 1987; O'Donoghue and Rommel, 1992). If infection levels are high and tissue cysts are > 500 µm, isolation can be performed by dissecting muscle samples using a stereomicroscope and recovering the cysts with dissecting needles (Yang et al., 2001). Intact tissue cysts can also be recovered using a sedimentation technique. The detection and isolation can then be done by stereomicroscopical examination of the sediment (Markus, 1979).

Table 1.2: Cyst characteristics based on gross, light microscopic and TEM examination

***Sarcocystis* species**	**Gross**	**Light microscopy**	**TEM**
S. cruzi	microscopic	thin-walled	hair-like protrusions
S. hirsuta	micro-/macroscopic	thick-walled	finger-like protrusions
S. hominis	microscopic	thick-walled	bulb-shaped protrusions

Histological examination is frequently used, but is not a sensitive detection method due to the small amount of sampled tissue (Collins et al., 1980). On the other hand, histology permits to study the sarcocyst morphology. Routine species identification is based on morphological examination of the cyst wall (Table 1.2). However, due to the low resolution by light microscopic examination, it is only possible to differentiate between thin- and thick-walled species (Figure 1.2). *S. cruzi* is a thin-walled species (<1 µm) (Figure 1.2 A) which can easily be distinguished from *S. hominis* and *S. hirsuta*, both thick-walled sarcocysts (Figure 1.2 B). Their walls are radially striated and 2.5 - 9.0 µm thick (Dubey et al., 1989a). Distinguishing *S. hominis* from *S. hirsuta* is unreliable by light microscopy (Odening et al., 1995).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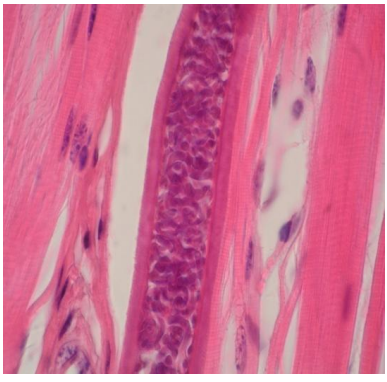
B

Figure 1.2: A: a thin-walled species (<1 µm) *S. cruzi*. **B**: a thick-walled sarcocyst is shown (*S. hominis* or *S. hirsuta*). HE staining, 400x.

Immunohistochemical methods have been developed in order to improve the sensitivity and specificity of histological detection of life cycle stages of *Sarcocystis* species in the intermediate hosts, especially in sheep and horses (Uggla and Buxton, 1990). However, these methods are complicated by the fact that polyclonal antisera show high cross-reactivity with antigens of heterologous species. Moreover, sera of rabbits immunized with *Sarcocystis* antigens

may also cross-react with antigens of other cyst-forming coccidians (Uggla and Buxton, 1990). Therefore, a species-specific diagnosis can be made only when specific monoclonal antibodies are used. These have been developed only for *S. cruzi* (Burgess et al., 1988) in cattle, but are not commercially available. In this report, Burgess et al. (1988) identified one monoclonal antibody that reacted with antigens of merozoites as well as bradyzoites and sporozoites of *S. cruzi*.

The most reliable method for morphology-based species identification is ***transmission electron microscopy*** (TEM) (Dubey et al., 1989b; Odening et al., 1995) (Table 1.2). The cyst wall of *S. hominis* (Figure 1.3A) is characterized by finger-like villar protrusions that are broadly seated on the cyst surface, containing long microfilaments in the core, that run from the base to the tip. *S. hirsuta* (Figure 1.3B) however, is characterized by diagonally bent, bulb-shaped, or club-shaped villar protrusions arising with a stalklet from the cyst wall and containing large osmiophilic granules (Dubey et al., 1989b; Odening et al., 1995).
Although routine identification is based on cyst wall morphology, a lot of controversy still exists on the reliability (Tenter, 1995). Since, morphological characters, such as cyst size and cyst wall structure may vary during cyst development, location or due to fixation (Dubey, 1989a). As there is no reliable way to determine the age of sarcocysts in naturally infected cattle, caution should be taken with the taxonomic value and with respect to describing a new species only based on the cyst morphology (Böttner et al., 1987; Tenter, 1995).

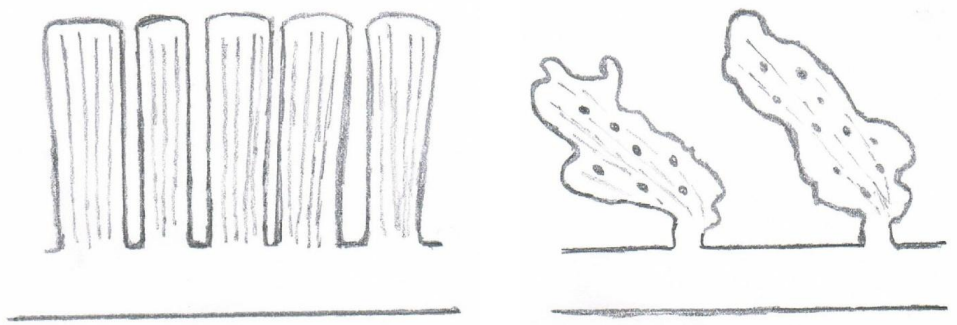

Figure 1.3: schematic representation of the cyst wall morphology of both thick-walled species.
A: *S. hominis.* **B**: *S. hirsuta*.

1.4.1.2 Digestion techniques

Peptic (Seneviratna et al., 1975) or tryptic (Boch et al., 1978) digestion techniques, with consequent microscopical examination, are frequently used, practical and sensitive methods for *Sarcocystis* detection (Boch et al., 1978; Collins et al., 1980; van Knapen et al., 1987). However, sarcocyst identification is not possible, because these methods result in lysis of not only muscle tissue but also of the sarcocyst walls, releasing the bradyzoites. The latter survive in the incubation fluid and can be examined at 400X magnification, metrocytes are however not resistant to digestion (Dubey et al., 1989a).

These methods are used in epidemiological studies, but they can also be used for purification of bradyzoites for antigen and nucleic acid preparations. To purify the bradyzoites, a subsequent step of density gradient centrifugation is needed. The density of *Sarcocystis* bradyzoites varies with the age of the tissue cyst and with the species, therefore optimizing the composition of the discontinuous density gradients is always needed (Heckeroth and Tenter, 2007).

1.4.1.3 Serology

Several serological tests based on crude antigen preparations have been developed and applied for the diagnosis of *Sarcocystis* infection in different intermediate host species (Tadros et al., 1980; Uggla and Buxton, 1990). In cattle, an indirect haemagglutination test (IHA) (Lunde and Fayer, 1977) and an enzyme-linked immunosorbent assay (ELISA) (Tadros et al., 1979; Savini et al., 1997) have been applied for serodiagnosis of sarcocystosis.

The usefulness of serological tests for the diagnosis of *Sarcocystis* infections has been limited however, by the high antigenic cross-reactivity among different *Sarcocystis* species. In addition, the *Sarcocystis* stages from which traditionally antigens have been derived are intracellular and therefore the antigen preparations lack standardization and are frequently contaminated with debris of host tissue (Tenter, 1995).

At present no serological assay for the demonstration of bovine *Sarcocystis* antibodies is commercially available.

1.4.1.4 Molecular techniques

To date, there are some molecular based methods that have been developed, though primarily in a research-based context.

Tests targeting rRNA, for example, ribosomal hybridization assay (Gajadhar et al., 1992), have been developed for some *Sarcocystis* species. These methods have been suggested to be more sensitive and accurate than assays targeting the rRNA genes, as there is up to 50 times more RNA than DNA in a cell, of which 90-95% is rRNA (Zarlenga and Higgings, 2001). Since RNA is not stable and easily degraded by enzymatic digestion, these assays are not suitable for the diagnosis of clinical or epidemiological samples (Heckeroth and Tenter, 2007). Moreover, standardization of RNA hybridization assays is difficult (MacPherson and Gajadhar, 1993) and the necessity of using radioactively labeled oligonucleotide probes restricts the use to specialized laboratories (Heckeroth and Tenter, 1999).

Since the development of the Randomly Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) fingerprinting technology, this method has been used in intermediate hosts to distinguish different *Sarcocystis* species (Macpherson and Gajadhar, 1994; Güçlü et al., 2004). An advantage of this technique is that short regions of genomic DNA can be amplified without prior knowledge of gene sequences. DNA sequence analysis of these PCR products can then be used to design species specific primers. However, this method has not been developed as a diagnostic test. The reason is that RAPD is laden by technical drawbacks, through which standardization of the assay between laboratories is difficult (Zarlenga and Higgings, 2001).

In recent years the small subunit rRNA gene has been used extensively to differentiate between apicomplexans and other eukaryotic species. Due to its abundance in the genome, the sensitivity of the tests increases. Moreover, its double feature of hypervariable regions interspearsed within highly conserved DNA sequences makes it a useful target for developing molecular-based diagnostics (Neefs et al., 1991; Tenter and Johnson, 1997; Fischer and Odening, 1998; Prichard and Tait, 2001). The rapidly growing database of 18S rDNA generated by phylogenetic studies of *Sarcocystis* species provides a rich source of sequences that can be exploited to develop specific PCRs (Fischer and Odening, 1998).

In the last decade, a number of PCR-based techniques (e.g., genus or species specific PCR, nested PCR, PCR-RFLP) have been established, especially for the identification of tissue cysts in livestock. They have been used however mostly in taxonomic studies (Tenter et al., 1994; Heckeroth and Tenter, 1999; Holmdahl et al., 1999; Yang et al., 2002; Li et al., 2002).

1.4.2 In the definitive host

1.4.2.1 Faecal examination

Diagnosis of intestinal sarcocystosis requires identification of sporocysts in the stool. Sporocysts can be observed by bright-field microscopy in a faecal flotation wet mount just beneath the coverslip. As an alternative, the ***traditional flotation technique*** based on concentration methods (specific gravity 1.15 or more) with satured sodium chloride (NaCl), sucrose or zinc sulphate ($ZnSO_4$) solutions can be used (Dubey et al., 1989a). The concentration step is necessary, because the number of sporocysts in the faeces is often low. The latter technique is more commonly used than the former. The determination of numbers of oocysts/sporocysts can be done by the McMaster technique (Heckeroth and Tenter, 2007). In addition, a modified Kato thick smear technique, commonly used for the diagnosis of helminthiasis, has also been tested successfully for the diagnosis of *Sarcocystis* sporocysts (Tungtrongchitr et al., 2007).
Morphological species identification/differentiation is not possible for the oocyst/sporocyst stages of this parasite. Sporulated oocysts are spherical, generally colorless, thin-walled (<1 µm) and contain two elongated sporocysts. The colorless wall is delicate and often ruptures. An oocyst residuum and micropyle are absent. Similarly, the morphology of sporocysts has no taxonomic value. Except for minor variations in size, the structure is the same (Mehlhorn and Heydorn, 1978). Each sporocyst contains four elongated sporozoites and a compact or dispersed granular residual body, but a Stieda body is absent. Because sporozoites are curved in the sporocyst, all four are often not seen in a single plane of focus. Each sporozoite has a central to terminal nucleus and several cytoplasmic granules, but there is no refractile body. The approximate size of the sporocyst (Figure 1.4) is 9 x 15 µm for *S. hominis*, 11-14 by 7-9 µm for *S. hirsuta*, and 14.5 – 17 by 9 – 11 µm for *S. cruzi* (Fayer, 1974; Heydorn et al., 1975; Dubey, 1976; Mehlhorn and Heydorn, 1978).

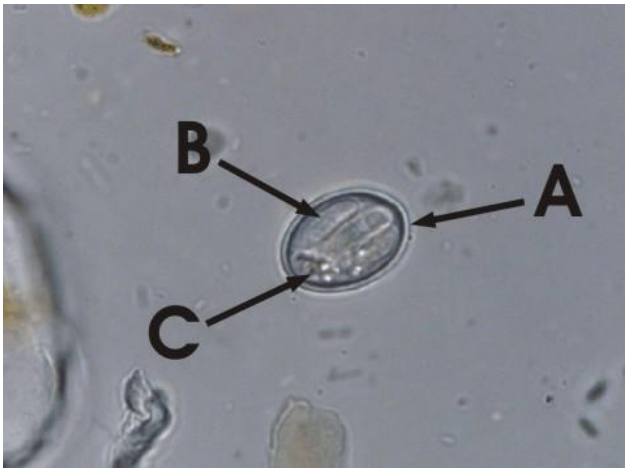

Figure 1.4: *Sarcocystis* spp. sporocyst, 100 x objective. A: thin cyst wall. B: sporozoite. C: residual body. (Source: http://people.upei.ca/sgreenwood/html/protozoa.html)

1.4.2.2 Serology

An indirect fluorescent antibody test (IFAT) is described to detect circulating antibodies against *S. hominis* in sera in man (Tadros et al., 1974). However, Heckeroth and Tenter (2007) assumed that data on *Sarcocystis* infections in definitive hosts have been complicated by the fact that these hosts do not develop parasite – specific antibodies.

1.4.2.3 Molecular techniques

Although DNA templates are stable targets for PCR amplification and diagnostic samples can be stored at ambient temperature for some time, each of the range of different sources of parasite material (faeces, tissue) involves different problems for PCR-based diagnosis, associated with inhibitors and sensitivity (Tenter et al., 1994). Therefore PCR assays that have been shown to be specific and sensitive on tissue in livestock probably cannot be used as such for the diagnosis of *Sarcocystis* species in faecal samples.

Recently, for intestinal sarcocystosis Xiang et al. (2009) have evaluated a genetic approach for the detection and identification of *S. hominis* sporocysts based on a seminested PCR-RFLP assay described by Yang et al. (2002). This method is based on the amplification of a fragment from the18S rRNA gene, using a semi-nested PCR combined with RFLP. Due to the low number of sporocysts excreted by the definitive host, Xiang et al. (2009) consider that

semi-nested PCR is necessary to increase the sensitivity of the assay. The sensitivity of this test was not established yet but this assay showed to be useful as a noninvasive diagnostic technique for human intestinal sarcocystosis. The primers described for this assay target relatively conserved portions of 18S rDNA. Therefore, successful amplification indicates only the presence of a *Sarcocystis* species. When used in conjunction with RFLP or sequencing, however, species identification is possible (Yang et al., 2002).

1.5 Pathogenicity

Little is known about the mechanisms of *Sarcocystis* species that cause pathogenicity in the intermediate as well as in the definitive hosts (Dubey et al., 1989a).

1.5.1 In the intermediate host

In cattle, infection can result in acute disease during the early phase of infection and chronic disease during the late phase of infection. The severity of clinical symptoms is related to the number of ingested sporocysts. There are no clinical symptoms that are pathognomonic for sarcocystosis (Tenter, 1995).

In general, species transmitted by canids are the most pathogenic for the intermediate host. Experimental infection studies with *S. cruzi* (infection dose ranging from 0 – 500 x 10^6 sporocysts) showed that an intake of less than 1000 sporocysts causes no clinical signs. Ingestion of a large dose (50.000 or more) results in clinical symptoms three to four weeks after infection (Dubey et al., 1982). It can cause fever, anorexia, anaemia, weight loss, hair loss, weakness, muscle twitching, prostration, abortion, reduced milk yield, hypersalivation, neurologic signs and death. Fever is usually associated with parasitaemia and clinical signs are generally seen during the second schizogonic cycle in blood vessels (Dubey and Lindsay, 2006). The other two bovine *Sarcocystis* species, *S. hirsuta* and *S. hominis* are only mildly pathogenic for cattle (Dubey, 1983a; Dubey et al., 1988). Experimental infection trials of these species were performed with a sporocyst infection dose ranging between 5000 – 25 x 10^6 for *S. hirsuta* and 10^5 - 10^6 sporocysts for *S. hominis*. Infection with 100.000 *S. hirsuta* sporocysts caused clinical symptoms including fever, diarrhoea and mild anaemia. Death was however, not described

(Dubey, 1983a). Inoculation of 1 million sporocysts of *S. hominis* caused only mild anaemia (Dubey et al., 1988).

Few field cases of acute sarcocystosis have been diagnosed however, in animals exposed to natural levels of infection. The first natural outbreak of acute sarcocystosis was reported in 1961 and was named Dalmeny disease, after the Canadian town where the outbreak occurred (Corner et al., 1963). Subsequently, cases have been reported in Canada (Meads, 1976), England (Clegg et al., 1978), Ireland (Collery and Weavers, 1981), Norway, Australia and the US. Cattle surviving the acute disease syndrome recover slowly although most succumb to chronic sarcocystosis, showing weight loss, hypersalivation, hyperexcitability and hair loss (especially on the neck, rump and tail switch). Data about the percentage of animals that develop chronic disease are not available. Some months after infection, animals can develop CNS symptoms (nystagmus, opisthotonus and lateral recumbancy with running gate) (Fayer and Dubey, 1986).

Chronic sarcocystosis can also result from the ingestion of a low dose of a pathogenic species and is more common than acute disease (Dubey et al., 1989a). It can cause reduced milk production, body weight, body weight gain (Frelier, 1977; Fayer and Dubey, 1986; Daugschies et al., 2000) and quality of meat (Fayer and Elsasser, 1991). It has been assumed that muscle metabolism is affected in these cattle (Telles de Jesus Filho and Miraglia, 1977; Daugschies et al., 2000).

1.5.2 In the definitive host

Infection in canids and felids does not cause any clinical symptoms (Dubey et al., 1989a). In man, the severity of the symptoms is correlated with the number of ingested sarcocysts (Fayer, 2004). The clinical course after infection with *S. hominis* in humans is usually mild and in many cases even asymptomatic (Fayer, 2004). It is characterized by transient nonspecific gastro-intestinal complaints, like nausea, stomach ache, abdominal pain and distension, and (watery) diarrhoea (Pena et al., 2001; Fayer, 2004). The symptoms appear 3 - 6 hours after eating infected beef and last about 36 hours (Fayer, 2004). In some cases abdominal discomfort, vomiting, diarrhoea, dizziness, fatigue, anaemia, and segmental necrotizing enteritis has been reported (Bunyaratvej and Unpunyo, 1992; Xiang et al., 2009).

1.6 Immunity

Cellular and humoral immune responses in infected animals indicate that *Sarcocystis* spp. are immunogenic in **intermediate hosts** (Dubey et al., 1989a). Due to the lack of schizogony in the **definitive host**, they acquire little or no immunity, and each meal of infected meat can thus initiate a new round of sporocyst production (Dubey and Lindsey, 2006).

In cattle (**intermediate host**), the major components of the humoral response are IgM and IgG_1. The IgM response appears early, but is transient. Experimental studies show that the IgM response begins to develop three to four weeks after infection and declines to low levels (near preinfection level) in two to three months, which is the time the sarcocysts mature. Three to five weeks post infection IgG_1 antibodies arise. A peak concentration is seen during the early sarcocyst formation and persists during the chronic infection for at least five to six months (Lunde and Fayer, 1977; Fayer and Prasse, 1981; Gasbarre et al., 1984).
Cattle surviving a primary *Sarcocystis* infection usually develop a transient immunity that protects them against acute disease after challenge with the homologous species, but not after infection with a pathogenic heterologous species (Uggla and Buxton, 1990; O'Donoghue and Rommel, 1992). The protection against acute disease appears to be maintained by continuous low dose infections or possibly intermittent exposure. Nevertheless, the mechanisms of protection are not yet understood (O'Donoghue and Rommel, 1992).
The predominant cells infiltrating visceral and muscular tissue are lymphocytes and macrophages (Johnson et al., 1975; Dubey et al., 1982). Sarcocysts present in muscle cells of naturally infected cattle however, are usually not accompanied by an inflammatory response (Dubey et al., 1989a).

1.7 Prevalence

1.7.1 In the intermediate host

Sarcocystis infection is common in many animals worldwide. In cattle the prevalence of *Sarcocystis* in adult bovine muscle is close to 100% (Table 1.3) in most regions of the world where it has been studied (Böttner et al., 1987; van Knapen et al., 1987; Vercruysse et al., 1989; Fortier et al., 1993; Woldemeskel en Gebreab, 1996; Latif et al., 1999; De Bosschere en Ducatelle, 2001; Pena et al., 2001).

Infections with more than one species are very common (78.2%) (Böttner et al., 1987). Nevertheless, little is known in mixed infections about the proportion of each species in cattle, due to difficulties in correct identification (Odening et al., 1995). Most surveys indicate that *S. cruzi* is the most prevalent species (Böttner et al., 1987; Dubey et al., 1989a; Fortier et al., 1993). Data about the prevalence of *S. hominis* show discrepancy between the surveys, though care should be taken in comparing those studies since they were based on different morphological methods. In two studies based on TEM examination in Brazil (Pena et al., 2001) and France (Solomon, 1991)*, S. hominis* was detected in 94 and 57% of the samples, respectively. In other studies carried out in India (Jain and Shah, 1987) and Germany (Boch et al., 1978) with light microscopic identification, *S. hominis* prevalences were 12.2 and 63%, respectively. The reliability of the latter studies is questionable, since Odening et al. (1995) stated that light microscopic examination is unreliable to distinguish *S. hominis* from *S. hirsuta*.

Table 1.3: Prevalence of bovine associated *Sarcocystis* species in cattle.

Country	Tech-nique	Thin-walled (%)	Thick-walled (%)	Overall prevalence (%)	Reference
Germany	b, c	65.5	63 *hominis* 35 *hirsuta*	99.7	Boch et al., 1978
New Zealand	a, e	98	79.8	100	Böttner et al., 1987
India	d	78.1	12.2	80.3	Jain and Shah, 1987
Netherlands	b, c	-	-	100	van Knapen et al., 1987
Belgium	a, e	97	56	97	Vercruysse et al., 1989
France	e, f	70.7	57 *hominis* 24 *hirsuta*	90	Solomon, 1991
France (Calvados)	e	75.4	4.6	80	Fortier et al., 1993
Ethiopia	e	-	-	82	Woldemeskel and Gebreab, 1996
Iraq	a	-	-	93.3	Latif et al., 1999
Belgium	a	-	-	100	De Bosschere and Ducatelle, 2001
Brazil	e, f	92	94 *hominis* 70 *hirsuta*	100	Pena et al., 2001
Iran	e	89	21	100	Nourani et al., 2010
Southern Italy	e	-	-	96	Bucca et al., 2011

[a] pepsin/HCl digestion, [b] tryptic digestion, [c] trichinoscopy, [d] compound microscope, [e] light microscopy, [f] TEM

1.7.2 In the definitive host

Data about prevalence of *Sarcocystis* species in the definitive hosts differ between the studies; moreover these reports do not differentiate the species. Prevalences in dogs range from 0% to 9% (Haralabidis et al., 1988; Aboshehada and Ziyadeh, 1991; Bugg et al., 1999; Barutzki and Schaper, 2003; Sager et al., 2006; Claerebout et al., 2009). In cats, 2.2% showed to be infected (Barutzki and Schaper, 2003). Regarding the prevalence of intestinal sarcocystosis in humans, published data on natural infections are limited. In a German study 13.64% of 403 patients with intestinal complaints were infected with intestinal parasites of whom 2% were shedding *Sarcocystis* sporocysts, but a distinction between *S. hominis* and *S. suihominis* was

not made (Gauert et al., 1983). In a Thai study 8.0% of 1124 rural residents were infected with *Sarcocystis* species (Tungtrongchitr et al., 2007).

1.8 Epidemiology

Although little epidemiological research is performed, a variety of conditions may permit the high prevalence of *Sarcocystis* species.

The **definitive host** gets infected by eating raw or undercooked meat, containing mature sarcocysts. In some countries, where the frequency of consumption of raw or undercooked meat is relatively common, humans should get easily infected (Dubey and Lindsay, 2006). At a temperature of 2-4°C bovine sarcocysts remain infective in meat for three weeks (Stephan et al., 1998). This implies that sarcocysts remain longer infective than the shelf life of raw meat. Although data are not available for *S. hominis*, Saleque et al. (1990) reported that sarcocysts of *S. miescheriana* in pork became non-infective after heating at 60°C for 20 min, at 70°C for 15 min or at 100°C for 5 min or after freezing for two days at -4°C or one day at -20°C.

Generally, as stated above, shedding of sporocysts by the **definitive host** can last for weeks or sometimes several months and numbers of excreted cysts can vary (Mehlhorn and Heydorn, 1978). The fact that *Sarcocystis* oocysts, unlike those of many other species of coccidia, are passed in faeces in the infective form, frees them from dependence on weather conditions for maturation and infectivity (Dubey, 1976). Sporocysts can survive in faeces of the definitive host for 2 years. Low temperatures or freezing does not influence their infectivity. However, *in vitro* investigations showed that sporocysts are sensitive to dryness, direct sunlight and strong fluctuations in temperature (Heydorn, 1980; Savini et al., 1996b). The sensitivity to dryness was also put forward as a possible explanation by Savini et al. in 1992 when they found that the prevalence of *Sarcocystis* was lower in cattle originating from arid and semi-arid regions in contrast to those from tropical and temperate regions.

Transmission of sporocysts can be direct or indirect.

Direct transmission is caused by contamination of feed and/or pasture of cattle with faecal material from the definitive hosts. The density of the **intermediate host** could influence the prevalence, since Savini et al. (1992) attributed the overall lower prevalence (52%) in Western Australia, in contrast to other countries, to the extensive manner of animal husbandry,

which results in less frequent contact between the intermediate and definitive host. Considering the bovine age, Savini et al. (1992) showed that only few animals younger than one year were infected in Western Australia, attributing this to a low contact in the suckling period with sporocysts. The infection rate has been shown to be higher in older cattle, up to four years, after which the prevalence decreases again (Savini et al., 1992).

Indirect spreading may occur via flooding or irrigation of pastures with water from sewage treatment plants. Furthermore, the contribution of agricultural use of sewage sludge has been considered as important, but has not been documented decisively (Burger and Wilkens, 1986). Finally, synanthropic flies, particularly the common house fly (*Musca domestica*) and cockroaches (order Blattaria or Blattodea) have been suggested as vectors of *Sarcocystis* (Markus, 1980). The transmission by synanthropic insects is predominantly via mechanical dislodgement from the exoskeleton, faecal deposition and regurgitation (Graczyk et al., 2005).

1.9 Economical impact

Not only acute sarcocystosis, but also chronic sarcocystosis can cause economic losses in livestock industry due to reduced milk production, body weight, body weight gain (Frelier, 1977; Fayer and Dubey, 1986; Daugschies et al., 2000) and quality of meat (Fayer and Elsasser, 1991). However, losses are difficult to calculate, because up to 100% of the cattle are infected and clinical disease is difficult to diagnose. Besides this, additional losses are caused by *S. hirsuta* which can cause macroscopic cysts in muscle tissue resulting in condemnation at slaughter (Dubey et al., 1990).

1.10 Prevention, control and treatment

The only recommendation that can be made in order to control *Sarcocystis* infections is interrupting the life cycle. Shedding of *Sarcocystis* sporocysts in faeces of the definitive hosts is the key factor in the spread of *Sarcocystis* infection (Dubey and Lindsay, 2006). Control is difficult to impossible, but attempts should be made to prevent animal pasture/stables, water and feed to become contaminated by faeces from dogs, foxes, cats, man and other definitive hosts.

Research on the viability of sporocysts of bovine associated *Sarcocystis* species is not performed so far, but viability tests for *S. neurona* showed that heat treatment is the most effective means of killing. Treatments with bleach (10%, 20%, and 100%), 2% chlorhexidine, 1% betadine, 5% *o*-benzyl-*p*-chlorophenol, 12.56% phenol, 6% benzyl ammonium chloride, and 10% formalin were not effective in killing sporocysts. However, treatment with undiluted ammonium hydroxide (29.5% ammonia) for 1 hour killed sporocysts (Dubey et al., 2002).

Dead animals should never be left for carnivores to eat and uncooked meat should never be fed to dogs and cats. Meat should be frozen or thoroughly cooked before it is eaten by pets or humans (Dubey and Lindsay, 2006).

There is no vaccine available to protect cattle or the definitive hosts against sarcocystosis (Dubey and Lindsay, 2006). Chemoprophylaxis using the anticoccidial drugs amprolium showed to be effective in preventing severe illness and death in experimentally infected calves (Fayer and Johnson, 1975). Administration of lasalocid, decoquinate and monensin on the other hand showed only minimal or no effect in calves (Foreyt, 1986).

In humans there is no known prophylaxis or therapeutic treatment for intestinal sarcocystosis (Fayer, 2004). But on the basis of results in experimental animals, it is plausible that sulfonamides and pyrimethamine may be helpful in treating sarcocystosis (Dubey and Lindsay, 2006).

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Chapter 2

Bovine Eosinophilic Myositis: a review of the literature

2.1 Introduction

Bovine Eosinophilic Myositis (BEM) is a histopathological descriptive term referring to a myositis in cattle in which the predominant inflammatory cell type is the eosinophilic granulocyte. Since cattle do not show any clinical symptoms, this pathological entity typically is observed in slaughterhouses and meat cutting plants.

2.2 Prevalence, legislation and economical impact

BEM appears worldwide in bovine carcasses and has been described in Europe, the United States, Canada, Iran, Korea and Japan (Reiten et al., 1966; Imes and Migaki, 1967; Van Hoof et al., 1972; Rimaila-Pärnänen and Nikander, 1980; Jensen et al., 1986; Gajadhar et al., 1987; Bundza and Feltmate, 1989; Fortier et al., 1993; Wouda et al., 2006; Do et al., 2008; Kimura, 2011; Javadi and Doustar, 2011). The prevalence varies according to the region, but recent data are missing (Table 2.1). Between 1960 and 1971, 0.003% of the slaughtered cattle in Belgium were condemned for BEM (Van Hoof et al., 1972). In the Calvados region of France (Fortier et al., 1993) and in Canada (Bradley and Taylor, 1993) a prevalence of 0.002% and 0.006%, respectively is reported. Regional differences were pronounced in the United States of America where the average of condemnations due to BEM is 0.011%, whereas exceptionally high BEM-associated carcass condemnations (up to 5%) have been reported in the western part of the USA (Jensen et al., 1986).

Table 2.1: prevalence of BEM in slaughtered carcasses in different countries

Country	Prevalence (%)	Reference
Belgium	0.003%	Van Hoof et al., 1972
France	0.002%	Fortier et al., 1993
Canada	0.006%	Bradley & Taylor, 1993
USA Colorado	0.028%	Reiten et al., 1966
USA	0.011%	Jensen et al., 1986
Western USA	5%	Jensen et al., 1986

Although BEM is not detectable *in vivo*, it results in economic losses at slaughter due to carcass condemnation as well as meat condemnation in meat cutting plants. Until the end of 2005, condemnation of BEM carcasses was based on the Royal Decree of March 9th 1953 (appendix 2, chapter II), in which is mentioned that meat with an abnormal visual aspect, whatever the cause, must be condemned. Since January the 1st of 2006, condemnation is based on the European Regulation (EC) no. 854/2004 (annex 1, section 2, chapter 5): "meat is to be declared unfit for human consumption if it indicates pathophysiological changes".

During meat inspection the lesions of BEM are often difficult to diagnose, since they are most of the time only visible on the cut surface of muscle tissue (the characteristics of the lesions are described below, "Lesions and symptoms"). Hence, they can pass unnoticed through carcass inspection (Stephan et al., 1998). This results in a shift of the problem to the level of the meat cutting plant, because anomalies are then found on an approved carcass. Data about the occurrence of BEM in meat cutting plants are not available, but it can be assumed that it is higher than in slaughterhouses.

2.3 Etiology

Inflammatory myopathies, to which BEM belongs, are a heterogeneous group of muscle diseases of which the causes described in literature are various. They can be caused by an autoimmune response, allergic reaction or following environmental exposure to xenobiotics, drugs or by infection with bacteria, viruses or parasites (Bruschi and Chiumiento, 2011).

The etiology of BEM is yet unknown. Neither helminths nor viral or bacterial microorganisms have been detected in the lesions (Oghiso et al., 1977; Jensen et al., 1986).

During investigations of experimental trichinosis in cattle, Smith et al. (1990) found focal lesions of eosinophilic myositis post infection. The gross aspect of these experimentally induced lesions was however not comparable with natural cases. Moreover, a survey by Smith et al. in 1991 of natural cases, based on ELISA, peptic digestion and histology, showed that trichinosis is unlikely to be associated with BEM. Since, larvae of *Trichinella* were not recovered by pepsin digestion, nor were they observed microscopically and only one animal was positive for *Trichinella* antibodies.

Besides infectious agents, host dependent factors also have been examined by comparing protein patterns between normal and BEM animals. Only a small difference in the α-globulin

region, which could point to a subtle difference in an enzyme or isoenzyme constitution in BEM animals, was reported. Nevertheless, it was not certain if this could be associated with the disease (Tengerdy and Imes, 1967).

Today, scientists believe that *Sarcocystis* species are associated with the development of this pathological entity (Gajadhar and Marquardt, 1992; Wouda et al., 2006). Following indirect indications have been found:

i. *Sarcocystis* species are frequently demonstrated in the centre of BEM lesions after semiserial histological examination (Jensen et al., 1986; Gajadhar et al., 1987; Gajadhar and Marquardt, 1992). Species identification of sarcocysts in BEM lesions is confusing as both thin- and thick-walled sarcocysts (Jensen et al., 1986), *S. hominis* or *S. hirsuta* (Rimaila-Pärnänen and Nikander, 1980), *S. cruzi* (Gajadhar and Marquardt, 1992), *S. hominis* (Wouda et al., 2006) and even an unidentified species (Gajadhar et al., 1987) have been observed. The identification on species level however, was based on the morphology of the cyst wall, which is hampered due to the fact that intralesional sarcocysts are mostly damaged (Wouda et al., 2006).
ii. Saito et al. (1993) showed positive labelling of the necrotic centre in granulomatous lesions with an anti-*S. cruzi* rabbit serum.
iii. Gajadhar and Marquardt (1992) and Wouda et al. (2006) observed that intralesional sarcocysts are damaged and this could evoke an immune response.
iv. Researchers detected functional *Sarcocystis cruzi* specific IgE using a passive cutaneous anaphylaxis test and local IgE binding in the environment of degenerating sarcocysts by immunofluorescence techniques (Granstrom et al., 1990a; Granstrom et al., 1990b). Ely and Fox (1989) and Granstrom et al. (1989) reported also an increase in IgG for *S. cruzi* in BEM animals. However, it is not clear if an increase of IgG is associated with this pathological entity (Ely and Fox, 1989).
v. It was shown that carcasses with BEM contain less bradyzoites than normal bovine carcasses (Gajadhar and Marquardt, 1992; De Bosschere and Ducatelle, 2001), assuming that this was due to a specific immune response towards the sarcocysts.

Nonetheless, due to the high prevalence of *Sarcocystis* in cattle, and the low prevalence of BEM, the presence of sarcocysts inside lesions could be a coincidence (Van Hoof et al., 1972). Experimental oral infection with *Sarcocystis* moreover, did not lead to BEM (Dubey et al., 1982; Dubey, 1983; Dubey et al., 1988).

2.4 Pathogenesis

Since *in vivo* diagnosis is not possible and experimental models are not available yet, research on the pathogenesis of BEM is hampered. Several hypotheses have however been put forward in the past.

First of all, Granstrom et al. (1989) hypothesized that BEM animals would be genetically predisposed to produce IgE in response to *Sarcocystis* bradyzoite antigens, and that BEM would be an abnormal response to sarcocyst degeneration.

Secondly, Jensen et al. (1986) hypothesized, based on the morphological characteristics of the lesions, that both type I and type IV hypersensitivity are involved in the pathogenesis of BEM.

Thirdly, a rupture of the cyst wall has been suggested as a possible trigger of BEM (Jensen et al., 1986; Gajadhar and Marquardt, 1992). Three different ways in which cysts can rupture have been proposed: (i) Jensen et al. (1986) suggested that some sarcocysts become overdistended during their development, which can cause weakening and rupture of the wall. They believe that an increase in the number of bradyzoites, an accumulation of metabolic products containing eosinophilic chemo-attractant and cytotoxins, and a passage of fluid into the cyst cavity due to enhanced osmotic pressure could cause the distension. Gajadhar and Marquardt (1992) assumed that (ii) a genetic defect in the cyst wall and/or (iii) repeated sensitization of the host against a certain *Sarcocystis* species could be a cause of cyst wall rupture.

Oghiso and Fujiwara already detected an eosinophilic chemotactic factor in BEM lesions in 1978. The chemotactic activity was correlated with the severity of the lesions, suggesting that it was produced locally. They showed that the factor had a protein nature, with a molecular weight smaller than bovine albumin. It was heat stable at 60°C, but inactivation occurred after trypsin or EDTA treatment. Oghiso and Fujiwara (1978) therefore assumed that the eosinophilic chemotactic factor consisted of two different active fractions, such as Eosinophil Chemotactic Factor of Anaphylaxis (ECF-A) and Eosinophil Stimulation Promoter (ESP). Nevertheless, if sarcocysts would possess or induce an eosinophilic chemotactic factor, the morbidity of BEM would be much higher, according to Granstrom et al. (1989).

2.5 Predisposing factors

Little is known on the disease profile of BEM, since data in literature are scarce and based on small scale studies.

2.5.1 Animal related factors

Until now it is assumed that an age predisposition for BEM does not exist. The age of the animals described in literature varies between 15 months and eight years (Imes and Migaki, 1967).

Research showed that the prevalence in heifers is statistically higher than in bulls (Reiten et al., 1966; Imes and Migaki, 1967). Reiten et al. (1966) could not show a difference in the prevalence between cows and heifers, while Imes and Migaki (1967) found that cows were less affected with BEM than heifers. In the survey of Reiten et al. (1966) a possible role of estrogens in the pathogenesis of BEM is suggested.

Purebred beef cattle (e.g. Japanese Black, Blonde d'Aquitaine, Hereford, Aberdeen Angus, and Shorthorn) as well as crossbreds have been shown to be affected by BEM (Hamilton and McCance, 1968; Oghiso and Fujiwara, 1978; Saito et al., 1993; Wouda et al., 2006). Imes and Migaki (1967) found in their survey a high percentage of BEM cases in Hereford or Hereford cross cattle. Although data in literature show that beef cattle are more often affected, BEM is also reported in dairy cattle (Reiten et al., 1966; Imes and Migaki, 1967; Oghiso et al., 1977).

Finally, Imes and Migaki (1967) reported that carcass finish of bovines condemned for BEM was listed good to very good by the farmer. Except for two cases, all had had a health history without any evidence of diseases.

2.5.2 Environmental and farm related factors

Data in literature suggest that geographical or farm related factors could play a role in BEM. Reiten et al. (1966) and Jensen et al. (1986) showed that the condemnation rate for BEM was higher in animals fattened in Western USA. Moreover, Wouda et al. (2006) reported cases of BEM in one beef cattle herd in the Netherlands with a high incidence (30%) of affected slaughtered older females.

Seasonally differences are described in literature, but do not show coherent results. Reiten et al. (1966) found during each of three years of their study of 173 condemned carcasses a higher incidence in animals slaughtered in the summer, suggesting that arthropods would play a role. These results however, were not confirmed in the survey by Imes and Migaki (1967) on 947 affected carcasses during a 14 month survey. They found the highest incidence in autumn and in the beginning of the winter, though mentioning that these results could have been influenced by abnormal weather conditions.

2.6 Lesions and symptoms

As described above, BEM is a subclinical myopathy that is not detectable *in vivo*. It is a pathological entity characterized by multifocal or diffuse grey-green discolorations in striated muscles (skeletal and cardiac muscle) (Figure 2.1 and Figure 2.2). Lesions are not seen in smooth muscles or internal organs. Generally, if only a few lesions are present in the carcass, the most active muscles have been shown to be affected, such as the muscles of the tongue, the masseter, myocard and diaphragmatic muscle. In severe cases, all striated muscles can be affected (Imes and Migaki, 1967). When BEM affected carcasses are exposed to air the color of the lesions fades to grey, probably due to oxidative enzymatic changes of myoglobin caused by the accumulations of the eosinophilic granulocytes (Hamilton and McCance, 1968; Stephan et al., 1998). In some cases lymph nodes can show green areas, due to accumulations of eosinophilic granulocytes (Imes and Migaki, 1967; Bundza and Feltmate, 1989).
The affected carcasses have been subdivided according to the gross pathological findings in two types, namely the multiple focal type and the multiple diffuse type (Imes and Migaki, 1967). Two decades later Jensen et al. (1986) established two other macroscopical categories, namely category A and B, with category A being less prevalent. Categories A (Figure 2.1) carcasses have few, large focal lesions in striated muscles, whereas category B (Figure 2.2) have multiple disseminated lesions in the tongue, oesophagus, heart, diaphragm or skeletal muscles.

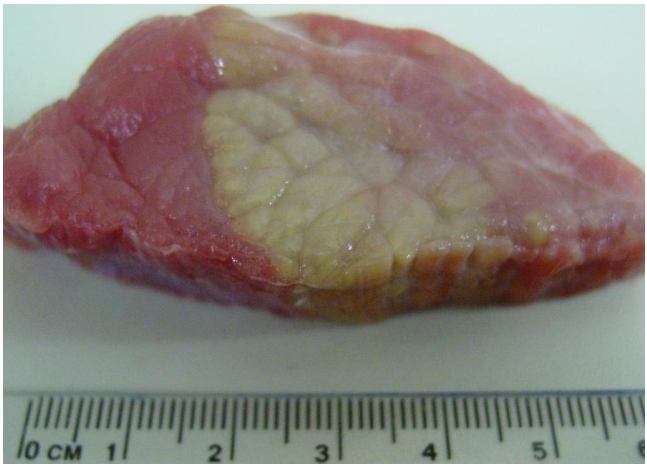

Figure 2.1: BEM, large focal grey green lesions in striated muscles

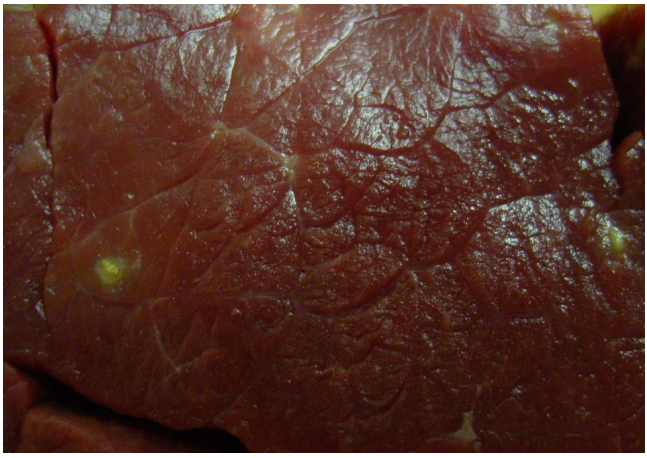

Figure 2.2: BEM, multifocal grey-green discolorations in striated muscles (skeletal and cardiac muscle).

Histopathologically (Figure 2.3), the lesions of BEM are characterized by an infiltration of eosinophilic granulocytes, lymphocytes, plasma cells, macrophages, multinucleated giant cells and mast cells (Imes and Migaki, 1967; Oghiso et al., 1977; Jensen et al., 1986). Most studies describe a granulomatous organization of the lesions (Figure 2.4), where the centre consists of degenerated muscle fibers and eosinophilic granulocytes (Jensen et al., 1986; Gajadhar et al., 1987; Wouda et al., 2006). Jensen et al. (1986) subdivided these lesions in early, intermediate and late stage granulomas and reported that all three stages can occur in the same

animal. In those lesions phagocytosis by multinucleated giant cells can be prominent. Extensive fibrosis or an increase of adipose tissue replacing muscle fibers can occur (Jensen et al., 1986; Oghiso et al., 1977). Vascular alterations are reported in small arteries or arterioles, such as oedematous swelling of the intima with cuboidal metaplasia of endothelial cells, degeneration of the media and cellular infiltration (Oghiso et al., 1977).

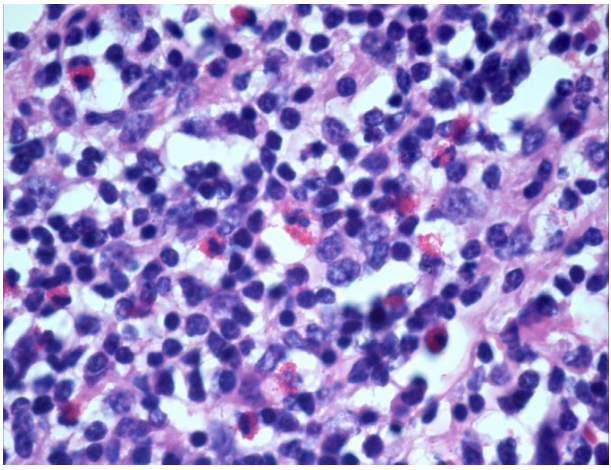

Figure 2.3: Microscopical BEM lesion, remark the infiltration of eosinophilic granulocytes, lymphocytes, and macrophages. HE staining, 1000x.

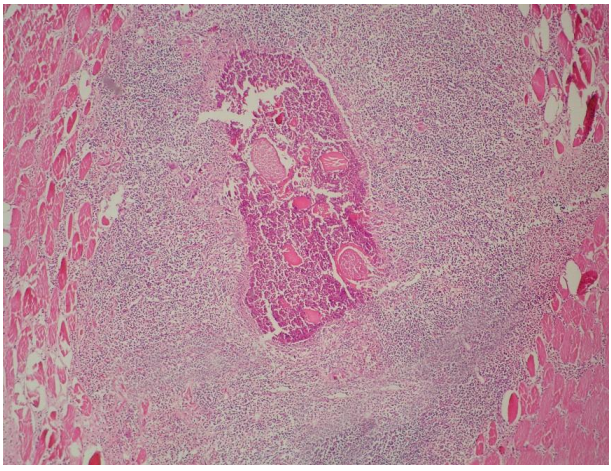

Figure 2.4: Microscopical BEM lesion, a granulomatous organization. HE staining, 50x.

2.7 Diagnosis and differential diagnosis

Up till now, *in vivo* diagnosis of BEM is not possible. A putative diagnosis can be made by macroscopical examination at meat inspection. There is the possibility however, to mistake BEM lesions with *Taeniid* cysts (Figure 2.5) having died, due to macroscopical similarities between the lesions (Gonzalez et al., 2006). Another differential diagnosis is the migration of *Hypoderma lineatum*, since the larvae can cause gross lesions characterized by foci of yellowish or greenish gelatinous oedema in the diaphragm, pleura, mediastinum and peritoneum (Panciera et al., 1993).

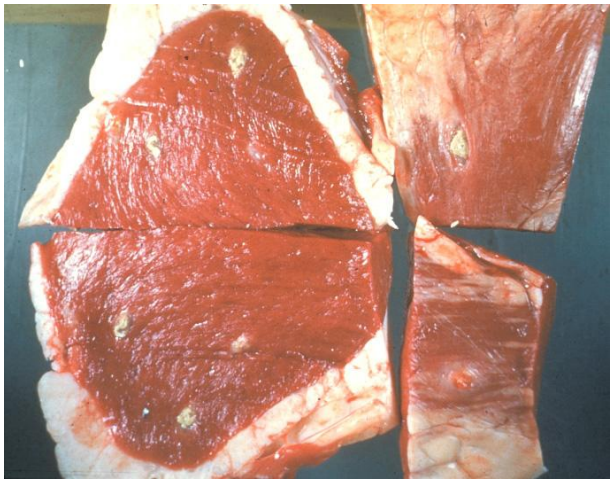

Figure 2.5: Bovine, skeletal muscle and heart. Both heart and skeletal muscle contain cysticerci (*Cysticercus bovis*). Cysticerci have undergone necrosis and mineralization.
(Source: http://www.cfsph.iastate.edu)

Definitive diagnosis of BEM in cattle is still based on the histological examination of post-mortem muscle samples. At this level, BEM lesions can be differentiated from those caused by *Taeniid* cysts and *Hypoderma lineatum*. Histological examination of *Hypoderma lineatum lesions* reveals oedematous tissue, infiltrated by eosinophilic granulocytes (Panciera et al., 1993). Although *Trichinella spiralis* in experimentally infected cattle may produce lesions that show microscopically the characteristics of eosinophilic myositis, it is not generally considered important as a differential diagnosis, because bovines usually do not consume infected dead rodents (Bundza and Feltmate, 1989).

2.8 Prevention and Treatment

For the time being, preventive measures and therapeutic treatment are unknown. However, since BEM is assumed to be associated with *Sarcocystis* species, it is realistic to advise the preventive measures that are being applied for *Sarcocystis* species.

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SCIENTIFIC AIMS

Bovine Eosinophilic Myositis (BEM) is a pathological entity of unknown etiology and pathogenesis. Despite the importance of BEM worldwide, resulting from financial losses at the level of slaughterhouses and meat cutting plants, little information is available on this topic.
It has been suggested that *Sarcocystis* species are associated with BEM. This protozoan parasite is however, present in the muscle tissue (without inducing microscopical lesions) of almost 100% of the bovine population, whereas the prevalence of BEM is low. Moreover, the information on this parasite in bovines is fragmentary.

The **main objective** of this thesis is to study the occurrence of bovine *Sarcocystis* species and to improve the understanding of their role in the etiology and pathogenesis of BEM.
Therefore, the **specific sub-objectives** are:

1. To develop a reliable molecular technique to identify bovine *Sarcocystis* species which is suitable to be applied both on fresh and formalin fixed/paraffin-embedded tissue in order to compare the occurrence of *Sarcocystis* species in normal cattle and in BEM carcasses (Chapter 3).
2. To determine the occurrence of *Sarcocystis* species in general and *S. hominis* in particular in raw minced meat in Belgium (Chapter 3).
3. To investigate the role of *Sarcocystis* species in BEM.
   - Test the hypothesis that BEM might be induced by a specific species of *Sarcocystis* (Chapter 4).
   - Characterize the inflammatory response and the presence of sarcocysts in lesions of spontaneous occurring BEM cases by histology and immunohistochemistry (Chapter 5).
   - Investigate the induction of muscular lesions by *Sarcocystis* antigens after intramuscular inoculation (Chapter 5).
   - Compare the lesions of spontaneous occurring BEM cases with lesions induced by experimental intramuscular injections with bovine *Sarcocystis* antigens (Chapter 5).

EXPERIMENTAL STUDIES

Chapter 3

Development of a molecular based assay and determination of the presence of bovine *Sarcocystis* species in minced beef in Belgium

Adapted from:

Vangeel, L., Houf, K., Chiers, K., Vercruysse, J., D'Herde, K., Ducatelle, R., 2007. Molecular-based identification of *Sarcocystis hominis* in Belgian minced beef. J. Food Prot. 70, 1523–1526.

3.1 Introduction

Sarcocystis is a genus of cyst-forming coccidia belonging to the phylum Apicomplexa. This genus is composed of more than 200 species (Frenkel and Smith, 2003). They have an obligatory two-host life cycle (Dubey, 1976). In the intermediate host, which can be herbivorous or omnivorous, merogony and cyst formation takes place, whereas gamogony and sporogony occurs in the carnivorous or omnivorous definitive host (Dubey, 1976; Tenter, 1995).

Cattle are common intermediate hosts of sarcocysts. The prevalence of *Sarcocystis* in adult bovine muscle is close to 100% in most regions of the world where it has been studied (Böttner et al., 1987; van Knapen et al., 1987; Vercruysse et al., 1989; Fortier et al., 1993; Woldemeskel and Gebreab, 1996; Latif et al., 1999; De Bosschere and Ducatelle, 2001; Pena et al., 2001).

Bovine muscle can harbor three species, i.e., *Sarcocystis cruzi*, *Sarcocystis hirsuta*, and *Sarcocystis hominis*, for which canids, felids, and primates (including humans), respectively, are the definitive hosts (Dubey, 1976; Tenter, 1995). Routine identification has been based on morphological examination of the cyst wall. *S. cruzi*, a thin-walled species, can easily be distinguished from *S. hominis* and *S. hirsuta*, both thick-walled sarcocysts, by light microscopy. The morphological distinction between *S. hominis* and *S. hirsuta*, however, is only possible by transmission electron microscopy (TEM) (Dubey et al., 1989; Odening et al., 1995), which is cumbersome.

S. hominis infection in humans is characterized by transient nonspecific gastrointestinal complaints. The clinical course usually is mild and in many cases asymptomatic, although the severity is correlated with the number of ingested sarcocysts (Fayer, 2004).

Because of the difficulties involved in correct identification, little is known about the relative distribution of these *Sarcocystis* species in bovine muscle. From a public health point of view accurate data on the prevalence of *S. hominis* in cattle are needed and should be derived from a reliable technique for identification. In some Western European countries, where consumption of raw (minced) bovine meat is common, the occurrence of *S. hominis* should be monitored.

The first objective of the present study was to develop a reliable molecular identification technique. The second aim was to use this technique to determine the presence of *Sarcocystis* spp. in general and of *S. hominis* in particular in raw minced beef in Belgium.

3.2 Materials and methods

3.2.1 Development of a molecular based identification technique

Samples (200 g) of diaphragm of nine slaughtered bovines were examined. The sedimentation technique developed by Markus (1979) was used with minor modifications to collect the cysts and cyst fragments. A glass funnel closed with a clip near the end of the rubber tube, was filled with physiological saline (37°C). A strainer in combination with a single layer of gauze was placed on the top of the funnel, and a thin layer of minced meat was put on the gauze and remained under the fluid level. This apparatus was then incubated at 37°C for 4 hours without stirring. The sediment in the stem of the funnel was drawn through the rubber tube by opening the spring clip and collected in a 60 ml screw-cap container (Deltalab, Barcelona, Spain).
This sediment was examined stereomicroscopically (50x) for the presence of cysts, both thin- and thick-walled. Thick-walled cysts were recovered, and each cyst was cut into equal parts by microdissection (Figure 3. 1). From each cyst, one half was stored in phosphate-buffered solution without Ca^{2+} and Mg^{2+} at – 20°C until further analysis by polymerase chain reaction (PCR), and the other half of each cyst was processed for TEM because this technique is until now accepted as the “gold standard” for identification.

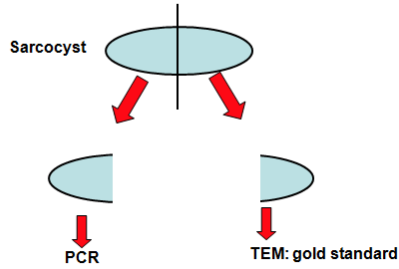

Figure 3. 1: Schematic representation of microdissection of thick-walled cysts for further PCR and TEM analysis.

For cyst lysis prior to DNA extraction, a series of different dilutions and different incubation periods of a pepsin/HCl solution were evaluated (Seneviratna et al., 1975; Gajadhar and Marquardt, 1992). The effect of 10-fold dilutions of 60 to 0.6 mg pepsin was evaluated every ten minutes.

Thick-walled cyst halves were defrosted at room temperature, and 50 µl of a fresh pepsin/HCl solution was added to each sample and incubated at 37°C under optimized conditions (see "Results"). Subsequently, DNA was extracted using a commercial genomic DNA purification kit (ChargeSwitch® gDNA Micro Tissue Kit, Invitrogen™, Carlsbad CA, USA) according to the manufacturer's instructions.

Sarcocystis genus specific primers were designed based on the 18S rRNA sequence of *S. cruzi* (GenBank accession no. AF017120), *S. hominis* (AF006470), and *S. hirsuta* (AF017122), which are deposited in the National Center for Biotechnology Information database (Bethesda, Md.).

Optimal PCR conditions were tested by using different concentrations of primers, $MgCl_2$, and *Taq* polymerase. Annealing was performed at different temperatures.

The specificity of the *Sarcocystis* genus specific primers was tested by PCR using DNA extracts of a bovine foetus (7 months of gestation), *Neospora caninum* tachyzoites (B. Losson, University of Liège, Liège, Belgium), and *Toxoplasma gondii* tachyzoites (P. Dorny, Institute of Tropical Medicine, Antwerp, Belgium).

The PCR products were size separated by electrophoresis of 2 µl of the reaction products in a 1% agarose (Invitrogen) gel in 1x Tris-borate-EDTA at 110 V..

The amplicons of the samples were enzymatically purified with ExoSapIt (USB, Cleveland, Ohio) and sequenced, using the same primers, with the BigDye®Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Lennik, Belgium). The sequencing products were purified with the CleanSEQ Dye-terminator removal (Agencourt Bioscience Corporation, Beverly Mass.) and run on a 3730 DNA Analyzer (Applied Biosystems). The electrophoregrams were exported and converted to the BioEdit v5.06 software package (Tom Hall, Ibis Therapeutics, Carlsbad, Calif.). The sequences were aligned to published *S. hominis* (accession nos. AF006470, AF006471, AF006477 and AF176945), *S. hirsuta* (accession nos. AF017122 and AF006469) and *S. cruzi* (accession nos. AF017120 and AF176935) 18S rRNA sequences obtained from NCBI GenBank by using Pairwise Alignment Algorithms (available from http://www.ebi.ac.uk/emboss/align/index.html).

The half-cysts for TEM were fixed in a solution of 2% glutaraldehyde (Fluka, Buchs, Switzerland), and subsequently a solution of 2% glutaraldehyde and 8% bovine serum albumin (BSA fraction V, Roche, Mannheim, Germany) was added in order to capture the half-cyst in a protein coagulate. After 2 hours, the sample was rinsed with sodium-cacodylate trihydrate buffer (Fluka) supplemented with 1% calcium chloride dihydrate (Merck, Darmstadt, Germany). Then a post fixation procedure was carried out with osmium tetroxide (Serva, Heidelberg, Germany) supplemented with Potassium ferricyanide (Merck) overnight. Afterwards a routine dehydration and embedding was done.

3.2.2 Detection of *Sarcocystis* spp. in raw minced beef.

From January through March 2006, 67 unrelated samples of 200 g raw minced beef were obtained from 25 retail stores. Samples were collected at different times in different retail stores and in different areas all over Belgium. The lack of relatedness of the samples was subsequent checked through the Belgian tracing system. Raw minced beef is prepared from the muscles of the thigh and loin region of cattle.

The extraction and stereomicroscopic identification of cysts was performed as described above. All cysts were collected and counted. Thick-walled cysts were stored individually in phosphate-buffered saline without Ca^{2+} and Mg^{2+} at -20°C until further analysis.

Cyst lysis and DNA extraction were carried out, and the genus specific PCR assay was performed using the optimized conditions (see "Results"), followed by sequence analysis as described above.

3.3 Results

3.3.1 Development of a molecular based identification technique.

For cyst lysis prior to DNA extraction, the optimal concentration was 60 mg of pepsin (Merck, Darmstadt, Germany), 85 mg of NaCl (VWR international, Fontenay-sous-Bois, France), and 220 µl of 37% HCl (Merck) diluted in 100 ml distilled water. The optimal incubation time was 30 minutes.

The primer set SARf (5'-TGGCTAATACATGCGCAAATA-3') and SARr (5'-AACTTGAATGATCTATCGCCA-3') allows detection of the three targeted *Sarcocystis* species. Depending on the species, amplicons of 164 bp for *S. hominis*, 172 bp for *S. cruzi*, and 186 bp for *S. hirsuta* are generated (Table 3.1).

Table 3.1: Localization of primers on the 18S rRNA gene sequences

species	GenBank no.	primer SARf position	primer SARr position	amplicon length (bp)
S. cruzi	AF017120	152 - 172	303 - 323	172
S. hirsuta	AF017122	163 - 183	328 - 348	186
S. hominis	AF006470	88 - 108	231 - 251	164

Optimal PCR reactions were obtained using 4 µl of DNA template, 5 µl of PCR buffer (200 mM Tris-HCl [pH 8.4] and 500 mM KCl) without Mg^{2+} (Invitrogen), 2.0 U of *Taq* polymerase (Invitrogen), 1.5 µl of a deoxynucleoside triphosphate mixture containing each deoxynucleoside triphosphate (Invitrogen) at a final concentration of 10 mM, 2.3 µl of 50 mM $MgCl_2$ (Invitrogen), and 47 pmol of both forward and reverse primers in a total volume of 50 µl. The optimal conditions for amplification were as follows: initial denaturation at 94°C for 3

min, followed by 39 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, elongation at 72 °C for 1 min, and a final elongation at 72°C for 3 min.

Amplification of the 18S rRNA gene using the SARf and SARr primers produced a fragment of the expected size range (150 to 200 bp) in all samples examined. No amplicon was obtained for *Toxoplasma gondii*, *Neospora caninum*, and bovine DNA (Figure 3.2).

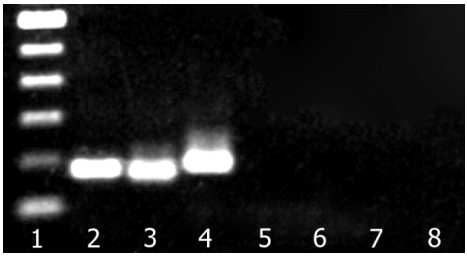

Figure 3.2: agarose (1%) gel electrophoresis of the amplified products for each species and negative controls. Lane 1: molecular size marker, 100 bp DNA ladder; lane 2: *S. cruzi*; lane 3: *S. hominis*; lane 4: *S. hirsuta*; lane 5: *Toxoplasma gondii*; lane 6: *Neospora caninum*; lane 7: *Bos taurus*; lane 8: negative control.

Sequence analysis of PCR products from five thick-walled half-cysts revealed a similarity of 96.8% to 100% with sequences deposit in the gene bank for *S. hominis* (accession nos. AF006470, AF006471, AF006477 and AF176945). In contrast, similarities of only 73.5% to 75.8% and 70.8% to 78.4% were obtained for *S. hirsuta* (accession nos. AF017122 and AF006469) and *S. cruzi* (accession nos. AF017120 and AF176935), respectively.

The other cyst halves from three of these five samples were examined by TEM. All three cyst walls (Figure 3.3) had finger-like protrusions, which were seated broadly on the cyst surface. In the core of the protrusions, long microfilaments were abundant, but no osmiophilic granules were seen. These findings are in accordance with published descriptions of *S. hominis* (Dubey et al., 1989; Odening et al., 1995).

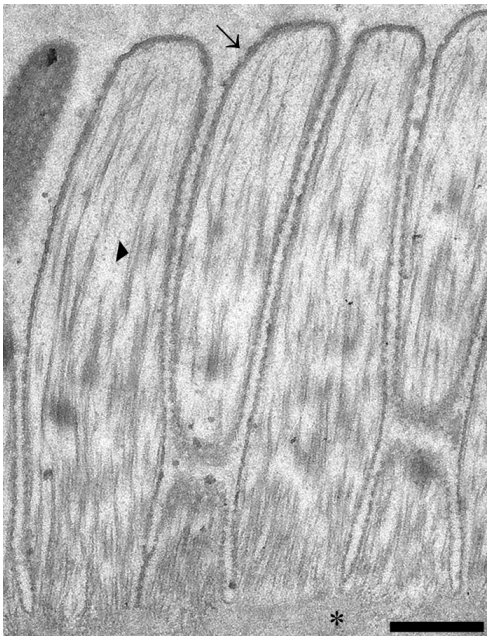

Figure 3.3: Transmission electron micrograph of the cyst wall of a sectioned *S. hominis*, showing (*) the ground substance, (→) the parasitophorous vacuolar membrane, (▲) microfilaments.

3.3.2 Detection of *Sarcocystis* spp. in raw minced beef

In 63 (94%) of the 67 samples, complete sarcocysts or fragments of sarcocysts were detected. Thick-walled cysts (*S. hominis* or *S. hirsuta*) were present in 61 (91%) and thin-walled (*S. cruzi*) were present in 33 (49%) of the samples. Mixed infections of thick- and thin-walled cysts were observed in 31 (46%) of the samples (Figure 3.4).

Amplification of the 18S rRNA gene using the SARf and SARr primers produced an amplicon of the expected size range in 60 of the 61 samples examined. From 50 samples, a sufficient amount of the amplicon was obtained for further sequence analysis.

The result of the sequence analysis of 39 samples was reliable for further species identification. The sequences of the amplified fragments from 38 of the 39 PCR products (97.4%) showed a similarity of more than 95% to those of *S. hominis* published in NCBI Genbank (accession nos. AF006470, AF006471, AF006477 and AF176945) in contrast to a similarity

of 67% to 75% to *S. hirsuta* and of 72.5% to 79.9% to *S. cruzi*. The sequence of one sample (2.56%) was 97% similar to those from *S. hirsuta* (accession nos. AF017122 and AF006469), 77.3% similar to *S. hominis*, and 70.8% similar to *S. cruzi* (accession nos. AF017120 and AF176935).

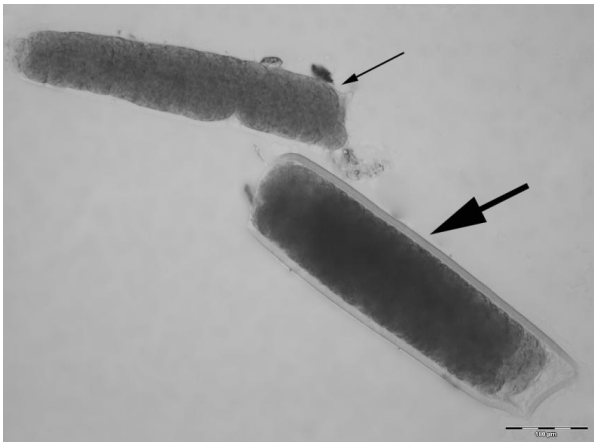

Figure 3.4: fragments of thin-walled (thin arrow) and thick-walled (thick arrow) cysts. Bar, 100µm.

3.4 Discussion

To our knowledge, this is the first report using a stereomicroscopical identification technique combined with a genomic assay for the identification of bovine sarcocysts. The results from the molecular assay were in accordance with those of the TEM examination as all TEM examined samples showed finger-like protrusions that were broadly seated on the cyst surface and contained no osmiophilic granules, which is consistent with the published descriptions of *S. hominis* (Dubey et al., 1989; Odening et al., 1995). However, *S. hirsuta* is characterized by diagonally bent, bulb- or club-shaped villar protrusions arising with a stalklet from the cyst wall and containing large osmiophilic granules (Dubey et al., 1989; Odening et al., 1995). Because TEM is laborious, expensive and not readily available, the PCR assay could be the method of choice for identification.

In the present study, sequence analysis of PCR products from cysts collected from minced beef in Belgium revealed that *S. hominis* was present in 97.4% of the samples. This result is in contrast with a molecular study conducted in Vermont, USA (Pritt et al., 2006), in which no *S. hominis* was found. However, data about the primers used in the latter PCR assay were not described to our knowledge. In two studies based on TEM examination in Brazil (Pena et al., 2001) and France (Solomon, 1991), *S. hominis* was detected in 94% and 57% of the samples, respectively. In other studies carried out in India (Jain and Shah, 1987) and Germany (Boch et al., 1978) with light microscopic identification, *S. hominis* prevalences were 12.18% and 63.3%, respectively. Because Odening et al. (1995) stated that light microscopic examination is unreliable for distinguishing *S. hominis* from *S. hirsuta*, care should be taken when comparing the latter studies.

The findings in the present study suggest the existence of a very efficient epidemiological cycle of *S. hominis*. Regarding the prevalence of intestinal sarcocystosis in humans, unfortunately, data in literature on natural infections are limited. In a study carried out in Germany (Gauert et al., 1983), 13.64% of patients with intestinal complaints were infected with intestinal parasites, and 2% of these patients were shedding *Sarcocystis* sporocysts. In two thirds of the patients, a connection of intestinal sarcocystosis and clinical symptoms could be made, but a distinction between *S. hominis* and *S. suihominis* was not made. The reason for limited information on *Sarcocystis* prevalence in humans may be explained by the fact that intestinal sarcocystosis is characterized by non-specific transient intestinal complaints, as shown in experimental infection trials (Heydorn, 1977; Pena et al., 2001). Thus, most cases of natural

infections may not be reported. However, further research is needed on the prevalence of *S. hominis* in humans to obtain a better insight into the epidemiological cycle of this protozoon.
In conclusion, this study showed a high occurrence of *S. hominis* in minced bovine beef at retail in Belgium. The genus specific PCR in combination with sequence analysis was a useful tool for *Sarcocystis* identification in bovine muscle. This technique allows a reliable species identification and can provide a better insight into the prevalence of *S. hominis*. This information is important from a public health point of view because minced beef is commonly consumed raw in some European countries, including Belgium.

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Chapter 4

Identification of *Sarcocystis* spp. associated with Bovine Eosinophilic Myositis

Adapted from:

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4.1 Introduction

Sarcocystis is a genus of cyst-forming coccidia belonging to the phylum Apicomplexa. They have an obligatory two-host life cycle. In the intermediate host the asexual cycle with cyst formation takes place, whereas the sexual cycle occurs in the definitive carnivorous host (Dubey, 1976).

Cattle are common intermediate hosts of sarcocysts. The prevalence of *Sarcocystis* in adult bovine muscle is close to 100% in most regions of the world where it has been studied (Böttner et al., 1987; van Knapen et al., 1987; Vercruysse et al., 1989; Fortier et al., 1993; Woldemeskel and Gebreab, 1996; Latif et al., 1999; De Bosschere and Ducatelle, 2001; Pena et al., 2001; Vangeel et al., 2007). Bovine muscle can harbor three species, namely *Sarcocystis cruzi* with canids as definitive hosts, *Sarcocystis hirsuta* with felids as definitive hosts and *Sarcocystis hominis* with primates as definitive hosts (Heydorn et al., 1975).

Sarcocystis species have been suggested to play a role in Bovine Eosinophilic Myositis (BEM), a specific inflammatory myopathy characterized by multifocal grey-green lesions in striated muscle of cattle. Histologically, the lesions consist of an eosinophilic inflammation with myofiber degeneration (Imes and Migaki, 1967; Jensen et al., 1986; Oghiso et al., 1977; Vangeel et al., 2012). The fact that *Sarcocystis* species are frequently found in the centre of the lesions (Jensen et al., 1986; Gajadhar et al., 1987; Gajadhar and Marquardt, 1992) and the evidence that antigens of *Sarcocystis* species can induce an immune response with predominantly eosinophilic granulocytes (chapter 5; Vangeel et al., 2012) is an argument in favor of their role.

Bovine Eosinophilic Myositis is hitherto not detectable in the living animal, because affected animals appear clinically normal (Imes and Migaki, 1967). It results however in economic losses due to carcass condemnation at slaughter as well as meat condemnation in meat cutting plants due to abnormal appearance. Worldwide, BEM prevalence reported range from 0.002% to 0.028% of slaughtered cattle (Reiten et al., 1966; Imes and Migaki, 1967; Van Hoof et al., 1972; Bradley and Taylor, 1993; Fortier et al., 1993). Exceptionally high BEM-associated carcass condemnations (up to 5%) have been reported in the western part of the USA (Jensen et al., 1986).

Remarkably, the prevalence of BEM is very low, while the prevalence of sarcocysts in cattle is extremely high. The reason for this discrepancy is not clear, but a possible explanation could be that BEM may be associated with one specific *Sarcocystis* species. In the past, *Sarcocystis* identification in BEM lesions has been performed by morphological methods (light and transmission electron microscopy). Nevertheless, Odening et al. (1995) stated that light microscopic examination is unreliable to distinguish *S. hominis* from *S. hirsuta*. Moreover, a pitfall of both techniques is the fact that intralesional sarcocysts are mostly damaged (Wouda et al., 2006), hindering the identification. Therefore the objective of the present study was to determine if BEM is associated with a particular *Sarcocystis* species using molecular tools.

4.2 Materials and methods

4.2.1 Sample collection and processing

From January 1994 through October 2007, striated muscle samples from 97 unrelated bovine carcasses (determined by the Belgian meat tracing system), condemned for BEM, were examined. From each carcass, two to ten muscle tissue samples with lesions and two without lesions were collected. Samples were taken from skeletal muscles, diaphragm and, when available, from the masticatory muscle, tongue, heart and oesophagus. The samples were fixed in 10% phosphate–buffered formalin for 24 hours and embedded in paraffin.

4.2.2 Histological examination

Tissue sections were cut at 4 µm and stained with haematoxylin and eosin (HE) for light microscopic examination.

The diagnosis of BEM at carcass inspection was confirmed on histology when eosinophilic granulocytes were the predominant cell type in the multifocal inflammatory myopathy (Van Vleet and Valentine, 2007).

The presence of sarcocysts laying inside the lesions (intralesional) as well as sarcocysts laying outside a lesion in normal muscle tissue (extralesional) was recorded for each carcass on semiserial sections from at random selected samples.

4.2.3 Molecular identification of *Sarcocystis* spp. at species level

All paraffin embedded formalin fixed tissues containing intralesional sarcocysts on the HE sections were further processed for Laser Capture Microdissection (LCM). Twenty serial sections from each sample were cut at 5 µm, HE-stained and dehydrated. The protocol by De Preter et al. (2003) was used. The intralesional sarcocysts or remnants, and the extralesional sarcocysts, if present in the same section, were microdissected using a PixCell II laser capture microscope equipped with an infrared diode laser (Arcturus Engineering, Santa Clara, CA, USA) as described by Emmert-Buck et al. (1996) and Bonner et al. (1997). Each sarcocyst was individually captured on a CapSure™ Macro LCM Cap (Arcturus Bioscience, Mountain View, CA, USA).

DNA from the laser captured sarcocysts was extracted from the LCM caps using the Pico pure DNA extraction kit™ (Arcturus Bioscience, Mountain View, CA, USA).

A fragment of the 18S rRNA gene (164 bp for *S. hominis*, 172 bp for *S. cruzi* and 186 bp for *S. hirsuta*) was amplified using the primer set SARf - SARr as described by Vangeel et al. (2007). The amplicons of the samples were purified (Qiaquick purification kit, Qiagen GmbH, Hilden, Germany) and sequenced using the dideoxy chain terminator method in a 3100 Genetic Analyzer (Applied Biosystems, Lennik, Belgium). The electrophoregrams were exported and converted to the DNAstar software (DNAstar Inc., Madison, WI, USA). If the identification of nucleotide positions was not possible due to nearly exactly overlapping peaks in the electrophoregrams, the PCR products were purified (Qiaquick purification kit, Qiagen GmbH, Hilden, Germany) and cloned into the plasmid pGEM-T Vector System (Promega corporation, Madison WI, USA). The sequences of one to six cloned copies per isolate were subsequently determined using the dideoxy chain terminator method in a 3100 Genetic Analyzer (Applied Biosystems, Lennik, Belgium). Sequence analyses and alignments were performed by the DNAstar software (DNAstar Inc., Madison, WI, USA). The sequences were aligned to the bovine *Sarcocystis* species: *S. hominis* (accession nos. **AF006470**, **AF006471** and **AF176945**), *S. hirsuta* (accession nos. **AF017122** and **AF006469**) and *S. cruzi* (accession

no. **AF017120**) 18S rRNA gene sequences published in the EMBL GenBank. Multiple alignments were made using CLUSTALW (http://www.ebi.ac.uk/clustalw), and phylogenetic analysis was carried out using the program Mega v.3.1 (Kumar et al., 2004) by bootstrap test of phylogeny (1000 replicates), neighbour-joining and analysing the consensus tree. Bootstrap values below 50% were not taken into account.

4.3 Results

4.3.1 Histological examination

In all 97 bovine carcasses the diagnosis of BEM was confirmed by histology. The inflammatory infiltrates in the muscle samples consisted mainly of eosinophilic granulocytes. Additionally, lymphocytes, plasma cells and macrophages were observed. In the majority of the lesions, the inflammation was accompanied by fragmentation of myocytes.

Intralesional sarcocysts (Figure 4.1) were detected in BEM lesions of 27 (28%) of the 97 bovine carcasses. In six carcasses, more than one sarcocyst was detected inside different BEM lesions. In total, 36 intralesional sarcocysts were detected. Fifteen of these 36 intralesional cysts were thick-walled (cyst wall: 4.28 ± 0.76 µm thick). The remaining 21 intralesional cysts were remnants of which the thickness of the wall could not be determined. All intralesional sarcocysts were damaged (Figure 4.2) and located centrally or paracentrally in the lesion. Nine of them were inside a muscle fiber, while 27 were extracellular.

Extralesional sarcocysts (Figure 4.1) were found in normal muscle tissue of 55 (57%) of the 97 BEM-carcasses. From those, thin-walled (cyst wall: 1.02 ± 0.18 µm thick) sarcocysts and thick-walled (cyst wall: 4.28 ± 0.76 µm thick) sarcocysts were found in 23 and 25 carcasses, respectively. A mixed infection (thin- and thick-walled) was found in 7 carcasses. These sarcocysts were always intracellular in intact muscle fibers and no inflammation was found surrounding these muscle cells.

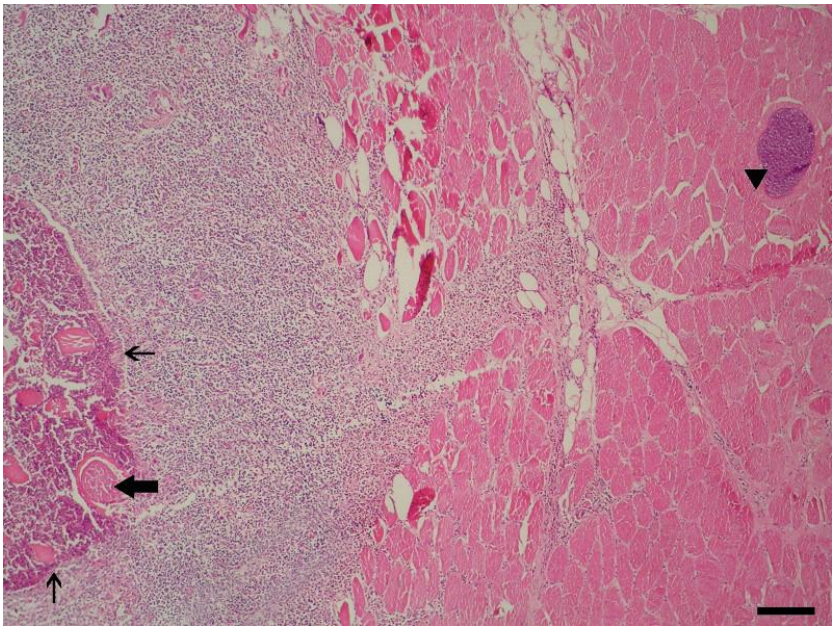

Figure 4.1: Muscle sample from a BEM carcass, showing a lesion (thin arrow) with a damaged intralesional sarcocyst (thick arrow). Remark the extralesional sarcocyst (arrowhead) lying in normal muscle tissue without surrounding inflammatory cells. HE-staining, bar = 200 µm.

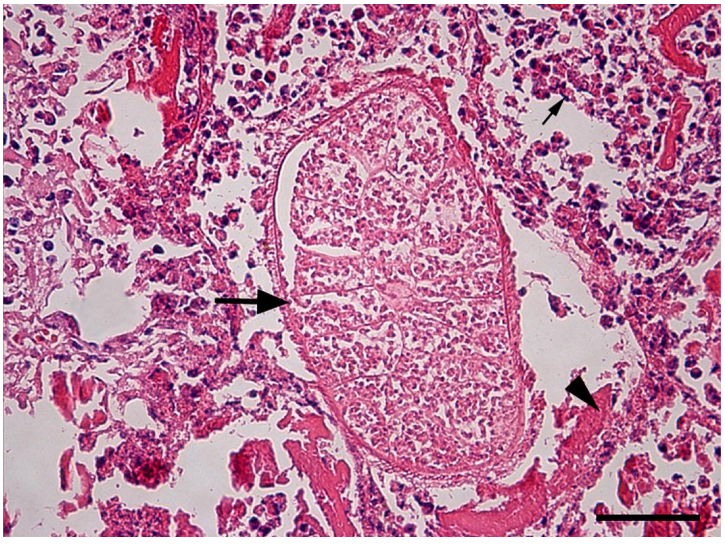

Figure 4.2: Damaged intralesional sarcocyst (thick arrow), lying free and surrounded by degenerated eosinophilic granulocytes (thin arrow) and muscle fibers (arrowhead). HE-staining, bar = 50 µm.

4.3.2 Molecular identification of *Sarcocystis* spp. at species level

A total of 38 sarcocysts (30 intralesional sarcocysts or remnants thereof, and eight extralesional sarcocysts) were successfully microdissected. After DNA extraction an amplicon of the expected size range (150-200 bp), based on the 18S rRNA gene, was generated for 36 (28 intralesional and eight extralesional) samples. Species identification by sequence analysis of the PCR product or the cloned copy was obtained for 27 samples (22 intralesional and five extralesional). The results are shown in Table 4.1.

Table 4.1: Molecular identification of intra- and extralesional sarcocysts in Bovine Eosinophilic Myositis lesions

	Intralesional sarcocysts		Extralesional sarcocysts	
carcass	**species**	**number**	**species**	**number**
1	*S. hominis* [a]	2	*S. hominis* [a]	2
2	*S. hominis* [a]	1		
3	*S. cruzi* [d]	1		
4	*S. hominis* [a]	1		
5	*S. hominis* [a]	1		
6	*S. hominis* [a]	2		
7	*S. hominis* [a]	1		
8	*S. hominis* [a]	1		
9	unidentified species [e]	2	unidentified species [e]	1
10	*S. hominis* [b]	1	*S. hominis* [b]	1
11	*S. hominis* [a]	1		
12	*S. hominis* [a]	1		
13	*S. hominis* [a]	1		
14	*S. hominis* [a]	1		
15	*S. hominis* [a]	2		
16	*S. hominis* [a]	1	*S. hominis* [a]	1
17	*S. hominis* [a]	1		
18	*S. hirsuta* [c]	1		

[a] ≥ 98.8% similarity to *S. hominis* **AF176945**; [b] ≥ 98% similarity to *S. hominis* **AF006470**; [c] 97.8% similar to *S. hirsuta* **AF006469**; [d] 92.4% similar to *S. cruzi* **AF017120**, [e] ≤ 86.7% similar to one of the bovine *Sarcocystis* species.

4.3.2.1 Species identification of intralesional *Sarcocystis* spp.

Eighteen intralesional samples (Table 4.1) showed a similarity of more than 98% to *S. hominis* accession number **AF176945** or **AF006470** (EMBL Genbank) in contrast to less than 73% similarity to *S. hirsuta* (accession no. **AF006469**) and less than 68% to *S. cruzi* (accession no. **AF017120**). The sequence of another sample showed a similarity of 97.8% to *S. hirsuta* (accession no. **AF006469**), 80% to *S. hominis* (accession no. **AF006470**) and 74.4% to *S. cruzi* (accession no. **AF017120**). One sample showed 92.4% similarity to *S. cruzi* (accession no. **AF017120**), 84.1% to *S. hominis* (accession no. **AF006470**) and 82.6% to *S. hirsuta* (accession no. **AF006469**). Two intralesional (Ssp13, Ssp14) samples originating from the same carcass (no.9) showed less than 86.7% similarity to a known bovine *Sarcocystis* species. Ssp13 showed 97.7% homology with Ssp14. The amplicons of Ssp13 and Ssp14 had a length of 174 and 177 bp, respectively. An alignment report and a phylogenetic tree of their sequences with published bovine *Sarcocystis* spp. are shown in Figure 4.3. The sequence data of Ssp13 and Ssp14 reported here have been submitted to EMBL and have been assigned accession numbers **FN394498**, **FN394499**.

4.3.2.2 Species identification of extralesional *Sarcocystis* spp.

Four extralesional samples (Table 4.1) showed a similarity of more than 98% to *S. hominis* accession no. **AF176945** or **AF006470** (EMBL Genbank) in contrast to less than 74% similarity to *S. hirsuta* (accession no. **AF006469**) and less than 69% to *S. cruzi* (accession no. **AF017120**). One extralesional (Ssp15) sample originating from the same carcass (no.9) as Ssp13 and Ssp14 also showed less than 86.7% similarity to a known bovine *Sarcocystis* species (Figure 4.3). Ssp15 showed 99.4% and 97.7% homology with Ssp13 and Ssp14, respectively. The amplicon of Ssp15 had a length of 176 bp. The sequence data of Ssp15 reported here have been submitted to EMBL and have been assigned accession number **FN394500**.

4.3.2.3 Pairwise alignment of cloned copies of single sarcocysts

From fourteen *S. hominis* samples, sequence analysis of more than one clone per isolate was possible. Pairwise alignment of these different cloned copies showed an intra-isolate sequence divergence between 0 – 5.5% (homology 94.5 – 100%). The sequence divergence consisted mostly of at random distributed single nucleotide substitutions or insertions. In one of the clones of four isolates an insertion of three nucleotides, TAA, located on bp 153-155 (based on reference accession no. **AF006470**) was found.

Unfortunately, it was not possible to clone the PCR product of the *S. cruzi* sample into the plasmid pGEM-T Vector System or to obtain more than one cloned copy from the Ssp13, Ssp14 and Ssp15 samples.

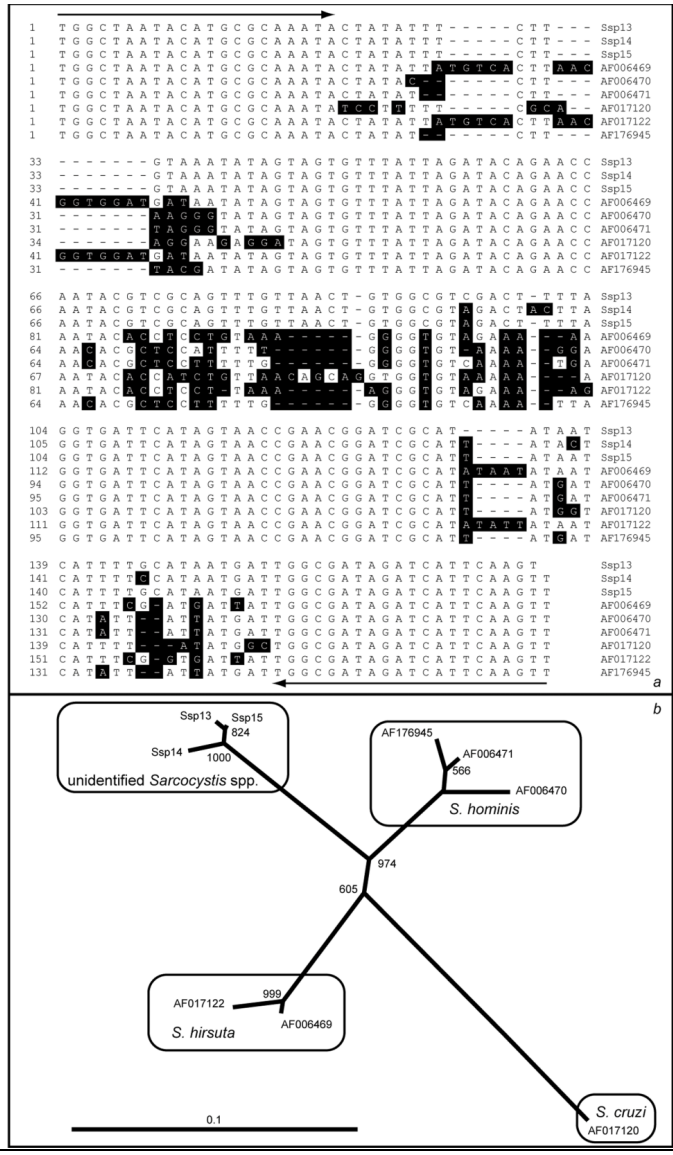

Figure 4.3: An alignment report (a) and an unrooted phylogenetic tree (b) are shown. The alignment report is generated from a nucleotide fragment of the 18S rRNA gene sequences of the three unidentified *Sarcocystis* species (Ssp13, Ssp14, Ssp15) with published sequences of bovine *Sarcocystis* species in the EMBL Genbank. *S. hominis* (accession nos. AF006470, AF006471 and AF176945), *S. hirsuta* (accession nos. AF017122 and AF006469) and *S. cruzi* (accession no. AF017120). Nucleotides that differ from Ssp13, one of the unidentified species, are shaded. The forward (SARf) and reverse primer (SARr) are indicated with an arrow. The unrooted phylogenetic tree shows the relationship of the different *Sarcocystis* species. Bootstrap resampling with 1000 replicates are indicated for the nodes. Lengths of the lines are proportional to the number of base changes.

4.4 Discussion

To our knowledge, this is the first report using microscopic detection combined with a genomically based assay for the identification of single microdissected sarcocysts in BEM.

Although sarcocysts or fragmented sarcocysts were present in 28% of the BEM lesions, this figure could be an underestimate. As Collins et al. (1980) showed that histological examination is an insensitive technique for the detection of *Sarcocystis*. Furthermore, a specific immune response against sarcocysts and subsequent destruction could also result in a decreased histological detection. This finding is supported by the fact that muscle cells close to BEM lesions expressed MHC class II at their cell membrane (chapter 5; Vangeel et al., 2012) and that BEM carcasses contained fewer bradyzoites than normal carcasses (Gajadhar and Marquardt, 1992; De Bosschere and Ducatelle, 2001).

In the present study, we tested the hypothesis that BEM might be associated with a specific *Sarcocystis* species. Literature on this topic is confusing as both thin- and thick-walled sarcocysts (Jensen et al., 1986), *S. hominis* or *S. hirsuta* (Rimaila-Pärnänen and Nikander, 1980), *S. cruzi* (Gajadhar and Marquardt, 1992), *S. hominis* (Wouda et al., 2006) or even an unidentified species (Gajadhar et al., 1987) have been observed in BEM lesions. All studies were based on light or transmission electron microscopy for species identification. The main disadvantage of these techniques is that morphological identification can be hampered due to damage of the cyst walls which is often present in BEM lesions (Wouda et al., 2006). In the present study however, LCM in combination with a PCR based identification technique (Vangeel et al., 2007) was used to bypass these difficulties. It turned out that different bovine *Sarcocystis* species were present inside BEM lesions, and that the species distribution inside these lesions was the same as those in normal bovine muscle. The majority (82%) of intralesional sarcocysts were found to be *S. hominis*, reflecting the high prevalence of this species in non-lesional muscles (Vangeel et al., 2007). However, also *S. cruzi* and *S. hirsuta* were found in lesions of BEM, as well as a hitherto unidentified species.

Although sequencing of 18S rDNA is being used for species identification of bovine *Sarcocystis* species, difficulties have been described since Fischer and Odening (1998) reported considerable differences in sequence between *S. hominis* isolates. At that moment, they could not differentiate if the unclear nucleotide positions were due to sequence variations between gene copies within a single sarcocyst, or due to strain variations or mixed infections. In contrast, Rosenthal et al. (2008) showed that variation among alleles is rare in *S. cruzi* 18S rDNA.

In the present study, sequence analysis of several cloned copies from single *S. hominis* cysts showed that sequence microheterogeneity (sequence divergence between 0 – 5.5%) exists in bovine *Sarcocystis* spp. as is also described in other Apicomplexan species (Reddy et al., 1991; Bishop et al., 2000; Caraguel et al., 2007). This is the first time that microheterogeneity in *Sarcocystis* spp. has been demonstrated. This finding may limit the utility of the 18S rRNA gene for strain identification in bovine *Sarcocystis* species.

In conclusion, our findings indicate that BEM is not associated with one particular *Sarcocystis* species. In addition, the results show that intragenomic variation occurs in *S. hominis* 18S rDNA. Therefore further research of other markers is necessary in order to identify strain variations in bovine *Sarcocystis* spp. which could be associated with BEM.

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Note: Nucleotide sequence data reported in this paper are available in the EMBL database under the accession numbers: **FN394498**, **FN394499** and **FN394500**.

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Chapter 5

Characterization of the inflammatory response in Bovine Eosinophilic Myositis and experimentally induced lesions.

Adapted from:

Vangeel, L., Houf, K., Geldhof, P., Nollet, H., Vercruysse, J., Ducatelle, R., Chiers, K., 2012. Intramuscular inoculation of cattle with *Sarcocystis* antigen results in focal eosinophilic myositis. Vet. Parasitol. 183, 224 – 230.

5.1 Introduction

Bovine Eosinophilic Myositis (BEM) is a rare, specific subclinical myopathy that occurs worldwide in clinically healthy cattle (Imes and Migaki, 1967). Although BEM is not detectable in the living animal, it results in economic losses due to carcass condemnation at slaughter as well as meat condemnation in meat cutting plants. It is known to meat inspectors as a well-established entity characterized by multifocal or diffuse white to grey-green discolorations in striated muscles. Histologically, BEM essentially consists of myofiber degeneration accompanied by an infiltration of large numbers of eosinophilic granulocytes (Imes and Migaki, 1967; Oghiso et al., 1977; Jensen et al., 1986).

Reported prevalences of BEM range from 0.002% to 0.028% of slaughtered cattle in different parts of the world (Reiten et al., 1966; Imes and Migaki, 1967; Van Hoof et al., 1972; Bradley and Taylor, 1993; Fortier et al., 1993). Exceptionally, high BEM-associated carcass condemnations (up to 5%) have been reported in the western part of the USA (Jensen et al., 1986).

The etiology and the pathogenesis of BEM are yet unknown, but in the centre of BEM lesions, often damaged *Sarcocystis* cysts are observed (Jensen et al., 1986; Gajadhar et al., 1987; Gajadhar and Marquardt, 1992; Wouda et al., 2006). Consequently, it has been suggested that these cyst-forming protozoa, having an obligatory two-host life cycle, are involved in BEM (Gajadhar and Marquardt, 1992; Wouda et al., 2006).

The involvement of *Sarcocystis* in BEM is still a matter of debate, since the prevalence of this protozoan in normal adult bovine muscle is close to 100% (Vangeel et al., 2007), while BEM prevalence is much lower. Moreover, experimental oral inoculation of calves with *Sarcocystis* sporocysts does not lead to BEM (Dubey et al., 1982; Dubey, 1983; Dubey et al., 1988). Animals presenting BEM, however, carry significantly fewer *Sarcocystis* bradyzoites in their muscles (Gajadhar and Marquardt, 1992; De Bosschere and Ducatelle, 2001). This may suggest that BEM constitutes an immunologic reaction against *Sarcocystis* antigens.

The objective of the present study was to investigate if experimental intramuscular inoculation of calves with *Sarcocystis* antigens (lysed sarcocysts) can induce a lesion with the characteristics typical to natural BEM.

5.2 Materials and methods

5.2.1 Natural BEM cases

5.2.1.1 Sample origin, collection and processing and characterization of the lesions

Striated muscle samples from 44 unrelated bovine carcasses (determined by the Belgian meat tracing system), condemned for BEM, were collected in four Belgian slaughterhouses from January 2005 to October 2007. The affected carcasses were from meat type, dairy type as well as mixed type animals. Clinical examination of the animals prior to slaughter revealed no clinical signs.

For each carcass, two to ten muscle tissue samples with BEM lesions were taken from skeletal muscles, diaphragm and, when available, from the masticatory muscle, tongue, heart and oesophagus. The samples were fixed in 10% phosphate-buffered formalin for 24 hours and embedded in paraffin.

Tissue sections were cut at 4 µm and stained with haematoxylin and eosin (HE), Giemsa, Von Kossa and Von Gieson for light microscopic examination. The lesions were assessed for the presence, distribution and organization of the inflammatory cells.

A panel of monoclonal and polyclonal antibodies, as shown in Table 5.1, directed against CD3 (T-cell), CD20 (pan-B cell antigen), MAC387 (Calprotectin, reactive macrophages), HLA-DR antigen (MHC class II) and COX-2 (cyclooxygenase-2) were used for immunohistochemical stainings. Staining was performed using the peroxidase streptavidine complex (Dakocytomation A/S, Glostrup, Denmark), diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, St. Louis, USA) and H_2O_2 followed by counterstaining with haematoxylin.

The lesions were assessed for the presence, distribution and organization of the inflammatory cells. The diagnosis of BEM at carcass inspection was confirmed by histology when eosinophilic granulocytes were the predominant cell type in the multifocal inflammatory myopathy (Van Vleet and Valentine, 2007). The presence of sarcocysts was examined within the lesions as well as in non-affected areas of the same sample.

Muscle samples from three non-affected bovine carcasses were also included as negative controls for the immunohistochemical stainings. Sample collection in this control group was from the same muscles as in the group of the natural BEM cases.

Table 5.1: Primary and secondary antibodies used for immunohistochemical labelling of the natural BEM lesions and of the experimental injection sites.

Specificity	Primary antibody					Secondary antibody			
	Description	Isotype	Dilution	Temp. (°C)	Incubation time (min.)	Description	Dilution	Temp. (°C)	Incubation time (min.)
CD3	Rabbit anti-human CD3[a]	Pc	1/100	25	120	Goat anti-rabbit IgG-biotinylated[a]	1/500	21	30
CD20	Rabbit anti-human CD20[b]	Pc	1/100	25	120	Goat anti-rabbit IgG-biotinylated[a]	1/500	21	30
MAC387	Mouse anti-human MAC387[c]	IgG1	1/800	25	120	Goat anti-mouse IgG-biotinylated[a]	1/200	21	30
MHC class II	Mouse anti-human HLA-DR antigen, alpha chain Clone TAL.1B5[a]	IgG1	1/40	25	120	Goat anti-mouse IgG-biotinylated[a]	1/200	21	30
COX-2	Goat anti-carboxy-terminus of rat COX-2 (M19, sc-1747)[d]	Pc	1/400	4	Overnight	Rabbit anti-goat IgG-biotinylated[a]	1/400	21	30

Pc, polyclonal; [a] Dakocytomation A/S, Glostrup, Denmark; [b] Neomarkers, CA, USA; [c] Serotec, Cergy Saint-Christophe, France; [d] Santa Cruz Biotechnology, Santa Cruz, US

5.2.2 Experimental intramuscular inoculations with *Sarcocystis* antigens

5.2.2.1 Antigen preparations

The sedimentation technique developed by Markus (1979), with minor modifications (Vangeel et al., 2007), was used to collect sarcocysts from fresh samples of 200 g diaphragm from slaughtered bovines not presenting any lesions of BEM. The sediment was examined stereomicroscopically (magnification 50x) and sarcocysts were recovered using a microclassic unopette with capillary pipettes (Brand GMBH, Wertheim, Germany). The cysts were washed three times with sterile PBS and 900 sarcocysts were suspended in PBS. The suspension contained a mixture of 450 thick- and 450 thin-walled sarcocysts. Soluble antigens were obtained by sonication of the 900 purified sarcocysts on ice using a Vibra-cell VC375 (Sonics and Materials, Danbury, CT, USA). Sonication conditions were 375 W, 20 kHz, 20 times for 30 s, pulser on, duty cycle: 50%, microtip limit: 4.5. After aliquotation in 6 doses of 450 µl, all antigen preparations were stored at -20 °C.

5.2.2.2 Animals

Two clinically healthy male Friesian calves (*Bos taurus*), aged four months, were used, indicated as calf A and B. They were reared indoors at the Faculty of Veterinary Medicine, Ghent University, Belgium and maintained on a ration of hay and concentrates. The animal experiment was approved (EC2007/049) by the Ethical Committee for Animal Experiments of the Faculty of Veterinary Medicine, Ghent University, Belgium.

5.2.2.3 Inoculation

Both calves were inoculated three times with intervals of three weeks with the *Sarcocystis* sonicate (450 µl antigen suspension + 500 µg Quil A). The negative control consisted of 450 µl PBS admixed with 500 µg Quil A and was injected in the contralateral muscle group. The inoculum was each time injected in a different muscle group (both gluteal muscles and both sides of the neck muscles) with a 26 Gauge, 1/2 inch needle. Prior to inoculation, the site of injection was shaved and marked with a skin marker pen in order to know the exact place for

the muscle biopsy. The general symptoms and local response at the injection site were observed for 7 days after inoculation.

5.2.2.4 Sample collection and histopathology of the muscle biopsy at the injection site

An open surgical muscle biopsy specimen (Ledwith and McGowan, 2004) was taken at the site of injection one week after each intramuscular inoculation. The sample collection was performed under sedation (0.5 ml/ 100 kg Rompun 2%, Bayer, Brussels, Belgium) and local anaesthesia of the skin and subcutis (1 ml procain chloridum 4%, Kela, Hoogstraten, Belgium). The specimen (1 cm × 1 cm × 2 cm) was taken surgically with the longitudinal axis aligned with the direction of the muscle fibers and placed into 10% neutral buffered formalin for 24 hours and thereafter embedded in paraffin.
Tissue sections were cut at 4 µm and different stainings were performed as described above (see 5.2.1.1.). The muscle biopsies were assessed for the presence, distribution and organization of the inflammatory cells.

5.3 Results

5.3.1 Natural BEM cases

Carcass inspection revealed typical macroscopical lesions of BEM (multifocal white to grey-green discolorations of muscles) in all 44 condemned carcasses. The number and distribution of foci in the different skeletal muscle groups, diaphragm, myocardium and the tunica muscularis of the oesophagus varied largely from one carcass to another. A predisposition for a specific muscle group was not observed.
Sarcocysts were detected inside BEM lesions (intralesional) of 12 (27.3%) of the 44 examined bovine carcasses (Figure 5.1). In six animals, sarcocysts were present in two or more BEM lesions. All intralesional sarcocysts were damaged and located centrally or paracentrally in the lesion. In non affected muscle areas of the BEM carcasses, sarcocysts were found in intact muscle fibers in 31 cases (70.5%).

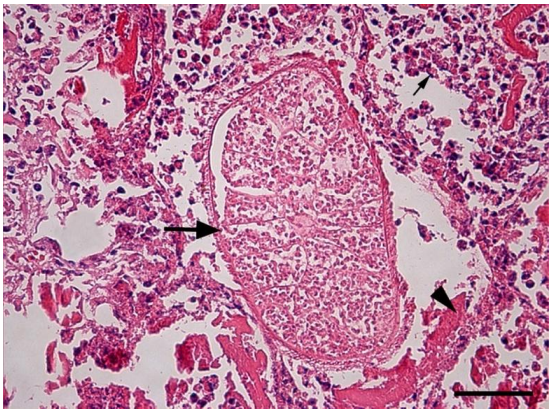

Figure 5.1: Intralesional sarcocyst (thick arrow), lying free and surrounded by degenerated eosinophilic granulocytes (thin arrow) and muscle fibers (arrowhead). HE staining. Bar, 50 µm.

The inflammatory infiltrates in the affected areas of the muscle samples of the 44 condemned bovine carcasses were typical of BEM, and consisted mainly of eosinophilic granulocytes. Additionally, lymphocytes, plasma cells and macrophages were observed. Giemsa staining showed a moderate infiltration of mast cells which were often partly degranulated. In the majority of the lesions, the inflammation was accompanied by fragmentation of myocytes. In many lesions, the infiltrate of the inflammatory cells was sharply demarcated (focal inflammatory organization of the lesion) (Figure 5.2). In other lesions, the infiltrate was scattered between muscle fibers (diffuse inflammatory organization of the lesion) and in some cases it was accompanied by an increase of collagen bundles (Figure 5.3 and Figure 5.4), as confirmed by Von Gieson staining. Some lesions had a central area with necrotic eosinophilic granulocytes and degenerated muscle fibers, lined with a rim of epithelioid macrophages surrounded by numerous eosinophilic granulocytes, some lymphocytes and some Langhans' type multinucleated giant cells (granulomatous organization of the lesion) (Figure 5.5). Semiserial sections of the lesions did not reveal a different organization of the inflammatory infiltrate. Different types of inflammatory organization frequently occurred in the same carcass.

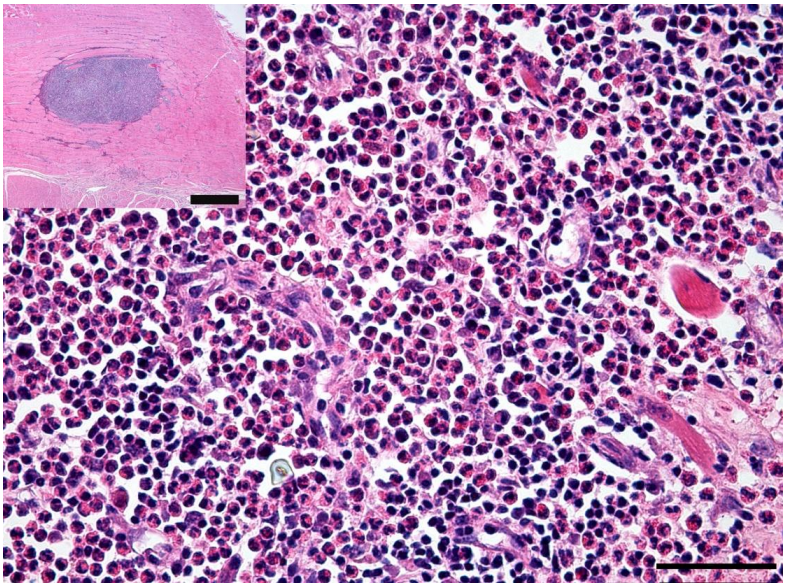

Figure 5.2: Focal inflammatory organization of a BEM lesion. Note the predominant infiltration of eosinophilic granulocytes. HE staining. Bar, 50 µm. *Inset*: overview of the lesion. Notice the well demarcated accumulation of inflammatory cells. HE staining. Bar, 500 µm.

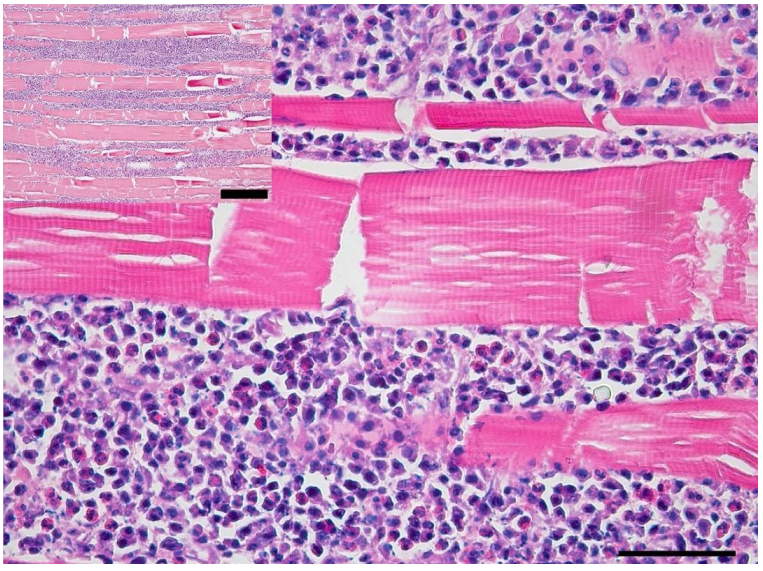

Figure 5.3: Diffuse inflammatory organization of a BEM lesion. The inflammatory cells (mainly eosinophilic granulocytes) are distributed between the muscle fibers. HE staining. Bar, 50 µm. *Inset*: overview of the lesion. HE staining. Bar, 250 µm.

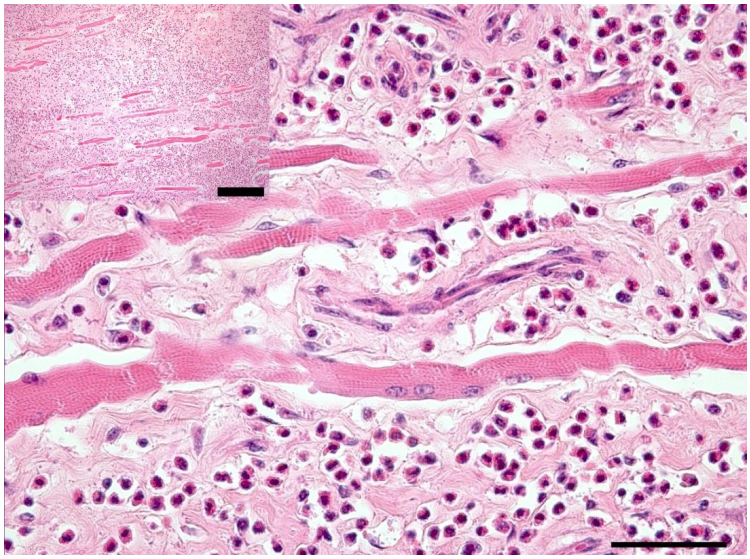

Figure 5.4: Diffuse inflammatory organization of a BEM lesion with fibrosis. The inflammatory cells (mainly eosinophilic granulocytes) are distributed between the muscle cells. Notice the increase of fibrous tissue and the atrophy of muscle fibers. HE staining. Bar, 50 µm. *Inset*: overview of the lesion. HE staining. Bar, 250 µm.

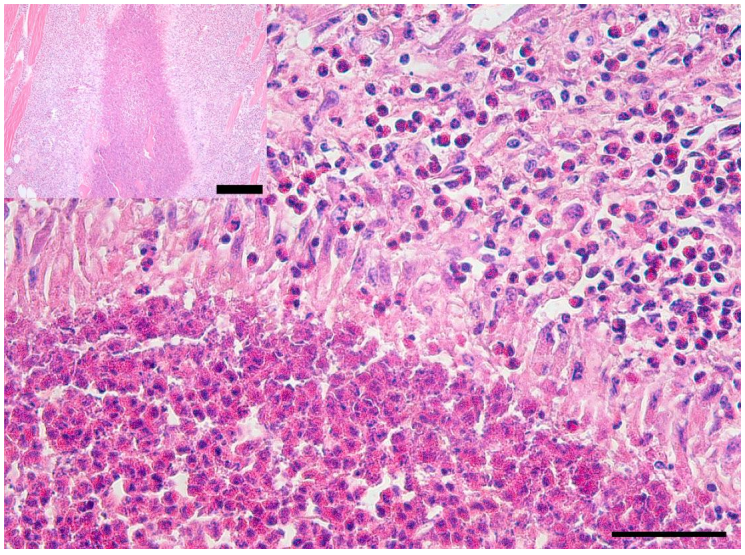

Figure 5.5: Granulomatous organization of a BEM lesion. The centre of the lesion consists of degenerated eosinophilic granulocytes. This is lined by a rim of epithelioid macrophages surrounded by a collar of eosinophilic granulocytes and lymphocytes. HE staining. Bar, 50 µm. *Inset:* overview of the lesion. HE staining. Bar, 250 µm.

All BEM lesions contained $CD3^+$ and $CD20^+$ cells. The $CD3^+$ and $CD20^+$ cells were randomly distributed in the lesions, except in lesions with a granulomatous organization, where $CD20^+$ cells were organised in a follicular pattern surrounding the rim of epithelioid macrophages. MAC387 positive cells were noticed in all types of lesions. In lesions with a focal organization, small numbers of MAC387 positive cells were present. In diffuse lesions, these MAC387 immunolabelled cells were numerous and randomly scattered inside the lesions. In granulomatous lesions, the necrotic centre of the lesion was always MAC387 negative, whereas the rim of epithelioid macrophages was positive. The periphery of these lesions was strongly infiltrated with MAC387 positive cells. When multinucleated giant cells were present, they also showed MAC387 labelling. Expression of MHC class II was demonstrated in large numbers of lymphocytes and macrophages as well as in epithelioid macrophages in granulomatous lesions. Multinucleated giant cells and eosinophilic granulocytes however, were negative. All capillaries and postcapillary venules in and adjacent to the lesion showed MHC class II positive endothelium. Some arteriolar endothelium in the lesions expressed MHC class II. The endothelium of arteries however, was negative. In almost all tissue sections of BEM lesions a small number of muscle cells close to the lesion showed a fine granular cytoplasmic and a plasma membrane MHC class II positive staining (Figure 5.6). COX-2 expression was detected in endothelial cells from capillaries and arterioles (Figure 5.7) and in a small number of macrophages in association with BEM lesions.

In the three animals of the negative control group, thick-walled sarcocysts were detected in one carcass and a mixed infection was found in the second carcass. In the third carcass no sarcocysts were observed in the examined histological sections. No inflammation was observed around sarcocysts and immunohistochemical staining for CD3, CD20, MAC387, MHCII (HLA-DR) and COX-2 were all negative.

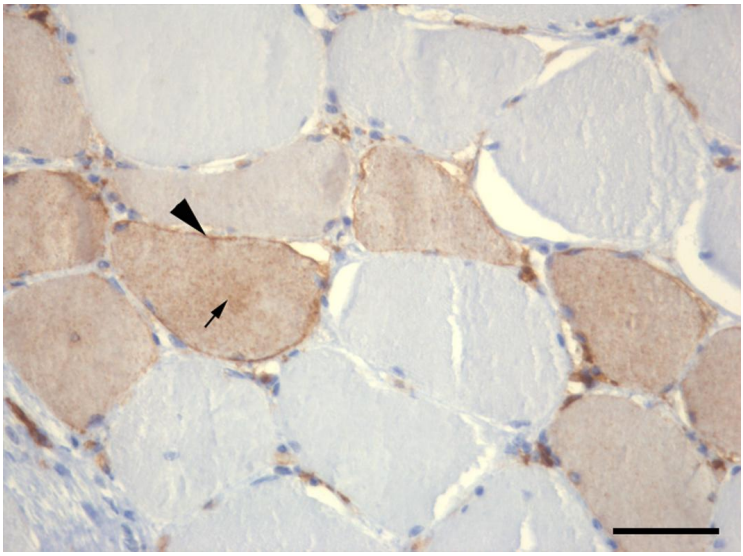

Figure 5.6: Note the fine granular cytoplasmic (arrow) and plasma membrane (arrow head) MHC class II positive staining of muscle fibers. Bar, 50 µm.

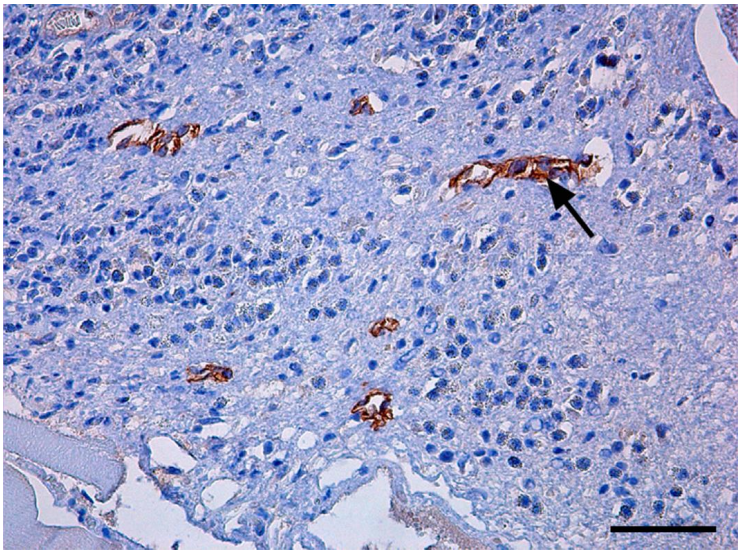

Figure 5.7: COX-2 positive immuno-labelling of endothelial cells (arrow) adjacent to the BEM-lesion. Bar, 50 µm.

5.3.2 Experimental intramuscular inoculations with *Sarcocystis* antigens

5.3.2.1 Clinical examination

The injection sites of the negative control inoculum (PBS + adjuvant) in both calves showed no alterations.

Four hours after the first inoculation of the *Sarcocystis* antigens the general health status of both calves was normal. Twenty-four hours later, calf A showed pain reaction upon palpation of the injection site with *Sarcocystis* antigen. After 48 hours, this calf showed a local, well circumscribed swelling of 5 mm diameter that was not attached to the skin and not movable. The nodular swelling was enlarged by 3 mm the day after and was present until the day of biopsy.

After the second and third inoculation, general and local symptoms were absent in both calves.

5.3.2.2 Characterization of the lesion at the injection site

The muscle biopsy samples collected at the negative control, the first and the second inoculation sites did not show any macroscopically visible alterations. The biopsy samples at the injection site of the third inoculation of both calves showed a slight white discoloration, which was not well-circumscribed.

Histological and immunohistochemical examination of negative control biopsy samples (PBS + adjuvant) showed no myofiber alterations. Sarcocysts were not found in the histological sections. Eosinophilic granulocytes, mast cells and $CD20^+$ cells were not present. Only a few $CD3^+$ and $MAC387^+$ cells were observed. MHC class II and COX-2 immunostainings were negative.

Histological examination of the biopsy samples after the first inoculation of *Sarcocystis* antigen revealed a moderate influx of inflammatory cells (Figure 5.8) the organization of which was a diffuse interstitial infiltration in calf B and a focal perimysial localization in calf A. The cells were identified as eosinophilic granulocytes (sometimes degranulated), activated macrophages ($MAC387^+$), $CD3^+$ cells (T-lymphocytes) and some $CD20^+$ cells (pan B cell antigen). Expression of MHC class II was demonstrated in lymphocytes and macrophages.

Giemsa staining showed an infiltration of some mast cells containing metachromatic granules, sometimes partly degranulated. In calf A endothelial cells of arterioles were cuboidal and showed COX-2 expression. In calf B a mild loss of cross striation of muscle cells was observed.

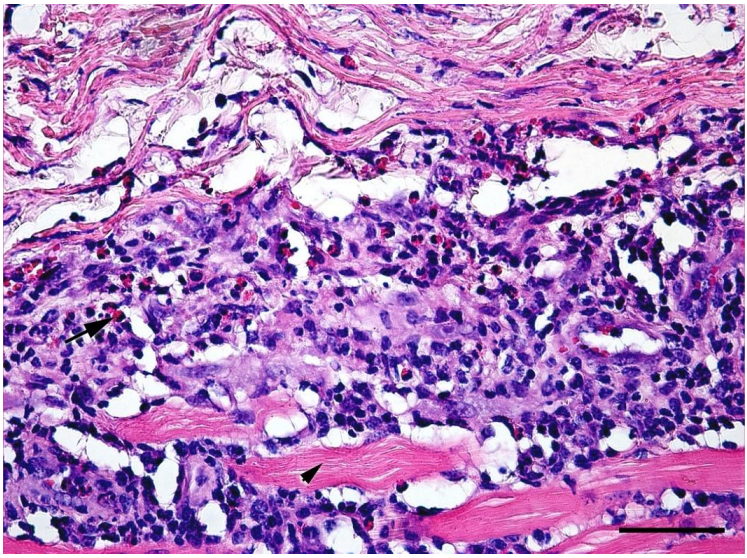

Figure 5.8: Muscle biopsy after the first *Sarcocystis* antigen inoculation. Note the infiltration of eosinophilic granulocytes (arrow), macrophages and some lymphocytes between the muscle fibers (arrow head). HE staining. Bar, 50 µm.

Muscle biopsy samples after the second inoculation (Figure 5.9) revealed more severe changes. In both calves numerous inflammatory cells: eosinophilic granulocytes, macrophages (MAC387$^+$, MHC class II$^+$), multinucleated giant cells with large phagolysosomes, CD3$^+$ cells and some CD20$^+$ cells and mast cells were present. Many necrotic myofibers, fibrin deposits and focal dystrophic calcification (Von Kossa positive) were observed. Some muscle cells showed a row of central nuclei and few muscle fibers showed a fine granular cytoplasmic and a plasma membrane MHC class II staining. Endothelial cells showed swollen nuclei and cytoplasm and frequently showed MHC class II and COX-2 labelling.

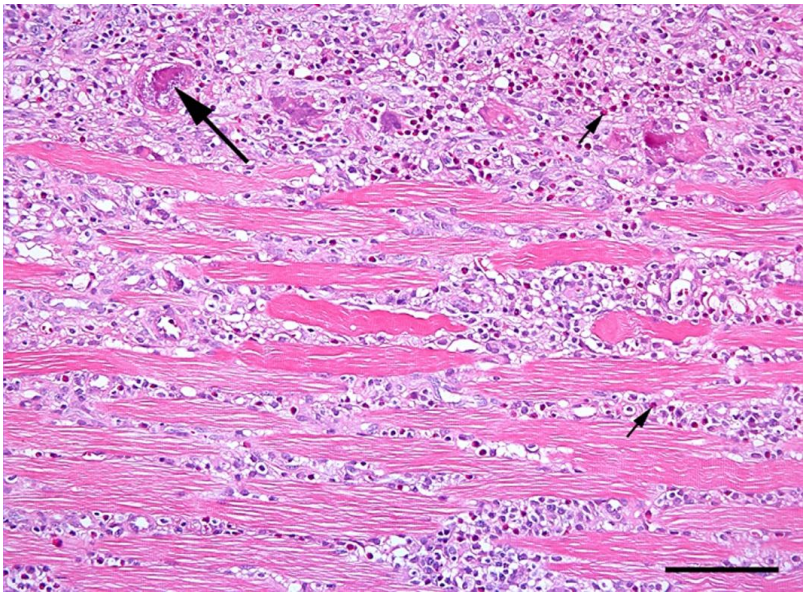

Figure 5.9: Muscle biopsy after the second *Sarcocystis* antigen inoculation. Note the infiltration of eosinophilic granulocytes (thin arrows) and the multinucleated giant cells with phagolysosomes (thick arrow). HE staining. Bar, 100 µm.

The third inoculation revealed profound histopathological alterations (Figure 5.10). There was focal necrosis of muscle fibers and inflammatory cells with oedema, surrounded by muscle cells with central nuclei containing up to two prominent nucleoli. These altered muscle cells frequently showed a fine granular cytoplasmic and a plasma membrane MHC class II^+ staining. Dystrophic calcification was present and inflammatory cells consisted of numerous eosinophilic granulocytes, $MAC387^+$ and MHC class II^+ cells, $CD3^+$ and $CD20^+$ cells and few mast cells. Vascular changes consisted of an increase in cross sections of capillaries and arterioles. The endothelium was frequently swollen and showed $COX\text{-}2^+$ and MHC class II^+ staining.

In all examined muscle biopsies, from the three antigen inoculations, the inflammatory cells showed an at random distribution in the lesions.

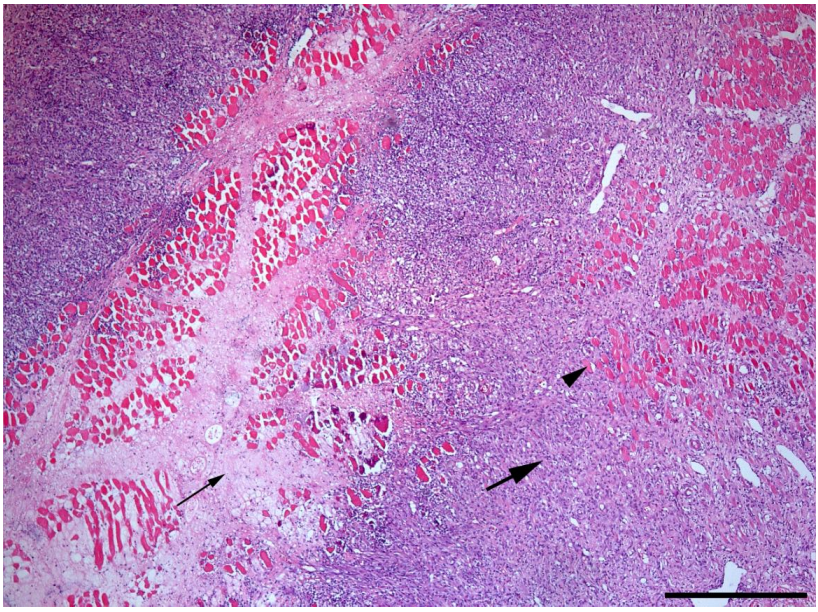

Figure 5.10: Muscle biopsy after the third *Sarcocystis* antigen inoculation. Note the necrosis (thin arrow) and the extensive inflammatory infiltration (thick arrow) between muscle fibers (arrow head). HE staining. Bar, 500 µm.

5.4 Discussion

The prevalence of sarcocysts in muscle of non-affected adult cattle, is extremely high (Vangeel et al., 2007) and no host reaction is observed around these cysts. This suggests that their intracellular localization in the muscle probably protects them from the immune system. The host cell must therefore not present any parasite antigen on its surface, or else *Sarcocystis* species are able to interfere with antigen presentation actively, as has been suggested for other protozoan pathogens (Lüder and Seeber, 2001).

If sarcocysts are involved in BEM, a possible trigger for the immune response may therefore be a rupture of the cyst wall as suggested by Jensen et al. (1986) and Gajadhar and Marquardt (1992). In the present study, calves were repeatedly injected intramuscularly with sarcocyst antigens to mimic antigen release. This resulted in a muscular lesion at the injection site that was histologically similar to the lesions observed in natural BEM cases. Yet already after the first injection, microscopical alterations were observed, including the presence of numerous eosinophilic granulocytes, inflammatory macrophages, T-lymphocytes, B-lymphocytes, MHC

class II expression and induction of COX-2 expression. These lesions aggravated with every injection. These results support the hypothesis that antigen release of sarcocysts elicit an immune response and therefore may be involved in the development of BEM.

In both natural as well as experimentally induced lesions, the results of the immunohistochemical stainings offer some insights in the immune response. The antibody recognizing the myelo-monocytic marker MAC387 (calprotectin), suggests that there is continuous, active recruitment of blood-derived macrophages in the lesions, because monocytes lose calprotectin gradually after migration from blood into tissue (Poston and Hussain, 1993). The inducible enzyme, COX-2, can be expressed locally in response to a proinflammatory stimulus (Seibert et al., 1994). COX-2 expression in lesion-associated vasculature has been reported in various conditions (Koki et al., 2002). It leads to vasodilatation (Vane and Corin, 2003) and the reinforcement of permeability-increasing and chemotactic effects of other mediators (Kumar et al., 2005), thus strengthening the inflammation. If antigens are released also the cross striated muscle cells can present the antigens to the immune system. Indeed, MHC class II expression was found on muscle cells close to the lesion in both natural cases as in the experimentally inoculated animals. Nevertheless, it should be stressed that MHC class II antigen presentation on muscle cells can only take place when co-stimulatory molecules are present (Wiendl et al., 2005), the presence of which was not investigated here. Normal muscle cells do not express MHC class II, but the induction of the expression on muscle fibers and its role in initiation or maintenance of inflammation also has been shown in human inflammatory myopathies (Englund et al., 2001).

In conclusion, we present evidence that antigens of sarcocysts can induce an immune response in bovines which is morphologically similar to natural cases of Bovine Eosinophilic Myositis.

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Chapter 6

GENERAL DISCUSSION

Introduction

The main objective of this thesis was to study the occurrence of bovine *Sarcocystis* species and their role in Bovine Eosinophilic Myositis (BEM). A better knowledge will provide a scientific basis for future control measurements and will help the food safety agencies in their decision making regarding this pathology and the impact of *S. hominis* on public health. In this final chapter, the following topics will be discussed in a more general context:

1. *Sarcocystis* species: food safety and public health perspective

1.1. Why is monitoring of zoonotic *Sarcocystis* species recommendable?

1.2. What is the impact of the confusion in terminology between Sarcosporidiosis and Bovine Eosinophilic Myositis?

1.3. Is the developed PCR technique applicable for the detection of *Sarcocystis* in meat inspection?

1.4. How can the differences in occurrence of *S. hominis* in cattle between countries be explained?

1.5. Why are infections of *S. hominis* in humans rarely reported?

2. Can the current epidemiological cycle explain the high occurrence of *S. hominis* in cattle?

2.1. Is indirect spread of *S. hominis* possible due to leaks in hygienic barriers?

2.2. Does another transmission route exist?

3. Pathogenesis of Bovine Eosinophilic Myositis

3.1. Do the morphologic findings indicate a unique inflammatory myopathy?

3.2. What triggers the inflammatory response?

4. General conclusions and further perspectives

6.1 *Sarcocystis* species: food safety and public health perspective

6.1.1 Why is monitoring of zoonotic *Sarcocystis* species recommendable?

From a public health point of view, *Sarcocystis* can be included in list B within the group 'other zoonoses' of directive 2003/99/EC, which means that they have to be monitored depending on the epidemiological situation in the member states. Regulation 854/2004/EC classifies *Sarcocystis* species however under the general term of 'zoonotic species' according to Taylor et al. (2010). There are to our knowledge, no reliable data on the prevalence of *S. hominis* in man and no data about the relevance for public health. Though data in literature show that infection with *S. hominis* in man is usually mild or asymptomatic (Fayer, 2004) a risk analysis study is needed to assess the impact of a zoonotic species for public health. EFSA concluded, based on their risk assessment (with the criteria from the Regulation 178/2002/EC) that the monitoring and clarification of the impact on public health of *S. suihominis* is prioritized over *S. hominis* (Taylor et al., 2010). In 2008 however, Fosse et al. showed that *S. suihominis* has a low risk for public health, using a quantitative approach for a comparative risk assessment in pig slaughter. Nevertheless, these authors mentioned that some clinical data for *S. suihominis* were lacking. As long as these gaps in the knowledge of zoonotic *Sarcocystis* species exist, it is difficult to determine the real impact of *S. hominis* and *S. suihominis* on public health.

From an economic point of view, the presence of *S. hominis* in meat affects the import and export of meat. Moré et al. (2011) already mentioned the rejection of beef from Brazil and Argentina due to *Sarcocystis* spp. infection. Moreover, during the present study, our assistance was asked in the diagnosis of *Sarcocystis* in beef from Honduras. Since *S. hominis* causes a (limited) risk for public health, the United States Department of Agriculture (USDA) and the Food Safety Inspection Service (FSIS) have forbidden import of Honduran beef due the presence of sarcocysts (personal communication Bart N., 2009). This is surprising, since Dubey et al. (1989) stated that the overall prevalence of *Sarcocystis* species in US is close to 100%. Such embargo is made possible by the World Trade Organization, in which a government can refuse import of food with a potential public health hazard. This could be a precedent for restricting also export of Belgian beef, given the high occurrence of *S. hominis* in Belgium.

These reflections emphasize the need for a harmonized and validated method that is interna-

tionally accepted, for the monitoring of zoonotic *Sarcocystis* species in cattle, pigs and humans.

6.1.2 What is the impact of the confusion in terminology between Sarcosporidiosis and Bovine Eosinophilic Myositis?

For a long time, confusion exists between the terminology 'Sarcosporidiosis' and 'Bovine Eosinophilic Myositis' (BEM). 'Sarcosporidiosis', used as a synonym for sarcocystosis, is the terminology for an infection with *Sarcocystis* species, causing no inflammatory response in muscle tissue. Since most sarcocysts are not macroscopically visible, gross inspection will not reveal any pathological changes and without any specific detection technique the sarcocysts are not detected in slaughterhouses, though the overall prevalence in cattle is close to 100% (Böttner et al., 1987; van Knapen et al., 1987; Vercruysse et al., 1989; Fortier et al., 1993; Woldemeskel and Gebreab, 1996; Latif et al., 1999; De Bosschere and Ducatelle, 2001; Pena et al., 2001; Vangeel et al., 2007). In rare cases, macroscopical *S. hirsuta* cysts develop that can be detected by visual meat inspection (Dubey et al., 1990). In contrast to Sarcosporidiosis, BEM causes typical grey-green lesions. Since the exact etiology is unknown (Dubey et al., 1989), carcasses and meat that show these lesions have to be declared unfit for human consumption due to pathophysiological changes (regulation 854/2004/EC). For already some decades, it has been suggested that *Sarcocystis* species play a role in this pathology, and probably this is the basis for the confusion in terminology. Due to the fact that the term Sarcosporidiosis is frequently used instead of BEM, not only problems occur in insurance cases, but also data published for sarcocystosis by the European member states in the "The Community Report of EFSA on Trends and Sources of Zoonoses, Zoonotic agents, Antimicrobial Resistance and Foodborne Outbreaks in the European Union" are not correct. Beside Luxembourg, Belgium is however the only European country that submits data about sarcocystosis, though the data submitted appear to be the number of carcasses declared unfit for human consumption due to BEM. They report a prevalence of Sarcosporidiosis as being 0.002% in cattle, which represents the prevalence of BEM and not the real occurrence of 97.4% as was demonstrated in chapter 3 (Vangeel et al., 2007).

In the future, these terminologies must be used properly in order to report correct data on zoonotic *Sarcocystis* species in the context of the regulation 854/2004/EC and to achieve a clear context in insurance cases.

6.1.3 Is the developed PCR technique applicable for detection of *Sarcocystis* in meat inspection?

At the start of this doctoral research, tests used for the detection and identification of bovine *Sarcocystis* species in many diagnostic or research laboratories were based on the morphology of sarcocysts by conventional light microscopical or laborious transmission electron microscopical techniques (Fortier et al., 1993; Pena et al., 2001). Moreover, researchers question the description of *Sarcocystis* species based on sarcocyst morphology alone, since this feature may vary depending on the developmental stage, location of the cyst and the degenerative stage of the parasitized host cell (Tenter et al., 1995). A direct diagnostic test based on the detection/identification of parasite specific molecules by molecular based methods could provide a valuable alternative in the diagnosis of *Sarcocystis* infection.

Therefore, a reliable molecular based identification technique was first developed. In order to study the occurrence of bovine *Sarcocystis* species in beef and in lesions of BEM, the assay had to be able to detect sarcocysts on both fresh and formalin fixed/paraffin-embedded samples. The use of formalin fixed tissue imposes however some restrictions on the primer selection, as the generation of amplicons of more than 300-400 bp is not successful (Casale et al., 2010). Nevertheless, a genus specific primerset has been designed, allowing the detection of the three known bovine *Sarcocystis* species and which by sequencing of the amplicon is able to identify sarcocysts at species level. The primers did not cross-react with related protozoa and the results were in accordance with TEM, still the gold standard at that moment (Vangeel et al, 2007). Moreover, the accuracy of the tool was confirmed by exchanging samples with Dr. Benjamin Rosenthal (Animal Parasitic Diseases Agricultural Research Service, USDA, Beltsville, Maryland, USA) (unpublished data). This laboratory used a different PCR assay followed by sequencing (Pritt et al., 2008) using the primers developed by Dubey et al. (2006) in the context of genetic characterization of *Sarcocystis* species in different intermediate hosts.

A disadvantage of our technique is that the PCR generates amplicons with only small differences in molecular weights, making differentiation of the three species by routine agarose gel electrophoresis impossible. For research purposes, identification is possible by clone- and/or sequence analysis of the generated amplicon. In food-safety control however, the latter techniques are expensive and labour intensive, and therefore not routinely implied.

All molecular techniques developed in the last decade for detection and identification of bovine (and porcine) *Sarcocystis* species (Gonzalez et al., 2006; Vangeel et al., 2007; Pritt et al.,

2008) were assessed as unsuitable for 'high routine inspection' of zoonotic *Sarcocystis* species by the EFSA's Scientific Panel (Taylor et al., 2010). An improvement for large scale monitoring of *Sarcocystis* species in cattle could have been the development of a multiplex PCR, which we also aimed to develop during this thesis. However, we encountered several problems like suboptimal sensitivity in case of co-infections (unpublished data). Another alternative can be the use of a PCR-RFLP technique as described by Yang et al. (2002). Nevertheless, Domenis et al. (2011) have modified our genus PCR, based on the EFSA recommendations, making it more suitable for large scale studies. They labelled the reverse primer with a Hex fluorescent dye making it possible to determine the size of the amplicon by capillary electrophoresis and thus making sequence analysis for identification of the *Sarcocystis* species redundant.

6.1.4 How can the differences in occurrence of *S. hominis* in cattle between countries be explained?

Using molecular techniques, the occurrence of *S. hominis* in bovine meat has recently been determined in three different studies. Surprisingly, the occurrence of *S. hominis* shows large variations between the USA and Europe, namely 0% (n=41) in US and Uruguayan samples (Pritt et al., 2008) in comparison to 42.7% (n=300) in Italy (Domenis et al., 2011) and 97.4% (n=39) in Belgium (Vangeel et al., 2007). Since the extreme high prevalence of *S. hominis* in Belgian beef samples, confirmation of our results using another assay was urged. Our results were confirmed by retesting our samples (unpublished data) in the laboratory of Dr. Benjamin Rosenthal (Animal Parasitic Diseases Agricultural Research Service, USDA, Beltsville, Maryland, USA) with the PCR technique described by Dubey et al. (2006). How can these variations than be explained?

First, these data can reflect **real existing differences** due to different epidemiological factors between countries.

Secondly, a **bias in the occurrence** could be caused by **differences in the sampling method**. Both Pritt et al. (2008) and Domenis et al. (2011) sampled a small amount of muscle, 8 mg and 25 mg respectively and performed immediately DNA extraction. This may imply two risks. **The first risk** is that, given the low sample volume, there will be no sarcocyst present in the sample. This hypothesis could explain why Pritt et al. (2008) had 45.5% samples in which no *Sarcocystis* DNA was detected, while the overall prevalence of *Sarcocystis* species

in US cattle reported is close to 100% (Dubey et al., 1989). This cannot be compared with Domenis et al. (2011) since they did not mention the number of negative samples in their survey. In our study, starting from 200 g beef and including an extra step for cyst collection prior to DNA extraction, we did not detect sarcocysts in only 6% of the samples. This indicates that our method of sampling is more sensitive. **The second risk** is that a mixed infection is present in a sample, as shown in 46% and 56% of the samples in Belgium and Italy, respectively (Vangeel et al., 2007; Domenis et al., 2011). Such co-infections result in overlapping peaks in sequence analysis giving uninterpretable results. These samples should than be further analyzed by cloning and sequencing to identify the different species, which is not performed in the study of Pritt et al. (2008). In our study, we have bypassed this problem by extracting DNA from individually isolated cysts in each sample. A better alternative is now the method of Domenis et al. (2011) where co-infestations can be clearly detected using a fluorescent labeled primer for size separation.

6.1.5 Why are infections of *S. hominis* in humans rarely reported?

The striking discrepancy between the high occurrence of *S. hominis* in cattle and the apparent low prevalence in humans raises the question: why is *S. hominis* so little reported in humans? One can put forward different hypotheses:

First, it is possible that the reported **prevalence in humans is reliable** and that there really exists a discrepancy between the prevalence in intermediate and definitive hosts. This could be caused by the life cycle of the parasite. Since humans can shed sporocysts during a long time and that these sporocysts can survive for a long period in the environment, it is possible that one person infects several cattle. The fact that *S. hirsuta*, as opposed to *S. hominis* and *S. cruzi*, has a low occurrence in cattle (Vangeel et al., 2007; Domenis et al., 2011) is an argument against this hypothesis as the patent periods of the three bovine *Sarcocystis* species are similar (Mehlhorn and Heydorn, 1978).

A second possibility is that data on the **prevalence in humans are underreported**, due to the following factors:

(i) The majority of infections are asymptomatic, possibly due to a low infection dose. However, data in literature on the infection dose are not present. In the study of Pena et al. (2001), consumption of 200 g raw meat resulted in gastro-intestinal symptoms in two of six

volunteers. Unfortunately, the corresponding number of *S. hominis* sarcocysts per 200 g was not reported. Regarding our study, in 67 samples of 200 g minced beef from retail we have found 42 and 25 samples containing respectively 0-10 and 10-30 thick-walled sarcocysts (Vangeel et al., unpublished data).

(ii) Symptomatic infections are difficult to detect, since symptoms of intestinal sarcocystosis in humans, if present, frequently occur during the prepatent period (Pena et al 2001; Fayer, 2004). This implies that faecal shedding of sporocysts is not detectable in stool at that moment and consequently most clinical cases will not be linked to intestinal sarcocystosis.

(iii) The sampling frequency of stool is insufficient. Mehlhorn and Heydorn (1978) stated that excretion of sporocysts is not constant during the long patent period. This points out that routine monitoring of several stool samples over the course of a few days would be needed to get better insight in the prevalence.

(iv) The conventional techniques are probably not sensitive enough and not species specific. Moreover, the sporocysts are colorless, which makes them difficult to detect (Mehlhorn and Heydorn, 1978). Therefore, molecular techniques, as described by Xiang et al. (2009) for the detection of *Sarcocystis* species in stool samples need to be further validated for their sensitivity and optimized for large scale application.

6.2 Can the current epidemiological cycle explain the high occurrence of *S. hominis*?

6.2.1 Is indirect spread of *S. hominis* possible due to leaks in hygienic barriers?

The high occurrence of *S. hominis* in cattle points to a successful epidemiological cycle. Assuming that the transmission of *S. hominis* only occurs via ingestion by cattle through food and/or water contaminated with human faeces, implies the existence of leaks in hygienic barriers.

Those supposed gaps in hygienic barriers for *S. hominis* can (only) be explained by the indirect spread of sporocysts caused by the fact that many sewers are still not connected to a water treatment system (causing dissemination of sporocysts during floods) or that farmers still use sludge on pastures that are grazed, despite the fact that in Flanders the VLAREM legislation prohibits the latter since 2004. In literature these hypotheses have already been put forward, but data on the presence of *Sarcocystis* sporocysts in sludge and surface water are scarce and not decisive (Burger and Wilkens, 1986).

In 2011, Domenis et al. performed a case-control study and found a significant correlation between *S. hominis* positive farms and raw meat consumption by the farmer, absence of sewage system and the contamination of pastures with human sewage, suggesting the maintenance of the epidemiological cycle by these factors. In a preliminary study, a questionnaire was used to collect information on possible risk factors for *S. hominis* (Vangeel et al., unpublished data). The results (in Table 6.1) suggest that the majority of the animals were exposed to at least one risk factor. Since we only received information from 26 farmers, these data are only indicative. However, four farms had zero-grazing and thus animals were not exposed to any of the above mentioned factors. This raises the following question: can *S. hominis* be present in well or drinking water? As this is already described for *Toxoplasma*, *Cryptosporidium* and *Giardia* (Betancourt and Rose, 2004; Sroka et al., 2006) further research is needed to clarify if *Sarcocystis* sporocysts are also present in well water and if they can pass the water purification system.

Table 6.1: Preliminary questionnaire-based data from 26 farms on risk factors for indirect transmission of *S. hominis*.

Factor	n farms / 26 farms
Possibility of grazing on pasture	22
Access to surface water	9
Regular flooding of pasture	4
Spreading sludge from sewers on pasture while cleaning	11
Spreading farm sewage on pasture	21
Sewers not connected to water treatment system	9
Common human and farm sewage system	3
Using sludge from water treatment plants as fertilizer on pastures	0

6.2.2 Does another transmission route exist?

Data in literature show that ingestion of sporocysts is the major mode for transmission, though taking into account the high occurrence of *S. hominis*, questions arise if other transmission routes exist (e.g. mechanical vectors). In 2005, Graczyk et al. reported that synanthropic flies can act as mechanical vectors of protozoan parasites. Another possibility could be that blood feeding flies transmit merozoites from cow to cow, as an experimental study showed that *Sarcocystis* can be transmitted by blood transfusion (Fayer and Leek, 1979). Moreover, data have suggested that hematophagous arthropod species could be vectors for *Besnoitia besnoiti* (a closely related protozoon) explaining the recent spreading of bovine besnoitosis (Alzieu et al., 2007; Jacquiet et al., 2010). A preliminary study to test this hypothesis was started in our research group (master thesis Tinneke Tuytschaever, 2008). However due to clotting of the blood, DNA extraction was not successful, and at this moment further optimalization of the DNA extraction is not yet performed.

6.3 Pathogenesis of Bovine Eosinophilic Myositis

6.3.1 Do the morphologic findings indicate a unique inflammatory myopathy?

Both type I and type IV hypersensitivity have been suggested to play a role in BEM (Jensen et al., 1986). The presence of eosinophils which is the most characteristic aspect of this inflammatory myopathy is an argument in favor of a type I hypersensitivity. The granulomatous organization of lesions in natural BEM, however, suggests that this myopathy may be a type IV hypersensitivity (delayed type hypersensitivity, DTH) reaction occurring in the setting of chronic antigen stimulation. Meeusen (1999) described three different types of DTH reactions in which sensitized T cells recruit macrophages (type I), eosinophilic granulocytes (type II) or induce fibrogenic cytokines (type III). The fact that eosinophilic granulocytes are present in BEM could thus suggest that it is a DTH type II reaction.

Based on the data obtained during the different studies, the main argument in favor of a role of *Sarcocystis* in BEM is the fact that intramuscular injection of *Sarcocystis* antigens in calves elicits a similar inflammatory response as the one seen in natural BEM cases. In contrast there are still aspects that question the possible role of sarcocysts in these lesions, namely:

(i) in only 28% of the BEM lesions, intralesional sarcocysts were demonstrated by means of histology,

(ii) in carcasses affected with BEM, intralesional as well as extralesional sarcocysts were present in muscle,

(iii) almost 100% of cattle are infected with *Sarcocystis* spp., in which no inflammatory response is observed, whilst the prevalence of BEM is low,

(iv) *Sarcocystis* species are intracellular protozoa, this implies that one would not expect these pathogens induce an eosinophilic inflammation, except if these parasites cause an immediate (type I) hypersensitivity. However, the fact that the experimentally induced BEM lesions in our study (chapter 5; Vangeel et al., 2012) were still present a week after antigen inoculation and no neutrophils were seen, may be an argument against immediate hypersensitivity. Since eosinophils, neutrophils, lymphocytes and macrophages recruited to the site of mast cell degranulation persist for only one to two days (Meeusen, 1999). Meeusen (1999) however described that a type I hypersensitivity reaction often occurs concurrently with a DTH type II, which can explain that the inflammatory response was still present at the time of muscle biopsy, since DTH reactions take two to three days to develop. Bearing in mind, however, that

DTH type II reactions are not described in association with protozoa and that *Toxoplasma* and *Sarcocystis neurona* cause a mononuclear inflammation or a DTH type I reaction (Goedegebuure, 1987), neither an immediate hypersensitivity nor DTH type II fits entirely within the frame of *Sarcocystis* playing a role in BEM. Thus BEM is a unique myopathy, unless the Th1/Th2 dichotomy does not fit in this pathology. This Th1/Th2 paradigm is first described in mice, but data in literature show that cattle do not follow this paradigm (Estes and Brown, 2002). Moreover, it has also become clear in mice that many complicated pathological situations cannot be simply explained by the Th1 cell and Th2 cell paradigm (Ouyang et al., 2008). Therefore, better understanding of immune responses in BEM is important to elucidate the role of sarcocysts.

6.3.2 What triggers the immune response?

At this moment, there is no explanation for the discrepancy between the occurrence of BEM and the occurrence of *Sarcocystis* species in cattle. Since sarcocysts are residing in a parasitophorous vacuole, just like *Toxoplasma*, they are expected to be mainly presented on MHC class II molecules, since they originate from the extracellular space and are intracellularly located within an endocytic vesicle (Lüder and Seeber, 2001). The fact that the prevalence of *Sarcocystis* species in bovine muscle is close to 100% suggests however that bovine *Sarcocystis* species are capable of suppressing the host immune response when present in muscle tissue. The development of BEM may after all be detrimental to the survival of the parasite. The mechanism of this host-pathogen interaction is not known for bovine sarcocysts, but in two related protozoa, *Toxoplasma* and *Sarcocystis neurona*, glycosylphosphatidylinositol (GPI)-anchored surface antigens called SnSAGs are described, covering the cell surface (Lekutis et al., 2001; Howe et al., 2008). The functional role of these surface antigens has not been fully defined, but data suggest that the SAG proteins are involved in host cell invasion, immune modulation and/or virulence attenuation (Saeij et al., 2008). Stage specific expression has been observed in *Toxoplasma gondii*, *Neospora caninum* and *Sarcocystis neurona* (Fernandez-Garcia et al., 2006; Risco-Castillo et al., 2007; Saeij et al., 2008; Gautam et al., 2011) suggesting that the bradyzoite-specific SAG molecules may be important for immune evasion and persistence of a chronic infection (Kim and Boothroyd, 2005; Saeij et al., 2008). Such mechanisms could also be present in bovine sarcocysts, but have not yet been described.

The immunohistochemical study in chapter 5 (Vangeel et al., 2012) clearly demonstrates that myocytes adjacent to BEM lesions express MHC class II. So, most likely, these muscle cells are capable of presenting antigens in BEM. But the question "what exactly triggers antigen presentation" in a low number of cattle remains unanswered. Basically, three hypotheses can be put forward:

- the trigger is a **parasite related factor** through which the immunosuppression disappears and MHC class II, as stated above, is expressed on this specific cell initiating an immune response,
- the trigger is a **host related factor** through which the primary trigger is host cell damage or rupture, causing release of the sarcocyst,
- the trigger is **multifactorial**

6.3.2.1 Parasite related factors

The main hypothesis for a role of parasite related factors in BEM, taking into account that extralesional intact sarcocysts are also present in BEM carcasses, is that intralesional sarcocysts have lost their capability of suppressing the host immune response. Three different mechanisms can be suggested:

First, it has been described that death of bradyzoites in *Sarcocystis* and *Toxoplasma* cysts can occur (Dubey et al., 1989; Pavesio et al., 1992). Also in our TEM examination of viable sarcocysts, apoptotic bodies were at random detected (Vangeel et al., unpublished data). One could suggest that if massive apoptosis occur, the downregulating mechanisms are lost.

Secondly, it could be that sarcocysts inducing BEM have specific features that interfere with the normal host-pathogen interaction, such as a lack of specific surface antigens that are important for immune evasion, as is already suggested for *S. neurona* (Howe et al., 2008). If so, this will not be a species specific feature since we have demonstrated that all three bovine *Sarcocystis* species can be involved in BEM (chapter 4; Vangeel et al., under review). However, the involvement of a specific strain is not excluded. Since this would be a disadvantageous feature, one would expect that these strains would disappear, because they will never be capable to complete their life cycle or on the other hand it can explain the low prevalence of this myopathy.

Finally, a mutation could arise in the genome of a sarcocyst, through which a modified surface antigen expression arises, causing a change in host-pathogen interaction.

6.3.2.2 Host related factors

The fact that only a small minority of infected animals develop BEM is an argument in favor of host related factors. Because studies show that cattle do not follow the Th1/Th2 paradigm in the strictest sense, it is suggested that their expression pattern can be impacted by breed or genetics, husbandry conditions or health status (Brown et al., 1998; Estes and Brown, 2002). Taking into account the data retrieved in a preliminary study on the profiles of 43 bovines with BEM (Vangeel et al., unpublished data), one may put forward some reflections:

First, neither in literature (Imes and Migaki, 1967) nor in our preliminary study one could show an age predisposition.

Secondly, data in literature suggest that meat producing animals are more frequently affected with BEM than dairy cattle (Reiten et al., 1966; Oghiso et al., 1977). In our preliminary study, the highest affected type was also beef cattle (one Blonde d'Aquitaine and 27 Belgian Blues). Nevertheless, this might not reflect a true number, since proportionately more beef cattle are slaughtered. Also a breed predilection was not clearly seen, since the BEM carcasses were from the following breeds: Belgian Blue, Blonde d'Aquitaine, West-Flemish Red breed, East-Flemish Red Pied and Holstein Friesian.

Thirdly, Imes and Migaki (1967) and Reiten et al. (1966) found that heifers, and heifers and cows, respectively are more affected than bulls. Also in our preliminary study, 70% of the carcasses were of the female sex. The fact that Roberts et al. (2001) described that sex-associated hormones have an influence on the immune response to protozoan parasites, namely that males develop stronger Th1 and females stronger Th2 responses (e.g. mast cells and eosinophils have estrogen receptors), suggests that death/degeneration of sarcocysts could result in a strong eosinophil influx, whereas normally a Th1 response would probably clean up the parasitized host cell. However, one would then also expect a higher prevalence of BEM. Moreover the calves used in our study, in which focal eosinophilic myositis was induced, were males (chapter 5; Vangeel et al., 2012).

Fourthly, the musculature/muscular development might be a factor involved. During sampling of the BEM carcasses, information on the category A-E and the SEUROP classification of the carcass was not always available, therefore the mean carcass weight was calculated based on the sex. For the male and the female BEM cases the mean carcass weight was 512 kg and 444 kg, respectively. These are higher than the mean carcass weights in 2007 of slaughtered cattle in Flanders (data obtained from Prof. Stefaan De Smet, UGent), namely 478kg for males (category A-C) and 371 kg for females (category D and E). Though firm conclusions cannot

be drawn, these data suggest that muscle hypertrophy or a rapid weight gain in case of dairy cattle could play a role in BEM. This may lead to subclinical muscle injury/damage which may result in a release of antigens that are normally hidden from the immune system. Another animal related factor could be genetic diversity in muscle physiology. Not only in humans but also in cattle, the calpain system, a family of calcium-dependent intracellular proteases and the endogenous inhibitor calpastatin, function as regulators of muscle protein accumulation (Muroya et al., 2012). In humans, tissue specific calpains have been implicated in diseases like diabetes, LGMD2A and idiopathic eosinophilic myositis (Krahn et al., 2006; Zatz and Starling, 2005). In cattle, it has been suggested that this enzyme system plays a role in muscle hypertrophy and can be influenced by e.g. β-agonist stimulation (Parr et al., 1992). A possible hypothesis is that in animals developing BEM, a genetic defect or an external factor exists that causes changes in calpain expression. This in turn, may lead to impaired protein turnover and muscle damage, releasing hidden antigens of *Sarcocystis*.

6.3.2.3 Multifactorial

Since no conclusive hypotheses for the parasite as well as the host related factors can be put forward, one can assume that the trigger for antigen presentation will be a combination of different elements.

6.4 General conclusions and further perspectives

During this thesis, particularly in the research area of *Sarcocystis*, a controversial issue arose, namely the high occurrence of *S. hominis* in minced beef in Belgium. In order to determine the exact impact of all zoonotic *Sarcocystis* species on public health and to further elucidate the high occurrence, the variation in prevalence and the modes of transmission, the development of a harmonized monitoring program with standardized tools for *Sarcocystis* species in cattle, pigs and man is needed. This would help to implement measures to prevent further circulation of these protozoa.

Concerning Bovine Eosinophilic Myositis, still many questions remain unanswered. Since the prevalence is under 1%, this pathology can be classified as a persisting rare event (Saegerman, 2004), of which the detection *in vivo* will be difficult. By using *Sarcocystis* sonicate for antigen inoculation, it is not clear to which specific antigen(s) the immune response is directed. The identification of immunodominant antigens is, however, necessary to develop an *in vivo* diagnostic test. Therefore, a first step is the purification of different antigens of bovine *Sarcocystis* species to determine immunodominant antigens by Western blot analysis. Finally, as the role of sarcocysts in BEM has not yet been fulfilled, also host related factors have to be examined. In order to study the mechanisms in BEM, research on the expression levels of genes involved in this specific myopathy will probably help to further elucidate the pathogenesis.

6.5 References

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Summary

Cattle are common intermediate hosts of *Sarcocystis* (*S.*) species, a genus of heteroxenous cyst-forming coccidia belonging to the phylum Apicomplexa. The prevalence of *Sarcocystis* species in adult bovine muscle is close to 100% in most regions of the world. Cattle can harbor three species as intermediate host, namely *Sarcocystis cruzi*, *Sarcocystis hirsuta* and *Sarcocystis hominis*, with canids, felids and primates (including humans) as definitive hosts, respectively. Infection in humans with *S. hominis* is characterized by transient nonspecific gastro-intestinal symptoms. However, the clinical course is usually mild and in many cases asymptomatic.

It has been suggested that *Sarcocystis* spp. are involved in the development of Bovine Eosinophilic Myositis (BEM). This is a specific subclinical myopathy with typical grey-green lesions, which occurs worldwide in clinically healthy cattle. Although BEM is not detectable *in vivo*, it results in economic losses due to carcass as well as meat condemnation in slaughter and meat cutting plants. Intriguing is the fact that though the prevalence of BEM is low, the prevalence of *Sarcocystis* in cattle is extremely high, and the reason for this discrepancy is still not clear.

The first part of the literature review (**chapter 1)**, starts with an overview of bovine *Sarcocystis* species. The second part (**chapter 2**) focuses on the present knowledge of BEM. Based on both chapters, it is clear that still little or obsolete information is available on bovine *Sarcocystis* species and their role in this multifocal myopathy.

Therefore the general aim of this doctoral thesis was to assess the occurrence of *Sarcocystis* species in normal bovine muscle, and to contribute to the understanding of their role in BEM.

In the study described in **chapter 3**, a reliable molecular based identification technique for bovine *Sarcocystis* species was developed. The genus specific PCR based on the 18S rRNA gene sequence, followed by sequencing of a specific amplicon, showed to be highly discriminatory. Moreover with this technique, *Sarcocystis* identification in both fresh and formalin-fixed/paraffin-embedded bovine muscle was possible.

Since little was known on the relative distribution of each *Sarcocystis* species in bovine muscle due to difficulties in correct identification and in view of the zoonotic importance of *S. hominis*, the second aim of the study in chapter 3 was to apply the developed identification tool to determine the presence of different *Sarcocystis* spp. in general and of *S. hominis* in particular in raw minced beef. This study showed a high occurrence of *S. hominis* (97.4%) in minced bovine beef at retail in Belgium. As the consumption of raw minced beef is common

in Belgium and in some other European countries, this finding may point to a potential risk for public health. Furthermore this finding also suggests the existence of a very efficient epidemiological cycle of *S. hominis*.

To examine the role of *Sarcocystis* species in BEM, two studies were performed (**Chapter 4 and 5**).

First, *Sarcocystis* species were identified inside lesions of BEM in order to test the hypothesis that BEM might be induced by a specific *Sarcocystis* species (**chapter 4)**. Therefore, cysts present in BEM lesions from 97 condemned carcasses were collected using laser capture microdissection. Subsequent, the cysts were identified at species level using the genus PCR developed in chapter 3, followed by clone- and/or sequence analysis of the amplicons. Intralesional sarcocysts or remnants were found in BEM lesions in 28% of the carcasses. The majority (82%) of intralesional *Sarcocystis* species were identified as *S. hominis*. However, *S. cruzi* and *S. hirsuta*, as well as an unidentified species were also present. These observations demonstrate that *Sarcocystis* species present in lesions of BEM are not restricted to a single species.

A second study (**chapter 5**), assessed whether experimental intramuscular inoculation of *Sarcocystis* antigens can induce similar lesions as those observed in natural cases of BEM. Therefore, two calves were repeatedly injected intramuscularly with adjuvanted *Sarcocystis* antigen. The morphological changes at the injection sites in these calves were then histologically and immunohistochemically compared to spontaneous lesions from 44 BEM condemned carcasses sampled in slaughterhouses. Experimental intramuscular injection of *Sarcocystis* antigen resulted in lesions at the injection sites similar to those of the lesions of spontaneous occurring BEM. They were characterized by massive infiltration of eosinophilic granulocytes, reactive macrophages ($MAC387^+$ cells), T-cells ($CD3^+$) and B-cells ($CD20^+$). Both in the experimental and in the natural cases, COX-2 expression was present in endothelial cells adjacent to lesional areas. MHC class II^+ staining was found amongst others in muscle cells surrounding the lesion. These findings show that *Sarcocystis* antigens, when presented to the host immune system, can induce an inflammatory response in bovine muscle similar to spontaneous occurring BEM.

The doctoral thesis concludes with a general discussion wherein first the results of the different studies are put into a broader context, and secondly the general conclusions and further perspectives are presented.

In conclusion, evidence is presented that antigens of sarcocysts can induce an immune response in bovines resulting in focal eosinophilic myositis. BEM is however not associated with one particular *Sarcocystis* species. Finally, a high occurrence of *S. hominis* is shown in minced bovine beef at retail in Belgium. Therefore, future research should focus on the possible impact on public health and on the epidemiological cycle of zoonotic *Sarcocystis* species.

Samenvatting

Sarcocystis (*S.*) species zijn heteroxene coccidia en behoren tot het phylum Apicomplexa. Het rund treedt op als tussengastheer en kan geïnfecteerd worden door drie species, namelijk *Sarcocystis cruzi*, *S. hirsuta* en *S. hominis*, met respectievelijk Canidae, Felidae en primaten (waaronder de mens) als eindgastheer. De prevalentie van *Sarcocystis* species bij runderen is in verschillende landen onderzocht en benadert bijna steeds 100%. Een infectie met *S. hominis* veroorzaakt bij de mens voorbijgaande niet-specifieke gastro-intestinale symptomen. Het klinisch verloop is echter meestal mild en in veel gevallen asymptomatisch.
Het wordt gesuggereerd dat *Sarcocystis* species een rol spelen in de ontwikkeling van Bovine Eosinofiele Myositis (BEM). Dit is een subklinische myopathie met typische groengrijze letsels die wereldwijd voorkomt in klinisch gezonde runderen. Hoewel BEM niet detecteerbaar is *in vivo*, resulteert het zowel op slachthuis- als op uitsnijderijniveau in economische verliezen als gevolg van afkeuring door afwijkend uitzicht van het karkas of van het vlees. Niettegenstaande de hoge besmettingsgraad van *Sarcocystis* species in runderen is de prevalentie van BEM laag. De reden voor deze discrepantie is tot nu toe niet duidelijk.

In het eerste deel van het literatuuroverzicht worden de verschillende boviene *Sarcocystis* species besproken (**hoofdstuk 1**). Het tweede deel van het literatuuroverzicht richt zich op de huidige kennis van BEM (**hoofdstuk 2**). Uit beide delen blijkt dat nog steeds weinig of reeds verouderde informatie beschikbaar is, zowel over *Sarcocystis* species in runderen als over hun rol in deze multifocale myopathie.
De algemene doelstelling van deze doctoraatsthesis is dan ook om betere inzichten te verwerven in enerzijds het voorkomen van deze parasiet in normale runderen en anderzijds in hun rol in BEM.

De eerste doelstelling van de studie beschreven in **hoofdstuk 3**, was de ontwikkeling van een betrouwbare moleculair gebaseerde identificatie methode voor de drie boviene *Sarcocystis* species. Er werd aangetoond dat identificatie tot op species niveau mogelijk was met de op punt gestelde genus specifieke PCR, gebaseerd op het 18S rRNA gen, gevolgd door sequentiebepaling. Daarnaast bleek met deze techniek de identificatie van *Sarcocystis* spp. in zowel verse als formolgefixeerde runderspieren mogelijk. Aangezien tot dan toe, als gevolg van identificatie moeilijkheden, weinig gekend was over de relatieve verdeling van de verschillende *Sarcocystis* species in runderen en omwille van het zoönotisch belang van *S. hominis*, was de tweede doelstelling van hoofdstuk 3 het bepalen van het voorkomen van *Sarcocystis* species in runderen en van *S. hominis* in het bijzonder in rauw rundsvlees. De resultaten toon-

den aan dat *S. hominis* in hoge mate voorkomt (97,4%) in rundsvlees in België. Aangezien de consumptie van rauw gehakt rundvlees (bijv. Steak tartaar) veel voorkomt in België en in een aantal andere Europese landen, duiden deze bevindingen op een mogelijks onderschat risico voor de volksgezondheid. Daarnaast suggereren de resultaten van deze studie dat de epidemiologische cyclus van *S. hominis* zeer efficiënt is.

Om de rol van *Sarcocystis* species in BEM te onderzoeken, werden twee studies uitgevoerd (**hoofdstuk 4 en 5**).

In een eerste studie (**hoofdstuk 4**) werd de hypothese, dat BEM veroorzaakt wordt door een specifieke *Sarcocystis* species onderzocht. Sarcocysten aanwezig in BEM letsels van 97 afgekeurde karkassen werden verzameld door middel van laser capture microdissectie. Vervolgens werd species identificatie uitgevoerd met de in hoofdstuk 3 ontwikkelde genus PCR, gevolgd door kloonanalyse en/of sequentiebepaling van het amplicon. In 28% van de karkassen met BEM letsels werden intralesionale sarcocysten of restanten teruggevonden. De meerderheid (82%) van deze intralesionale *Sarcocystis* species werden als *S. hominis* geïdentificeerd, hoewel ook *S. cruzi*, *S. hirsuta* en een niet gekende *Sarcocystis* species werden gedetecteerd. Hieruit kan worden besloten dat het ontstaan van BEM niet gerelateerd is aan één specifieke *Sarcocystis* species.

In een tweede studie (**hoofdstuk 5**) werd nagegaan of experimentele intramusculaire inoculatie van kalveren met *Sarcocystis* antigenen een letsel kan induceren met dezelfde kenmerken als BEM. Hiertoe werden twee kalveren herhaaldelijk intramusculair geïnjecteerd met geadjuveerde *Sarcocystis* antigenen. De morfologische veranderingen ter hoogte van de injectieplaats in deze kalveren werden histologisch en immunohistochemisch onderzocht en vergeleken met de BEM letsels van 44 afgekeurde karkassen. Experimentele intramusculaire injectie van *Sarcocystis* antigenen resulteerde in letsels op de injectieplaats die vergelijkbaar waren met de letsels van natuurlijk voorkomende BEM. Ze werden gekenmerkt door een massale infiltratie van eosinofiele granulocyten, reactieve macrofagen ($MAC387^+$ cellen), T-cellen ($CD3^+$) en B-cellen ($CD20^+$). Zowel in de experimentele als in de natuurlijke gevallen werd COX-2 expressie waargenomen in endotheelcellen gelegen in de nabijheid van aangetaste zones. Daarnaast werd MHC II^+ aankleuring vastgesteld in onder andere spiercellen gelegen rondom de letsels. Deze resultaten tonen aan dat *Sarcocystis* antigenen, wanneer zij gepresenteerd worden aan het immuunsysteem, een ontstekingsreactie kunnen induceren in de spieren van runderen. Bovendien heeft deze inflammatoire respons de kenmerken die ook in BEM karkassen worden geobserveerd.

Deze doctoraatsthesis besluit met een algemene discussie (**hoofdstuk 6**) waarin de bevindingen van de verschillende studies in een bredere context worden geplaatst en waarin tenslotte de algemene conclusies en verdere perspectieven besproken worden.

Tot besluit kan gesteld worden dat *Sarcocystis* antigenen in staat zijn om in runderen een immuunrespons te induceren die resulteert in focale eosinofiele myositis. Het ontstaan van BEM is echter niet geassocieerd met één specifieke *Sarcocystis* species. Tenslotte werd aangetoond dat *S. hominis* in hoge mate voorkomt in rundsvlees in België. Verder onderzoek naar het mogelijk belang voor de volksgezondheid en naar de epidemiologische cyclus van zoönotische *Sarcocystis* species is daarom aangewezen.

Curriculum Vitae

Lieve Vangeel werd geboren op 26 december 1979 te Lier. Na het behalen van het diploma voortgezet wetenschappelijk onderwijs, richting wiskunde - wetenschappen, aan de Europese School te Mol, begon ze in 1997 met de studies Diergeneeskunde aan de Universiteit van Antwerpen. In 2000 slaagde ze aan deze universiteit met onderscheiding. Haar studies werden verder gezet aan de Universiteit Gent, waar ze in 2003 afstudeerde als dierenarts (optie paard), met onderscheiding.

Onmiddellijk daarna trad zij in dienst bij het Laboratorium voor Pathologie van de Huisdieren. Van oktober 2003 tot einde 2004 volgde zij het Veterinary Pathology Residency Program. In 2005 behaalde ze vervolgens een IWT beurs voor het uitvoeren van wetenschappelijk onderzoek, met als titel “De rol van *Sarcocystis* species in Boviene Eosinofiele Myositis”, aan het Laboratorium voor Pathologie van de Huisdieren, in samenwerking met de vakgroep Veterinaire Volksgezondheid / Voedselveiligheid en het Laboratorium voor Parasitologie van de Huisdieren.

In 2006 behaalde ze aan de Universiteit Gent het FELASA certificaat type C in de proefdierkunde en in 2008 voltooide ze haar doctoraatsopleiding in de Diergeneeskundige Wetenschappen.

In 2010 trad zij in dienst als kwaliteitsverantwoordelijke aan het laboratorium Pathologische Ontleedkunde van UZ Leuven en behaalde in 2011 aan de KULeuven het certificaat “Kwaliteitszorg, een multidisciplinaire aanpak in een biomedische, biotechnologische of farmaceutische omgeving”.

Lieve Vangeel is auteur of mede-auteur van een aantal wetenschappelijke publicaties in internationale tijdschriften. Zij was meermaals spreker op internationale congressen.

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