Universidade de Trás-os-Montes e Alto Douro

Sanitary evaluation of large game hunted in Idanha-a-Nova county.

Pilot study on evaluation of Sarcocystis spp. in muscular samples from large game harvested for human consumption**.**

Mestrado Integrado em Medicina Veterinária

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Vila Real, 2013

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"Agir, eis a inteligência verdadeira. Serei o que quiser. Mas tenho que querer o que for. O êxito está em ter êxito, e não em ter condições de êxito. Condições de palácio tem qualquer terra larga, mas onde estará o palácio se não o fizerem ali?" Fernando Pessoa

Abstract

In the last three decades, game hunting activity assumed a major role at social, cultural and economic level in several regions of Portugal. Large game hunting, with focus on red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*), is a significant part of that hunting activity. The study area of this survey, Idanha-a-Nova County, is one of the most representative places of this hunting activity.

In this circumstances, the knowledge on the health of those animals become an essential matter, mainly if their meat is used for human consumption, being zoonotic agents the major target of veterinary attention. For that reason, presently in the Portuguese epidemiological risk area for tuberculosis in large game, it is mandatory the presence of a veterinarian in each driving hunt to do a sanitary evaluation in the field. The geographic area of this study is included in this risk area.

This survey occurred in hunting season 2011/2012 and had as main objectives the evaluation of game meat preparation conditions in the field and the *Sarcocystis* spp. occurrence in muscular samples collected from large game harvested for human consumption.

In order to fulfill the previous issues it were attended 24 drives hunting in 14 hunting areas in which were harvested 500 animals (345 red deer and 155 wild boars). From those, 52 red deer (15.1%) and 45 wild boars (29.0%) were declared unfit for human consumption. The main cause of condemnation observed was Tuberculosis compatible lesions, underlining the persistence and importance of this disease in large game hunted in Idanha-a-Nova County.

The evaluation of the game meat preparation conditions in the field, made on the evisceration place, gathered information from 3 main domains: structural requirements, hygienic requirements and transportation of game and carcasses, and by-products destination. The structural requirements domain was the one with the lowest score. In two cases, it was observed that it would be possible to overcome this problem if these Hunting Areas share the evisceration place with one geographically close with better classification. Within each domain several parameters were analyzed, being personal cleaning/disinfection devices, knives utilization, garment, footwear and mask the ones with the lowest punctuations, indicating the need for its improvement to ensure better protection of handlers and better hygiene of game meat.

Through histological examination of the oesophagus, diaphragm, and heart, *Sarcocystis* spp. cysts were detected in 65.6% red deer, 38.9% wild boar, 100% fallow deer and mouflon. In red deer and wild boar oesophagus was the less often affected sample.

The applied diagnostic approach revealed high level of *Sarcocystis* spp. occurrence, underlining that life cycle and zoonotic potential should be further investigated and

indicates that several issues should be addressed when planning surveillance and prevention actions.

Key Words: *Sarcocystis*, Red Deer, Wild Boar, Idanha-a-Nova, Tuberculosis.

Resumo

Nas últimas três décadas a caça assumiu um papel importante a nível social, cultural e económico em várias regiões de Portugal. A caça maior, sobretudo veados (*Cervus elaphus*) e javali (*Sus scrofa*), é uma parte relevante dessa atividade. E a área de estudo deste trabalho, Idanha-a-Nova, é um dos concelhos mais representativos dessa importância.

Nestas circunstâncias, o conhecimento do estado sanitário destes animais torna-se uma questão essencial, sobretudo se para consumo humano, sendo os agentes zoonóticos o principal alvo da atenção veterinária. Por essa razão, na Área Epidemiológica de Risco para a Tuberculose dos Animais de Caça Maior é obrigatório a presença de um veterinário em cada montaria para efetuar a avaliação sanitária. A área geográfica deste estudo está incluída nesta área epidemiológica de risco.

Este estudo ocorreu na época venatória 2011/2012 e tinha como principais objetivos a avaliação das condições do local de evisceração e a ocorrência de *Sarcocystis* spp. em amostras musculares de animais de caça maior destinados ao consumo humano.

Para cumprir os objetivos foram acompanhadas 24 montarias, em 14 zonas de caça, nas quais foram caçados 500 animais (345 veados e 155 javalis). Destes animais, 52 veados (15.1%) e 45 javalis (29.0%) foram rejeitados para consumo. A principal causa da rejeição foram lesões compatíveis com Tuberculose, enfatizando a importância desta doença da caça maior em Idanha-a-Nova.

Para a avaliação das condições do local de evisceração foram recolhidas informações em 3 domínios: requisitos estruturais, requisitos higiénicos e transporte de animais caçados e carcaças e destino de subprodutos. O domínio dos requisitos estruturais foi o que teve o resultado mais baixo. Em dois casos foi observado que seria possível melhorar este resultado através da partilha, por parte das Zonas de Caça, do local de evisceração mais próximo e com melhor classificação. Cada domínio foi ainda dividido em parâmetros, sendo que os que obtiveram pontuações mais baixas foram: Dispositivos de Limpeza/Desinfeção Pessoal, Utilização das Facas, Vestuário, Calçado e Máscara. Este resultado indica a necessidade de melhorar estes parâmetros para assegurar uma maior proteção dos intervenientes e salubridade da carne de caça.

Através do exame histológico de esófago, diafragma e coração foram detetados quistos de *Sarcocystis* spp. em 65,6% dos veados, 38,9% dos javalis, 100% dos gamos e muflões. Tanto no veado como no javali o esófago foi a amostra de tecido menos afetado.

Este exame revelou um nível elevado de ocorrência de *Sarcocystis* spp., reforçando a necessidade de investigar o ciclo de vida e o potencial zoonótico, indicando a necessidade de contemplar estas questões na elaboração de planos de vigilância e prevenção.

Palavras-chave: *Sarcocystis*, Veados, Javalis, Tuberculose Bovina, Avaliação Sanitária.

General Index:

Image Index:

Table Index:

Abbreviation, Acronym and Symbol Index:

µm – micrometers
ANMP – National Association of Portuguese City Halls
BD – By-products Destination
CT – Carcass Transportation
DA – Dog Access
EFSA – European Food Safety Agency
ELISA – Enzyme-linked immunosorbent assay
FW – Footwear
G – Garment
g – Grams
GH - Game Heaping
GIS – Geographical Information Systems
Gl – Gloves
GU - Gloves Utilization
h – Hour
ha – Hectares
HA – Hunting Area
HE – hematoxylin-eozine
HPS – Human Hypereosinophilic Syndrome
IFAT – Immunofluorescent Antybody test
IHA – Indirect Hemagglutination test
INE – National Institute of Statistics
IUCN – International Union for the Conservation of Nature
Kg – Kilogram
km^2 – square kilometres
KnU – Knives Utilization
M – Mask
M. – *Micobacterium*
ºC – Centigrade
OK – Own Knives
PAS – Periodic Acid-Schiff
PC – Pest Control
PCDD – Personal Cleaning/Disinfection Devices
PD – Proper Drainage
TB – Tuberculosis
Tcl – Tuberculosis compatible lesion
USA – United States of America
WK - Work Organization

1. Introduction

In the last three decades, large game hunting activity assumed an increasing economic, social and cultural importance in several regions of Portugal, being the red deer and the wild boar the most important game species hunted.

These game species may be infected by several zoonotic agents requiring some veterinarian concerns and attention mainly if their meat is used for human consumption. For that reason, presently in the Portuguese epidemiological risk area for tuberculosis in large game, it is mandatory the presence of a veterinarian in each driving hunt to do a sanitary evaluation in the field. The geographic area of this study, Idanha-a-Nova County, is included in this risk area.

One of the zoonotic agents that may affect red deer and wild boar is *Sarcocystis* spp., a unicellular parasite that may be found in muscles of the infected animals. In *Sarcocystis* life cycle, humans may act as definitive host (gastrointestinal sarcocystosis) for *S. suihominis* and *S. hominis* (Heydorn, 1977, cited by Mohammadi & Petri, 2006) or as an intermediate host (muscular sarcocystosis) for a considerable number of species, some of them not yet determined (Mohammadi & Petri, 2006; EFSA, 2010; Esposito, D. H, *et al*,. 2012). In fact, according to several authors, it is necessary to make clear the impact in public health of *Sarcocystis* species to clarify their importance and the need of their monitoring (EFSA, 2010**)**.

The lack of information of *Sarcocystis* spp. prevalence in large game species, the importance that *Sarcocystis* spp. may assume to the public health, the importance of large game as a source of meat for human consumption and the importance of sanitary evaluation of game species in the field, justify the development of this study which was carried in wild boars and red deer hunted in a Idanha-a-Nova County during the hunting season 2011/2012.

The main objectives of the present study include:

- The characterization of the number of wild boar and red deer hunted in Idanha-a-Nova County and the main cause of condemnation of animals for human consumption;
- The evaluation of game meat evisceration place;
- Identification of the *Sarcocystis* spp. infection occurrence in red deer and wild boar hunted in Idanha-a-Nova County through histopathological analyses.

2. Bibliographic revision

2.1. Large Game Hunting in Portugal

In the last three decades, large game hunting is increasing its importance on economical, social and cultural features of several regions of Portugal. To underline the increasing importance of large game hunting in Portugal is the size of hunting area, which was about 5 million ha in 2004. From those 5 million ha, 3.2 million ha (1/3 of Portugal) were divided for 1700 Association Hunting Area and 720 Tourist Hunting Area and 1.8 million ha dived for 600 of other types of hunting areas (Lopes *et al.*, 2004). Other fact that emphasizes the increasing importance of large game hunting is the number of hunter's licenses issued, which almost doubled from season 1999/2000 to season 2004/2005, (Vingada *et al.*, 2010).

Hunting and game management are mainly regulated by Act 173/1999, of September 21st, which was updated to Act n. º 2/2011, of January 6th. To both activities there are several specific directives emitted to regulate specific cases. In accordance with these legal diplomas the large game species hunted in Portugal are the wild boar (*Sus scrofa*), the fallow deer (*Cervus dama*), the red deer (*Cervus elaphus*), the roe deer (*Capreolus capreolus*) and the mouflon (*Ovis ammon*). These species can be hunted by sit and wait hunting, stalking, drive hunting, battue hunting and spear hunting. While the red deer, the roe deer, and the wild boar are native species, the fallow deer and the mouflon were introduced, the first one centuries ago and the second one two decades ago (Lopes *et al.*, 2004).

The present study was mainly focused on red deer (*Cervus elaphus*) (Image 1) and wild boar (*Sus scrofa*).

Image 1 - Red deer grazing in a field in Idanha-a-Nova County.

2.1.1. Red deer (*Cervus elaphus* Linnaeus, 1758)

Taxonomy: Kingdom-Animalia; Phylum-Chordata; Class-Mammalia; Order-Artiodactyla; Family-Cervidae; Genus-*Cervus*; Specie-*C. elaphus*.

The red deer (Image 2) is the largest wild ungulate hunted in Portugal. Its existence has suffered some ups and downs through history. While, in the medieval period, it was widespread in Portugal (Mendonça, 2003, cited by Vingada *et al.*, 2010), in the last part of the nineteenth century, red deer was already on the edge of extinction (Bugalho, 2002, cited by Vingada *et al.*, 2010). In the 1970's red deer presented a reduced and spotted distribution confined to fenced areas (Tapada de Mafra, Torre Bela, Tapada de Vila Viçosa, Parâmio, Montesinho e Contenda) and was practically extinct in their natural habitat. Nonetheless, in the following decades, were made several deliberated attempts to restocking by releasing animals from those fenced herds (Vingada et al, 1997, cited by Vingada *et al.*, 2010; Fonseca, 2004a, cited by Vingada *et al.*, 2010). In addition, several animals dispersed naturally from Spain into Portuguese territory in the Montesinho Mountains, Contenda-Barrancos region, Tagus International and more recently Gerês Mountains (Vingada *et al.*, 2010). Furthermore, in the border regions were taken a series of measures to improve habitat quality allowing red deer populations to settle permanently in our country. And, currently, this specie is widespread throughout Portugal, especially to south of the river Mondego, with emphasis on the populations of the International Tagus, Lousã, Alentejo (Moura, Mourão and Barrancos) and Silves. In the north of the country, it is evident the population of the north-eastern border (Lombada National Hunting Area). The expansion of red deer, seen in the last decades in Portugal, is for sure rare in the recent history of Western Europe. The totality of causes of this fact still to explain but is possible to mention some: the enormous plasticity and adaptation to different habitats; a progressive abandon of the rural lands, providing shelter; possible increase of the tolerance to man's presence; and a strong sense of territoriality, that doesn't allow great densities but forces the increase of the distribution area (Salazar, 2009).

In literature, is quoted that the number of red deer (*Cervus elaphus*) in Portugal should be approximately 15 000 to 20 000 animals and almost half of them are restricted to fenced areas. It was the importance of this specie in the large game that led to the promotion of red deer (*Cervus elaphus*) populations in Portugal. The largest proportions of animals harvested are from the South of Portugal (tourist hunting reserves, Tapada da Contenda) and Tagus International (Vingada *et al*., 2010). According to the official game statistics, in Idanha-a-Nova County, were harvested 962, 1479 and 1243 (data only until 31-12-2009) red deer in seasons 2006-07, 2007-08 and 2008-09 respectively (DGAVc, 2010).

Because it is not possible to determine if the reintroduction of the animals were a success (different origins of animal from Spain, Scotland and Hungary) and because of the lack of studies on red deer genetics, it is generally accepted that *Cervus elaphus hispanicus* is the dominant subspecies in Portugal (Salazar, 2009).

Image 2 - Red deer.

2.1.2. Wild boar (*Sus scrofa* Linnaeus, 1758)

Taxonomy: Kingdom-Animalia; Phylum-Chordata; Class-Mammalia; Order-Artiodactyla; Family-Suidae; Genus-*Sus*; Species-*S. scrofa*.

Wild boar (*Sus scrofa*) is also one very important large game species hunted in Portugal. The distribution of this specie has also known several ups and downs through the Portuguese history. A serious decrease, in wild boar population, was prominent in the 19th century and in the beginning of the 20th century. During this period the population was constrained to mountainous areas contiguous to Spain and some Royal Hunting Grounds (Fonseca, 2004a, cited by Vingada *et al.*, 2010; Lopes *et al.*, 2004). Taking into account the low density of populations, in 1967 the wild boar hunting was prohibited outside enclosure areas (act 47 847, of August 14th in Vingada, J. *et al.* 2010). Also, in 1969, in the VIIth IUCN (International Union for the Conservation of Nature) Resource Technical Meeting (Fonseca, 2004a, cited by Vingada *et al.*, 2010), the designation of endanger species was allocated to wild boar. The possible re-colonization by the clusters which remained next to the perimeter of Portugal, mainly those on south of Tagus Rive, were then responsible for the spreading out and the nowadays national distribution of wild boar populations (Lopes *et al.*, 2004; Vingada *et al.*, 2010). In the present days, wild boar populations are again widespread through all Portuguese territory, apart from the huge metropolitan and seashore areas (high human density), as according to the data reported by hunting areas

associations (Fonseca *et al*., 2004, cited by Vingada *et al.*, 2010; Lopes *et al.*, 2004). According to the official game statistics, in Idanha-a-Nova County were harvested 367, 708 and 817 (data only until 31-12-2009) wild boars in seasons 2006-07, 2007-08 and 2008-09 respectively (DGAV, 2010c).

Image 3 - Wild boar traces.

2.2.*Sarcocystis* spp.

2.2.1. History and Taxonomy

The first report on *Sarcocystis* was made by Miescher in 1843, who described white threadlike cysts in striated muscles of a mouse (*Mus musculus*) (Dubey *et al.*, 1989, cited by, Dahlgren, 2010). No scientific name was given at the time and in the following twenty years it was referred as "Miescher's Tubules". In 1865 similar structures were found in pig muscle by Kühn and named *Synchrytium miescherianum* (Dahlgren, 2010). Lankester in 1882 introduced the name *Sarcocystis* (Greek: sarkos - flesh, kystis - cyst) but only in 1899 the denomination *Sarcocystis meischeriana* was proposed to identify this species (Dubey, 1989, cited by Fayer, 2004). Afterwards, for every intramuscular cyst found in a new host was proposed a new specie name. Throughout this time there was the controversy of which taxonomic group did it belong to (protozoa or fungi), on the account that sarcocyst stage was the only identified and that in several culture media there were seen hyphae and mycelia after a few days (result of contamination). In 1967, 124 year after the first report of *Sarcocystis*, there was a study using electronic microscopy on the bradyzoites, which revealed organelles like those seen on the apicomplexan protozoa in species such as *Toxoplasma* and *Eimeria* (Senaud, 1967, cited by Fayer, 2004). In1970, with the inoculation of bradyzoites from *Sarcocystis* of birds into cultured mammalian cells, there was development of sexual stages and oocysts (Fayer, 1970, cited by Fayer, 2004; Fayer, 1972, cited by Fayer, 2004). In 1972 the heteroxenous life was revealed when the life-cycles of several *Sarcocystis* species of sheep, swine, and cattle were determined (Heydorn *et al.*, 1972, cited by Dahlgren, 2010; Rommel *et al.*, 1972, cited by Dahlgren, 2010).

In the present, *Sarcocystis* is a unicellular parasite with the following classification: **Kingdom:** Protozoa > **Phylum:** Apicomplexa > **Class:** Sporozoea > Subclass: Coccidia > **Order:** Eucoccidiorida > Suborder: Eimeriorina > **Family:** Sarcocystidae > Subfamily: Sarcocystinae > **Genera: *Sarcocystis*** (Dalhgren, 2010).

Several coccidian species were revealed in the last two centuries, and its classification was mainly based on intermediate and definitive host species, life cycle and phenotypic characters, such as oocyst morphology (Tenter, 1995, cited by Tenter *et al.*, 2002). For example, transmission studies using *Sarcocystis* cysts from three divergent morphological types found in cattle were fed to different potential definitive hosts: dogs, cats, and humans. These three genuses were infected and new species names were proposed: *S. bovicanis*, *S. bovifelis*, and *S.*

Bovihominis, respectively (Heydorn, *et al.*, 1972 cited by Fayer, 2004; Rommel *et al.*, 1972, cited by Fayer, 2004; Rommel, *et al.*, 1972, cited by Fayer, 2004). These species classification scheme, together with others misinterpretation took to a lightly misconstruction of the taxonomic classification within the genus *Sarcocystis.* In the following lines are listed those possible mistakes: **1 -** the limited number of morphological characters, which were often misidentified or incompletely described; **2** - deficient awareness of the life-cycle of many *Sarcocystis* species; **3** - the fact that intermediate and definitive hosts are generally infected by more than one *Sarcocystis* species; **4** - the fact that sarcocysts with similar morphology may appear in different host species; **5** - and the imperfect acquaintance of the intermediate host range of a particular *Sarcocystis* species (Dahlgren, 2010).

But with the application of molecular methods the classification of protozoa group is now seen in a different way and is possible that genetic information will sustain a new classification of Coccidia group in coming future. In fact, there have been a growing number of publications making the review and reclassification of the Apicomplexa group (Tenter, 1995, cited by Dahlgren, 2010; Tenter *et al*, 2002, cited by Dahlgren, 2010). Today, about 200 *Sarcocystis* species have been described from many species of reptiles, birds, and mammals from all over the world and some species have the definitive hosts determined but not all species have been assigned a name (Dahlgren, 2010).

2.2.2. Life Cycle

Sarcocysts spp. has an obligatory heteroxenous life cycle (Image 4), with a sexual stage in enteroepithelial cells of the definitive host (predator), and asexual generation in the tissues of the intermediate host (prey) (Dubey *et al.*, 1989, cited by Fayer, 2004; Kia, 2011).

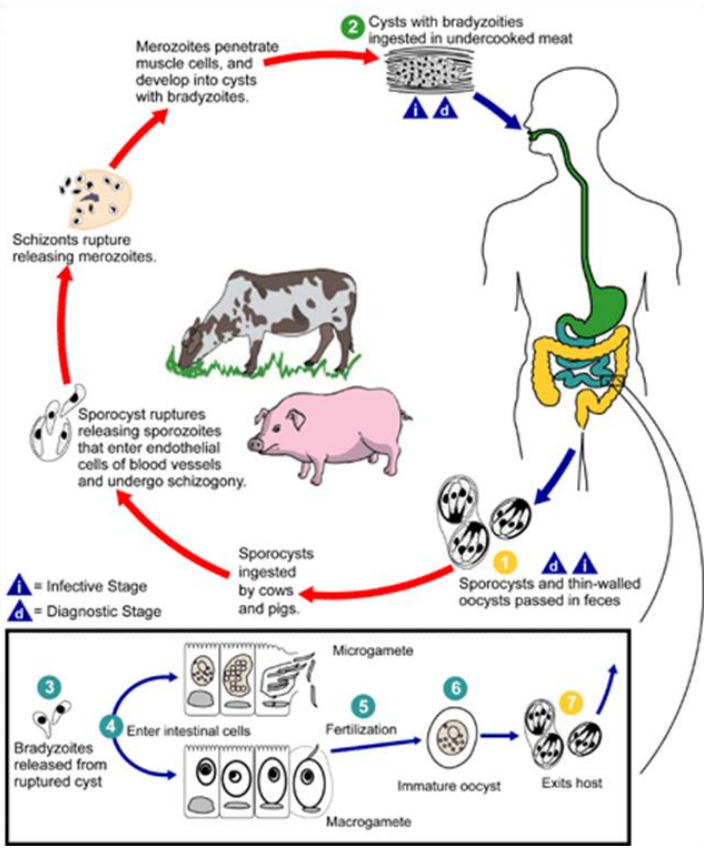

Image 4 - *Sarcocystis* spp. life cycle (Source: http://dpd.cdc.gov/dpdx/html/imagelibrary/s-z/sarcocystosis/body_sarcocystosis_il5.htm, data: 22/02/2013 10:45).

2.2.2.1. Intermediate Host

The following description of the *Sarcocysts* spp. life cycle is based on studies of *S. cruzi* in cattle (Fayer, 1972, cited by Fayer, 2004; Fayer, 1982, cited by Fayer, 2004). Oocysts or free sporocysts from the definitive host are ingested by a susceptible intermediate host and pass to the small intestine. The sporocyst walls separate releasing four sporozoites. Motile sporozoites migrate through the gut epithelium entering, eventually, in endothelial cells on small arteries. Here they undergo the first of four asexual generations (called schizogony or merogony), producing numerous merozoites (cells morphologically similar to sporozoites and bradyzoites) about 15 to 16 days after ingestion of sporocysts. It is possible to see merozoites constituting the second generation in the peripheral blood 27 days after ingestion of sporocysts. The third asexual generation appears as multinucleate schizonts in capillaries. Merozoites from this generation enter muscle cells to form metrocytes (mother cells), and originate sarcocyst formation (Fayer, 2004). The tissue-cysts in the intermediate host are divided into compartments and contain two types of reproductive stages, metrocytes and bradyzoites (=cystozoites) (Dahlgren, 2010). At the beginning Sarcocysts is a unicellular bodie containing a single metrocyte. Through repeated

asexual multiplication, numerous metrocytes accumulate and the sarcocyst increases in size. Then sarcocysts mature from noninfectious metrocytes to infectious forms called bradyzoites (Greek: brady-slow, zoite-small animal) (Image 5).

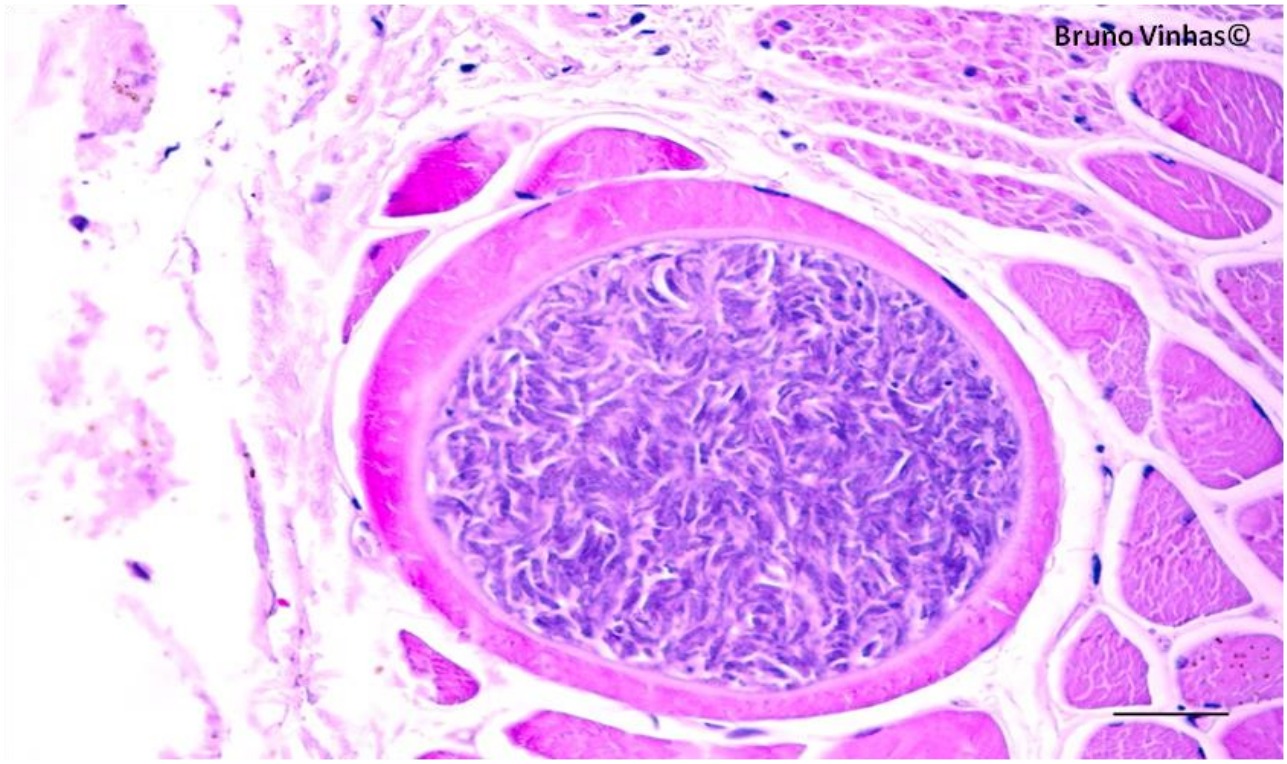

Image 5 - Wild boar, oesophagus. Image of a *Sarcocystis* spp. bradyzoite in transversal cut. H&E. Bar=30µm.

The time that maturation takes varies between species and may take 2 months or more to sarcocysts become infectious for the definitive host. Mature sarcocysts may diverge in walls structures and thickness and villar protrusions size and organization. Sarcocysts may be found in all striated muscles of the body including the tongue, oesophagus, diaphragm, as well as cardiac muscle and, to a lesser extent, in smooth muscle. It is also possible to find sarcocysts, in lesser degree, in neural tissue (spinal cord, brain and Purkinje fibers of the heart). It seems that humans are accidental hosts when sarcocysts develop in striated muscles as there is little or no opportunity to maintain a life cycle (Fayer, 2004).

Considering the prepatent period, it varies with the *Sarcocysts* species. For example, *Sarcocystis wapiti* has a 10 to 12 day prepatent period (Speer *et al.*, 1982; Foreyt, 1995) and *S. sybillensis* has a 14-day prepatent period (Dubey *et al.*, 1983; Foreyt, 1995).

Other significant fact quoted in literature is that generally, species transmitted by canids are more pathogenic than those transmitted by felids (William, M. Samuel *et al*, 2001).

2.2.2.1.1. Clinical Symptoms

In the intermediate host the clinical symptoms depend significantly on the infection occurrence and *Sarcocystis* species. For example, ingestion of less than 1 million sporocysts of *S. miescheriana* generally causes subclinical illness in pig (*Sus scrofa domesticus*) (Dubey *et al.*, 1988) but ingestion of 50.000 sporocysts of *S. suihominis* cause illness in 100% of pigs and 50% of those fed with 1 million died (Dubey *et al.*, 1988). Other factor is the animal condition. For example, according to Dubey *et al.* (1988) the ingestion of 50.000 *S. Miesheriana* sporocysts by a pregnant female is enough to cause abortion, become moribund or even die. Other study on mule deer (*Odocoileus hemionus hemionus*) with different sporocists dosage mentioned that all fawns orally inoculated became ill and 9 in 11 died (Hudkins, 1977) and relating the dosage levels with mortality rates we have : 1.0×10^6 - 100%, 2.5×10^5 -75% and 5.0×10^4 - 75%. The intermediate host symptoms reported by several studies were anorexia, pyrexia, weakness, weight loss (Hudkins, 1977), less weight gain (Foreyt W. J., 1995), lethargy, anaemia, icterus, lymphadenopathy and abortion 2-5 weeks after infection (Dalghren, 2011). Being the main possible source of clinical signs the second generation of meront/schizont in endothelial cells of capillaries in most tissues and organs (Dalghren, 2011).

2.2.2.1.2. Pathophysiology

In literature, it is possible to find several studies describing histological alterations caused by *Sarcocystis* in the intermediate host. Resuming, we may find mild to severe edema in all muscle tissues examined and several muscles with mild to moderate congestion and haemorrhages together with sarcocysts (Khatkar *et al.*, 1993 cited by Avapal, 2003). In the kidneys, lungs, heart and liver we may see moderate to severe hemorrhages on serosal surfaces and we may also find serous atrophy of pericardial and perirenal fat reflecting starvation, anorexia and cachexia (Dubey *et al*, 1989 cited by Avapal, 2003; Thomson, 1989 cited by Avapal, 2003). In addition were also observed haemorrhages on pericardium, endocardium and on serosal and mucosal surface of intestine, (Avapal, 2003).

2.2.2.1.3. Prophylaxis

Regarding prophylaxis there were made several studies. Those studies reported that immunity to one species of *Sarcocystis* does not seem to give rise to significant protection against another species (Fayer, 1984 cited by Abdel-Baki, 2009; Munday, 1981 cited by Abdel-Baki, 2009) and can be limited in time (Dubey *et al.*, 1988). Those studies also refer that animals surviving to a primary infection get protection against lethal or acute clinical disease in a secondary infection, month's later (Dubey, 1981 cited by Abdel-Baki, 2009; Dubey, 1983 cited

by Abdel-Baki, 2009; Leek *et al.*, 1983 cited by Abdel-Baki, 2009; Fayer, 1984 cited by Abdel-Baki, 2009; Ford,, 1985, cited by Abdel-Baki, 2009; O'Donoghue, 1988, cited by Abdel-Baki, 2009). In this case the acquired immunity does not eliminate nor does it prevent further establishment of sarcocysts (O'Donoghue, 1988, cited by Abdel-Baki, 2009). Other fact quoted in literature is that sporozoites, as well as merozoites, are probably more immunogenic than bradyzoites (Lindsay *et al.*, 1995). Furthermore, is referred that cell mediate immunity is probably more important than humoral immunity (Lindsay *et al.*, 1995). Those studies also refer that the presence of *Sarcocystis* is not necessary for the maintenance of protective immunity (Lindsay *et al.*, 1995). Referring now to chemoprophylaxis, there are three studies conducted in the USA mentioning that amprolium and salinomycin can diminish the symptoms of acute illness and act as a prophylactic measure (Fayer *et al*., 1975; Leek *et al*., 1980; Leek *et al*., 1983). Nevertheless, in one of the studies an additional group of five lambs were treated therapeutically with salinomycin beginning 21 days after sporocists inoculation. All five died from acute sarcocystosis (Leek *et al.*, 1983).

2.2.2.2. Definitive Host

Concerning definitive host, the ingestion of meat with a sarcocyst by the definitive host, initiate this stage of the life cycle. In first place there must be the rupture or digestion of the sarcocyst wall which becomes bradyzoites motile. Bradyzoites leave the sarcocyst and penetrate the intestinal cells to develop into the male stadium called microgamete or the female stadium denominated macrogamete. In order to evolve for the next stage microgamete and macrogamete need to fuse. Subsequently the macrogamete initiates the maturation with the cytoplasm sequential development (sporogony) into an oocyst containing two sporocysts. Then the oocysts transpose to the intestinal lumen and appear in the fecal smears where may be find intact oocysts, only in the first few days of patency, or two adjacent sporocysts with the oocyst wall barely visible. Usually, the oocyst wall ruptures releasing sporocysts. This may be the only stadium observed in feces. Each sporocyst has four sporozoites which are the infective stage for susceptible intermediate hosts (Fayer, 2004).

2.2.2.2.1. Clinical Symptoms

Generally the infections do not cause clinical symptoms. Still, the *Sarcocysts* species which infect man have been reported to cause abdominal discomfort, vomiting, diarrhea, and respiratory distress (Dubey et al. 1989; Fayer 2004; Dalghren, 2011).

2.2.2.2.2. Prevention and Prophylaxis

There is no known and approved therapeutic prophylaxis or treatment for intestinal sarcocystosis, and avoid the ingestion of infected meat is the only measure recognized to be effective. (Fayer, 2004). For instance, in literature is mentioned a study with co-trimoxazole (Croft, 1994, cited by Fayer, 2004) or furazolidone (Mensa *et al.*, 1999, cited by Fayer, 2004) but the efficacy of this two drugs remains to be demonstrated. And is also quoted that in Thailand six persons were submitted to surgical resection of the small intestine followed by antibiotic treatment. However this extremely aggressive treatment has not been applied in other cases (Bunyaratvej *et al.*, 1982 cited by Fayer, 2004).

2.2.3. Specificity of hosts

Concerning the specificity of intermediate hosts, we may find many transmission studies, mostly in bovid, which indicate that there are some species without specificity and some with specificity. For example, *S. hominis* sporocysts infect cattle but not pigs while sporocysts from *S. suihominis* infect pigs but not cattle (Damriyasa, 2004). On the other hand, *S.cruzi* from dogs infect cattle (*Bos taurus*), water buffalo (*Bubalus bubalis*), and bison (*Bison bison*) (Fayer, 1982). Moreover, in a recent study, molecular data from *Sarcocystis* species of cattle and water buffalo stoutly suggested that the same *Sarcocystis* species infect both intermediate host species (Jehle *et al.*, 2009, cited by Dahlghren, 2011). In other study, naturally infected cattle meat was fed to a human volunteer, who shed sporocysts. Then sporocysts were ingested by a water buffalo that was necropsied 119 days later. In the necropsy was found a larger number of *sarcocystis* in skeletal muscles that were infective when ingested by two human volunteers (Chen, 2003). This study proved that water buffalo can serve as intermediate host, as cattle, for *S. hominis* (Damriyasa, 2004). This data induce us to think that *Sarcocystis* is genetically predisposed to infect specific intermediate hosts or within closed related hosts (Solaymani-Mohammadi, *et al.*, 2006). The same may happen to humans by ingesting sporocysts of predators of nonhuman primates. One study with a captive-born rhesus monkey, with an acute fulminant disease caused by *Sarcocystis*, report us that there is susceptibility to a primate have an acute infection although there is no apparent typical definitive host (Lane, *et al.*, 1998 cited by Fayer, 2004). Other studies made in the 1990ies on *Sarcocystis* infections in exotic animals, born and raised in German zoos, indicated that these intermediate hosts had become infected by German *Sarcocystis* species, since they did not have the opportunity to become infected with the species usually occurring in its natural habitat (e.g., Stolte *et al.*. 1996, cited by Dahlghren, 2011;

Stolte *et al.*, 1997 cited by Dahlghren, 2011). Furthermore, new intermediate hosts of *S. neurona* have continuously being found during the last decade (Elsheikha, 2009).

Analogous specificity can also be established for definitive hosts. For example, dogs and coyotes serve as definitive hosts for *S. cruzi*, but humans and cats do not (Leek, 1978 cited by Damriyasa, 2004). Humans, baboons, and rhesus monkeys can serve as definitive hosts for *S. hominis* (Heydorn, 1976 cited by Damriyasa, 2004), and humans, chimpanzees, rhesus and cynomolgus monkeys can serve as definitive hosts for *S. suihominis* (Fayer, 1979 cited by Damriyasa, 2004). No other definitive hosts have been identified for *S. hominis* or *S. suihominis* (Damriyasa, 2004). Other important fact reported in literature is that *Sarcocystis* uses either felids or canids, but not both (Huong *et al.*, 1997, cited by Chen, 2011; Odening *et al.*, 1995, cited by Chen, 2011; Odening *et al.*, 1996, cited by Chen, 2011; Zuo *et al.*, 1995 cited by Chen, 2011).

In resume, it is important to keep our mind open as every new study on this issue brings new facts. And studying on this issue is the only way to increase the knowledge and know for sure how each *Sarcocystis* cycle works.

2.2.4. Diagnostic

The first clinical traits to consider in diagnostic of sarcocistosis are reports of previous prevalence, if they exist, symptoms and ecopathology of affected animals (Tenter, 1995). This knowledge is an important complement to the laboratorial methods that may include a stool exam in the final host, direct observation of cysts in the carcass, histological examination, and digestion method and muscle squash, Enzyme-linked immunosorbent assay (ELISA), Indirect Hemagglutination test (IHA) and Immunofluorescent Antybody test (IFAT) in intermediate host.

Considering the stool exam, the best method is flotation based on high-density solutions rather than those based on formalin–ethyl acetate and other sedimentation methods (Saito Odening *et al.*, 1998, cited by Solaymani-Mohammadi, 2006). In a stool smear it is possible to find three structures: **1 -** intact oocysts (only in the first few days of patency) **2 -** two adjacent sporocysts (oocyst wall may be visible or not) or **3 -** individual sporocysts (sometimes the only observed) (Fayer, 2004). Sporocysts can be seen by microscopy as an oblong or cylindrical shape. Within the sporocysts may be observed long and teardrop-shaped sporozoites. Because sporocysts of different species have common characteristics in size and shape is not possible to distinguish species using microscopy (Saito Odening *et al.*, 1998, cited by Solaymani-Mohammadi, 2006).

In slaughtered animals diagnose of *Sarcocysts* spp. may be done by the direct observation of cysts in the carcass if they are macroscopically visible. In these cases cysts appears as cream-colored, cylindrical cysts that resemble grains of rice running in parallel streaks through the muscle tissue (Friend, M. & *et al.* 1999).

But if the cysts have microscopic size, it can only be found through histological examination. In the histological examination can be used haematoxylin and eosin stain or periodic acid-Schiff (PAS) reaction. Nonetheless, a variability may be find in staining as the sarcocyst wall may be very thin or not clearly visible and in others the intensity of staining may not be sufficient to clearly determine that the wall is PAS positive (Fayer, 2004).

Other method, preferable if there is a large quantity of meat, is grinding it followed by an artificially digestion and centrifuge. After centrifuge the pellet can be stain or just examined microscopically for the presence of bradyzoites (Fayer, 2004; Hamidinejat, 2006). In the literature the digestion method is described as the gold standard for diagnoses of bradyzoites (Hamidinejat, 2006).

The same author also describes a method denominated muscle squash. In this method the collected muscle is cut in small pieces and strongly crushed between two slides. One of the slides is fixed with methanol and stained by giemsa, then examined with the optical microscope looking for bradyzoites (Hamidinejat, 2010).

It is also possible to use an ELISA test, with *S. miescheriana* as antigen, an IHA, with antigens from *Sarcocystis gigantean* and IFAT test for the detection of antibodies to *S. cruzi* bradyzoites (Moré *et al.*, 2008).

2.2.5. Species affecting red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*)

Sarcocystis spp. can be found worldwide and infect birds, poikilothermic animals and mammals (Xinwen Chen, 2011) (Table 1).

Table 1 - Examples of *Sarcocystis* species with respective Intermediate Host and Definitive Host (Yang *et al.*, 2002; Uggla *et al.*, 1990).

Sarcocystis Specie	Intermediate Host	Definitive Host
S. cruzi	Cattle	Dog, wolf, coyote, fox
S. gigantea	Sheep	Cat, fox
S. capracanis	Goat	Dog, coyote, fox
S. cervicanis	Red deer	Dog
S. fusiformis	Water buffalo	Dog
S. meischeriana	Pig	Dog

2.2.5.1. Red deer

The first report of *Sarcocystis* in a red deer (*Cervus elaphus*) was done by Hessling in 1854 (Drost *et al.*, 1975, cited by Kutkienè, 2003). After that, major studies were performed in Europe and North America (Kutkienè, 2003) and eight species were described for red deer. In the following lines are described the eight species and its finding.

Previous to 1995 there were only thin-walled cysts described in red deer and the specie was named *S. grueneri* by Yakimoff and Sokoloff (1934) (Kutkienè, 2003). After, a study derived on the thin-walled cyst ultrastructure (Hernández-Rodríguez *et al.*, 1981), this specie name changed to *S. cervicanis* (Kutkienè, 2003). Different studies derived in North America refer as *S. waipiti* to this specie (Dubey *et al.*, 1989; Largerquist *et al.*, 1993). In fact, there are some literatures whose authors describe *S. gruneri*, *S. cervicanis* and *S. waipiti* as single specie with low specificity to the intermediate host (Fayer *et al.*, 1982; Matuschka, 1983; Balbo *et al.*, 1988; Santini, 1997). Some of them even formulate the hypothesis that this type of cysts founded in cervids inhabiting all the Holarctic belong to the same species (Wesemeier *et al.*, 1995 cited by Kutkienè, 2003). For these authors it was opportune to give this species the first name, *S. grueneri* (Kutkienè, 2003).

Another *Sarcocystis* species reported in red deer was denominated as *S. capreolicanis* (Wesemeier *et al.*, 1995 cited by Kutkienè, 2003). On the other hand, studies in North America refer to this species, which infect elk (*Cervus canadensis*), as *S. sybillensis* (Dubey et al., 1989; Largerquist *et al.*, 1993).

Another species reported in literature was *S. hofmanni* (Wesemeier *et al.*, 1995 cited by Kutkienè, 2003) also denominated as a *Sarcocystis* sp. similar to *S. hofmanni* (Stolte *et al.*, 1996 cited by Dahlghren, 2009).

Several studies made in Norway described 5 more species found in red deer which were common to other cervids: *S. hjorti* (described for the first time and named in this study and also found in moose), *S. tarandi* (also in reindeer) *S. rangiferi* (also in reindeer), *S. hardangeri* (also in reindeer) and *S. ovalis* (also in moose) (Dalhgren, 2009).

Concerning the prevalence of *Sarcocystis* infection in red deer, the ones found in literature are resumed in the following table (Table 2).

Table 2 - Prevalence of *Sarcocystis* infection in red deer found in literature (in muscular samples).

Prevalence of *Sarcocystis* infection (%)	Country	Reference
98	Hungary	Kavai *et al.*, 1976 cited by Goldová, 2008
63	Spain	Navarrete *et al*, 1978 cited by Goldová, 2008
25	Czechoslovakia	Lukesová *et al.*, 1989 in Goldová, 2008
98.0	Germany	Partenheimer-Hannemann, 199, cited by Goldová, 2008
90.2	Germany	Spickschen *et al*., 2002 cite for Goldová, 2008
100	USA	Largerquist *et al.*, 1993 cited by William, 1995
94.3	Poland	Tropilo *et al*., 2001 cited by Goldová, 2008
70.2	Lithuania	Kutkiené, 2003
78.6	eastern Slovakia	Goldová, 2008
50	eastern Slovakia	Hvizdošová 2009
100	Norway	Dalhgren, 2009

2.2.5.2. *Sus scrofa*

Referring to wild boar (*sus scrofa*), there are three known species that infect those animal specie: (**1**) *S. miescheriana* (synonym: *Sarcocystis suicanis*) (Kühn, 1865, cited by Tenter, 1995; Labbé, 1899, cited by Tenter, 1995; Erber, 1977 cited by Tenter, 1995), (**2**) *S. porcifelis* (Dubey, 1976 cited by Tenter, 1995) and (**3**) *S. suihominis* (Tadros *et al.*, 1976 cited by Tenter, 1995; Heydorn, 1977 cited by Tenter, 1995) with final hosts being dog, cat and man, respectively.

Concerning the prevalence of *Sarcocystis* infection in wild boar, the ones found in literature are resumed in the following table (Table 3).

Table 3 - Prevalence of *Sarcocystis* infection in wild boar found in literature.

Prevalence of *Sarcocystis* infection (%)	Country	Reference
48	Hungary	Kavai *et al.*, 1976, cited by Goldová, 2008
100	Netherlands	Tadros *et al.*, 1976 cited by Malakauskas, 2002
100	Germany	Erber 1978 cited by Malakauskas M., 2002
95.6	Lithuania	Grikienienë *et al.*, 1995 cited by Malakauskas, 2002
96.3	Lithuania	Grikienienë *et al.*, 1999 cited by Malakauskas, 2002
24.7	Poland	Tropilo *et al.*, 2001 cited by Malakauskas, 2002
89.1	Lithuania	Malakauskas, 2002
85	eastern Slovakia	Goldová, 2008
83.3	eastern Slovakia	Hvizdošová, 2009

1.1.1. *Sarcocystis* as a Zoonotic Agent

In *Sarcocystis* world there are two known species that may involve humans as definite host (gastrointestinal sarcocystosis) in their life cycle: *S. suihominis* and *S. hominis* (Heydorn, 1977 cited by Mohammadi *et al.*, 2006). Considering men as an intermediate host (Muscular

sarcocystosis) the number of species is not determined nor their name (Mohammadi *et al.*, 2006; EFSA, 2010; Esposito, D. H, *et al.*2012).

1.1.1.1. Gastrointestinal Sarcocystosis and Prevention

Humans are a final host to *S. suihominis* and *S. hominis*. Generally, infections are self-limiting, of short duration, and often asymptomatic (Fayer, 2004), being, the main symptoms caused by these two species in man, gastrointestinal symptoms such as abdominal pain, nausea, vomiting and diarrhoea (Mohammadi *et al.*, 2006). Anyway, it is referred in literature that *S. hominis* may cause more stern symptoms like circulatory problems (tachycardia), drowsiness, fatigue, anaemia and dyspnoea (Mohammadi *et al.*, 2006; EFSA, 2010). *S. hominis* is also described in literature as the cause of human Hypereosinophilic Syndrome (HPS) as well as chronic diarrhoea (Nichpanit *et al*, 2010). Despite this vulgar form of infection in the definitive host, there are some recent studies reporting an alternative pathway. These studies distinguishes the vulgar non-invasive cycles from an invasive cycle, being S. *fusiforms* and S.*meischeriana* referred as possible species with the invasive cycle (Bunyaratvej *et al.*, 2007). This alternative pathway may be the cause of an asexual phase in the definitive host, being a possible source of muscular sarcocystosis or chronic inflammation in the intestinal mucosa in man (Bunyaratvej *et al*, 2007). So, as a recent report in Europe concludes, is necessary to make clear the impact in public health of these *Sarcocystis* species to clarify it importance and the need of monitoring (EFSA, 2010). For that, additional studies are needed to clarify the *Sarcocystis* spp. cycles (Bunyaratvej *et al*, 2007).

Referring to prevention, avoiding the ingestion of cysts by the definitive host is the most effective way of prevent infection. When meat may be harbouring cysts, we should thoroughly frozen (-4ºC to -5ºC – 48h; 20ºC – 24h) or thoroughly cook (60ºC-20’; 70ºC-15’; 100ºC-5’) the meat to kill infectious bradyzoites (Fayer, 2004; Mohammadi *et al.*, 2006). These measures will prevent the development of intestinal stages, where humans might serve as definitive hosts or host from erratic cycles (Fayer, 2004).

1.1.1.2. Muscular Sarcocystosis and Prevention

There are many unknown *Sarcocystis* species that may affect man with muscular sarcocystosis but the study of life cycle and epidemiology of these species is incomplete (Bunyaratvej *et al*, 2007; EFSA, 2010; Esposito *et al*., 2012). In these cases, symptoms in humans range from self-limiting to chronic or moderately severe vasculitis and myositis (Fayer,

2004; EFSA, 2010). Southeast Asia is one of the most affected areas with human sarcocystosis (Bunyaratvej *et al*, 2007; Mohammadi *et al.*, 2006; EFSA, 2010; Esposito, D. H, *et al*,. 2012), and a recent worldwide outbreak (Germany, France, Spain, Singapore, Belgium, Netherlands, Switzerland) of an acute muscular *Sarcocystis*-like illness affected 100 persons who had a recent travel to Tioman Islands in Malaysia. During the epidemiological study there were identified intramuscular cysts (Sarcocystis) in two of the involved persons in the outbreak (Esposito *et al*,. 2012). For that reason, the researchers believed that a *Sarcocystis infection seems to be the most likely cause of this outbreak* (Esposito *et al*,. 2012). Before this outbreak, there were only about 92 cases of muscular sarcocystosis in humans reported worldwide (Fayer, 2004). In these cases humans are a dead end host (EFSA, 2010).

Referring to prevention, as it is hard to prevent and eliminate the animal's infection, the most important to do is aware people to the health risk of each travel destination and the importance of appropriate hygiene and safe food and water consumption (Esposito *et al*,. 2012).

3. Materials and Methods

3.1. Area of study

This survey was made in Idanha-a-Nova County which is located in southeast of central region of Portugal (1416.3 km^2 and 10 147 habitants (AMNP, 2012) where occurs the transition of landscape from northern mountains to the south plateau of Portugal (Image 6).

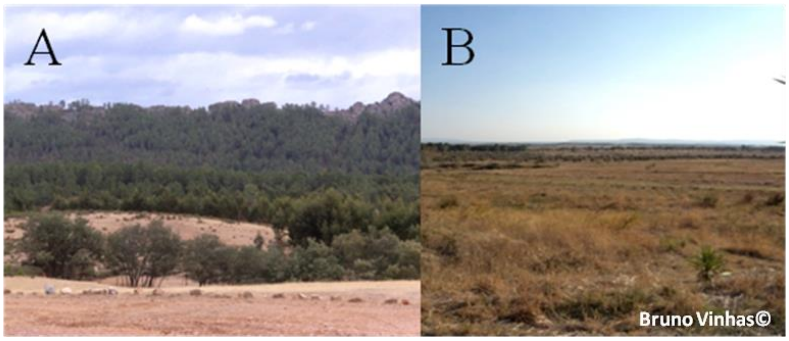

Image 6 – Representative image of the two landscapes found in Idanha-a-Nova. A northen mountains. B South plateau.

Its limits are Penamacor County on north, Castelo Branco and Fundão Counties on west and Erges and Tejo Rivers on east and south (respectively), which corresponds to the Spain border (Extremadura). In the following image it is possible to observe the location of Idanha-a-Nova County in the Portugal map (Image 7).

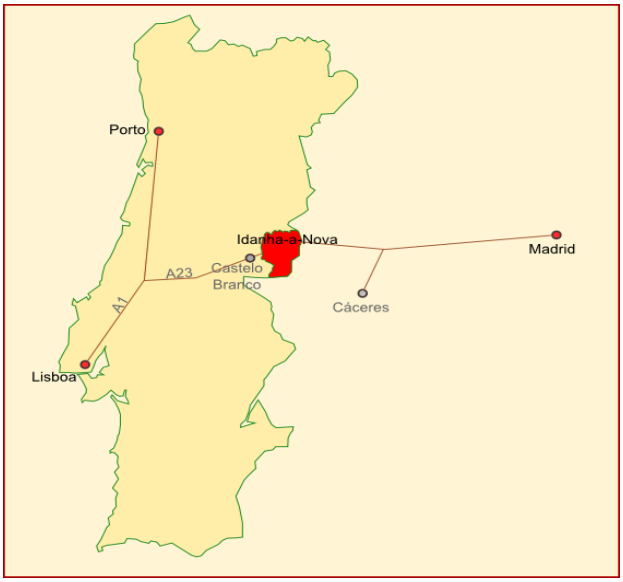

Image 7 - Location of Idanha-a-Nova County in the Portugal map. (source: httpwww.hortasdidanha.ptsiteindex.phpas-terras, data: 12-03-2013, 11:10).

Idanha-a-Nova has a considerable rural area with approximately 50% of land dedicated to agriculture (43% dry agriculture, 9% watered agriculture, and 5% graze land), 30% are forested areas, mainly oaks, and 13% is shrub land and sparse vegetation. Domestic animal production (bovine and small ruminants, especially sheep), generally based on outdoor extensive production, has foremost significance in local economy.

Other important activity to local economy is hunting, emphasized by the continuous decreasing of the agriculture. In fact, this County is known as one of the best places to hunt in Portugal and large game hunting is the most significant part of it. Overall, there are 119816.39 ha of Hunting Areas (84.8% of the County) corresponding to 42 Association Hunting Areas with 51 702.65 ha, 31 Tourist Hunting Areas with 44 375.82 and 14 County Hunting Areas with 23 737.92 (DGAV, 2010a). Most of this land is shared by livestock and wild ungulates, which lead us to other important aspect to have in account; Idanha-a-Nova is in the Epidemiologic Risk Area for Bovine Tuberculosis (Image 8) in large game and has a high infection occurrence in wild boar and red deer (Santos *et al.*, 2009; Vieira-Pinto *et al*, 2011; DGAV, 2010b; DGAV, 2010c). Additionally to the identification of the risk areas, in 2011, the Portuguese Veterinarian Authority published an internal law (edital n.º 1) describing mandatory rules that must be observed during each driving hunt organized in this Epidemiologic Risk Area for Bovine Tuberculosis. For example:

- "…There must be a place to the evisceration of harvested animals…with proper conditions to carry out that task…"
- There must be present a veterinary responsible by the initial examination of every harvested animal presented in the evisceration spot to come up with one of the following results:
- Animals which present alterations that may suggest an health risk must go to byproducts or if the Hunting Area requests to a specific establishment for game meat preparation to a final decision been taken;
- Animals which do not present alterations that may suggest a health risk go to self consumption or to a specific establishment for game meat preparation to be inspected and then placed in the market…"

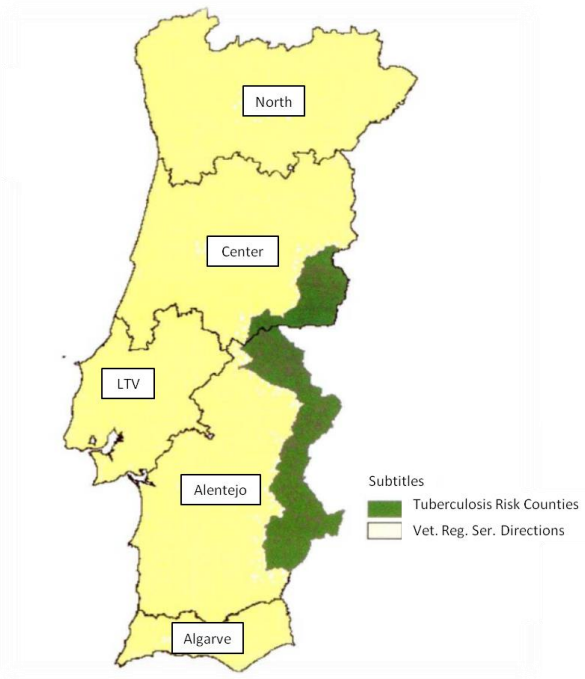

Image 8 - Epidemiologic Risk Area for Bovine Tuberculosis map. (adapted from Edital nº1)

The present study was derived during the hunting season 2011-2012 from October to February, in 24 hunting campaigns (sampling plots) organized in 14 hunting areas from Idanha-a-Nova (Image 9, Table 4).

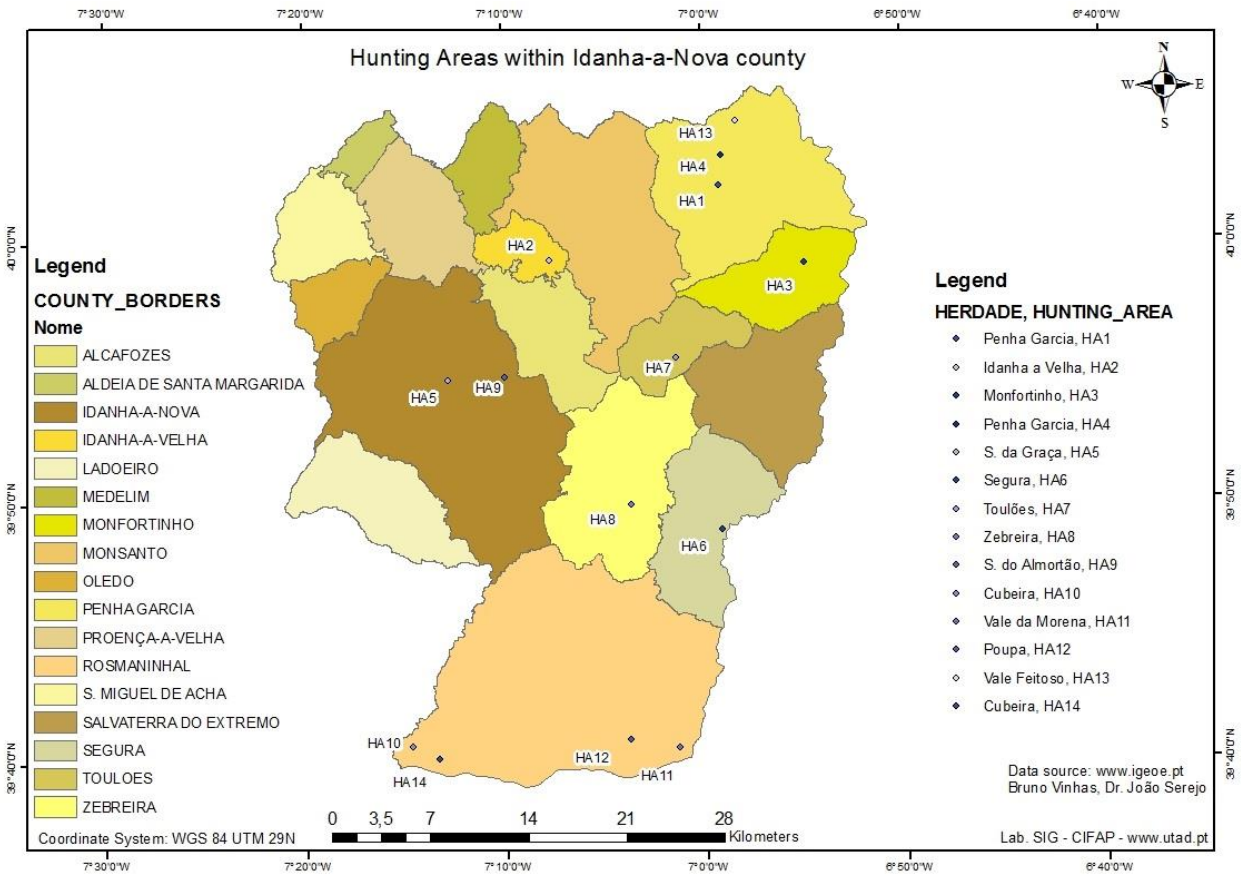

Image 9 – Surveyed hunting areas (HA) within Idanha-a-Nova County.

Table 4 – Number of hunting areas and correspondent type, sanitary evaluation place, council, number of driving hunts and harvested animals.

Hunting Area	Hunting Area Type	Sanitary Evaluation Place	Council	Drive Hunting Number	Harvested animals		
					Red deer	Wild boar	TOTAL
1	As	A	Penha Garcia	1	4	4	8
2	As	B	Idanha-a-Velha	1	1	15	16
3	As	C	Monfortinho	1	8	36	44
4	As	D	Penha Garcia	1	1	1	2
5	As	E	Idanha-a-Nova	1	0	10	10
6	As	F	Segura	4	17	20	37
7	As	G	Toulões	1	1	4	5
8	As	H	Zebreira	2	4	11	15
9	As	I	Idanha-a-Nova	1	0	3	3
10	To	J	Rosmaninhal	1	8	1	9
11	To	K	Rosmaninhal	2	98	5	103
12	To	L	Rosmaninhal	4	107	11	118
13	To	M	Penha Garcia	4	66	29	95
14	To	J	Rosmaninhal	1	30	5	35
TOTAL		14	8	24	345	155	500

(As – Associative Hunting Area; To – Touristic Hunting Area)

3.2. Game Sanitary Evaluation

In each driven hunting action, the large game specimens hunted were collected and gathered in the evisceration place, where the veterinarian carried out the sanitary evaluation referred as initial examination of wild game on the spot. This procedure was based in the veterinary experience and in the Regulation (EC) 854/2004, which establish rules for the organization of official controls to animal origin products intended for human consumption. In brief, were pursued the following procedures:

1. questions to the hunters about abnormal behaviour;
2. carcass external analyses, that included evaluation of corporal conditions, wounds (shot, dog bite, others), secretions and joints palpation;
3. Carcass internal analyses, that included visual analyses of the carcass internal surface, looking for the generalized presence of tumours or abscesses, parasites (Image 10) and weird bodies not resulting from the hunting, incision of lymph nodes (ln.) (mandibular (Image 11A) ln. in wild boar and prescapular (Image 13B) and mesenteric ln. (Image 13A) in red deer) and viscera analyses with incision of kidney (Image 11A), heart (Image 11B), spleen (11C), liver, lung, bronchial ln. and mesenteric ln..

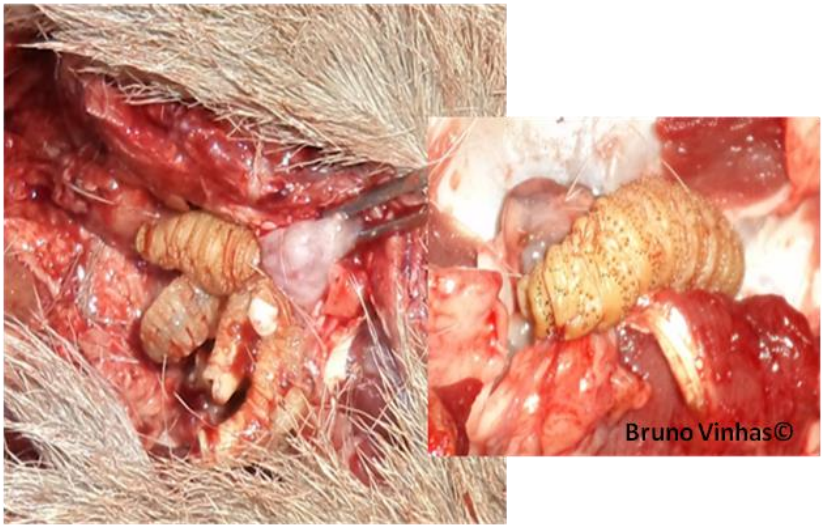

Image 10 – Image of parasitic larvae of oestrids in red deer.

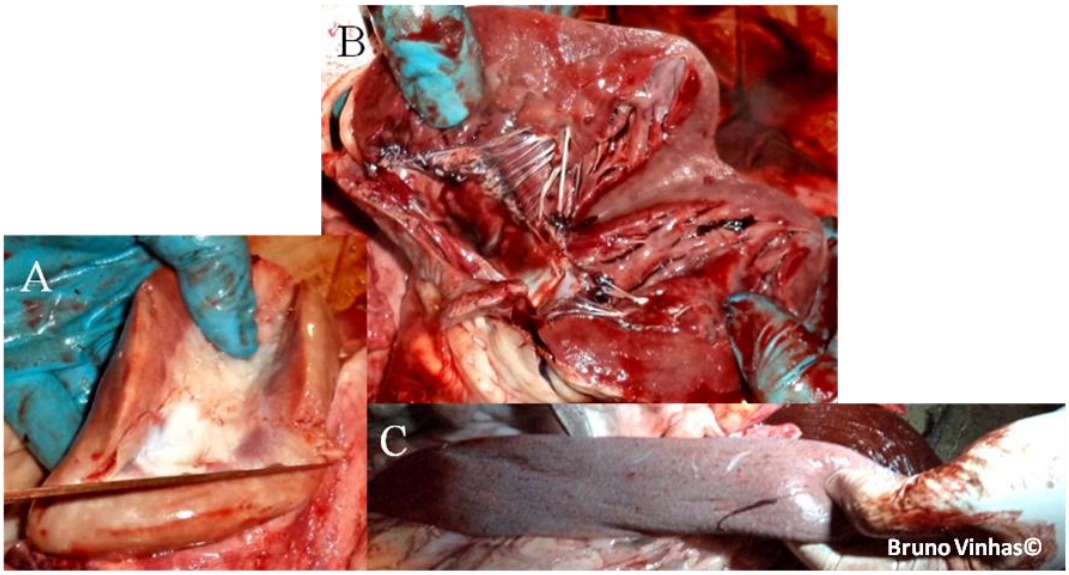

Image 11 - Inspection of several tissues. A – Kidney incision. B – Heart incision. C - Spleen inspection.

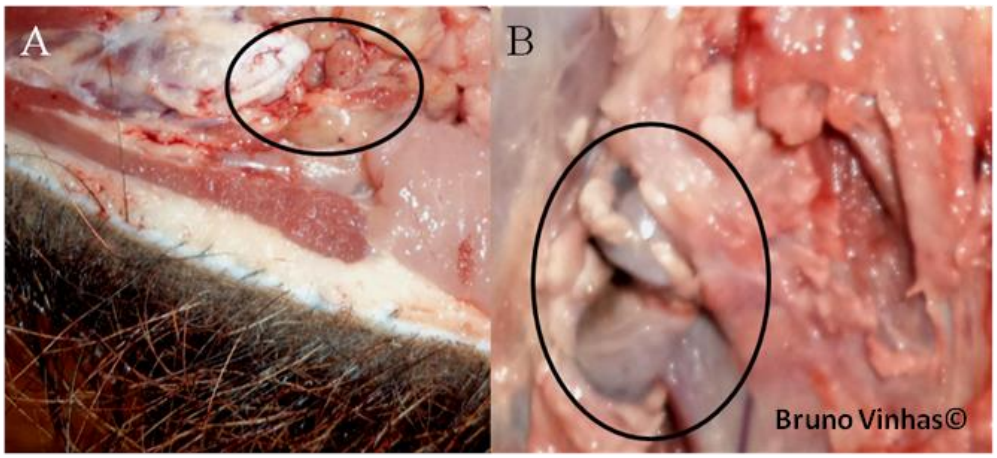

Image 12 – Lymph nodes Inspection. A - mandibular lymph nodes incision. B - pre-escapular lymph nodes incision.

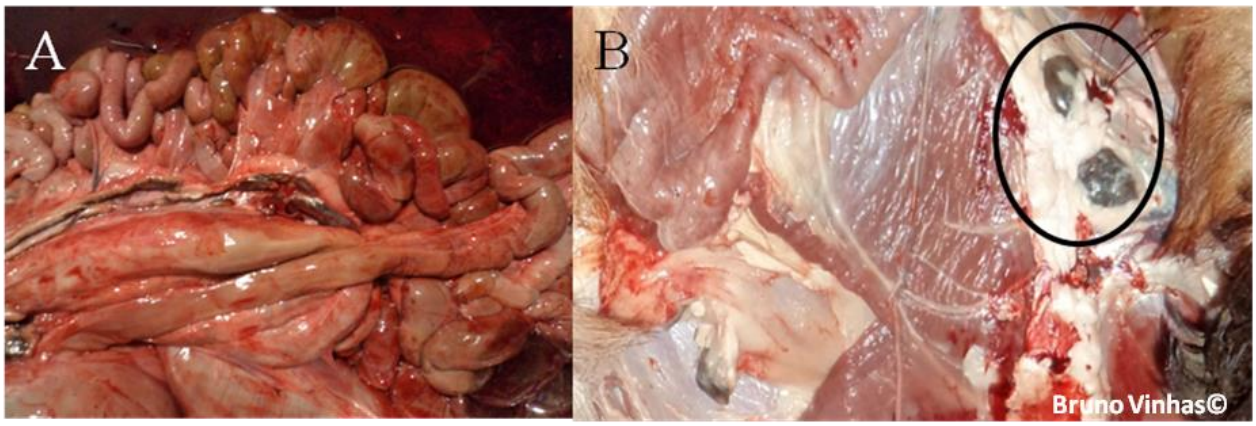

Image 13 - Lymph nodes Inspection. A - Mesenteric lymph nodes. incision. B - Inguinal lymph nodes incision.

According to the Edital Nº1, at the end of the procedure a sanitary decision was emitted as previously described. Then, the carcasses intended to commercial purpose were transported to a specific establishment for game meat preparation, where it was submitted to a sanitary inspection performed by the Official Veterinarian which included the *Trichinella* spp. test on wild boar. The carcasses intended for self consumption were taken by the hunters. In this context there were collected the data about the number of harvested animals and all the cases where meat was declared unfit for human consumption were registered.

3.3.Evaluation of game meat preparation conditions in the field

Additional to the number of harvested animals and the condemned cases declared unfit for human consumption, were collected data concerning the game meat preparation conditions in the field. This study was derived in 14 hunting areas in which were made 24 drives hunting. Due to the physical (geographical) difference between hunting areas and evisceration places (image 15), both were defined with specific assignments as presented in Table 5. In this topic was mainly gathered information related to evisceration, sanitary evaluation, elimination of animal by-products and derived products not intended for human consumption and carcass transportation. These data were grouped into three main domains:

- Structural requirements (Table 6);
- Hygienic requirements (Table 7);
- Transportation of game and carcasses, and by-products destination (Table 8).

Table 5 - Hunting areas and correspondent assignment.

Hunting Area	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Corresponding Evisceration Place	A	B	C	D	E	F	G	H	I	J	K	L	M	J

In order to objectively rank each site/procedure used for game meat preparation in the field it was created a table with parameters to analyse and the punctuation for each analysed parameter, as it is described in the following tables.

Table 6 - Classification of the structural requirements parameters present during game meat preparation in the field (maximum punctuation: 12).

Parameter		Classification	
Water		0 -	Absent
		1 -	Present – Insufficient Quantity or Availability
		2 -	Present in Suitable Conditions
Light		0 -	Absent
		1 -	Present – Insufficient
		2 -	Present in Suitable Conditions
Pest Control		0 -	Absent
		1 -	Present
Dogs Access		0 -	Uncontrolled
		1 -	Controlled(1)
Floor (Image 14)(2)		0 -	Soil
		1 -	Plastic Protection
		2 -	Floor (Tile or Concrete)
Cleaning/ Disinfection Devices	Personal	0 -	Absent
		1 -	Present
	Knives	0 -	Absent
		1 -	Present
	Infrastructures	0 -	Absent
		1 -	Present
Proper Drainage Runoff		0 -	Without Drainage
		1 -	With Drainage

(1) It was considered controlled when sanitary evaluation place had a physical barrier; (2) Floor where the game meat sanitary evaluation was made.

Image 14 - Example of the three types of floor found in the study. A – soil. B – plastic protection. C - tile.

Image 15 - Image of the several evisceration places.

Table 7 - Classification of the hygienic requirements parameters present during game meat preparation in the field (maximum classification: 12).

Parameter	Classification	
Level of cleanliness[1]	0 -	Absent
	1 -	Present
Own Knives	0 -	Absent
	1 -	Present
Knives utilization	0 -	Without Regular Cleaning During Activity
	1 -	Regular Cleaning[2]
Garment (Image17)	0 -	Without Specific Clothing
	1 -	Non-disposable Specific Clothing
	2 -	Disposable Specific Clothing
Footwear (Image17)	0 -	Without Specific Footwear
	1 -	With Specific Footwear
Gloves (Image17)	0 -	Do not use
	1 -	Use
Gloves Utilization	0 -	No Change
	1 -	With Regular Change[3]
Mask	0 -	Do not Use
	1 -	Use
Game Heaping[4]	0 -	Present
	1 -	Absent
Work Organization[5]	0 -	Bad
	1 -	Good
Final Wash	0 -	Absent
	1 -	Present

(1) Before sanitary evaluation; (2) There was some action for cleaning the knives during evisceration, some of which coincident with the opening of animals with lesions compatible with tuberculosis; (3) E.g. - There was gloves change when rupture occurs; (4) In the evisceration place (Image 16); (5) If there was already someone to eviscerate, work too close, carcasses too close, order in animals evisceration, etc.

Image 16 – Image of absence of game heaping in the evisceration place.

Image 17 - Representative image of the hygiene requirements.

Table 8 - Classification of the parameters related to game and carcass transportation and to by-products destination (maximum classification: 4).

Parameter	Classification	
Game transportation for the collection Site (Image 18)	0 -	Not Specific
	1 -	Specific but not Exclusive
	2	Specific and Exclusive
Carcass Transportation after sanitary evaluation	0 -	Non-Specific Vehicle
	1 -	Specific Vehicle
By-products destination(1)	0 -	Non Regulated Destination
	1 -	Regulated Destination (1)

(1)Burial or Collected by City Hall Services (storage)

Image 18 - Image of animal's transportation to the collection site.

Image 19 - Representative image of animals (A), carcass transportation (B) and by-products destination (C).

The information collected in the report sheets (Annex I) was crossed with the previous tables originating punctuation. This evaluation was made by Hunting Area and Driving Hunt, what, in some cases, originates several punctuations in each place. The final classification of each Hunting Area is the average between those punctuations

3.4. Analysis of the presence of *Sarcocystis* spp. in muscular samples of large game hunted in Idanha-a-Nova County

3.4.1. Sampling procedure

In this study were collected muscular samples from 32 red deer (*Cervus elaphus*) and 36 wild boar (*Sus scrofa*) from the several drive hunts attended. After knowing the first and

interesting results, samples from 11 fallow deer and 10 mouflons were additionally collected following the same protocol.

The following table presents the numbers of collected muscles by specie and tissue (Table 9).

Table 9 - Numbers of collected muscles by specie and tissue.

	Heart	Oesophagus	Diaphragm	Total
Red Deer (32)	32	25	29	86
Wild Boar (36)	31	30	35	96
Fallow Deer (11)	11	11	11	33
Mouflon (10)	10	9	9	28
Total	81	75	84	243

The samples were collected by convenience from animals that were approved for human consumption after the sanitary evaluation in the spot. The number of samples was determined by the availability of the veterinarian and driving hunt logistic.

The protocol established for each animal was the collection of samples from (Dahlgren, 2010):

- Oesophagus (portion near the cardia: ~60g)
- Heart (left ventricular apex - ~100g)
- Diaphragm (diaphragm pillars: ~100g);

After collection, a portion of about 15g of each muscle was preserved in 10% formaldehyde and the remaining portion was frozen for further analyses (Image 20).

Image 20 – Representative image of samples. A - Samples ready to freeze. B - Containers where tissues were preserved in 10% formaldehyde.

Data concerning the gender and age of each animal were also collected. Unfortunately there were ten wild boars whose data was not possible to assemble. The red deer and wild boar

age was established by the veterinarian, according with his experience, in two classes: adult and young.

3.4.2. Histopathological Analysis

The muscular portions fixed in 10% commercial formalin were processed and included in paraffin. Three cuts were made with 3µm thick, having then been proceeded to conventional staining haematoxylin-eosin (HE). After that, was made a systematic observation of tissues on a light microscope in all its extension and an animal was considered positive when, at least, one parasitic cyst compatible with *Sarcocystis* spp. was detected in one of the muscles (heart, diaphragm or oesophagus) (Image 21) (Fayer, 2004). In this study, the data related with the Sarcocystis spp. compatible cysts will be described as Sarcocystis spp.. The parasitic cyst features observed were:

- cysts containing many bradyzoites and a small number of metrocytes;
- a generally thick and striated wall that occasionally could be thin and smooth;
- long protusions which are in general folded over and could be noted within the capsule.

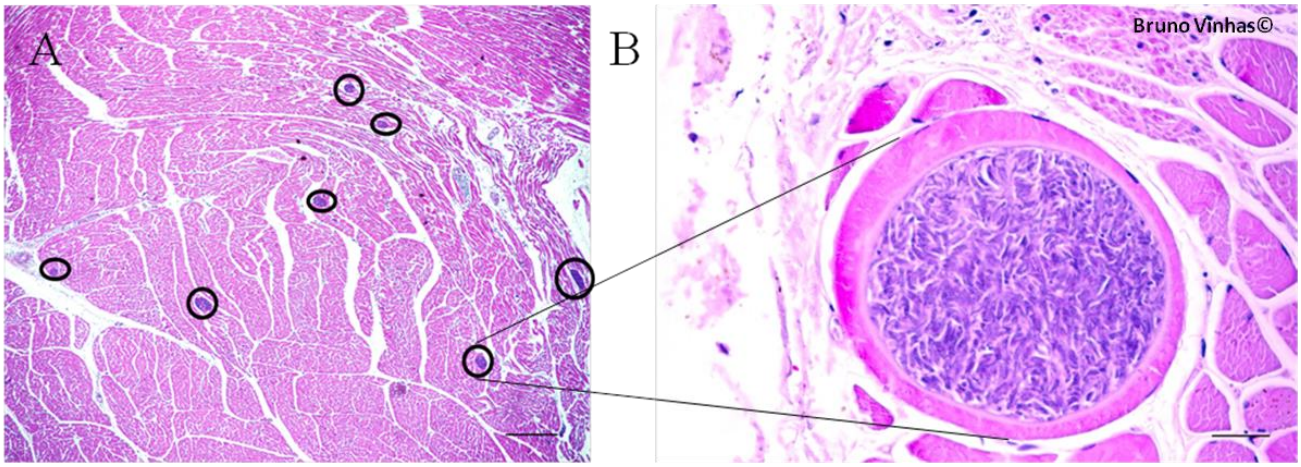

Image 21 – A - Wild boar heart. Evidence of a high number of parasites observed. H&E. Bar=300µm. B - Wild boar, oesophagus. Image of a *Sarcocystis* spp. bradyzoite in transversal cut. H&E. Bar=30µm.

3.4.3. Statistical Analysis

In wild animal's disease study, it is common to analyze the relationship that could be established between disease occurrence and Sex (male, female), Age (young, adult) or simultaneous diseases occurrence (tuberculosis, *Sarcocystis*). Because these are dichotomous variables, it is mandatory to use non parametric tests in order to analyze the degree of association the variables.

The phi coefficient is a statistic test that enables to evaluate the degree of association between two dichotomous variables. This measure is similar to the correlation coefficient in its interpretation. Two dichotomous variables are considered positively associated when most of the data falls along the left upper to downright diagonal cells. In opposite , if most of the data falls along the down left to upper right diagonal cells the two dichotomous variables are negatively associated (D' Hainaut, 1992).

Table 10 – Table example.

Variable A \ Variable B	0	1	Total A
0	a	b	G
1	c	d	H
Total B	e	f	N

Formula for the phi coefficient.

The formula for Phi is

Phi = (a d –b c) / sqrt(e f g h)

Phi result could be analysed by means of comparison to Phi significance, which is calculated by:

Phi 0,05 = 1,960 / sqrt(n) ; Phi 0,01 = 2,576 / sqrt(n)

However, in some cases, in disease associated variables research, they are used categorical variables classified in more that two classes. In these cases, phi coefficient could not be used, and it is necessary to apply a contingency test.

The contingency test is a statistic test that enables to evaluate the degree of association between two variables classified into more than 2 classes. This test requires creating a contingency table (Table 11) with original data and the calculation of expected values, as below presented, in order to calculate a correlation value (D' Hainaut, 1992).

Table 11 - Contingency table example.

Variable A \ Variable B	1	2	...	N	Total A
1	F11	F12		F1n	SA1
2	F21				SA2
…					
p	Fp1			Fnp	
Total B	SB1	SB2			Snp

C = sqrt((M - 1)/M) and M = sum (Fnp^2 / SAp SBn)

C max = sqrt((k - 1)/k) and R = C / C max

Where:

- C is the contingency coefficient
- M is the sum of expected values
- C max is the maximum expected contingency coefficient
- K is a value calculated according the number of classes for the variables (number of rows or columns), as presented in Table 12
- R is the estimated correlation value

Table 12 – k values for C max calculation.

K	3	4	5	6	7	8	9
C max	0,816	0,866	0,894	0,913	0,926	0,935	0,949

3.5. GIS Methodology

Using information produced and presented by Portuguese cartographic authorities (National Geographic Institute – www.igp.pt; Military Geographic Institute – ww.igeoe.pt) such as topographic plans, contours and CORINE Land Cover, it was created a GIS (ArcGis 9.x, Arcinfo version) dedicated to this research.

Using GPS collected positions, it was created a vector file (point shape file) with all sampling plots location. Data collected during fieldwork (e.g. tuberculosis compatible lesions) was assigned to each sampling plot location, in order to enable descriptive statistics analysis and geostatistical analysis (Schröder, 2006). Using the percentage of tuberculosis compatible lesions calculated to each sampling plot and geostatistical analysis (performed using Geostatistical Analyst 2.0 for ArcGIS 9.x. ArcInfo version) a continuous map related to disease spread was created, in order to extent the results to all study area and to create continuous disease intensity maps represented by a colour intensity gradation (Schröder, 2006).

4. Results and Discussion

During the hunting season from October 2011 to February 2012, 24 organized hunting campaigns (sampling plots) were surveyed in Idanha-a-Nova in 14 hunting areas (Image 9).

A total of 500 animals were presented for sanitary evaluation. Table 13 and Image 22 present the number and species of harvested animals in each hunting area and the respective level of condemned animal for human consumption.

Table 13 - Number and species of harvested animals in each hunting area and the respective level of condemned animal for human consumption.

Hunting Area	Type As/To	Red Deer		Wild Boar		Total	
		Harvested animals	Condemned animals (%)*	Harvested animals	Condemned animals (%)*	Harvested animals	Condemned animals (%)*
1	As	4	0 (0)	4	0 (0)	8	0 (0)
2	As	1	0 (0)	15	0 (0)	16	0 (0)
3	As	8	0 (0)	36	8 (22.2)	44	8 (18.2)
4	As	1	0 (0)	1	0 (0)	2	0 (0)
5	As	-	-	10	6 (60)	10	6 (60)
6	As	17	1 (5.9)	20	3 (15)	37	4 (10.8)
7	As	1	0 (0)	4	0 (0)	5	0 (0)
8	As	4	0 (0)	11	4 (36.4)	15	4 (26.7)
9	As	-	-	3	0 (0)	3	0 (0)
10	To	8	2 (25)	1	1 (100)	9	3 (33.3)
11	To	98	10 (10.2)	5	3 (60)	103	13 (12.6)
12	To	107	23 (21.5)	11	9 (81.8)	118	32 (27.1)
13	To	66	1 (1.5)	29	6 (20.7)	95	7 (7.4)
14	To	30	15 (50.0)	5	5 (100)	35	20 (57.1)
Total		345	52 (15.1)	155	45 (29.0)	500	97 (19.4)

(*Condemned animals for human consumption after sanitary evaluation)

From the 500 animals hunted and examined in loco, 97 (19.4%) were condemned for human consumption: from those 52 were red deer and 45 were wild boar.

The main cause of carcass condemnation was the presence of Tuberculosis compatible lesions (Tcl) (Image 23), what was expected since Idanha-a-Nova is in the Epidemiologic Risk Area for Tuberculosis, as mentioned before.

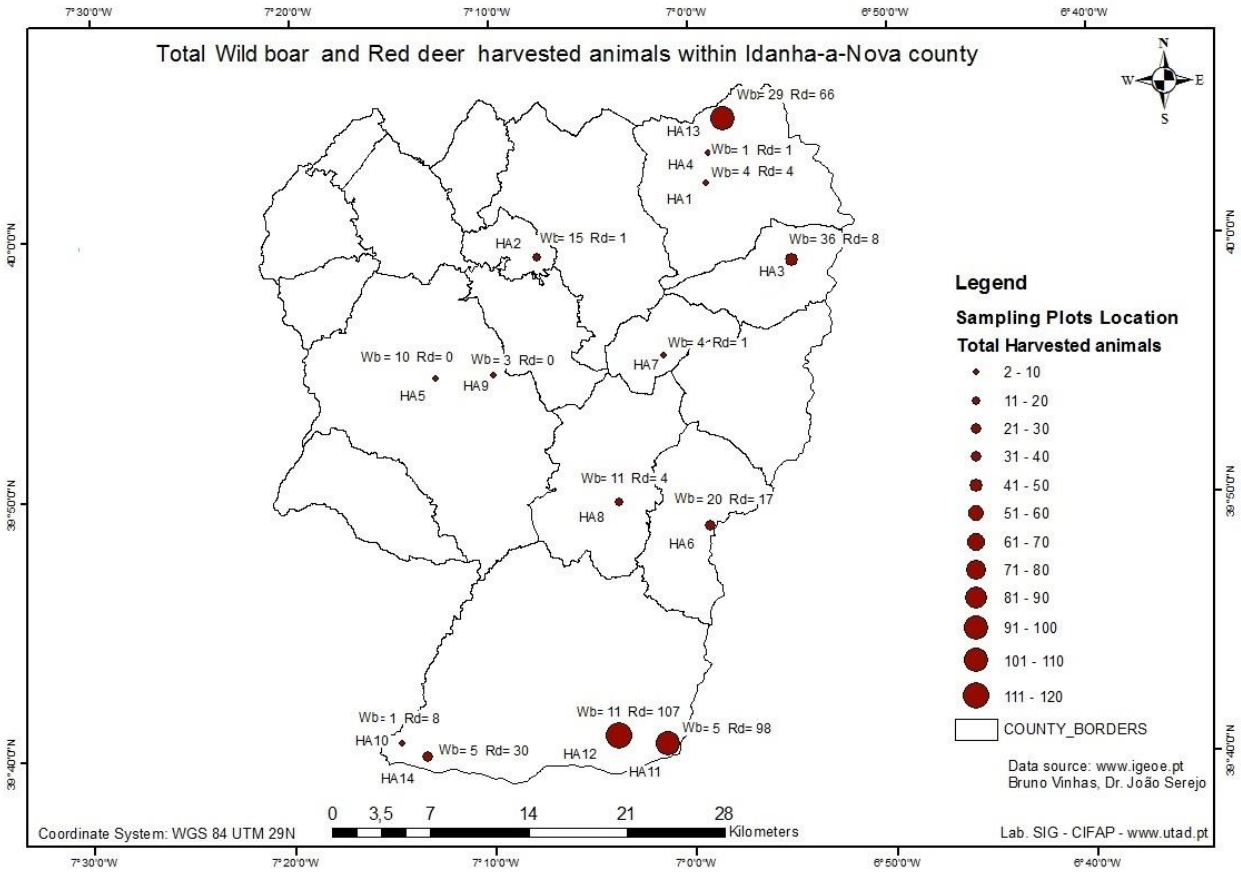

Image 22 - Harvested animals in each surveyed Hunting Area within Idanha-a-Nova County.

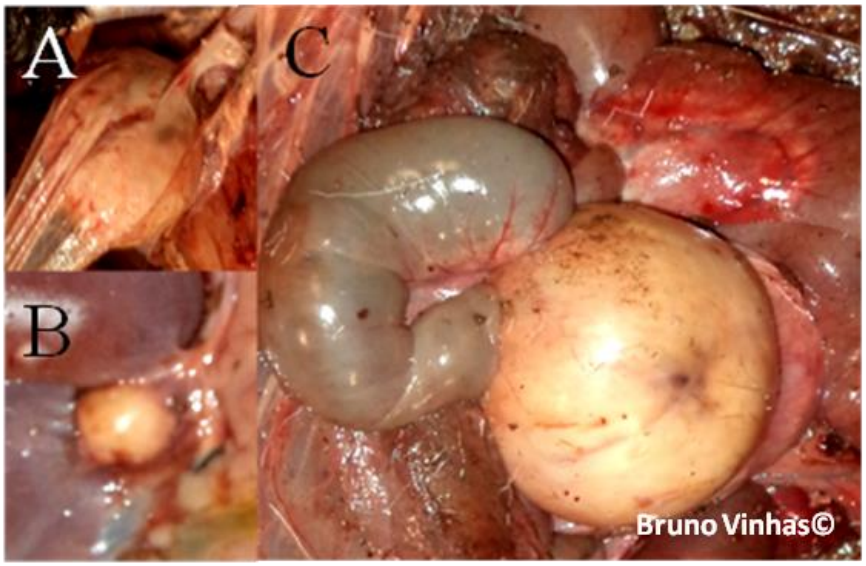

Image 23 –Tcl images. A – Bladder. B – Liver. C - Mesenteric ln..

From the total of the 97 condemned carcasses, only 18 red deer and 1 wild boar were reproved by a different cause: 13 red deer and 1 wild boar by dog bites (Image 24A), 4 red deer by repugnant meat due to action of scavenger birds, mainly vultures, and 1 red deer by emaciation (Image 24B).

Image 24 - Condemned carcasses. A - Condemned carcass due to dog bite. B - Condemned carcass due to emaciation.

In the present study, 78 (15.6%) of the harvested animals were condemned for human consumption due to Tcl. This result is coincident to the one (14.7%) found two seasons before (2009-2011) for the same geographical areas (Vieira-Pinto *et al*, 2011). When we focus our attention in the species, we observe that the lesions were mainly detected in wild boar with 28.4% of the harvested animals with Tcl while only 9.9% of hunted red deer presented Tcl. Comparing these results with the study made by Vieira-Pinto *et al.* (2011), again we found similar occurrences respecting Tcl in wild boar (22%) and red deer (11.8%). In this study were also sampled 67 of 69 Tcl and then made bacterial isolation and identification. And different results were obtained, fixing the tuberculosis prevalence in 15.9% to wild boar and 10.3% to red deer (Vieira-Pint *et al*, 2011). Another study made in seasons 2005-06 and 2006-07, in the same geographical area, only analysed wild boar and obtained a prevalence value of 6,3 %. But this lower prevalence value was based in M. *bovis* isolation from wild boar samples with and without lesions (Santos *et al.*, 2009), while ours and Vieira-Pinto studies were based in the number of Tcl identified by the veterinarian which could have increased the prevalence value. Also, this difference could be explained by the time gap between studies, 4 seasons, in which the tuberculosis prevalence may have raised. This two studies remember us the need of distinguish

Tcl prevalence from TB prevalence. And the values of Tcl prevalence reported in this study may be different from tuberculosis prevalence.

Now, when the Tcl prevalence analysis was done by hunting areas (image 25 e 26) there were found five areas above 15% (HA5 60.0%, HA10 27.3%, HA12 27.1%, HA8 26.7% and HA3 18.2 %.). Adding to this analysis the animal species, wild boar or red deer in each hunting area, it was possible to observe that wild boar leads with an overall Tcl occurrence of 28.4% and reaching 80% in two hunting areas (HA10 with 83.3% and HA12 with 81.8%) and 60% in other two (HA11 and HA5 with 60.0%). Respecting red deer the overall percentage was lower, 9.9%, being HA12 with 19.6%, HA10 with 18.4% and HA11 with 10.2% the areas with the highest values.

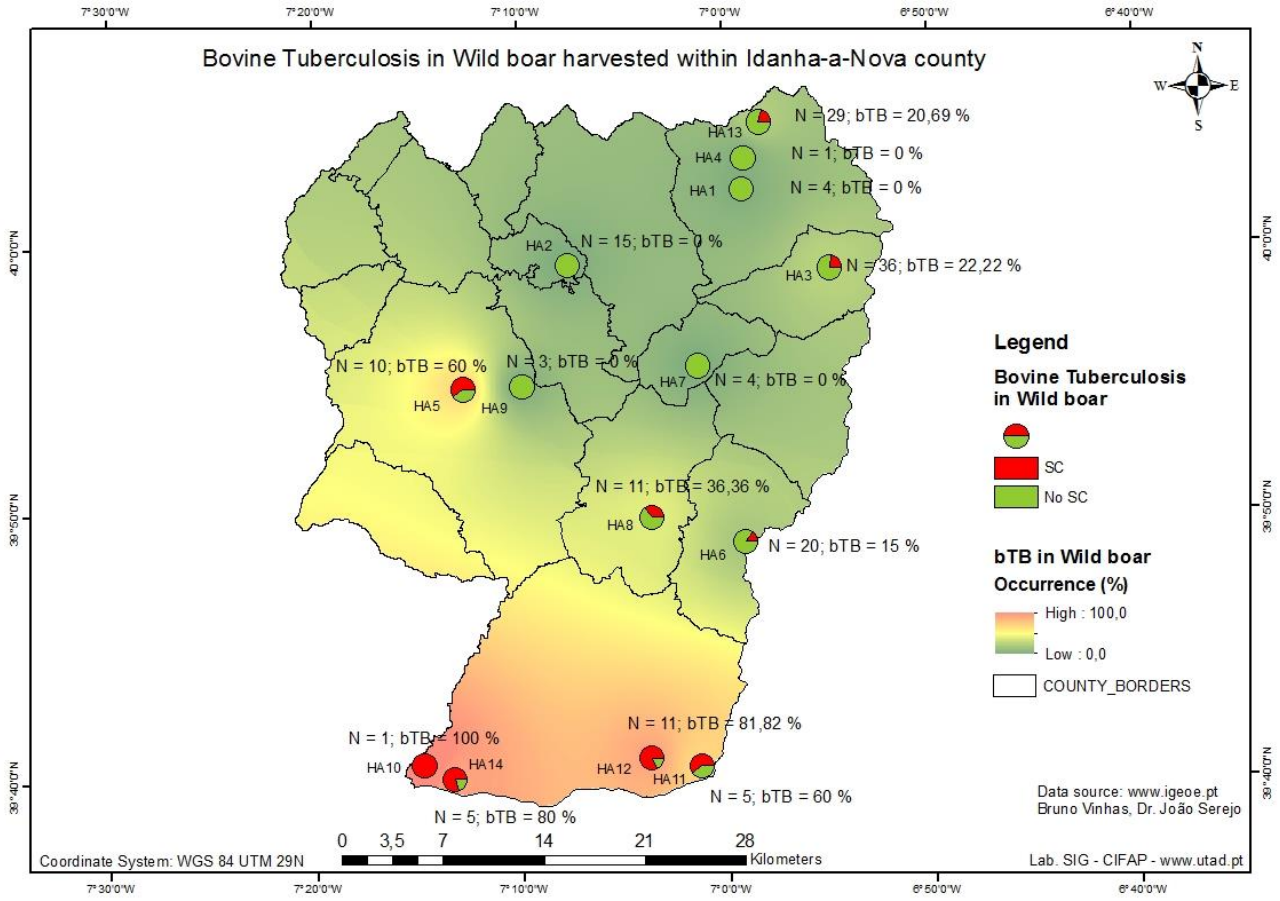

Image 25 – Geographical distribution of TB in wild boar harvested within Idanha-a-Nova County.

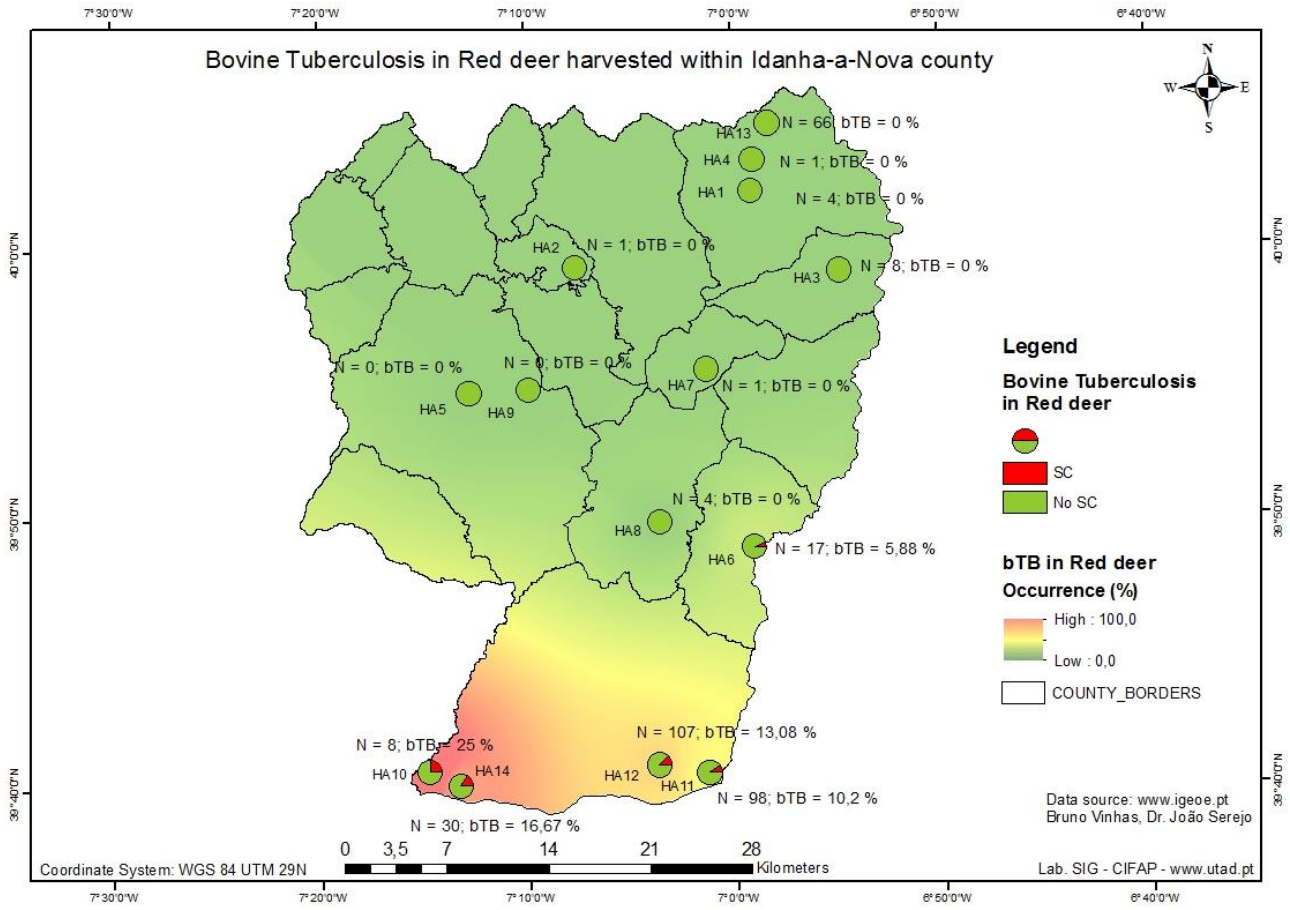

Image 26 – Geographical distribution of TB in red deer harvested within Idanha-a-Nova County

The area which is the headspring is along the border with Spain (DGAV, 2010a; DGAV, 2010b) where we can find HA6, HA10, HA11 and HA12. These limits are also coincident with a region of Spain (Extremadura) with an important problem in TB prevalence (Vicente *et al.*, 2005; Ministerio de Medio Ambiente y Medio Rural y Marino, 2010). Several authors indicate the high population density and the large game management (artificial feeding and watering) as the possible reasons to the high tuberculosis prevalence's (Vicente *et al.*, 2005; Vicente *et al.*, 2007). Other fact to have in account is the occurrence found in HA5 (Idanha-a-Nova County centre) that is the only place which is not located in the previous described zone and that may be a sign to have into consideration in the spreading of the infection.

The severe, growing and spreading problem of tuberculosis in the region, already identified by the national veterinarian authorities, is presently controlled by a monitoring program that includes the mandatory presence of a veterinarian to do the sanitary evaluation of large game animals harvested in each drive hunting (Edital n.1, 2011; DGAV, 2010a; DGAV, 2010b,)

Concerning to the evaluation of game meat preparation conditions in the field, the results obtained are summarized in Table 14, Table 15 and Table 16.

This evaluation was carried out due to the fact that season 2011/2012 was the first when Edital nº 1 of Tuberculosis was applied. This regulation, as mentioned before, defines the obligation of sanitary evaluation to all hunted animals by a veterinarian, the hygienic field conditions necessary to the sanitary evaluation, the specific rules for sampling diseased suspected animals and the obligation of supervising animal by-products disposal in the Epidemiologic Risk Area for Tuberculosis, which covers the entire Idanha-a-Nova County.

The tables used are complex and comprise a great number of aspects as it tries to be the most reliable possible to the requirements of the Edital nº 1. It would be possible to divide in more tables, including more details and some details may be a little subjective. But after taking in account the collected data and being important to analyse this significant aspect, this tables seemed the most adequate.

Table 14 - Structural requirements score for each evisceration place.

		Evisceration Place Classification												
Parameter (maximum punctuation)		A	B	C	D	E	F	G	H	I	J	K	L	M
Water (0-2)		1	1	1	0	1	2	1	2	1	1	1	2	2
Light (0-2)		2	1	2	1	1	2	1	2	2	2	1	2	2
Pest Control (0-1)		0	0	0	0	0	1	0	1	0	0	0	0	0
Dogs Access (0-1)		0	0	0	0	1	1	0	1	0	1	0	1	0
Floor (0-2)		2	0	2	0	2	2	1	2	0	2	1	2	2
Cleaning /Disinfection devices	Personal (0-1)	0	0	0	0	0	0	0	0	0	0	0	0	0
	Knives (0-1)	1	1	0	0	1	1	0	1	1	1	1	1	1
	Infrastructures (0-1)	1	1	1	0	0	1	0	1	0	1	1	1	1
Proper Drainage (0-1)		0	0	1	0	0	1	0	1	0	1	0	1	0
Final Score (0-12)		7	4	7	1	6	11	3	11	4	9	4	10	8

Table 15 - Hygienic requirements score for each evisteration place.

	Evisceration Place Classification												
Parameter (maximum punctuation)	A	B	C	D	E	F	G	H	I	J	K	L	M
Level of cleanliness (0-1)	1	0	1	1	1	1	1	1	0	1	1	1	1
Own Knives (0-1)	1	0	1	1	1	1	1	1	1	1	0,5	1	1
Knives Utilization (0-1)	0	0	0	1	0	1	1	0	1	1	0,5	1	1
Garment (0-2)	0	0	0	0	0	2	0	0	0	1	0	1	1
Footwear (0-1)	0	0	1	0	0	0	0	0	0	1	0	1	1
Gloves (0-1)	1	1	1	1	1	1	1	0,5	1	1	1	1	1
Gloves Utilization (0-1)	1	0	0	1	0	0	1	0	1	1	0,5	1	1
Mask (0-1)	0	0	0	0	0	0	0	0	0	0	0	0	0
Game heaping*(0-1)	1	1	0	1	1	1	1	1	1	1	1	1	1
Work Organization (0-1)	1	1	1	1	1	1	1	1	1	1	1	1	1
Final Wash (0-1)	1	0	1	0	1	1	0	1	0	1	1	1	1
Final Score (0-12)	7	3	6	7	6	9	7	5.5	6	11	6.5	11	11

Table 16- Transportation of game and carcasses, and by-products destination score for each evisceration place.

	Evisceration Place Classification												
Parameter (maximum punctuation)	A	B	C	D	E	F	G	H	I	J	K	L	M
Game transportation*(0-2)	0	1	0	0	-	2	1	1	-	1	0.5	1	1
Carcass Transportation**(0-1)	0	0	0	0	0	0	0	0	0	1	0	1	1
By-products destination (0-1)	1	1	1	1	1	1	1	1	1	1	1	1	1
Final Score (0-4)	1	2	1	1	1	3	2	2	1	3	1.5	3	3

(* Conditions during game transportation to the collection site; ** Carcass Transportation after sanitary evaluation)

The final score for each place resulted from the sum of the classification of each one in the three main domains analysed (Table 17).

Table 17 - Final score for each evisceration place.

	Evisceration Place Classification												
Parameter (maximum score)	A	B	C	D	E	F	G	H	I	J	K	L	M
Structural requirements (0-12)	7	4	7	1	6	11	3	11	4	9	4	10	8
Hygienic requirements (0-12)	7	3	6	7	6	9	7	5.5	6	11	6.5	11	11
Transportation of game and carcasses, and by-products destination (0-4)	1	2	1	1	1	3	2	2	1	3	1.5	3	3
Final Score (0-29)	15	9	14	9	13	23	12	18.5	11	23	12	24	22

To understand the results, the previous classification table was converted into a percentage scale (Table 18). The results were hued with the following grades: < 40% - red – to improve; 40% ≥ 70% - grey – acceptable; > 70% - green – good.

Table 18 - Classification in percentage.

	Evisceration Place Classification												
Parameter	A	B	C	D	E	F	G	H	I	J	K	L	M
Structural requirements	58	33	58	8	50	92	25	92	33	75	33	83	67
Hygienic requirements	58	25	50	58	50	75	58	46	50	92	54	92	92
Transportation of game and carcasses, and by-products destination	25	50	25	25	25	75	50	50	25	75	38	75	75
Final Score	52	31	48	31	45	79	41	64	38	79	41	83	76

Analysing each Evisceration Place, we found 5 places with low punctuations in the structural requirements and 1 place in hygienic requirements.

This outcome was expectable, in our opinion, as structural requirements involve a considerable investment from the HA's entities. Other fact explaining the low result in structural requirements is that during the study period there were two HAs where new facilities were under construction. To minimize this problem and considering the geographical distribution of the HA/Evisceration place, it would possible to suggest that HAs 4 (place D) and 11 (place K), could share the place with HAs 1 (place A) and 12 (place L) respectively, which seems to be a less expensive and efficient solution.

Now, the low result in the hygiene requirement was found in one hunting area in which sanitary evaluation was made by the first time. So this result may be the consequence from the unawareness of the organization entity, although the guidance made in Edital nº 1. In this case would be important to aware the organization of its obligations defined in Edital nº1.

On the general score, in the overall results, there were two places with punctuation below 10 (in a maximum of 29), where it is need to ensure considerable changes to meet the rules defined in the Edital nº 1, that are necessary to guarantee the safety of game meat and adequately protect human health. On the other side there were four places above 20 points which are a good example to follow.

Considering now each analysed parameter, the ones which presented a lower punctuation were: Pest Control (PC), Dog Access (DA), Personal Cleaning/Disinfection Devices (PCDD), Proper Drainage (PD), Knives Utilization (KnU), Garment (G), Footwear (Fw), Mask (M) and Carcass Transportation (CT). In first place we will refer the less dangerous parameters which are PC, DA and CT.

Although PC and DA (Image 27) are important parameters that could be related to deficient hygiene and disease spread, those could be mitigated if additional measures are present namely, the existence of a closed structure (Image 28), the non abandonment of the game carcass and viscera throughout the work period and the existence of a good final wash that would help to avoid the presence of rodents

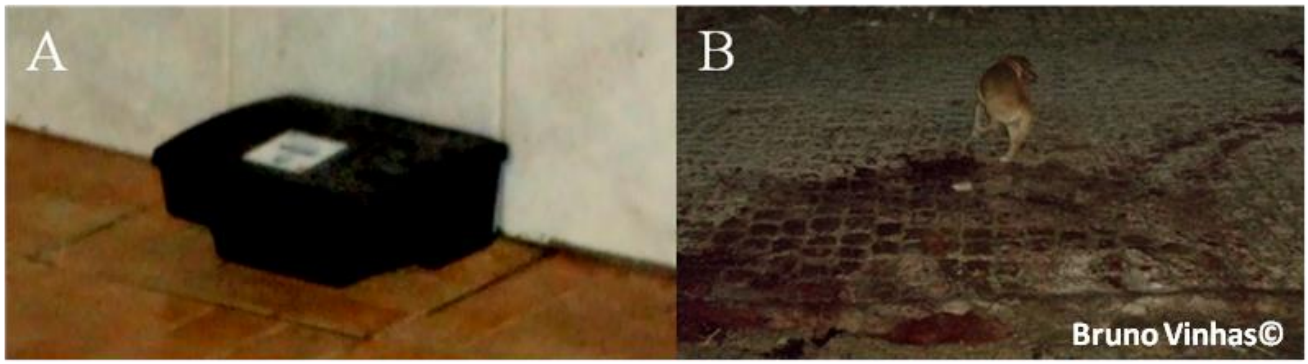

Image 27 - A - Image of pest control devices. B - Image of a dog with access to blood from game animals.

Image 28 - Image of an evisceration place where is easy to control dog access during evisceration and sanitary evaluation.

CT (Image 29) low punctuations was expectable because in most HAs carcass destination is for self-consumption and people are not concerned with the potential risk related to improper transportation. So the continuous awareness of people may be the only way to modify the punctuations of this parameter.

Image 29 - Representative image of carcass transportation. A - Carcasses intended to commercial propose. B - Carcasses intended to self-consumption.

In our opinion, according to the results reached in this study, the parameters important to focus on and modify in the shortest period possible are: PCDD, KnU, G, Fw, M and PD.

PCDD (Image 30) and KnU have a crucial role on creating a barrier between clean and unclean environment or between carcasses. For example when evisceration or sanitary evaluation ends, in order to avoid contamination of clean spaces and objects, or when a suspicious carcass is cut (image 31) to avoid contamination of the next carcass or even to diminish the microbial load (Image 32) during the evisceration and sanitary evaluation. These aspects gain significance as this study takes place in an Epidemiologic Risk Area for Bovine Tuberculosis.

Image 30 - Representative image of several cleaning and disinfection devices.

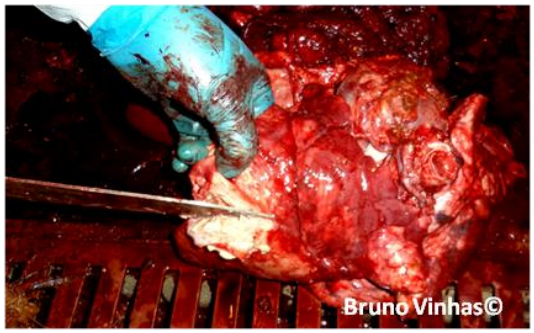

Image 31 – Red deer lung. Image of Tcl being cut.

Image 32 - Representative image of ongoing evisceration and sanitary evaluation.

G, Fw (Image 33) and M have a crucial role in prevent the infection of each person during evisceration or sanitary evaluation. Another important function is to prevent the contamination spreading for other places as car, home or other hunting areas.

Image 33 - Representative image of evisceration. A - Intervenient do not use gloves neither own knives, footwear or garment. B - Example of how to be dressed to evisceration.

PD has an essential role in stop the environment contamination (Image 34). This aspect gain significance as this study takes place in an Epidemiologic Risk Area for Bovine Tuberculosis.

Image 34 - Representative image of drainage.

In our opinion, these 6 parameters are the ones that should be improved in first place in order to increase the health protection of the intervenient and the safety of game meat. On the other hand, we would like to highlight four parameters which had a very positive punctuation: Own Knives (OK), Game Heaping (GH) Work Organization (WK) and By-products Destination (BD) (Image 35).

Image 35 - Representative image of the possible by-products destination. A - Storage of by-products to City Hall services collect. B - Burial.

The parameter Gloves (Gl) (Image 36) had also a very positive result but, in our opinion, this parameter should not be seen alone but matched with the parameter Gloves Utilization (GU). And GU punctuation was reasonable.

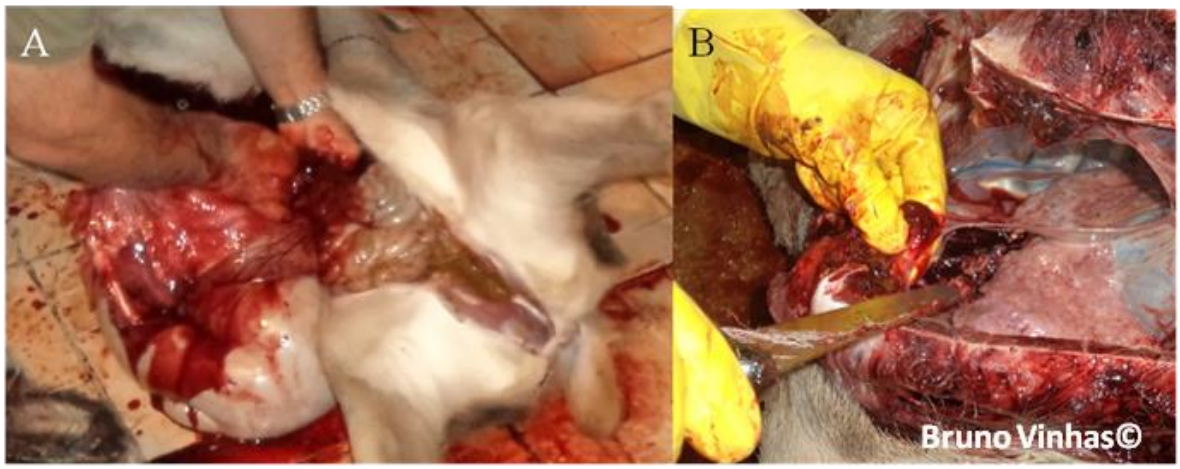

Image 36- Representative image of the use of gloves. A - Not wearing gloves. B – Wearing gloves.

In our opinion the overall result is a fine start but not excellent. And to the achievement of this overall positive result we must emphasize the tradition in large game in the County with

the veterinarian advice (more than 2 decades) and the City Hall efforts by offering a service for the correct elimination of the by-products. These two aspects were very important to HAs got this overall positive result. Nevertheless it is important to recognise what must be improve in order to keep ensuring the safety of game meat as well as the protection of animal and human health. This would be a crucial strategy and needed leverage to the economic development of this activity.

In this study were analysed muscular samples from 32 red deer (*Cervus elaphus*), 36 wild boar (*Sus scrofa*), 11 fallow deer (*Cervus dama*) and 10 mouflons (*Ovis ammon musimon*) for the presence of *Sarcocystis* spp.. A total of 243 muscular samples were submitted to histopathological analysis.

The *Sarcocystis* spp. occurrence found in each sampled animal species is summarized in Table 19 and the following figure shows the sampling location with the respective samples sizes (n) and the *Sarcocystis* spp. occurrence.

Table 19 - *Sarcocystis* spp. found in each sampled animal species.

Hunting Area	Type A/T	Red Deer		Wild Boar		Fallow Deer		Mouflon	
		Sampled	Inf. Sarc. n (%)	Sampled	Inf. Sarc. n (%)	Sampled	Inf. Sarc. n (%)	Sampled	Inf. Sarc. n (%)
1	As	4	1 (25)	-	-	-	-	-	-
2	As	-	-	3	0 (0)	-	-	-	-
3	As	7	2 (28.5)	15	5 (33.3)	-	-	-	-
4	As	-	-	-	-	-	-	-	-
5	As	-	-	-	-	-	-	-	--
6	As	2	1 (50)	11	7 (63.6)	-	-	-	-
7	As	1	1 (100)	1	0 (0)	-	-	-	-
8	As	1	1 (100)	3	1 (33.3)	-	-	-	-
9	As	-	-	3	1 (33.3)	-	-	-	-
10	To	9	8 (88.9)	-	-	-	-	-	-
11	To	4	4 (100)	-	-	-	-	-	-
12	To	-	-	-	-	-	-	-	-
13	To	2	1 (50)	-	-	11	11 (100)	10	10 (100)
14	To	2	2 (100)	-	-	-	-	-	-
Total		32	(65.6)	36	(38.9)	11	11 (100)	10	10 (100)

In this study, the overall results for the *Sarcocystis* spp occurrence were 65.63% (16/32) for red deer (*Cervus elaphus*), 38.89% (14/36) for wild boar (*Sus scrofa*), 100% (11/11) for fallow deer and 100% (10/10) for mouflon.

Unfortunately there are no *Sarcocystis* studies in Portugal, whether in hunting species whether in livestock, which could allow us to comparatively evaluate the results, obtained in this study.

When we compared our study with those studies in other countries it is possible to notice that the infection occurrence found in red deer (*Cervus elaphus*), was higher in Germany (98.0%, Partenheimer-Hannemann, 1991 cited by Kutkiene, 2003), United States (near 100%, Largerquist *et al.*, 1993; Foreyt, 1995) and Norway (100%) (Dalhgren, 2009). Concerning the eastern part of Slovakia there were found two different studies with two different results, one that presented an higher occurrence, (78.6%) (Goldová, *et al.*, 2008) and another one that presented an lower one (50%) (Hvizdošová, 2009). Referring now to wild boar (*Sus scrofa*), like

in red deer (*Cervus elaphus*), the infection occurrence was higher in the majority of studies found in literature, as the ones reported in the Netherlands and Germany (high up to 100%) (Tadros *et al.*, 1976 cited by Malakauskas, 2002; Erber 1978 cited by Malakauskas, 2002), in Lithuania (89.1%) (Malakauskas, 2002) and in eastern Slovakia (85.0%) (Goldová, 2008) and (83.3%) (Hvizdošová, 2009). Meanwhile a more resemble result was found in Poland, with 24.7% of specimens infected with *Sarcocystis* (Tropilo *et al*., 2001, cited by Malakauskas, 2002).

Nevertheless, in these studies found on literature some did not have information about the method and muscle sampled and in the ones which that information was given differed from our study (differ in method or muscle sampled or in both). For this reason, the differences from the values found in our study when compared with those found in literature may be explained for the non-concordant methodology.

A curious fact observed in this study (Table 19) was the occurrence found in fallow deer (100%) and mouflon (100%). After knowing the first results in red deer and wild boar we found interesting to collect samples from these two large game species although it were not contemplated in the initial study protocol. These samples were all collected in HA13 where only 2 red deer were analysed. From those, one was positive to *Sarcocystis* spp.. The results found in fallow deer and mouflon are expressive although the small number of samples. These results are coincident with the ones reached in two studies from Slovakia Republic that also revealed the presence of *Sarcocystis* in 100% of mouflons and fallow deer tested (Goldová, *et al.*, 2008) (Hvizdošová *et al.*, 2009). To further discussion would be important to determine the *Sarcocystis* specie involved and match its cycle with the environment and game species biology.

In the following images is presented the geographical distribution of the *Sarcocystis* spp. occurrence by drive hunting place in red deer and wild boar.

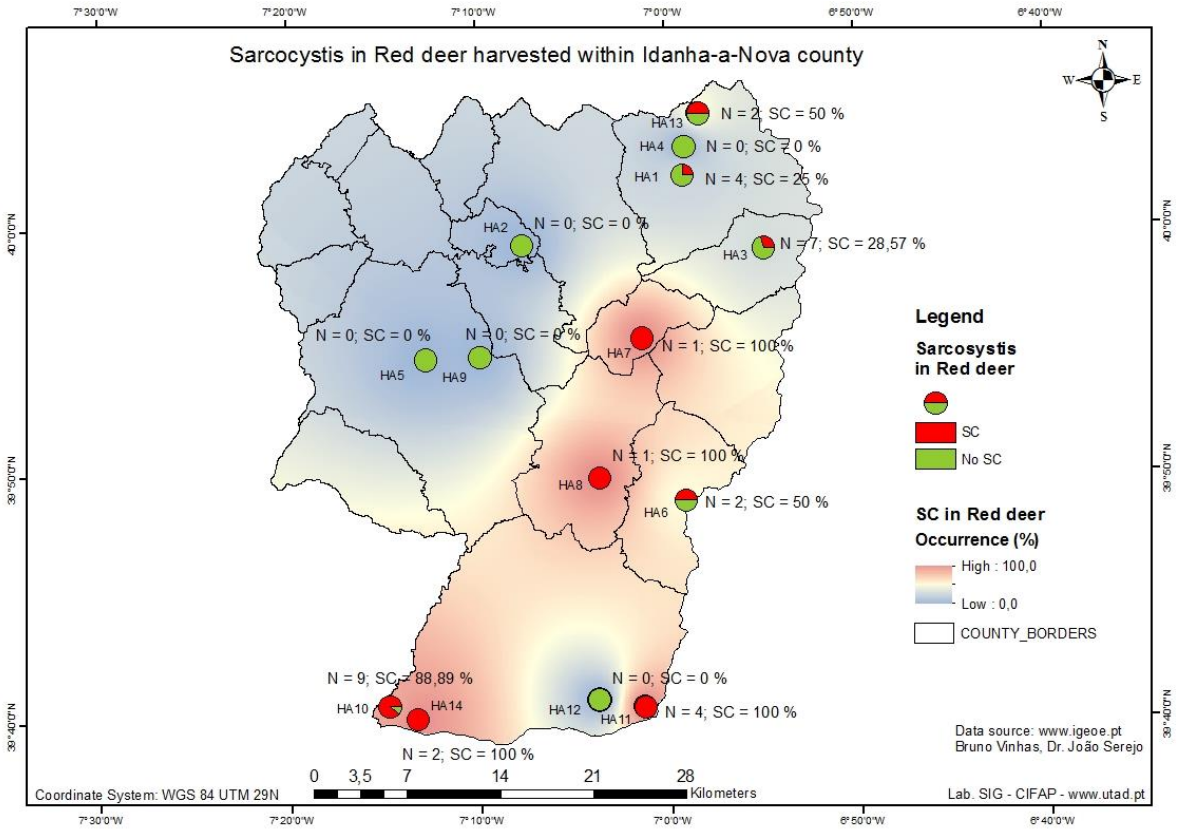

Image 37 – Geographical distribution of *Sarcocystis* occurrence in red deer harvested within Idanha-a-Nova county.

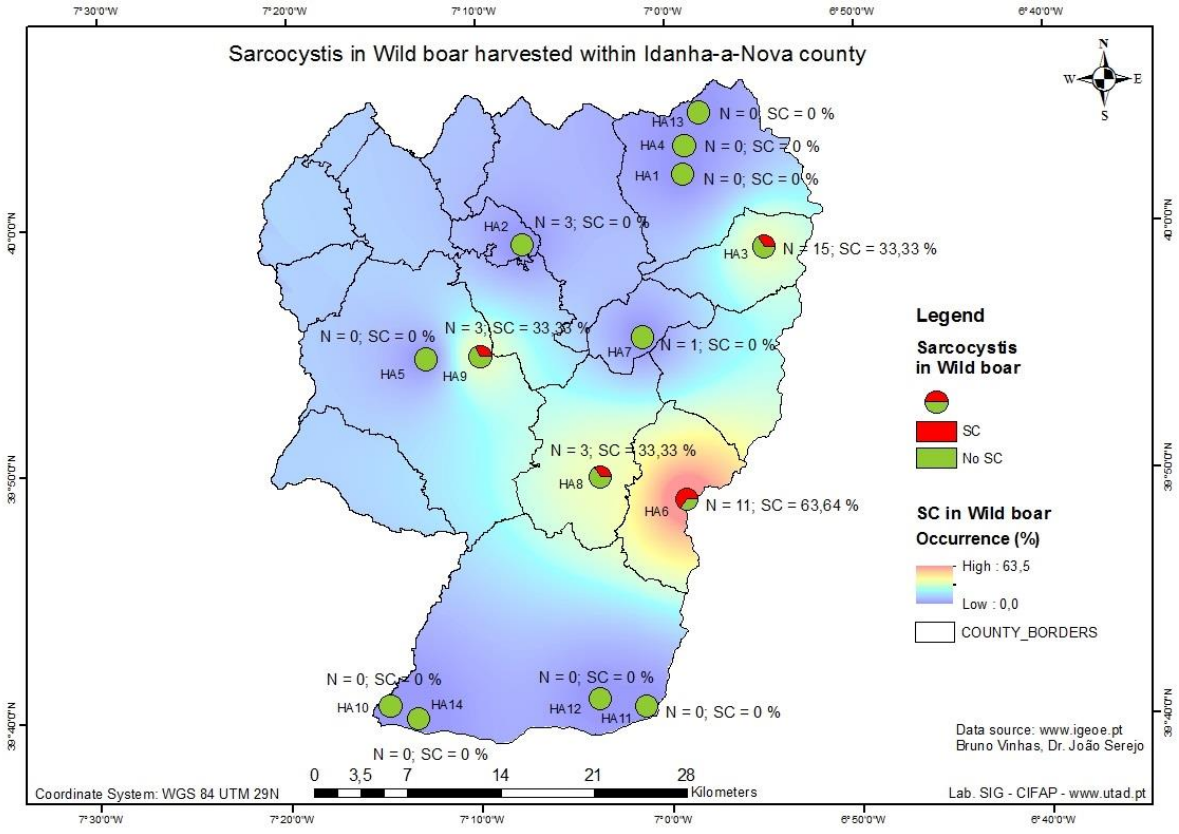

Image 38 – Geographical distribution of *Sarcocystis* occurrence in wild boar harvested in Idanha-a-Nova county.

Regarding Red Deer, when we analyzed the previous image we found a low number of samples per hunting area. But if we take into consideration only the two hunting areas with more samples we have HA10 and HA3 with an occurrence of 88.9% and 28.6% respectively. The occurrence in red deer found in HA10 is in accordance with the results found in most studies: 98.0% in Germany (Partenheimer-Hannemann, 1991 cited by Kutkiene, 2003), near 100% in United States (Largerquist *et al.*, 1993; Foreyt, 1995), 100% in Norway (Dalhgren, 2009), 78.6% in Slovakia (Goldová, *et al.*, 2008) and is higher than the overall red deer prevalence in this study (65.63%). In order to explain part of this occurrence found in HA10 (88.9%) we should have into consideration its location. HA10 is placed in the south of Idanha-a-Nova County in Tagus Natural Park, which is the area with highest population densities and high Tuberculosis prevalence (DGAC, 2010a; DGAV, 2010b).

Taking now into consideration the occurrence established in HA3 (28.6%), we found a similar result in literature: a study in Poland in which 24.7% specimens were infected with *Sarcocystis* (Tropilo *et al.*, 2001 cited by Malakauskas, 2002). Just to notice the result of HA3 is lower than the overall occurrence (65.63%). Other important fact to notice, when we analyse the red deer results by driving hunt place, is that in all places sampled were found *Sarcocystis*.

Referring to wild boar, we found the same problem as in red deer: the low number of samples in most places. But if we analyse, as in red deer, only the two hunting areas with more samples we have HA6 and HA3 with an occurrence of 63.6% and 33.33% respectively. These results are discrepant from the ones found in most literature: high up to 100% in Netherlands and Germany (Tadros *et al.*, 1976 cited by Malakauskas, 2002; Erber, 1978, cited by Malakauskas, 2002), 89.1% in Lithuania (Malakauskas, 2002) and 85.0% (Goldová, 2008) and 83.3% (Hvizdošová, 2009) in eastern Slovakia. Being the highest difference found to the HA3 results. On the other hand a study developed in Poland, shown a prevalence of 24.7% (Tropilo *et al.*, 2001 cited by Malakauskas, 2002), a result not far from the one found in HA3 (33.33%). Moreover, diverging from the red deer result, in wild boar there were drive hunting places where *Sarcocystis* was not found. Those places were HA2 and HA7 although in HA7 *Sarcocystis* was found in red deer.

As it was previously refereed, to further discussion of these results would be important to determine the *Sarcocystis* specie involved and match its cycle with the environment and game species biology.

Table 20 summarizes the occurrence of *Sarcocystis* spp. according to the age and gender of the sampled animals

Table 20 – *Sarcocystis* spp. occurrence according to the age and gender of the sampled animals.

Animal species	Male						Female					
	Adult		Young		Total		Adult		Young		Total	
	n	Pos (%)	n	Pos (%)	n	Pos (%)	n	Pos (%)	n	Pos (%)	n	Pos (%)
Red Deer (n=32)	21	16 (76.2)	2	1 (50)	23	17 (73.9)	9	4 (44.4)	0	0 (0)	9	4 (44.4)
Wild Boar (n=26)	5	1 (20)	6	1 (16,7)	11	2 (18.2)	9	5 (55.6)	6	1 (16.7)	15	6 (40)

Although it is not possible to make consistent analyses on this data due to the low number of samples of several classes in both species, we would like to draw attention to four aspects. First: as expected, older animals had a higher infection occurrence. Nevertheless, although wild boar adults showed to be more infected with statistic significance (p=5%, r=0,455*), in red deer there was no statistic significance (p=5%, r=0,138). According to the ecological law formulated by V.A. Dogel, the prevalence and intensity of parasites infection increases with age (Kutkiene, 2003). Second: referring to the occurrence between red deer genders (73.9% in males; 44.4% in females), in which were observed lower occurrences than the ones found in literature, 96% in males and 100% in females (Largerquist, 1993). Statistically, red deer males tend to be more infected with statistic significance (p=5%, r=0,452*) but in wild boar there was no relation between presence and absence of sarcocystis with statistic significance (p=5%, r=0,376). Third: the occurrence in young male and female wild boar was the same. This fact may be explained by the species behaviour, as young females and males live together in the first years of life (Acevedo, P. et al., 2006). Fourth: in red deer the males were the class with the highest occurrence while in wild boar were the females. In wild boar the fact may be explained by behaviour as adult females live in group and adult males are usually lonely (Acevedo, P. et al., 2006).

In Table 21 are presented *Sarcocystis* spp. distribution among the analysed muscle samples collected from both infected animals species.

Table 21 - *Sarcocystis* spp. distribution among the analysed muscle samples collected from both infected animals species

Animal species	% Inf. Heart		% Inf. Oesophagus		% Inf. Diaphragm	
	n	n Pos (%)	N	n Pos (%)	N	n Pos (%)
Red Deer	32	19 (59,4)	25	11 (44)	29	19 (62,1)
Wild Boar	31	8 (25,8)	30	1 (3,3)	35	9 (25,7)
Fallow Deer	11	11 (100)	11	9 (81,8)	11	8 (72,7)
Mouflon	10	10 (100)	9	9 (100)	9	9 (100)
Total	84	48 (57,1)	75	30 (40)	84	41 (48,8)

The results found in literature are, once again, higher than in our study. It was established 95% in red deer heart (Largerquist, 1993), 84.2% in red deer diaphragm (Malakauskas, 2002) and 89.1% in wild boar diaphragm (Malakauskas, 2002). Other interesting aspect is that diaphragm occurrence was higher than heart occurrence in red deer while the result was equal between these two muscles in wild boar. In these analyses we must also refer the zoonotic danger associated to the consumption of heart (57.1% infected) and diaphragm (62.1% infected) muscles, above all in rural areas. And to estimate the real danger present in game meat would be essential to determine the species involved in this infection. Because if this infection is perpetuated by one of the zoonotic *Sarcocystis* species previously referred a notice must be given to the veterinarian authorities so they may take the necessary measures to prevent human's infection. Those measures, already referred before, are avoiding the ingestion of cysts. When meat may be harbouring cysts we must thoroughly froze (-4ºC to -5ºC – 48h; 20ºC – 24h) or thoroughly cook (60ºC-20'; 70ºC-15'; 100ºC-5') the meat to kill infectious bradyzoites (Fayer, 2004; Mohammadi *et al.*, 2006).

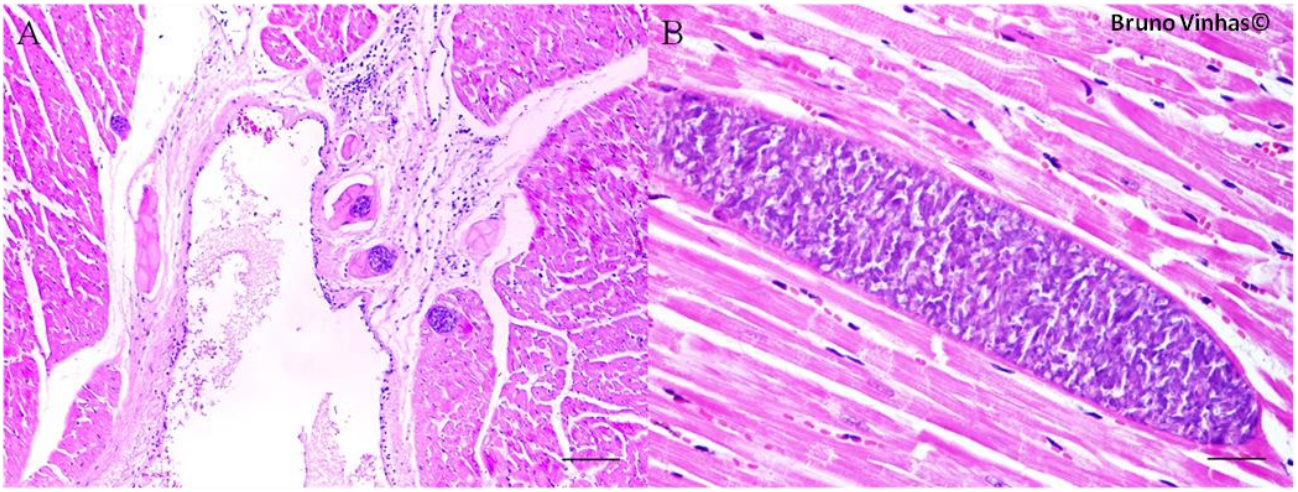

Image 39 – Representative image of meat harboring *Sarcocystis* spp. cysts. A - Mouflon Heart. Muscular tissue and Purkinje fibers with parasitic cysts. H&E. Bar=120µm. B – Red Dear Heart. *Sarcocystis* spp. cyst in a longitudinal cut. H&E. Bar=30µm.

Keeping the line of thought, determining the species would also be important in the management of herds. With that knowledge it would be possible to know which intermediate host is involved and try to establish measures to diminish the prevalence of *Sarcocystis* spp. in wild animal's populations. Because, as some field studies, prompted for the declining of mule deer population in USA, revealed there is a coincidence between increasing *Sarcocysts* infection rate in fawns with major fawn mortality (Hudkins, 1977). Others implications are that some species of *Sarcocysts* cause acute and chronic infections that may diminish the growth rate and/or health status of the cervid host (Dalghren, 2011). A study in Rocky Mountain elk revealed

that the mean weight gain of inoculated elk (27.1 ± 1.6 kg) was significantly ($P < 0.05$) less than that of controls (40.2 ± 4.9 kg) (Foreyt, 1995). So *Sarcocystis* spp. infection may be the cause of declining or a minor growth in population densities. This fact may diminish the quality of animals and thereby the value of the hunt. Other problem is the macroscopically detectable sarcocysts which may cause condemnation of meat, or even the entire carcass, during meat inspection. This fact has several reports in the 1970ies and 1980ies (Korbi, 1982, cited by Dalghren, 2011; Poppe, 1977, cited by Dalghren, 2011; Rognerud, 1978, cited by Dalghren, 2011) and leads to diminish the profit to hunting business. To a further discussion would also be important to have more information about the wild animals inhabiting the surveyed area.

In Table 22 it is presented *Sarcocystis* spp. distribution throughout matched samples collected from infected animals in order to analyse and understand the importance of each muscle as a source of human infection and as an elective sample for further diagnosis.

Table 22 – Presence of *Sarcocystis* spp. in matched samples collected from infected animals.

Specie	Number of Infected Animals (n)	Matched samples from infected animals (n)		
		Heart	Oesophagus	Diaphragm
Red Deer	3	3	-	3
	1	-	1	1
	8	8	8	8
TOTAL	12	11	9	12
Wild Boar	4	-	-	4
	4	4	-	-
	1	1	1	-
	1	1	-	1
TOTAL	10	6	1	5
Fallow Deer	7	7	7	7
	2	2	2	-
	1	1	-	-
	1	1	-	1
TOTAL	11	11	9	8
Mouflon	9	9	9	9
TOTAL	9	9	9	9

There were 14 positive animals that didn't present *Sarcocystis* in oesophagus: 3 red deer (heart and diaphragm infected), 9 wild boars (4 only heart infected, 4 only diaphragm infected, 1 heart and diaphragm infected) and 2 fallow deer (1 only heart infected and 1 heart and diaphragm infected). So in further studies the sample procedure could be restricted to heart and diaphragm mainly in wild boar. This restriction may help increase the number of samples.

Within infected red deer matched samples (12), there was only 1 case in which heart and diaphragm weren't infected and 11 in which both were infected. So, we cannot say that one of the muscles is more important to determine the infection in red deer. And, if it was only collected heart or diaphragm the result would be very similar. The statistical analysis on all red deer matched samples revealed that the relation between the presence and absence of *Sarcocystis* in:

a) heart and oesophagus is highly significant (p=5%, r=0.647***);
b) heart and diaphragm is highly significant (p=5%, r=0.931***);
c) oesophagus and diaphragm is highly significant (p=5%, r=0.760***).

Within infected wild boar matched samples (10), only in 1 case heart and diaphragm were infected in the same animal while there were 8 cases in which only heart or diaphragm were infected. So we can say, from this data, that both muscles have significant importance to determine infection in wild boar. The statistical analysis on all wild boar matched samples revealed that the relation between the presence and absence of *Sarcocystis* in:

a) heart and oesophagus has no statistic significance (p=5%, r=0.361);
b) heart and diaphragm has no statistic significance (p=5%, r=-0.059);
c) oesophagus and diaphragm has no statistic significance (p=5%, r=-0.112).

Within infected fallow deer matched samples (10), only in 1 case heart and diaphragm were infected in the same animal while there were 8 cases in which only heart or diaphragm were infected. So we can say, from this data, that both muscles combined have significant importance to determine a more accurate infection occurrence in wild boar. The statistical analysis on all wild boar matched samples revealed that the relation between the presence and absence of *Sarcocystis* in:

a) the fallow deer heart and oesophagus were all infected, two only in heart and nine in heart and oesophagus which unable the statistic analysis;
b) the fallow deer heart and diaphragm were all infected, two in heart and nine in heart and oesophagus which unable the statistic analysis;
c) the relation between the presence and absence of *Sarcocystis* in oesophagus and in diaphragm has no statistic significance (p=5%, r=0.241).

Within infected mouflon matched samples (11), all animals were infected as all muscles. So, we cannot say that one of the muscles is more important to determine the infection occurrence. The statistical analysis on mouflon matched samples confirms this statement as it was unable by the inexistence of negative samples.

5. Conclusion

The Tcl occurrence assumed by our study stands in 9.9% to red deer and 28.4% to wild boar. These high occurrences underline the tuberculosis as a very serious sanitary problem in large game species in Idanha-a-Nova County. This matter gets even more severe if we analyze by driving hunt place. In some cases tuberculosis prevalence reaches 18.2% or 15% in red deer and 83.3%, 81.8% or 60% in wild boar. This fact exalt the importance of the continuing monitoring (prevalence, by-products destination, hygiene rules respect in hunting activities) and inspection of all large game animals hunted in this County in order to avoid the consumption of infected carcasses or by-products by humans or animals. Monitoring, prevention and inspection are the only measures we have to control the disease and avoid the spreading of the infection, as wild animals are free and not possible to be regularly controlled alive.

Still regarding the tuberculosis problem, the assessment of the hygiene conditions of the site where sanitary evaluation takes place demonstrated that Idanha-a-Nova HA's had a good starting point. Nevertheless would be important to keep the good work with the improvement of parameters, such as Personal Cleaning/Disinfection Devices (PCDD), Knives Utilization (KnU), Garment (G), Footwear (Fw), Mask (M) and Proper Drainage (PD). These aspects are vital to diminish as most as possible the probability of contamination and spreading of tuberculosis, as well as other infectious diseases, to humans and animals.

Concerning the main matter aimed by this survey, the *Sarcocystis* spp. occurrence in Idanha-a-Nova County, it stands in 65.6% to red deer and 38.9% to wild boar. In some specific driving hunts it reaches 90.9% or even 100% in red deer and 63.6% in wild boar. The result obtained in the analyses of the fallow deer (100%) and mouflon (100%) samples was an important factor to unveil the interest in spread the study to these species in order to try to understand the high occurrence observed. To that understanding would be also crucial, in further surveys, determining the *Sarcocystis* species involved. Knowing the specie involved is essential to understand the prevalence in which the parasite life cycle has a key role, with an especially spotlight on the intermediate hosts implicated. In the same way, determining the *Sarcocystis* species involved is also important for the zoonotic point of view. As being conscious if we are dealing with a zoonosis is essential to take the necessary measures to monitoring, identify the risk of game meat and preventing human infection. From the zoonotic point of view would also be important to deepen the knowledge in *Sarcocystis* spp. cycle referred previously in "Gastrointestinal Sarcocystosis and Prevention". In particular, would be important to determine the invasive cycle species, the cycle itself and clarify the public health impact. This study also reminds the importance of crossing this kind of studies with veterinarian sanitary work in game

animals in order to be aware of the public health risk in game meat. Other interesting deduction is that the higher occurrences of *Sarcocystis* spp. coincide with higher population densities and higher occurrences of tuberculosis located in the south of the County. A study trying to comprehend if there is a correlation between these factors may have some interest. Other important deduction of this study is that in future sample collection the oesophagus muscle may be withdrawn as it is not crucial to determine *Sarcocystis* prevalence.

Additionally, this kind of studies is important to the consistent and conscious management of the hunting herds. As *Sarcocystis* spp. infection may be the cause of some declining or a minor growth in population densities. This parasite may diminish the quality and quantity of game animals and thereby the value of the hunt. And if we want to turn this area in an economic efficient business, being the source to local economic development in poor regions, as some actors in hunt activity pronounce, is vital to support that wish in investment in specialized knowledge and technicians.

To summarize, more studies are need and to go further in the discussion and conclusions those studies should be spread to other species, have a larger number of samples and determine the *Sarcocystis* species involved in the infection. The applied diagnostic approach revealed high level of *Sarcocystis* spp. occurrence, underlining that life cycle and zoonotic potential should be further investigated and indicates that several issues should be addressed when planning surveillance and prevention actions. I would like to finish with the sentence: we only find what we look for.

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6. Bibliography

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7. Annex

Annex I

Conditions Report Sheet

Condições do local da Inspecção - Ficha Individual

Local:_____ Data:_____

Hora da caçada:_____ Hora da I.S.:_____

Infra-estruturas

- Água:_____
- Iluminação:_____
- Acesso de Animais:_____
- Escorrência dos Solos:_____
- Suspensão do Animal:_____

Higiene

- Local
  - Início:_____
  - Final:_____
- Pessoal
  - Comportamento:_____
  - Protecção Individual:_____
- Utensílios:_____

Carcaças

- Condições de Transporte:_____
- Correspondência com as vísceras:_____
- Inspecção da cabeça:_____
- Destino:_____
- Marcação (se para consumo):_____

Subprodutos

- Destino:_____
- Condições de transporte:_____

Obs:_____

_____

_____

_____

_____

Annex II

Harvest Report Sheet

Universidade de Trás-os-Montes e Alto Douro

Relatório de caçada

Local: _____ **Data**: _____ **Nº animais caçados**: _____

Espécie	Total	Sexo		Jovem	Sub Adulto	Adulto
		M	F			
Veado						
Javali						
Nota (Gamo/Muflão)						

Amostragem

Espécie	**Nº**	**Idade**	**Sexo**	**Colheita**			**Observações/Lesões/Achados**	**Foto**	**Refª da Amostra**
				Eso	**Cor**	**Dia**			

ANOTAÇÕES:

<u>COLHEITA DE AMOSTRAS:</u> Esófago (porção mais junto ao cárdia - formol) – cerca de 60gr; **Coração (ventrículo esquerdo, ápice - formol) – cerca de 100g; Diafragma (dá-se preferência aos pilares do diafragma) – cerca de 100gr**