MORPHOMETRIC AND MOLECULAR CHARACTERIZATION OF *SARCOCYSTIS* SPP. IN CATTLE (*BOS* SPP.) AND GOATS (*CAPRA HIRCUS*) COLLECTED FROM THE SHAH ALAM ABATTOIR

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ABSTRACT

Sarcocystosis in meat-producing animals is a major cause of reduced productivity in many countries, especially those that rely on agriculture. Although several diagnostic methods are available to detect sarcocystosis, many are too time-consuming for routine use in abattoirs and meat inspection centres, where large numbers of samples need to be tested. Current establish method (transmission electron microscope) for *Sarcocystis* spp. identification is expensive and time-consuming. Alternatively, tissue compression technique can rapidly detect sarcocysts in tissue samples. Additionally, cysts measurement in corroboration with genotyping of 18S rDNA allows comprehensive species identification. Sequences of *Sarcocystis* spp. can also be analysed via phylogenetic tree to provide better understanding of the evolution of *Sarcocystis* spp. corresponding to the respective intermediate hosts. Furthermore, phylogeny analysis enables effective species differentiation of sarcocyst, especially for those closely related species that are morphologically similar. Tissue samples of cattle and goats were collected from the Shah Alam abattoir, Selangor. Samples were pre-screened for the presence of sarcocysts via tissue compression technique (group of unstained (methylene blue) and stained condition). Positive sample tissues were further confirmed by nested PCR by targeting the 18S rDNA regions. Statistical calculations were done to determine the efficacy of stain-based technique compared to the standard unstained technique of sarcocysts detection in tissue samples. Besides, sarcocysts detected in tissues were measured for morphometric study and then subjected for genotyping to determine the identity. Sequences were then used for phylogenetic tree analysis. A total of 583 tissue samples were collected. Of these, 245 samples were positive under pre-screening phase. Mc Nemar's and Cohen kappa analysis show that stain-based technique is able to detect three-folds higher sarcocysts and it is statistically in concordance with nested PCR outcome (gold standard). Morphometric study deduced that possibly one *Sarcocystis* sp.

infects cattle whereas two species in goats. Molecular BLAST analysis and morphometric findings confirmed that *Sarcocystis cruzi* was found in cattle, whereas goats were infected by *Sarcocystis capracanis* and *Sarcocystis tenella*. Phylogenetic analysis using nine representative sequences confirmed the identity of the two species, in addition of *S. tenella* where the corresponding sequences were separated from the reference gene and formed a subclade. All *Sarcocystis* spp. found shared dog as a definitive host. Dogs are often used in cattle farms to monitor the herds. Poor management in the farmlands potentially boosts up the dynamics of disease transmission between the hosts. The "*S. tenella*-like", maybe refers to *Sarcocystis hircicanis* rather than *S. tenella* where it also uses dogs as a definitive host. Insufficient or lack of the gene information of *S. hircicanis* in the GenBank explains the failure of BLAST to match the 18S rDNA of the *Sarcocystis* sp. with *S. hircicanis*.

ABSTRAK

Sarkositosis di dalam haiwan penternakan merupakan punca utama pengurangan produksi di banyak negara, terutamanya bagi negara yang berasaskan pertanian agrikultur. Walaupun terdapat beberapa kaedah diagnostik untuk mengesan penyakit ini, tetapi kebanyakan mengambil masa yang lama untuk penggunaan rutin di rumah sembelih dan pusat pemeriksaan daging, di mana kuantiti sampel daging yang tinggi perlu diperiksa. Kaedah terkini (mikroskop elektron transmisi) untuk tujuan pengenalpastian *Sarcocystis* spp. mahal dan mengambil masa. Secara alternatif, teknik pemampatan tisu dapat mengesan sarcocysts di dalam sampel tisu dalam jangka masa yang pendek. Selain itu, pengukuran sist bersertakan dengan genotip 18S rDNA membolehkan proses pengenalpastian parasit lebih terperinci. Jujukan gen *Sarcocystis* spp. juga boleh dianalisis melalui pokok filogenetik bagi meningkatkan pengetahuan terhadap evolusi *Sarcocystis* spp. secara serentak serta hos perantaraan masing-masing. Tambahan pula, analisa filogenetik juga boleh membezakan *Sarcocystis* spp. dengan lebih berkesan terutamanya bagi kumpulan yang berkait rapat serta mempunyai morfologi sarkosist yang sama. Sampel tisu daripada lembu dan kambing dikumpulkan di rumah penyembelihan Shah Alam, Selangor. Semua sampel menerusi pemeriksaan pra-penyaringan untuk mengesan kehadiran sarkosist melalui teknik pemampatan tisu (Dalam kumpulan berwarna (methylene biru) dan tidak berwarna untuk perbandingan). Sampel tisu positif disahkan dengan lanjut dengan menggunakan PCR bersarang yang berfokus kepada 18S rDNA. Pengiraan statistik telah digunakan untuk menentukan keberkesanan teknik berwarna berbanding dengan teknik tidak berwarna bagi pengesanan sarkosist dalam sampel tisu. Di samping itu, sarkosist yang dikesan dalam tisu telah digunakan dalam kajian morphometri dan kemudian ditentukan spesies parasit menerusi genotip genetik. Jujukan gen juga digunakan dalam analisa pokok filogenetik. Sejumlah 583 sampel tisu telah dikumpul. Daripada jumlah ini, 245 sampel disahkan

positif dalam pemeriksaan peringkat awal. Analisa Mc Nemar and Cohen kappa menunjukkan kaedah penggunaan warna dapat mengesan lebih tiga kali ganda sarkosist dan berkonkordansi secara statistik dengan PCR bersarang (piawai emas). Kajian morphometri mengesahkan terdapat satu *Sarcocystis* sp. yang menjangkiti lembu manakala dua species dikesan dalam kambing. Analisa molekular BLAST dan kajian morphometri menunjukkan *Sarcocystis cruzi* dikesan dalam lembu, manakala kambing dijangkiti oleh *Sarcocystis capracanis* and *Sarcocystis tenella*. Analisa filogenetik menggunakan sembilan wakil jujukan gen telah mengenalpastikan dua spesies, selain daripada *S. tenella* di mana jujukan-jujukan gennya terpisah daripada gen rujukan dan membentuk klad kecil. Semua *Sarcocystis* spp. yang dijumpai dalam kajian ini menggunakan anjing sebagai hos muktamad. Anjing sering dijumpai dan digunakan dalam ladang lembu untuk membantu memantau kumpulan lembu. Pengurusan yang tidak prihatin di ladang berpotensi untuk meningkatkan dinamik jangkitan penyakit di antara hos perantaraan (lembu) dengan hos muktamad (anjing). Jujukan menyerupai "*S. tenella*", berkemungkinan besar merujuk kepada *Sarcocystis hircicanis* di mana anjing juga digunakan sebagai hos muktamadnya. Kekurangan informasi gen *S. hircicanis* dalam GenBank menjadi punca utama kegagalan analisa BLAST untuk memadankan 18S rDNA daripada *Sarcocystis* sp. dengan *S. hircicanis*.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

:	ratio
µm	micro meter
µl	micro litre
mg	milligram
k	kilo
kg	kilogram
kb	kilobase
+ve	positive
-ve	negative
<	less than
%	percent
˚C	degree Celsius
mM	millimolar
M	molar
mm	millimetre
h	hour
g	gram
g	standard gravity
ml	millilitre
m	meter
min	minute
n	sample size
s	second
S	svedberg unit
U	unit

x	time
χ^2	chi-square
p	p value
κ	Cohen kappa
$\tilde{x}$	median

IFAT	Indirect Fluorescent Antibody Test
BLAST	Basic Local Alignment Search Tool
bp	base pair
$CaCl_2$	calcium chloride
CI	confidence interval
COXI	cytochrome c oxidase subunit I
ddH_2O	distilled and deionised water
DNA	deoxyribonucleic acid
dNTPs	nucleotide triphosphate
DPI	days post infection
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
et al.	*Et alii/alia* ("and others")
GTR	generalised time reversible model
IQR	Inter-quartile range
ITS 1	internal transcribed spacer 1
ITS 2	internal transcribed spacer 2
LB	Luria-Bertani
$MgCl_2$	magnesium chloride
$MgSO_4 \cdot 7H_2O$	magnesium sulfate heptahydrate

NJ	neighbour-joining
NNI	nearest neighbour interchange
NPV	negative predictive value
OTU	operational taxonomical unit
MP	maximum parsimony
ML	maximum likelihood
PCR	polymerase chain reaction
PEG	polyethylene glycol
pmoles	pikomoles
PPV	positive predictive value
psi	pound-force per square inch
rDNA	ribosomal DNA
rRNA	ribosomal RNA
rpm	revolutions per minute
RT	room temperature
sp.	species (singular)
spp.	species (plural)
ssu	small subunit
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
UV	ultraviolet
V	version
Vn	Variable region where n corresponding to the numerical figure

LIST OF APPENDIXES

CHAPTER 1: INTRODUCTION

1.1 Overview

Sarcocystosis infection has become a global concern since the reports of several outbreaks involving tourists who visited Malaysia, especially Tioman and Pangkor islands between 2012 to 2014 (Esposito *et al*., 2012; Esposito *et al*., 2014; Italiano *et al*., 2014). *Sarcocystis* are cyst-forming parasites, normally found in organs such as tongue, oesophagus, diaphragm, heart and skeletal muscle (Prakas & Butkauskas, 2012). Like *Toxoplasma gondii*, *Sarcocystis* requires two hosts to complete their life cycle, based on a prey-predator relationship. The complex interactions between prey (intermediate hosts) and predator (definitive hosts) contribute to the diversity of *Sarcocystis* spp. and these impel the parasites to co-evolve with their corresponding hosts, particularly in the definitive hosts (Dolezel *et al*., 1999). To date, there are more than 220 *Sarcocystis* spp. that have been documented which are able to infect a wide range of animals ranging from mammals, avian, fishes, and reptiles (Prakas & Butkauskas, 2012).

Sarcocystosis causes deterioration in the wellbeing of infected animals and results in reduced milk production, spontaneous abortions, and even death of heavily infected animals (Fayer, 2004). In addition, infected carcasses can transmit the disease to other hosts via the faecal-oral route, including humans, and symptoms include nausea, vomiting, and enteritis (acute, chronic and severe) (Fayer *et al*., 2015). Unfortunately, this disease has often been neglected due to the difficulties in diagnosis. Sarcocysts, a prominent form of *Sarcocystis* spp., are usually found embedded deeply into the host muscle tissues. This has often been overlooked during meat quality inspection. Although there are several methods for microscopic diagnosis of sarcocystosis, these techniques are too time-consuming for routine diagnosis, especially in meat inspection centres. In contrast, the muscle compression method is considered to be a rapid direct method for detecting sarcocysts, but there are concerns that its sensitivity is lower than

the pepsin digestion technique, indirect fluorescent antibody test (IFAT), and squeezing (Latif *et al*., 1999). Alternatively, this technique can be improved by utilising methylene blue. Methylene blue binds strongly to nuclei of parasites, including sarcocysts (consisting of hundreds to thousands of bradyzoites). This dye has been widely applied in the field of parasitology to diagnose diseases including Malaria, Chagas disease and sarcocytosis (Ferreira *et al*., 2006; Malakauskas & Grikienienė, 2002; Mohapatra *et al*., 2014; Prakas *et al.*, 2011; Pyziel & Demiaszkiewicz, 2009).

In addition to the above methods, molecular detection of sarcocysts by the polymerase chain reaction (PCR) has improved the efficiency of diagnosis and allowed for accurate species identification (Yang & Zuo, 2000). PCR requires only small amounts of template DNA, and therefore, ideal for highly sensitive and specific *Sarcocystis* spp. detection. In addition, the sensitivity and specificity can be further enhanced by using nested PCR, in which a second round of amplification is used to amplify target within the first product using a second set of primers (Pereira *et al*., 2008). Nested PCR has been successfully used to detect and identify *Sarcocystis* spp. in snakes and ruminants by partial 18S rRNA gene genotyping (Heckeroth & Tenter, 1999; Lau *et al*., 2013; Tenter *et al*., 1994). In addition, this technique has also now transformed the taxonomical organisation of organisms. An unidentified species can be determined by comparing specific genes, such as the small subunit ribosomal RNA, with an established gene database. Furthermore, phylogenetic tree analysis can lead to the discovery of new species or grouping of closely related species. The tree analysis has been proven useful in determining *Sarcocystis* spp. in ruminants and snakes (Lau *et al.*, 2013; More *et al*., 2008; Slapeta *et al.*, 2003).

1.2 Problem statements

Cattle and goats are some of the main sources of meat for human. The demand for these meat produce has increased enormously. However, cattle and goats are reservoirs of several *Sarcocystis* spp. For instance, *Sarcocystis cruzi*, *Sarcocystis hominis*, *Sarcocystis hirsuta* (Dubey *et al*., 1989c) and recently discovered *Sarcocystis sinensis*/*Sarcocystis rommeli* (Dubey *et al.*, 2015a; More *et al.*, 2014), are known to infect cattle, whereas goats are susceptible to *Sarcocystis capracanis*, *Sarcocystis hircicanis* and *Sarcocystis moulei* (Dubey *et al*., 1989d). However, not all *Sarcocystis* spp. are pathogenic to their hosts (Dubey *et al*., 1989b). *Sarcocystis cruzi* is the most pathogenic species to cattle in comparison to *S. hominis*, *S. hirsuta* and *S. sinensis/S. rommeli*. Likewise, goats are vulnerable to *S. capracanis* but not to the other species. Experimental infection demonstrated that the lethal dose of *S. capracanis* sporocysts could be as low as 100000, in contrast to the 10 million of sporocysts of *S. hircicanis* (Dubey *et al.*, 1989d).

Prevalence of sarcocystosis is closely associated with choices of meat eating, and cultural practices, as well as livestock (food resources) management. In Malaysia, the demands on red meats, particularly cattle and goats, are increasing dramatically. The high consumption of meats leads to an increase in demand, with the increment of 17.33% and three-folds in cattle and goats respectively, between 2010 and 2014 (Department of Veterinary Services, Ministry of Agriculture & Agro-based Industry Malaysia, 2015). Changing eating habits, such as the consumption of raw or lightly cooked meats are some of the main drivers affecting the emergence or re-emergence of many food-borne parasitic diseases including the risk of sarcocystosis transmission. Previous study has highlighted that the sarcocystosis in cattle in Malaysia were mainly caused by three species, notably *S. cruzi*, *S. hominis* and *S. hirsuta* (Dissanaike & Kan, 1978). However, recent investigation has shown only *S. cruzi* in imported Droughtmaster cattle (Latif *et*

al., 2013). Information on goat sarcocystosis cases is scarce and the extent of infection in goats is unknown.

The lack of information in these animals raises some important questions such as what is the prevalence and diversity of *Sarcocystis* spp. present in the meat of the livestock, particularly cattle and goats, that are sold in Malaysia? This is especially important as these livestock are the main meat resources to boost the economic status of the agricultural sector of Malaysia.

1.3   Objectives

These are the objectives to answer the research questions of current study:

1) To evaluate the efficacy of the methylene blue stained muscle compression technique compared to the unstained muscle compression technique and the nested PCR technique for the detection of *Sarcocystis* cysts in muscle tissue.

2) To identify *Sarcocystis* spp. in cattle and goats via phylogenetic analysis of 18S rDNA sequences and to evaluate their genetic variants.

3) To determine the usability of morphometric measurements data for *Sarcocystis* spp. size variations of sarcocysts detected in different organ muscle tissues.

CHAPTER 2: LITERATURE REVIEW

2.1   Background

Sarcocystis, previously known as Miescher's tubules, was discovered by Miescher in the year of 1843. The tubule appears to be a "milky white threads" which parasitised house mouse (*Mus musculus*). Miescher found these threads on the striated of muscles through autopsy (Dubey *et al.*, 1989b; Fayer, 2004). Similar organisms were discovered by Kühn in 1865 from swine and named as *Synchytrium miescherianum*, which were then re-named as *Sarcocystis meischeriana* by Labbe in 1899 since the genus of *Synchytrium* has been used to name another organism (Dubey *et al.*, 1989b).

Sarcocystis is a protozoan that belongs to the family Sarcocystidae. Like *Toxoplasma gondii* and *Neospora caninum, Sarcocystis* spp. are coccidian parasites with heterogeneous life cycles that involve intermediate and definitive hosts. Briefly, definitive hosts (predators) become infected when they consume infected intermediate hosts. The intermediate hosts get infected when they consume the silages contaminated with the faeces containing oocysts of *Sarcocystis* spp. from infected definitive hosts (Fayer *et al*., 2015). Livestock, including cattle, goats and sheep, are susceptible to sarcocystosis (Latif *et al*., 1999), and symptoms include fever, haemorrhages, low meat and milk yields, abortion, encephalomyelitis, and even death in severely infected individuals (Fayer *et al*., 2015).

2.2   Life cycle of *Sarcocystis* spp.

The life cycle of *Sarcocystis* spp. consists of sexual and asexual stages, which can be further divided into three phases known as sporogony, schizogony and gametogony. Sporogony occurs when sporozoites begin to invade the intermediate host. Schizogony is a form of asexual reproduction in the intermediate host where multiple fissions are involved. Lastly, gametogony, a sexual reproduction phase, occurs after

fertilisation of male and female gametes in the definitive hosts (Dubey, 1976; Fayer, 1972).

Figure 2.1 illustrates the details of a life cycle of *S. cruzi*, one of the three known *Sarcocystis* spp. which parasitises cattle besides *S. hirsuta* and *S. hominis*. Definitive hosts mostly are carnivorous animals from the family of Canidae (e.g. foxes and dogs) and Felidae (e.g. tigers and cats). Some omnivorous animals, especially those from the family of Hominidae like monkey and human beings, are likely to be the definitive hosts as well. On the other hand, intermediate hosts are mostly herbivorous which originated from the family of Bovidae such as cattle, goat, and sheep. *Sarcocystis cruzi* exhibits both sexual and asexual reproduction in the definitive and intermediate hosts, respectively.

2.2.1 Sexual reproduction

The life cycle begins when the bradyzoites of *S. cruzi*, liberated from sarcocyst due to the digestion process of definitive host's acidic enzymes in the stomach. The bradyzoites move and penetrate actively through the mucosa layer, generally known as lamina propria in the small intestine. These bradyzoites then undergo gametogony, where multiple gamonts differentiate into microgamonts (male) and macrogamonts (female), in the ratio of 5:95 (Dubey *et al.*, 1989b). Microgamonts then rupture and release the microgametes. These microgametes then migrate and approach the macrogametes and by fertilisation form the zygotes which eventually develop into oocysts. Each matured oocyst contains two identical sporocysts. The thin cell wall of oocysts ruptures easily. Commonly, an infected definitive host sheds up to millions of oocysts and sporocysts, but the number is highly dependent on the *Sarcocystis* sp. itself. Faeces which contained infective oocysts and sporocysts can contaminate the silages and infect intermediate hosts through digestion.
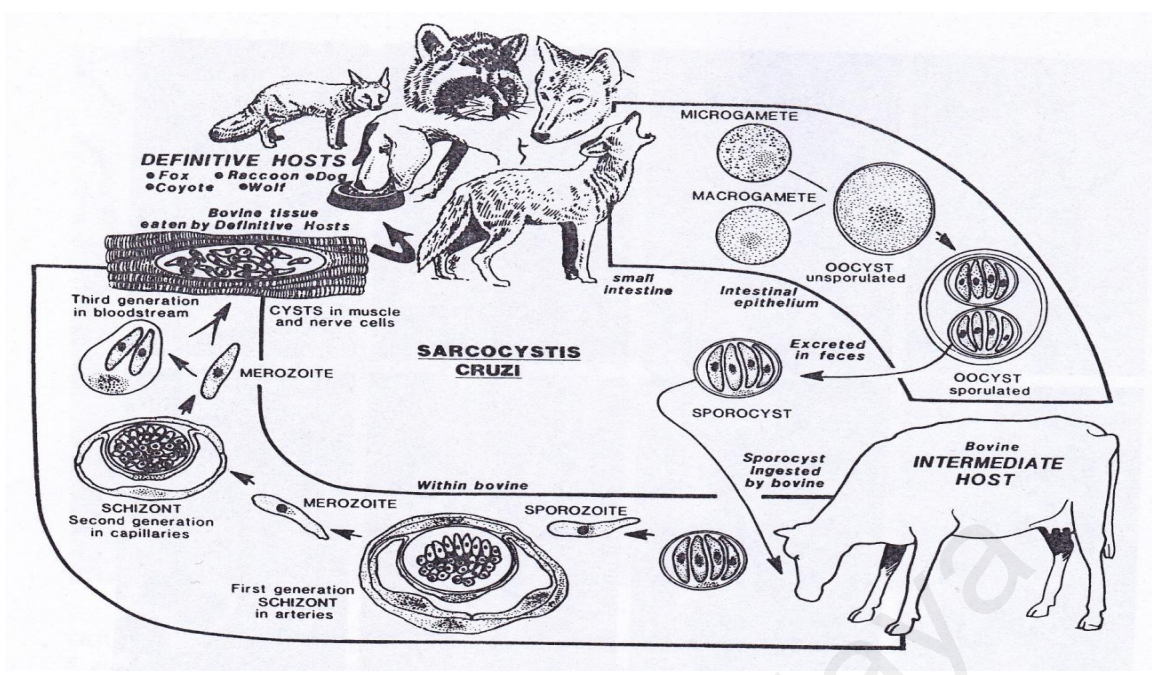
Figure 2.1: Life cycle of *Sarcocystis cruzi* (Dubey *et al.*, 1989b)

2.2.2   Asexual reproduction

When intermediate hosts consume the contaminated water and silages with the sporocysts, four sporozoites will be liberated from each sporocyst in the small intestine of the intermediate hosts. These sporozoites invade the endothelial cells and form schizonts through schizogony. This initiates the asexual reproduction cycle. There are two generations of schizonts development in *S. cruzi* where schizogony happens in endothelial cells on 7-15 days post infection (DPI) and 19-46 DPI (Dubey *et al.*, 1989b). *Sarcocystis cruzi* merozoites can be seen in between 24-46 DPI (Dubey *et al.*, 1989b). Merozoites migrate to the muscle tissues and ultimately form sarcocysts. Metrocytes, the early stage of sarcocysts, divide rapidly through binary fission to produce large number of bradyzoites (Lindsay *et al.*, 1995). Matured bradyzoites are liberated from the mature sarcocysts when the meat is consumed by definitive hosts such as dogs or wolfs and this completes the entire life cycle.

2.3   Epidemiology

Sarcocystosis can be found in majority of animals (Dubey *et al.*, 1989b). Prakas and Butkauskas (2012) noted large numbers of hosts (intermediate/definitive hosts) that are vulnerable to *Sarcocystis* spp., which are categorised into groups of mammals, birds, reptiles and others organisms. Of these, mammals (both intermediate and definitive hosts) are most susceptible to sarcocystosis.

Definitive hosts, such as canine and feline, are reservoirs for a variety of *Sarcocystis* spp. Due to the sustainability of these parasites in definitive hosts, they can constantly produce large amount of oocysts. In addition, *Sarcocystis* oocysts are able to tolerate harsh environmental conditions (Cawthorn *et al.*, 1984) and sporulate conveniently without relying to the weathers and seasons (Dubey *et al.*, 1989b; Fayer, 2004). Consequently, this increases the risk of infection and raises prevalence of

sarcocystosis in endemic area.

The prevalence of *Sarcocystis* is dependable on the species itself. For instance, *Sarcocystis* spp. infecting feline appear to be rare when compared to those infecting canids (Dubey *et al.*, 1989b). There are few explanations about this phenomenon. Firstly, infection in feline has significant lower yield of sporocysts. Additionally, the duration required for infective stages to mature is also significantly longer (several months to years). Lastly, some hosts are genetically more resistant towards the *Sarcocystis* spp. particularly in feline thus impeding the parasite's growth (Dubey *et al.*, 1989b).

2.3.1 South America and Europe

Ruminants in countries such as Argentina, Lithuania, and Spain have reported high prevalence of sarcocystosis. In Argentina, More *et al.* (2008) detected sarcocysts of *S. cruzi* in cattle by microscopy in all cardiac muscles (100.0%), followed by oesophagus and diaphragm muscles with infectivity rate of 71.0% and 28.0%, respectively. Similarly, all myocardium samples were reported positive with sarcocysts by the same corresponding author years later (More *et al.*, 2011). Besides, a study by Italians also indicated that there is a relatively high prevalence (78.1%) of sarcocystosis among semi-breed cattle in north-western Italy (Domenis *et al.*, 2011). There were also multiple reports on the prevalence of sarcocystosis in cattle ranging from 77.0% to 100.0% in other European countries such as Belgium, Croatia, Czechoslovakia, Holland, and Southern Germany (Boch & Erber, 1981; Cerna *et al.*, 1981; Van Knapen *et al.*, 1987; Vercruysse *et al.*, 1989; Wikerhauser *et al.*, 1981).

2.3.2. Iran

Iran is reported to be one of the highest prevalence nation in sarcocystosis where the affected livestock are mostly cattle, goats and sheep. Hamidinejat *et al.* (2010)

reported 94.7% of the examined cattle were infected with sarcocystosis in South-West of Iran. In addition, a recent finding highlighted that cattle slaughtered at the Karaj abattoir, Iran, were heavily infected with *S. cruzi* and *S. hominis*, with the prevalence of 96.8% and 34.7%, respectively (Nourollahi-Fard *et al.*, 2013). Similarly, high prevalence of goat sarcocystosis was reported (94.4%) in the slaughter house in Shiraz (Shekarforoush *et al.*, 2013), meanwhile Dehaghi *et al.* (2011) found out that approximately 99.0% of goats in the Kerman abattoir were diagnosed positive with sarcocystosis. Likewise, the prevalence of sheep sarcocystosis was also very high (97.1%), from the slaughtered sheep in Yarz Province (Hajimohammadi *et al.*, 2014).

2.3.3   East Asia

Sarcocystosis has been extensively reported in Asian countries such as China, Tibet, Mongolia and Japan. In South China, all domestic animals from cattle (*Bos taurus*), buffaloes (*Bulbalus bulbalis*) and swine (*Sus domestica*), were infected by *Sarcocystis* spp. (Olias *et al.*, 2010). In Mongolia, Fukuyo *et al.* (2002) highlighted a very high prevalence (100.0%) of sarcocystosis among meat-producing animals including cattle (*Bos taurus*), yak (*Bos grunniens*), hainag (a crossbreed animal between cattle and yak), sheep (*Ovis aries*), horses (*Equus ferus caballus*), and camels (*Camelus* spp.). In Tibet, a 21.8% of human faecal samples were tested positive with intestinal sarcocystosis (Yu, 1991); while sarcocystosis in beef in Tibetans' market was moderately high (42.9%; Yu, 1991), and these could possibly be closely associated with consuming partial or raw meats.

Several other surveys were carried out in Japan. One of the surveys compared infections on local and imported beef in Japan, and revealed that both sources were positive with sarcocystosis (local: 6.3%; imported: 66.3%) (Neefs *et al.*, 1990). Similarly, in East Hokkaido, higher prevalence of sarcocystosis was revealed in

imported cattle compared to the local Japan cattle (*Bos taurus*) (Yang *et al.*, 2002). Additionally, Saito *et al.* reported muscular sarcocystosis of 12.5%, 80.0% and 0.8% in sheep, cattle and swine, respectively (Saito *et al.*, 1997; Saito *et al.*, 1998; Saito *et al.*, 1999).

2.3.4 Southeast Asia

A high prevalence of sarcocystosis was reported in Northern Vietnam through combination of microscopy and molecular studies, with infectivity rates of 63.0% in cattle (*B. taurus*) and 90.0% in water buffaloes (*B. bubalis*) (Jehle *et al.*, 2009). Besides, Claveria *et al.* highlighted multiple sarcocystosis cases in Philippine found in water buffaloes (*Bubalus bubalis*), cattle (*B. taurus*), domestic pig (*S. sucrofa*), and goats (*Capra hircus*) with the prevalence 55.0%, 16.0%, 0.4% and 42.3%, respectively (Claveria *et al.*, 2004; Claveria *et al.*, 1997).

In Malaysia, human accidental sarcocystosis has been reported extensively since 1970 (Arness *et al.*, 1999; Thomas & Dissanaike, 1978) with a few outbreaks recently (Esposito *et al.*, 2012; Esposito *et al.*, 2014; Italiano *et al.*, 2014). Unfortunately, the knowledge of ruminant sarcocystosis in Malaysia still remains limited. Only one study has been conducted in animal, where high prevalence (40.8%) of sarcocystosis was reported in cattle and water buffaloes (Latif *et al.*, 2013).

2.4 Transmission

Sarcocystis spp. are obligatory heterogeneous parasites that can propagate based on the transmission to the intermediate and definitive hosts. In general, there are two modes of transmission, one involves transmission from animals to animals, whereas the other involves the parasite transmission from animals to human (Dubey *et al.*, 1989b).

2.4.1 Animals to animals

In an ecosystem, definitive hosts (predators) develop intestinal sarcocystosis after consumption of infected meat of intermediate hosts (prey) (Fayer, 2004). Mature cysts contain multiple bradyzoites that capable to break through the intestinal wall to form sporocysts which are then excreted out from definitive hosts in the faeces.

Animals get infected after ingestion of contaminated silages and this initiates the development of *Sarcocystis* spp. from merozoites which end as sarcocyst form within the muscle tissues. Additionally, flies can serve as vectors for transmitting sarcocystosis (Markus, 1980).

2.4.2 Animals to humans

Man, as one of the omnivorous animals, acts as a definitive host in zoonotic sarcocystosis transmission. Man usually develops intestinal sarcocystosis through consumption of raw meats from animals that harbour sarcocysts, particularly beef and swine, which have already been infected by zoonotic *Sarcocystis* spp. such as *S. hominis* and *Sarcocystis suihominis* (Fayer, 2004). Muscular sarcocystosis is acquired through consumption of contaminated water with sporocysts of *Sarcocystis nesbitti* as recently reported in Pulau Tioman and Pulau Pangkor, Malaysia (Esposito *et al.*, 2012; Esposito *et al.*, 2014; Italiano *et al.*, 2014).

2.5 Sarcocystosis in intermediate hosts

Intermediate hosts play an important role in aiding *Sarcocystis* spp. to effectively propagate, such as cattle and goats can simultaneously harbour multiple species of *Sarcocystis* despite some of these parasites are host specific (Prakas & Butkauskas, 2012).

2.5.1 Cattle

Three species of *Sarcocystis* have been reported in cattle namely *S. cruzi*, *S. hominis*, and *S. hirsuta* that correspond to their definitive hosts canids, primates, and felids, respectively (Dubey *et al.*, 1989b). Recently, a fourth *Sarcocystis* spp. was reported in cattle, *S. sinensis* or *S. rommeli* (Dubey *et al.*, 2014; Dubey *et al.*, 2015a; More *et al.*, 2014; Zuo & Yang, 2015). Furthermore, another species, *Sarcocystis heydorni*, was detected in cattle where humans are the definitive host (Dubey *et al.*, 2015b).

2.5.1.1 *Sarcocystis cruzi*

Sarcocystis cruzi, also known as *S. bovicanis*, was firstly described by Hasselmann in 1926. It is distributed widely around the world. *Sarcocystis cruzi* infects both cattle (*B. taurus*) and bison (*Bison bison*) which the definitive hosts could be a wide range of canids and mustelids such as domesticated dog (*Canis familaris*), coyote (*Canis latrans*), red fox (*Vulpes vulpes*), raccoon (*Procyon lotor*) and wolf (*Canis lupus*).

The sarcocyst is less than 500 μm in length and can be found in all striated muscles in several organs comprising of tongue, oesophagus, diaphragm, Purkinje fibres of hearts, skeletal muscles and even in the central nervous system (Dubey, 1982). Transmission electron microscope study showed that the sarcocyst wall is thin (less than 1 μm) with long narrow ribbon-like protrusions (up to 3.5 μm long and 0.3 μm wide), covering the surfaces of the cyst (Fujino *et al.*, 1982; Pacheco *et al.*, 1978).

Out of three species infecting cattle, *S. cruzi* is the most pathogenic towards the respective host (Fayer *et al.*, 1982). Symptoms of infected cattle including anorexia, pyrexia, anaemia, loss of weight, weakness, muscle twitching, prostration, abortion, reduced milk production and hypersalivation (Dubey *et al.*, 1989c; Fayer, 2004). Besides, it can also cause neurological disease and death depending on the number of

ingested sporocysts (Dubey *et al.*, 1989b). In addition, cattle infected with a high number of sporocysts (i.e. 200000) developed clinical illness and died due to acute sarcocystosis (Fayer & Dubey, 1986).

2.5.1.2 *Sarcocystis hominis*

Sarcocystis hominis was first discovered in 1976 by Dubey, which is synonymous to *S. bovihominis*. Definitive hosts of this species are mainly primates including man (*Homo sapiens*), rhesus monkey (*Macaca mulatta*), baboon (*Papio cynocephalus*), and chimpanzee (*Pantro glodytes*) (Dubey *et al.*, 1989c).

The sarcocyst has a thick wall (6 μm) with numerous villar protrusions causing it to be radically striated (Mehlhorn *et al.*, 1976). Bradyzoites are 7 to 9 μm in length and are packed within the sarcocyst (Mehlhorn *et al.*, 1974). This species only causes mild pathogenicity in cattle, where only mild anaemia develops when inoculated with one million of sporocysts, with no fatal individuals (Dubey *et al.*, 1988).

2.5.1.3 *Sarcocystis hirsuta*

Sarcocystis hirsuta, also known as *S. bovifelis*, was discovered by Moule in 1888. It appears to be the only species found macroscopically in cattle. This species can be found probably worldwide where the cat (*Felis catus*) is the definitive host. In naturally infected cattle, the sizes of sarcocyst could grow as large as 8 mm long and 1 mm wide (Böttner *et al.*, 1987), while in experimentally animals, the length of the sarcocyst is 800 μm and 80 μm in width (Dubey, 1982b). Similar to *S. hominis*, the sarcocyst wall is thick and can approach to 7 μm and appears to be radially striated or hirsute (Dubey, 1982b). Ultrastructure study showed the villous protrusions contain multiple microtubules which are measured at 70 μm long and 1.5 μm wide.

Sarcocystis hirsuta only causes mild symptoms to the cattle (Dubey, 1983).

Calves inoculated by 100000 of sporocysts developed symptoms such as febrile, diarrheal, and mild anaemia.

2.5.1.4 *Sarcocystis sinensis/ Sarcocystis rommeli*

Sarcocystis sinensis was initially reported to be specific to water buffaloes (*B. bulbalis*), but More *et al*. (2014) also reported high prevalence of this species in cattle in Argentina. This species was later renamed as *S. rommeli*, as the fourth *Sarcocystis* spp. infecting cattle (Dubey *et al.*, 2015a).

Sarcocystis rommeli was characterised by a microscopic sarcocyst with a wall measuring at 5 μm thick, with slender villar protusions. The villar protusions were up to 5 μm long, and up to 0.5 μm wide. Interestingly, the definitive host(s) is/are still unclear since oral ingestion of this sarcocyst failed to exhibit symptoms of intestinal sarcosystosis in human. Nevertheless, the author managed to prove the distinctness of this species compared to *S. cruzi*, *S. hominis* and *S. hirsuta* via genotyping the 18S rDNA (Dubey *et al.*, 2015a).

2.5.1.5 *Sarcocystis heydorni*

Sarcocystis heydorni is characterised by a thin cyst wall (<1 μm). Despite cyst size is similar to *S. cruzi*, the overall structure is different (Dubey *et al.*, 2015b). The sarcocyst is 1060 μm in length and 80 μm wide, with short, conical villar protrusions on the wall measuring at 0.5 μm in length and width (Dubey *et al.*, 2015b). To date, this is the fifth species reported in cattle and zoonotic in nature apart from *S. hominis*.

2.5.2 Goat

Goat (*C. hircus*) serves as an intermediate host for three *Sarcocystis* spp. namely *S. capracanis* and *S. hircicanis* with corresponding definitive host from family of canids,

whereas felids are the definitive host for *S. moulei*.

2.5.2.1 *Sarcocystis capracanis*

This species was firstly introduced by Fischer (1979). Its definitive hosts are Canidae comprising of the dog (*C. familiaris*), coyote (*C. latrans*), red fox (*V. vulpes*) and crab-eating fox (*Cerdocyon thous*) (Dubey *et al.*, 1989b).

This species measures up to 1000 μm long and 100 μm wide, with a cyst wall up to 3 μm thick. Ultrastructure study shows the presence of radial striations and finger-like villar protrusions on the cyst wall (Dubey *et al.*, 1989b). The cysts are found throughout skeletal muscles, central nervous system and myocardial muscles (Dubey, 1984).

Sarcocystis capracanis has been considered as the most pathogenic species in goats (Collins & Charleston, 1979; Dubey *et al.*, 1981). Infected goats developed symptoms including fever, weakness, anorexia, weight loss, tremors, irritability, abortion, and even death dependent on total number of sporocysts ingested. Clinical symptoms can appear after ingestion of only 5000 sporocysts and become lethal when 100000 sporocysts are ingested (Dubey *et al.*, 1981).

2.5.2.2 *Sarcocystis hircicanis*

Heydorn and Unterholzner discovered *S. hircicanis* in 1983, where the dog (*C. familiaris*) is the only definitive host. It was recorded in India (Pethkar & Shah, 1982), Turkey (Erber & Goksu, 1984) and the Federal Republic of Germany (Heydorn & Unterhlzner, 1983).

In terms of morphology, the sarcocyst is microscopic, but can grow up to 2500 μm in length. The sarcocyst wall is thin (< 1 μm), hairy, with long filaments, which are folded over the cyst wall but it lacks tubules (Dubey *et al.*, 1989b).

Information regarding the pathogenicity of this species remains unclear, however, inoculation of the sporocysts (10 millions) into goats have resulted in death on day-45 and -35 post inoculation (Dubey *et al.*, 1989d).

2.5.2.3 *Sarcocystis moulei*

Cat (*F. catus*) is the definitive host of *S. moulei*/*S. caprafelis*. This species was reported in Saudi Arabia (Al-Hoot *et al.*, 2005). The primary cyst wall consists of cauliflower-like protrusions with multiple fibellar structures (Al-Hoot *et al.*, 2005). The pathogenicity of this species is unknown.

2.6 Treatment

Several drugs that were previously used to treat diseases in poultry are found to be effective against muscular sarcocystosis, especially in cattle and goats when applied regularly for a month during pre and post-inoculation (Fayer & Johnson, 1975; Leek & Fayer, 1980, 1983). Among the drugs used to treat include amprolium and halofuginone, were effective to *S. cruzi* and *S. capracanis* infections. Cattle treated with amprolium (100 mg/kg) in time interval of 0 to 30 DPI, prevents acute disease caused by *S. cruzi*, whereas calves treated with same dosage from 21 to 35 DPI developed only mild sarcocytosis even when inoculated with 100000 sporocysts. This shows that amprolium is effective against *S. cruzi* and successfully impede the second generation of schizogony or any asexual stages before the formation of sarcocyst (Fayer & Dubey, 1984).

On the other hand, halofuginone with a dosage of 0.22 mg/kg body weight is claimed to be useful for reducing and preventing acute sarcocystosis caused by *S. capracanis* in goats. Drug application on day-5 till 36 DPI was reported to be effective against this parasite in goats (Voigt & Heydorn, 1981). Even though multiple drugs

were developed to counter sarcocystosis in livestock, however, these drugs only work against specific *Sarcocystis* spp. For instance, amprolium works effectively against *S. cruzi* but fails to have any significant impact on *Sarcocystis muris* in mouse (Dubey *et al.*, 1989b).

2.7   Control and prevention

Since there are no available specific drug candidates for treating the clinical muscular sarcocystosis, prevention is the only current solution to control the outbreak of this disease. According to Dubey, the main factor causing the extensive spreading of sarcocystosis is through transmission of sporocysts from faeces of definitive hosts. In order to minimize the transmission, several strategies have been suggested by the authors:

a) Limit interactions between intermediate hosts with definitive hosts as this can greatly reduce the transmission dynamic between hosts.

b) Avoid feeding carnivorous animals with raw meats. A proper management of meats is also mandatory. For instance, repeated freezing and heating the meats above 55 ℃ continuously for 20 min can destroy sarcocysts within the muscle tissues.

c) Deceased livestock should be handled properly by burying or incinerating as they may harbour live sarcocysts which can be consumed by wild carnivores.

2.8   Diagnosis

Diagnosis for sarcocystosis is often a challenge to researchers and meat inspectors, due to the difficulty of detecting the cysts. *Sarcocystis* spp. embed deep within the muscle tissues particularly for microscopic species. Livestock animals are normally been diagnosed for muscular sarcocytosis by observing the symptoms. Till date, there are several techniques which have been developed to diagnose *Sarcocystis*

spp. These techniques can be categorised into microscopic, molecular and serology. Microscopy is the most common method to diagnose sarcocystosis on meat samples.

2.8.1 Gross and microscopy

Gross inspection of meat is the most common method used in abattoirs and meat inspection centres to detect sarcocystosis. It is the cheapest and fastest way to detect abnormalities on the carcasses. However, it is the least sensitive because it is only able to detect macroscopic sarcocysts such as *S. hirsuta* and *S. moulei*. Microscopic *Sarcocystis* spp. such as *S. cruzi*, *S. hominis*, *S. capracanis* and *S. hircicanis* are often missed due to their small size and often hidden within the muscle tissues. In order to overcome this, several techniques such as muscle compression, pepsin digestion and histology methods were introduced.

Muscle compression technique or muscle squash involves excising a small portion of suspected muscle tissues, usually 2 mm thick and compressed between two microscopic slides. This spreads the muscle tissues evenly on the slides while increasing the chances of exposing the sarcocysts embedded in the tissues.

Pepsin digestion method was reported to be the most sensitive method (93.32%) in diagnosing sarcocystosis (Latif *et al.*, 1999). In brief, this technique digests the muscle tissues by using enzyme pepsin, a method that mimics the digestion process in the digestive system of hosts. The digested supernatants containing bradyzoites can be observed by a simple wet mount on a microscope slide. Fixation and staining also improve the detection rate of the bradyzoites.

In the histology method, tissue samples were fixed and stained to enhance cellular structures so that cell abnormalities can be easily detected via contrast staining (Cook, 2006) using a combination of haematoxylin and eosin stain. This counterstaining method clearly distinguishes sarcocysts from neighbouring host tissues.

Methylene blue (3,7-bis(dimethylamino)-phenothiazin-5-ium chloride) is a common dye used in parasitology (Ferreira *et al.*, 2006; Malakauskas & Grikienienė, 2002; Mohapatra *et al.*, 2014; Prakas *et al.*, 2011; Pyziel & Demiaszkiewicz, 2009). Since methylene blue is cationic in aqueous form and DNA contains negatively charged phosphate groups, the dye binds strongly to the nucleus and other phosphate-containing organelles. Therefore, this dye has been used to detect the nuclei of parasites such as *Plasmodium* spp. trophozoites (Mohapatra *et al.*, 2014) and *Trypanosoma cruzi* trypomastigotes (Ferreira *et al.*, 2006). Additionally, this dye also has been widely used in diagnosing sarcocystosis (Malakauskas & Grikienienė, 2002; Prakas *et al.*, 2011; Pyziel & Demiaszkiewicz, 2009).

2.8.2 Morphometry

Morphometry is a quantitative-based technique to describe morphology of an organism. This technique is especially useful in both medicine and taxonomy. For an example, neurologists use this approach to determine the abnormalities in patients suffering from schizophrenia and multiple sclerosis (Ballester, 1999). On the other hand, archaeologists utilise the operational taxonomical unit (OTU), that is measuring the size of a skull from a pile of bones to deduce its origins whether from the modern human, *Homo sapiens* or the earlier human, *Homo erectus*. Interestingly, morphometry is also useful to differentiate *Hepatozoon* spp. in snakes (Moco *et al.*, 2012; Moco *et al.*, 2002; O'Dwyer *et al.*, 2013), by measuring their distinct nucleus as well as the area (cm^2) of infected erythrocytes via light microscope (Figure 2.2). Apart from *Hepatozoon* spp., Obijiaku *et al.* (2013) successfully characterised the *S. cruzi* and *S. hominis* in cattle by measuring their sarcocyst sizes.
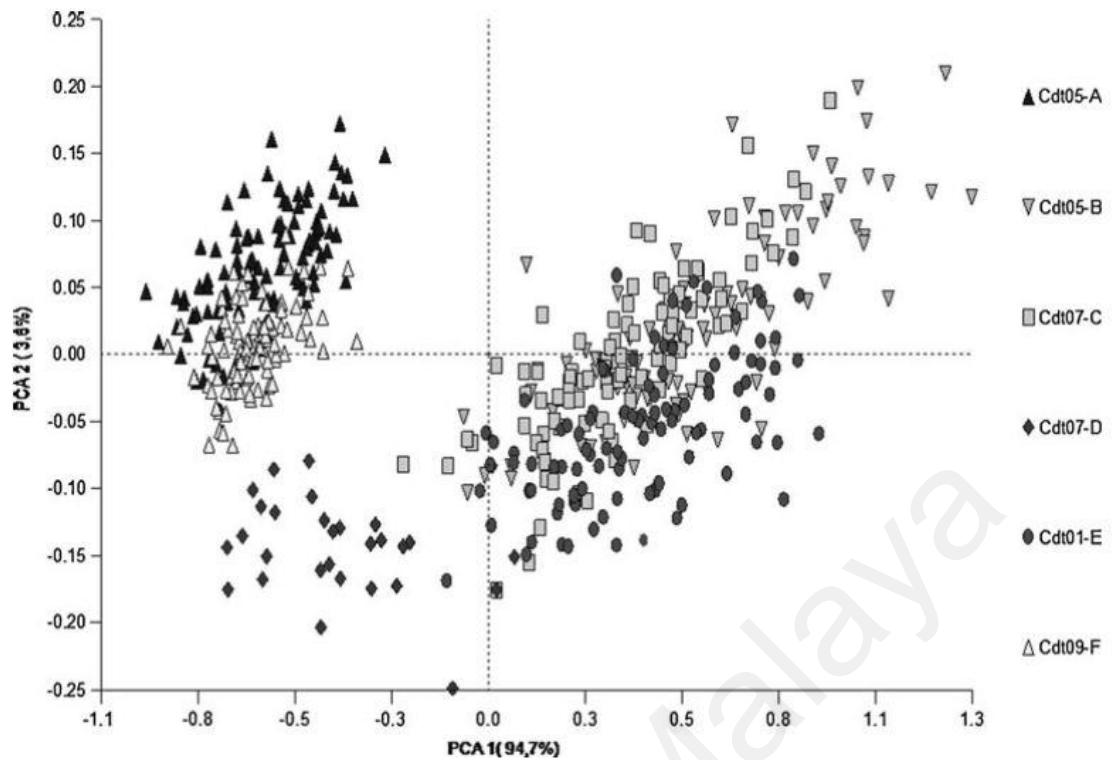
Figure 2.2: Morphometric scatterplot of *Hepatozoon* spp. isolated in snake (Moco *et al.* 2012)

The scatterplot displays the clustering of multiple *Hepatozoon* spp. The parasites are clustered based on the multivariate analysis of the parameters including parasite area, length, width, area of the nucleus of the parasite and length of the nucleus of the parasite. Each shape corresponds to *Hepatozoon* spp. gamonts (A-F) infecting the host *Caudisona durissa terrifica* (Cdt).

Identification of *Sarcocystis* spp. is not sufficed on single method. For example, the light microscopy observation of sarcocysts in muscle tissues is fast and cheap, however it is not possible to identify the species as the detail of the cysts i.e. cyst walls can hardly be seen through the basic light microscopy.

2.9   Serology

Serological studies have been carried out by using enzyme-linked immunosorbent assay (ELISA) to detect antibodies against *Sarcocystis* spp. in animals such as mice and pigs (O'Donoghue & Weyreter, 1983). This method has been proven useful for diagnosing acute sarcocystosis in naturally infected animals (Dubey *et al.*, 1989a). Furthermore, recent study on *S. cruzi* reported a higher prevalence of sarcocystosis in water buffaloes when applying serological method compared to microscopic observations (Metwally *et al.*, 2013).

Despite serology detects higher prevalence, the sensitivity and specificity of this test is still questionable. This is due to the cross-reaction among closely-related *Sarcocystis* spp., especially when crude antigens are involved. Consequently, this technique is not ideal to pin-point the exact *Sarcocystis* spp. infection.

2.10   Molecular

Molecular techniques such as PCR is much more convenient and sensitive due to only small amounts of DNA template as low as pictogram is required, therefore, ideal for *Sarcocystis* spp. detection even in low infected animals.

To date, multiple genes have been used to diagnose sarcocystosis. These markers are mostly from ribosomal repeated chains units which comprise of small ribosomal subunit (18S rDNA), large ribosomal subunit (28S rDNA) and internal transcribed spacer 1 and 2 (ITS-1 & 2) (Kutkiene *et al.*, 2011; Odening *et al.*, 1996; Olias *et al.*,

2010; Slapeta *et al.*, 2003; Stojecki *et al.*, 2012; Yang & Zuo, 2000). Besides the ribosomal repeated chain unit genes, cytochrome c oxidase subunit 1 (COX 1) has also been found useful for discriminating *Sarcocystis* spp. due to its conserved properties (Gjerde, 2013, 2014a; Gjerde *et al.*, 2015).

2.10.1 18S rDNA

Ribosomal small subunit gene (18S rDNA), is one of the most commonly used marker in molecular studies as well as diagnosis of ruminant muscular sarcocystosis. It is indeed an ideal gene marker for phylogenetic studies because it is highly conserved, and analysis of its variable regions allows effective speciation of organisms within a genus (Neefs *et al.*, 1990). There are nine variable regions across the entire *ssu rRNA* gene (Figure 2.3). Of these, region four and nine are the most prominent candidates for species differentiation (Hadziavdic *et al.*, 2014).

2.10.2 Phylogenetic tree analysis

Phylogenetic tree creates a network framework for determining the relatedness of organisms corresponding to ancestors, based on the gene sequences. Generally, phylogenetic tree clusters those closely related organisms into a single clade. Phylogenetic analysis of genomic data allows discovery of an unknown or cryptic species in the studied sample pools.

With the advancement in bioinformatics, researchers can easily perform complexes of multiple sequence alignment (MSA) in large scale, by using sequence alignment software such as MEGA V6 or Seaview V4 (Gouy *et al.*, 2010;Tamura *et al.*, 2013). Meanwhile, multiple models corresponding to the phylogenetic tree further allows researchers to study in-depth into their gene sequences.
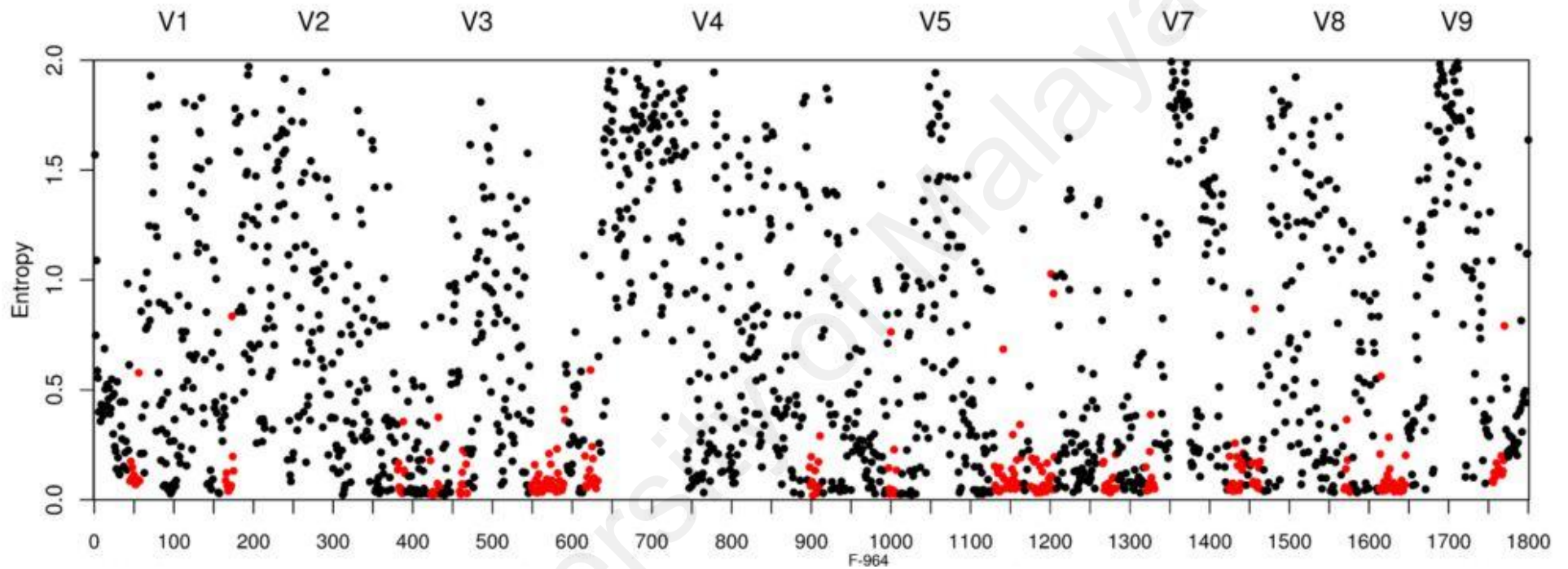
Figure 2.3: Nucleotide variable regions in the ribosomal small subunit (18S rDNA) (Hadziavdic *et al.* 2014)

The red dots mark the consecutive nucleotide positions where at least 90% of nucleotides having an entropy value less than 0.2. On the other hand, high nucleotide variable regions are denoted V1-9 (black dots).

Neighbour-joining (NJ) method, which is based on genetic distance matrix, is a time efficient model that allows phylogeneticists to quickly observe the tree topology. On the other hand, maximum parsimony (MP), maximum likelihood (ML) based technique emphasises on each sequences in details which therefore is time-consuming. Nevertheless, this analysis is more robust compared to the distance-based method (Saitou & Imanishi, 1989).

By genotyping the 18S rDNA and corroborating it with the phylogenetic tree analysis, Holmdahl *et al.* (1999) has managed to deduce the evolution of ruminant *Sarcocystis* spp. among the family of Sarcocystidae. This type of analysis has been updated over the years and is still useful in determining *Sarcocystis* spp. in animals (Dahlgren & Gjerde, 2008; Dahlgren *et al.*, 2008; Gjerde, 2012, 2013, 2014a, 2014b; Gjerde *et al.*, 2015; Gjerde & Josefsen, 2014; Gjerde & Schulze, 2014; Lau *et al.*, 2013; More *et al.*, 2008; Slapeta *et al.*, 2003).

2.11 Structures of *Sarcocystis* spp.

2.11.1 Sarcocyst

Sarcocyst is a structure which functions to "house" the bradyzoites. The size and shape of sarcocyst can be used for species differentiation. For instance, the average length of *S. cruzi* is about 500 μm, which appears to be a microscopic compared the macroscopic *S. hirsuta* with length of 8000 μm and width of 1000 μm (Dubey *et al.*, 1989b). However, both size and shape of the sarcocysts are greatly affected by the age, type of organs infected and the techniques used for visualising the sarcocysts. For instance, sarcocyst isolated from the cardiac muscle and central nervous tissue is smaller compared to those found in skeletal muscle (Dubey, 1982b; Dubey *et al*., 1984). In addition, fixatives and the process involved in histological studies in *Sarcocystis* spp., also affects the size of sarcocyst. The dehydration steps cause the sarcocyst to shrink

(Dubey *et al.*, 1989d).

Apart from size, taxonomic of the sarcocyst wall is important for classical species identification (Dubey *et al.*, 1989b), noting a total of 24 types of cyst walls discovered until the year of 1989, however many more have yet to be discovered. Each type of cyst wall is unique and corresponds to different species. For example, type one cell wall has a characteristic of multiple minute undulations, which can only be found in small mammals, whereas wall type 14, is characterised by having a tightly packed, cylindrical shaped like protrusions. This type of cyst wall can be found mostly in goats and sheep (Dubey *et al.*, 1989b). Cyst wall type 15 with tombstone-like protrusions, on the other hand, is found mainly in deer or closely related ungulate mammals (Dubey *et al.*, 1989b).

Although sarcocyst is a remarkable structure that aids in the taxonomical identity of *Sarcocystis* spp., it still remains a challenge for researchers to study the morphology of sarcocyst, due to lack of knowledge on determining the maturity of a sarcocyst, especially in the natural infected hosts (Dubey *et al.*, 1989b). Therefore, researchers need to interpret with care the structure of sarcocysts to prevent unnecessary biases.

2.11.2 Bradyzoites

The number of bradyzoites in each sarcocyst varies, ranging from hundreds to thousands. Their sizes are affected by the number present within the sarcocyst. i.e., the bradyzoite sizes tend to be smaller in a tightly packed sarcocyst, while significantly bigger in sarcocyst that contains lesser number of the zoites. Due to inconsistency in size, bradyzoite is therefore not a good candidate for taxonomical evaluation of *Sarcocystis* spp. (Dubey *et al.*, 1989b).

2.11.3 Schizont

This stage is not ideal for use to differentiate the *Sarcocystis* spp. because of its irregular size and shape, partly because it is affected by its developing processes (multiple generation of schizogony) and/or neighbouring muscle tissues that contract and relax, antagonistically. For an example, schizont of *S. cruzi* isolated from skeletal muscle is slender and elongated in shape compared to those isolated from renal glomeruli, which is globular in shape.

2.11.4 Oocyst and sporocyst

Oocyst and sporocyst were once used for determining species, however the size variations between species are limited, and similarity in structures are high throughout all *Sarcocystis* spp. Therefore, these stages are omitted from taxonomical studies of *Sarcocystis* spp.

CHAPTER 3: METHODOLOGY

3.1 Chemicals and reagents

All common chemicals were of Analar or higher grade available from Amresco Inc., U.S.A.; Amersham Pharmacia Biotech Inc., Sweden; APS Finechem, Australia; Difco, U.S.A.; BDH Ltd, England; Gibco BRL, Life Technologies Inc., U.S.A.; Invitrogen Corp, U.S.A; MBI Fermentas, U.S.A., Promega Corporation, U.S.A., and Sigma Chemical Co., U.S.A.

These chemicals included methylene blue powder, acetic acid glacial, acetone, boric acid, 95% ethanol, polyethylene glycol, magnesium chloride, D-glucose monohydrate, glycerol, hydrochloric acid, sodium hydroxide, Tris, and Tris hydrochloride. Agarose powder used to make the gel for electrophoresis was purchased from Promega Corporation, U.S.A. Besides, PCR kits and PCR cloning kit, pGEM®-T vector, were also from Promega Corporation, U.S.A. One Shot® TOP 10 *Escherichia coli* competent cells were purchased from Invitrogen Corporation, U.S.A. Ampicillin was purchased from Bio Basic Canada Inc., Canada. In addition, materials for preparation of growth medium for the Top 10 *E. coli* strain such as yeast extract, tryptone powder and sodium chloride were all purchased from Laboratories CONDA S.A., Spain.

3.2 Sterilisation

3.2.1 Clorox and alcohol sterilisation

Ethanol (70%) was used to sterilise blades used to cut the sample tissues, and Clorox was used to disinfect the contaminants on the blades. Dissecting apparatus were soaked in diluted Clorox overnight before being washed and re-used.

3.2.2 Moist and dry heat

Distilled and deionised water, microcentrifuge tubes, micropipette tips, phosphate buffer saline (PBS), centrifuge tubes (polycarbonate and polypropylene), and Schott bottles attached with plastic caps were sterilised by autoclaving 15 pound-force per square inch (psi) at 121 ℃ for 15 min. Autoclaved apparatus were put into 65 ℃ for drying purposes. Besides, glassware including pipettes, measuring cylinders, and conical flasks were sterilised by heating at 180 ℃ for 1 h in the oven.

3.2.3 Membrane filtration

Ampicillin solution and methylene blue stock solutions were filtered and sterilised by using disposable syringe filters with pore size of 0.22 μm (Sartorius Corporation, U.S.A).

3.3 Solutions for tissues sample staining

3.3.1 Methylene blue stock solution (0.2%)

Methylene blue powder (Unilab, Australia)   0.2 g

ddH_2O top up to   100 ml

3.3.2 Methylene blue working solution (0.025%)

Methylene blue stock solution (0.2%)   25 ml

ddH_2O top up to   200 ml

3.4 Medias and solution for bacterial cell culture

All solutions and media used for culturing the bacterial cell were prepared aseptically using a Bunsen burner.

3.4.1 Ampicillin (100 mg/ml)

One gram of ampicillin sodium powder was dissolved in 10 ml of ddH$_2$0. The mixture was filter-sterilised (section 3.2.3). Aliquots were separated into microcentrifuge tubes and kept at -20 °C for long terms storage.

3.4.2 Luria-Bertani (LB) medium in 400 ml

Tryptone		4.0 g
Yeast extracts		2.0 g
Sodium chloride		4.0 g
ddH$_2$O	top up to	400 ml

3.4.3 Luria-Bertani agar

Tryptone		4.0 g
Yeast extract		2.0 g
Sodium chloride		4.0 g
Bacteriological agar		6.0 g
ddH$_2$O	top up to	400 ml

The mixture was autoclaved and 400 µl of ampicillin (100 mg/ml) was added to a final concentration of 100 µg/ml when the medium was cooled down to about 55 °C.

3.5 Solutions for agarose gel electrophoresis

3.5.1 50x Tris-acetate-EDTA (TAE) buffer stock solution

Tris base		242.0 g
Glacial acetic acid		57.1 ml
500 mM EDTA (pH 8.0)		100.0 ml
ddH$_2$O	top up to	1000 ml

3.5.2 1x Tris-acetate-EDTA (TAE) buffer working solution

50X TAE buffer stock solution	20 ml
ddH$_2$O	980 ml
Total	1000 ml

3.6 Tissue samples collection

3.6.1 Sampling site

A cross-sectional study was conducted to examine the number of cattle and goats infected with *Sarcocystis* spp. Sampling was carried out at the Shah Alam abattoir, Selangor (GPS location: 3 ʹ03'25.5"N 101 ʹ31'10.8"E) (Figure 3.1). Local cattle sampled were from Kedah-Kelantan (KK). In addition, imported cattle (Droughtmaster) and goats (Feral goats) were also sampled.

3.6.2 Sample collections

Random sampling was carried out. About 5 g of muscle tissues were taken from the tongue, oesophagus, diaphragm, heart, and skeletal muscle. A total of 90 cattle (11 local; 79 imported from Australia) and 55 imported goats were sampled between April-June 2013. The sampled tissues were placed in polyethylene bags and in ice before bringing back to the laboratory. The samples were kept in -20 ℃ until further processing.

3.7 Microscopic wet mount screening

3.7.1 Tissue compression technique

Samples were screened for the presence of sarcocysts via tissue compression technique.
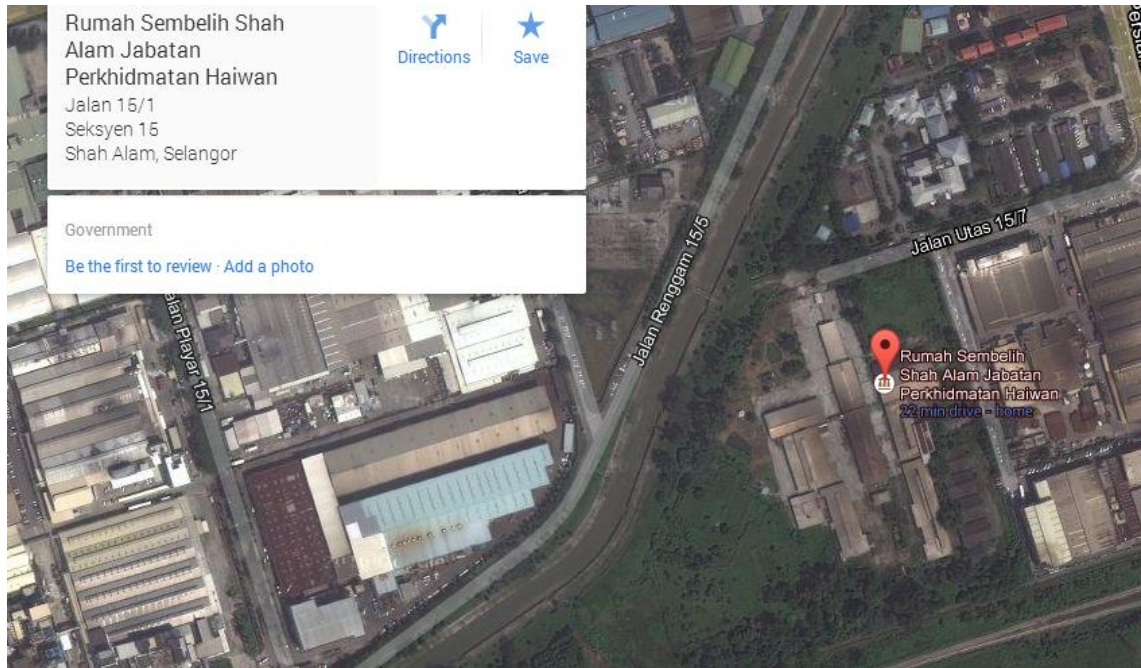
Figure 3.1: Sample collection site located at Shah Alam, Selangor Darul Ehsan (Google map, 2014)

Approximately, 2-3 mm of tissues were cut from each sample. These tissues were laid on a clean slide and briefly spread using scalpel blade (Appendix A), followed by compression with another slide (Figure 3.2). This maximises the tissue spreading for clear observation through a light microscope.

3.7.2 Microscopy examination

In addition to the standard compression technique used in the previous study (Latif *et al.*, 1999), methylene blue staining was also used (Pyziel & Demiaszkiewicz, 2009), with a minor modification. A total of 0.025% of methylene blue stain working solution was prepared from the stock solution of 0.2% and filtered through 0.2 μm filter before use. This application was done simultaneously with the standard tissue compression technique. Each organ tissue was cut and a drop of methylene blue stain was applied to each tissue. The tissues were stained for 10 min before washing with distilled water. The stained tissues were dried briefly and compressed between slides as standard compression technique before observation via the light microscope (Leica DM750, attached with Leica ICC50 colour camera) under 4x and 10x magnifications. Each slide consisted of three replicates of compressed tissues, labelled with host and organ codes. Additionally, tapes were applied to immobilize both slides to prevent unnecessary slides dislocations during microscopy observation. In this case, both unstained and stained groups were observed to compare the sarcocysts detection rate.

3.8 Molecular characterisation of *Sarcocystis* spp.

3.8.1 DNA extraction

The DNA extraction process was carried out by using Qiagen Blood and Tissue DNA extraction kit (Qiagen, Germany). Briefly, 180 μl of tissue lysis buffer (ATL) was added into tissue sample (about 25 mg) followed by 20 μl of Proteinase K.
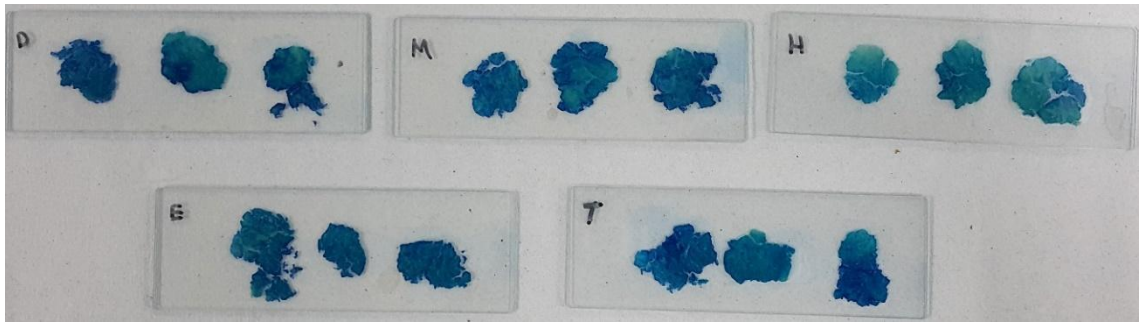
(a)
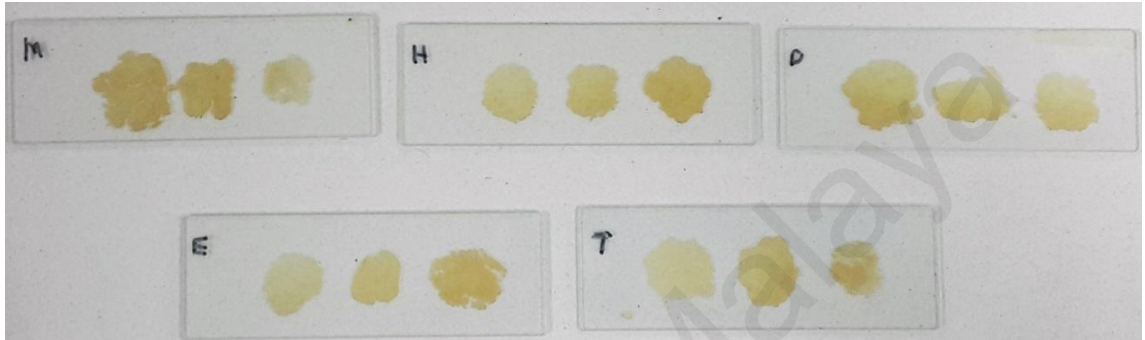
(b)

Figure 3.2: Tissue compression

(a) Methylene blue stained tissues (b) Unstained tissues. T: Tongue; E: Oesophagus; D: Diaphragm; H: Heart; M: Skeletal muscle.

The mixture was then vortexed for few seconds and incubated at 56℃ overnight. Meanwhile, the mixture was vortexed occasionally every half an hour to enhance the tissue lysis. After overnight incubation, 200 μl of buffer AL was added. The mixture was vortexed and further incubated at 56℃ for 10 min. Absolute ethanol, 200 μl was added to the mixture and vortexed thoroughly. Centrifugation was performed using bench top centrifuge (Eppendorf) at 8000 rpm for 1 min. Buffer AW1, 500 μl was added and the mixture was centrifuged at 8000 rpm for 1 min. The flow-through and collection tube were discarded and the DNeasy Mini spin column was placed in a new 2 ml collection tube. Buffer AW2, 500 μl was added and the mixture was centrifuged at 14000 rpm for 3 min to dry the DNeasy membrane. The flow-through and collection tube were discarded. The DNeasy Mini spin column was placed in a clean 1.5 ml microcentrifuge tube. Buffer AE, 100 μl was pipetted directly onto the DNeasy membrane and incubated at room temperature for 1 min, and then centrifuged at 8000 rpm for 1 min for elution of DNA. The extracted DNA was kept in -20℃ for long term storage.

3.8.2   Primers

The information of oligonucleotide used in this study is shown in Table 3.1. The stock concentrations of oligonucleotide were diluted to 100 pmoles/μl.

3.8.3   Nested Polymerase chain reaction (PCR)

Nested Polymerase chain reaction (PCR) was carried out using a thermo cycler (Bio-Rad, U.S.A.) with specific primers (forward and reverse) (Table 3.1). Briefly, 1L and 1H were used in primary PCR amplification, whereas 3L and 2H were used in nested PCR amplification.

Table 3.1: Primers used in polymerase chain reaction (PCR)

Oligonucleotide	Sequence (5'-3')	Purposes	Source
1L	CCATGCATGTCTAAGTATAAGC	Primary PCR reaction forward primer	Yang *et al.*, 2001
1H	TATCCCCATCACGATGCATAC	Primary PCR reaction reverse primer	Yang *et al.*, 2001
3L	CTAGTGATTGGAATGATGGG	Nested PCR reaction forward primer	Yang *et al.*, 2001
2H	ACCTGTTATTGCCTCAAACTTC	Nested PCR reaction reverse primer	Yang *et al.*, 2001
M13 F	GTTTTCCCAGTCACGAC	For sequencing (forward)	Universal
M13 R	AGCGGATAACAATTTCACACAGGA	For sequencing (reverse)	Universal

The PCR was carried out using the following reagents:

Reagents	Volume (μl)
5x Go Taq® Flexi Buffer	5.0
MgCl$_2$ (25 mM)	4.0
dNTPs (10 mM per nucleotide)	0.5
Forward primer (1L/3L)	0.5
Reverse primer (1H/2H)	0.5
Taq polymerase (5U/μl)	0.2
ddH$_2$O	10.3
DNA template/ ddH$_2$O	4.0
Total	25.0

A negative control was included for each PCR process. The PCR reaction was carried out at 95 ℃ for 2 min, followed by 35 cycles of 94 ℃ for 40 s, 50 ℃ for 30 s, 72 ℃ for 1.5 min, and followed by 72 ℃ for 6 min. The final step of PCR was on hold at 4 ℃.

3.8.4   Gel electrophoresis

One point three percent of agarose was prepared, heated, and added with SYBR® Safe DNA Gel Stain (Invitrogen™, U.S.A.) before being placed horizontally in the gel tank (Bio-Rad, U.S.A.). TAE (1x) was used as running buffer. Five μl of PCR products were mixed with 1 μl of loading dyes (Thermoscientific) and loaded on each well. The samples underwent electrophoresis at 100 mA for 30 min using Power Pac Basic Power Supply (Bio-Rad, U.S.A.). Both 1 kb and 1 kb plus O' gene ruler DNA ladder (Thermoscientific) were used to estimate the DNA fragments sizes. Gel images of electrophoresed samples were documented through Molecular Imager® ChemiDoc™ XRS Imaging System (Bio-Rad, U.S.A.).

3.8.5 PCR products purification

Amplified PCR products were purified either using QIAquick PCR purification kit (catalogue no: 28106) or QIAquick Gel Extraction Kit (catalogue no: 28706) depending on the number of DNA fragments/bands observed in gel electrophoresis.

3.8.5.1 Direct PCR products purification

Briefly, 20 µl of PCR products were added with 60 µl PB buffer in 1:3 ratio. The solutions were pipetted into spin column included in the purification kit and centrifuged for 13000 rpm for 60 s. Then 750 µl of PE buffer was added, followed by two additional centrifugations to remove PE buffer. The DNA was then eluted by adding 30-50 µl of ddH$_2$O.

3.8.5.2 Gel extraction PCR products purification

A total of 20 µl of PCR products were loaded on the 1% agarose gel together with 1kb DNA ladder (O' generuler ladder supplied by Thermoscientific). Electrophoresis band with expected sizes were cut using slide coverslips and put into each microcentrifuge tube. All tubes were weighed and QG buffer was added in the ratio of three volumes corresponding to the gel weight (e.g. 100 mg ~ 100 µl). The mixtures were incubated at 50 ℃ for approximately 10 min or until the gel has fully dissolved. Occasional vortexing was done to speed up the dissolving of the gel. Isopropanol, equal to gel volume, was added right after the heating process and mixed briefly before transferring into spin columns. The columns were spun at 13000 rpm for 1 min. The supernatant was removed and 750 µl of washing buffer (PE) was added. Two more centrifugation steps were conducted to remove washing buffer supernatants and residuals. For elution, 35 µl of elution buffer (EB) was added into each tube right in the middle of the column to maximise the yield. The tubes were incubated for 4 min at

room temperature, followed by 13000 rpm centrifugation for 1 min. The eluted solution was stored at -20 ℃ for downstream applications.

3.8.6 Ligation

The purified PCR products were ligated into the pGEM®-T vector (Promega Corp., U.S.A). The ligation mixture consisted of the reagents shown below following the manufacturer's instructions:

Reagents	Volume (μl)
2x ligation buffer	5
pGEM®-T Vector	1
PCR amplicons/insert (20 – 50 ng)	3
T4 DNA ligase (400,000 U/ml)	1
Total	10

The mixture steps were done in ice as the reagents were highly sensitive to temperature. The ligation mixture was incubated at 16 ℃ in a water bath for 16 h. The ligated products were transformed into TOP10F' competent *E. coli* strain cells.

3.8.7 Preparation of TOP10F' competent *E. coli* cells

Escherichia coli TOP10F' competent cells were used for inoculation. Ten μl of competent cells were pipetted into 50 ml falcon tubes with 5 ml of LB broth and grown overnight for approximately 16 h in a 37 ℃ shaking incubator at speed of 200 rpm. In the case of *E. coli* competency treatment, polyethylene glycol (PEG) method was applied (Nishimura *et al.*, 1990). This technique basically involved two media (A and B) which were prepared prior to the competent cells preparation. Briefly, 500 μl of overnight *E. coli* culture was pipetted into a conical flask containing freshly prepared medium A. The medium A was prepared by mixing of LB broth, 0.01 M sterile

magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) and 0.2% of glucose in a final volume of 50 ml. The conical flask was placed in the shaking incubator for approximately 1.5 h until the absorbance value at wavelength of 600 nm reached 0.4-0.6. The mixture was transferred into 50 ml falcon tubes and kept in ice for 10 min in order to slow down the exponential growth rate. The mixture was then centrifuged at 1500 x g at 4 ℃ and the supernatant was removed. Next, 3 ml of medium B was used to re-suspend the pellet. Medium B was prepared by mixing the LB broth, 0.12 M $MgSO_4 \cdot 7H_2O$, 12 g of polyethylene glycol (PEG), and 36% of glycerine in a final volume of 50 ml. The re-suspended pellets were aliquoted into microcentrifuge tubes with each tube containing 100 µl of competent cells. The cells were stored in extreme low temperature freezer (-80 ℃).

3.8.8 Transformation

The ligated product was added into 100 µl of TOP10F' *E. coli* competent cells followed by incubation for 30 min in ice. The vector was transformed into competent cells by heat shock technique. While ligation products and competent cells were incubated in ice, the water bath was set to 42 ℃. The competent cell was then placed in the water bath for 1 min followed by incubation in ice for 2 min. After incubation, 1 ml of LB broth was added to the tube, and incubated at 37 ℃ in a shaking incubator for 1 h. Then, the tube was centrifuged at 5000 rpm for 1 min. The supernatant was discarded and 200 µl of LB broth was added. The re-suspended cells were pipetted into LB agar plates containing ampicillin (100 µg/ml). The plates were incubated at 37 ℃ for overnight.

3.8.9 Screening for transformants through colony PCR

Escherichia coli colonies that grew on the plates were selected for screening of

positive clones. Prior to *E. coli* colony selection, PCR tubes with the following reaction were prepared:

Reagents	Volume (μl)
DreamTaq Green PCR Master Mix (2x) (Cat no: K1081)	5.0
M13 Primer Forward (-40)	0.2
M13 Primer Reverse (-48)	0.2
ddH$_2$0	4.6
Total	10.0

Pipette tips were used to pick a single colony and this was mixed with 10 μl of PCR reagents in the PCR tubes. The universal primers M13 F (-40) and R (-48) were used to detect the positive insertion of 18S rRNA gene (Table 3.1). A negative control without colonies was included.

PCR was carried out under the following conditions: Initial denaturation at 95 ℃ for 10 min; 30 cycles of denaturation at 95 ℃ for 30 s, annealing at 50 ℃ for 30 s, extension at 72 ℃ for 30 s; final extension at 72 ℃ for 5 min followed by infinite time on hold at 4 ℃. Amplified PCR products underwent electrophoresis to confirm positive insertion.

3.8.10 Extraction of plasmid DNA

Selected bacteria colonies with positive recombinant plasmids were cultured in 5 ml of LB with ampicillin (100 μg/ml) for subsequent plasmid extraction. Plasmid DNA was extracted from the overnight *E. coli* cells using QIAprep Spin Miniprep Plasmid Isolation Kit (Qiagen, Germany). Briefly, supernatant and cells were separated by centrifugation at 6,800 x g for 3 min at room temperature. The pellets corresponding to *E. coli* cells were re-suspended with 250 μl buffer P1 followed by transferring them into sterile microcentrifuge tubes. Two hundred and fifty μl buffer P2 was added into the

tube and the mixture was mixed thoroughly by inverting the tubes approximately four to six times. Three hundred and fifty μl of neutralisation buffer N3 was added, and mixed immediately together by inverting several times (four to six times). A cloudy white substance was obtained and was centrifuged for 10 min at 13,000 rpm. The supernatant was pipetted out into QIAprep spin column and centrifuged for 1 min at the same speed used for plasmid DNA binding. For each centrifugation, the supernatant was removed and the collection tubes reused. After plasmid DNA binding, 750 μl washing buffer PE was added and centrifuged for 1 min. One last centrifugation was performed in order to remove the residual buffer. For elution, 50 μl of buffer EB was pipetted right in the centre of each spin column, followed by 1 min incubation. After incubation, the tube was spun for 1 min and the plasmid DNA was aliquoted for sequencing or stored at -20 °C for storage.

3.8.11 DNA sequencing

Extracted plasmid DNA were labelled and sent for sequencing using M13 forward and reverse primers (Table 3.1).

3.8.12 Long-term storage of recombinant clones

A single well-defined colony of a chosen clone was inoculated in 1 ml of LB broth containing 50 μg/ml of ampicillin and incubated at 37°C in a shaking incubator until the culture reached mid-log phase. This was then transferred to a vial containing 1 ml glycerol solution. This was mixed gently and stored at -80°C until further use.

3.9   Data analysis

3.9.1   Sensitivity and specificity evaluation of tissue compression technique

The detection of sarcocyst in nested PCR, stained and unstained tissue

compression technique, were determined by using a binary scoring system. The sensitivity and specificity of microscopic methods comprised of unstained and stained groups were calculated based on reference test by means of nested PCR. Percentages of sensitivity and specificity (%) were calculated according to the formulae in Appendix C.

3.9.2 Positive predictive value (PPV) and negative predictive value (NPV)

Positive predictive value (PPV) and negative predictive value (NPV) were calculated based on previousl literature on prevalence of sarcocystosis (41%) in Selangor district (Latif *et al.*, 2013), by software from http://epitools.ausvet.com.au. (Sergeant, 2015). Both percentages of PPV and NPV were estimated according to the formulae in Appendix C.

3.9.3 Statistical tests

McNemar's test was carried out to test the discordance between the nested PCR, stained and unstained samples. In addition, Cohen's kappa statistics was applied to determine the concordance between the three techniques. All statistical analyses were conducted using SPSS V20.

3.9.4 Principal component analysis

The length and width of sarcocysts were analysed using principal component analysis (PCA), via R software, using modified scripts.

3.9.5 Sequencing data preparation for multiple sequence alignment

Random picked PCR positive samples were used in the phylogenetic study. Phylogenetic tree analysis was applied to study the overall diversity of *Sarcocystis* spp. infecting both cattle and goats through identification of potential clustering of individual

sequences in branches.

Sequence results obtained were initially analysed using Bioedit alignment software. Nested PCR primers were utilised to find both forward and reverse sequences (expected sequences length ~920 bp) and to trim the target sequences. Both forward and reverse sequences were aligned using MUSCLE algorithm implanted in Seaview V4 (Gouy *et al.*, 2010) to form consensus sequences. BLAST was used to identify sequence identity. Multiple sequence alignment was performed including out-group reference sequences obtained from the GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

3.9.6 Phylogenetic tree analysis

The phylogenetic tree was constructed using maximum likelihood (PhyML) under default settings. A total of 41 sequences were used in the analysis. These included nine sequences obtained in the present study, 31 *Sarcocystis* spp. reference sequences, and *Eimeria tenella* sequence (U67121) as outgroup. The *Sarcocystis* spp. sequences were: *S. cruzi* (GenBank: AF017120, JX679468), *S. capracanis* (GenBank: L76472), *S. tenella* (GenBank: KC209737, KC209735, KC209734), *S. hominis* (GenBank: AF176944, KF954731), *S. truncata* (GenBank: GQ251030), *S. silva* (GenBank: EU282016), *S. buffalonis* (GenBank: AF017121), *S. hirsuta* (GenBank: AF017122), *S. moulei* (GenBank: L76473), *S. gigantea* (GenBank: L24384), *S. nesbitti* (GenBank: JX661499, KC878476, HF544324), *S. zuoi* (GenBank: KC878487, KC878488, JQ029113), *S. singaporensis* (GenBank: KC878483, AF434059, KC878489), *S. columbae* (GenBank: GU253883), *S. cornixi* (GenBank: EU553478), *S. anasi* (EU553477), *S. neurona* (U07812), *S. suihominis* (GenBank: AF176936, AF176937), *S. elongata* (GenBank: GQ251014) and *S. hjorti* (GenBank: EU282017). The tree was constructed using Seaview V4 software (Gouy *et al.*, 2010).

CHAPTER 4: RESULTS

4.1 Tissue compression technique

A total of 583 tissue samples were sampled from 90 cattle (11 from local Malaysia Kedah-Kelantan breed; 79 imported from Australia, Droughtmaster breed) and 55 Australian feral goats. Preliminary screening by tissue compression technique (unstained and stained tissue preparations) revealed 245 samples containing sarcocysts. The positives samples were then cross-checked with nested PCR and subjected to the comparative detection techniques.

4.2 Prevalence of sarcocystosis in cattle and goats

The prevalence of sarcocystosis in both cattle and goats collected from the abattoir in Shah Alam was high. A total of 70 out of 90 cattle [77.8% (95% CI: 69.2-86.4)] (Appendix D) and 39 out of 55 goats [70.9% (95% CI: 58.9-82.9)] (Appendix E) were infected with *Sarcocystis* spp.

4.3 Sarcocysts morphometric characterisation

Sarcocysts were found in all five organs of both animals. A total of 357 sarcocysts were detected from 245 tissue samples (332 were involved in morphometry study, 22 broken sarcocysts were omitted). All sarcocysts were microscopic and spindle shape (rice grain size macrocysts were not found) (Figure 4.1). Table 4.1 presents the morphometric measurement of sarcocysts recovered from various organs from both animals. Cattle sarcocysts measured between 468.9-545.0 μm x 70.1-86.2 μm in the tongue, oesophagus, diaphragm and skeleton muscle while those found in the heart muscle were significantly smaller, with length and width in median of 283.5 μm x 84.7 μm. In goats, the median sarcocysts ranged between 445.2-600.1 μm x 76.8-103.5 μm.
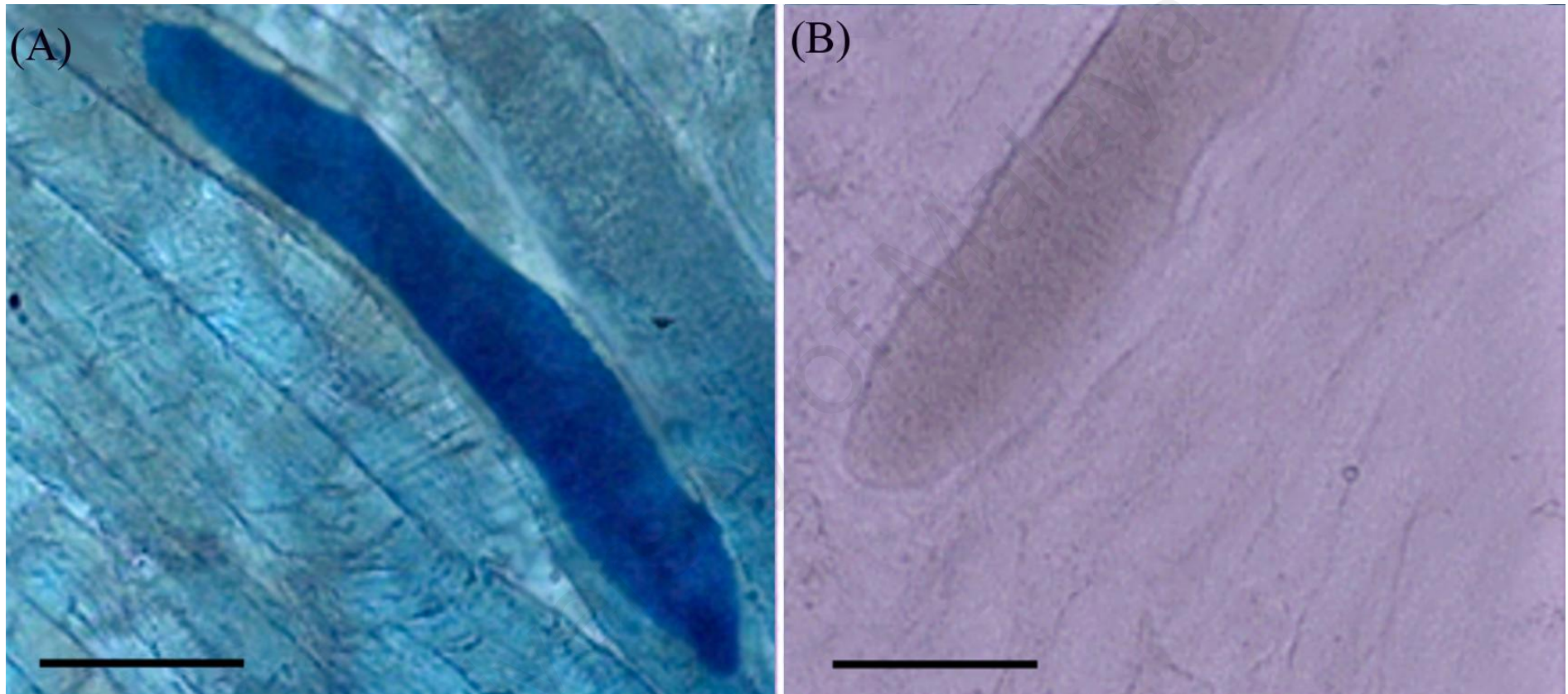
Figure 4.1: Sarcocysts within a tissue under unstained and methylene blue stained tissue preparations

(A) Spindle-like sarcocyst stained dark blue by methylene blue tissue compression preparation. (B) Brownish coloured sarcocyst in muscle tissue under unstained tissue preparation. Scale bars represent 50 μm and 100 μm corresponding to (A) and (B), respectively.

Table 4.1: Median (inter quartile range) (length and width in μm of sarcocysts) from different organs/body parts of cattle and goats

Host	Organs/Body parts	Length $\tilde{x}$ (IQR)	Width $\tilde{x}$ (IQR)	Sarcocyst size	Suspect *Sarcocystis* spp.
Cattle	Tongue (n = 72)	479.4 (261.6)	70.1 (24.4)	microscopic	*S. cruzi*
	Oesophagus (n = 48)	545.0 (278.9)	86.2 (26.8)	microscopic	*S. cruzi*
	Diaphragm (n = 61)	505.1 (240.1)	73.2 (28.2)	microscopic	*S. cruzi*
	Heart (n = 111)	283.5 (92.7)	84.7 (38.5)	microscopic	*S. cruzi*
	Skeletal muscle (n = 40)	468.9 (216.8)	73.8 (14.9)	microscopic	*S. cruzi*
Goat	Tongue (n = 48)	580.8 (240.6)	89.1 (40.6)	microscopic	*S. capracanis*/*S. hircicanis*
	Oesophagus (n = 53)	600.1 (103.5)	103.5 (52.0)	microscopic	*S. capracanis*/*S. hircicanis*
	Diaphragm (n = 20)	445.2 (76.8)	76.8 (17.7)	microscopic	*S. capracanis*/*S. hircicanis*
	Heart (n = 14)	218.6 (40.0)	59.7 (24.8)	microscopic	*S. capracanis*/*S. hircicanis*
	Skeletal muscle (n = 31)	598.3 (224.2)	80.5 (22.6)	microscopic	*S. capracanis*/*S. hircicanis*

n = sarcocysts count

$\tilde{x}$ = Median

IQR = Inter-quartile range

Similar to cattle, sarcocysts of the heart muscles in goats were smaller, measuring 218.6 μm x 59.7 μm. The PCA scatterplot (Figure 4.2) showed no significant clustering except for those found in the heart muscles. The Kruskal-Wallis test showed that sarcocysts in the heart muscle tissues were significantly ($p < 0.05$) smaller from other organs. Dunn's multiple pair-wise post-hoc variation test (Table 4.2) also indicates the smaller sarcocysts in the heart muscles, particularly in terms of length of the cysts.

4.4   Efficacy of methylene blue stain for the detection rate of sarcocysts

4.4.1   Unstained and stained tissue compression technique

Morphologically, the unstained bradyzoites appeared brownish, and less visible under a light microscope, whereas the methylene blue dye stained bradyzoites dark blue hue allows better differentiation of sarcocysts with the background (Figure 4.3A). Conversely, the brown unstained sarcocysts is also similar the host muscle tissues (Figure 4.3B). Positive sarcocysts detected by both unstained and stained techniques were 26.5% (95% CI: 21.0–32.0) and 76.3% (95% CI: 71.0–81.6), respectively (Table 4.3). Additionally, no macroscopic cysts were observed in either samples. As the nested PCR scored highest detection between the three techniques (Table 4.3), it was then set as the standard for statistical calculations.

4.4.2   Nested PCR assay

The nested PCR product for sarcocyst-positive samples produced a band at 950 bp and no amplification was observed for samples without cysts (Figure 4.4). The nested PCR assay successfully amplified 200 out of 245 positive samples (Table 4.3), with the detection rate of 81.6% (95% CI: 76.7–86.5).
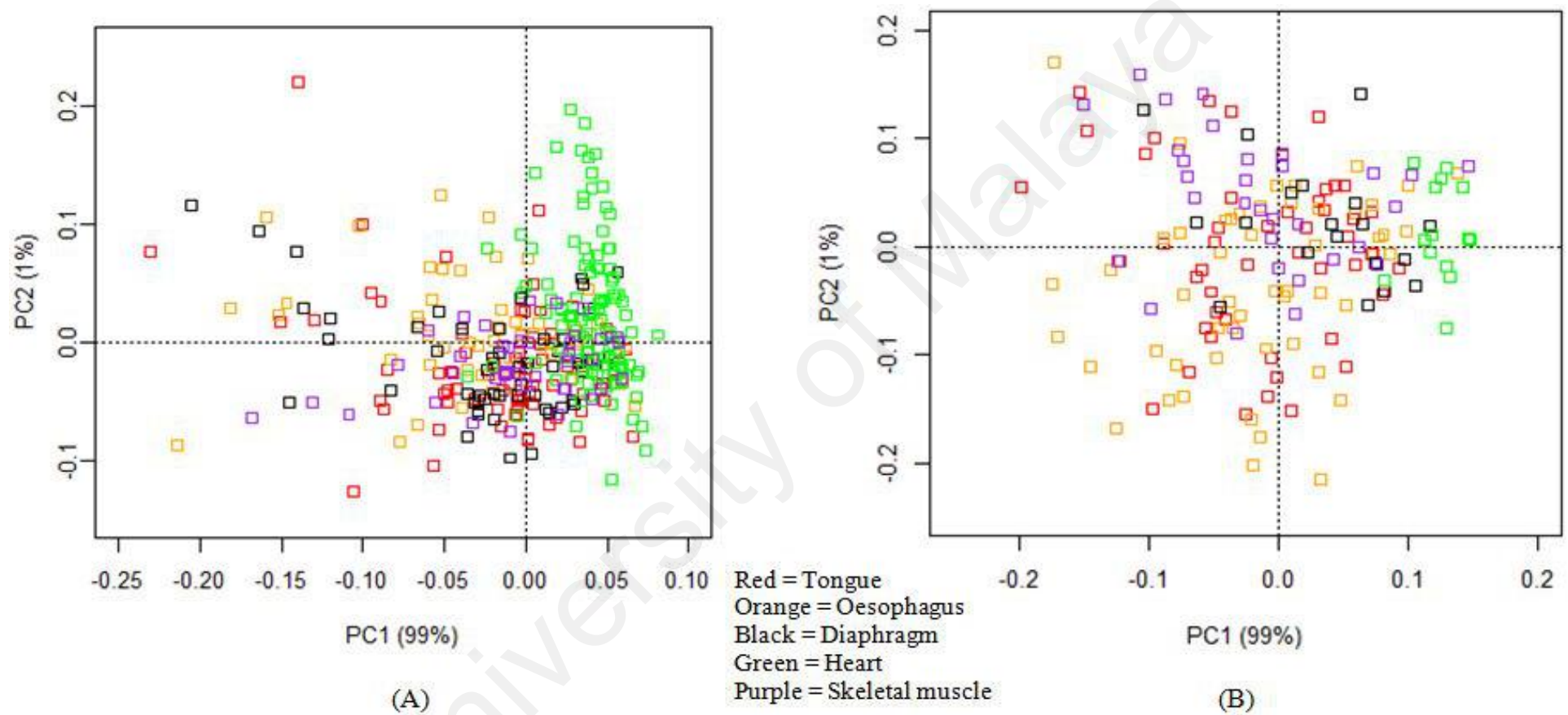
Figure 4.2: Morphometric data analysed by principal component analysis using parameters such as length and width measurements from sarcocysts in cattle (A) and goats (B)

Sarcocysts isolated from multiple organs are labelled with different colours: Red = Tongue; Orange = Oesophagus; Black = Diaphragm; Green = Heart; Purple = Skeletal muscle.

Table 4.2: Krukal-Wallis multiple pair-wise with Dunn's post-test in terms of sarcocysts sizes comparison

Host	Parameter	Comparison	*p* value	Parameter	Comparison	*p* value
Cattle	Length	T / E	1.000	Width	T / E	0.001*
		T / D	1.000		T / D	1.000
		T / H	0.000*		T / H	0.002*
		T / M	1.000		T / M	1.000
		E / D	1.000		E / D	0.065
		E / H	0.000*		E / H	1.000
		E / M	0.400		E / M	0.054
		D / H	0.000*		D / H	0.217
		D / M	1.000		D / M	1.000
		H / M	0.000*		H / M	0.178
Goat	Length	T / E	1.000	Width	T / E	1.000
		T / D	0.359		T / D	0.203
		T / H	0.000*		T / H	0.000*
		T / M	1.000		T / M	0.251
		E / D	0.436		E / D	0.012*
		E / H	0.000*		E / H	0.000*
		E / M	1.000		E / M	0.009*
		D / H	0.000*		D / H	0.399
		D / M	0.524		D / M	1.000
		H / M	0.000*		H / M	0.111

T = Tongue; E= Oesophagus; D = Diaphragm; H = Heart; M = Skeletal muscle
p = Adjust significant value where * indicates significant at level 95%
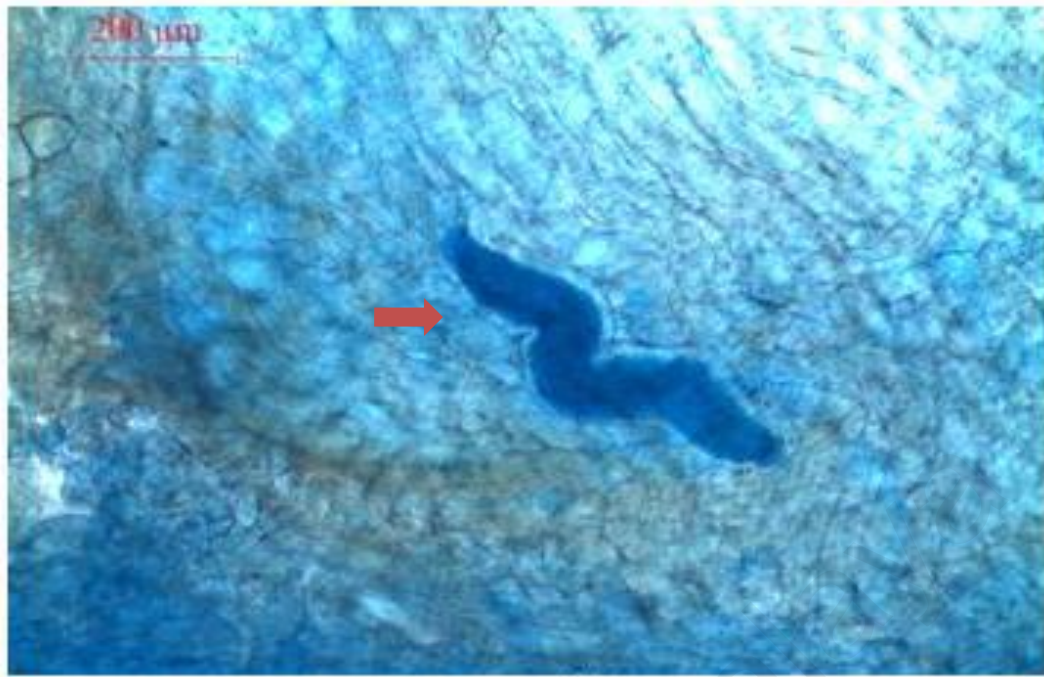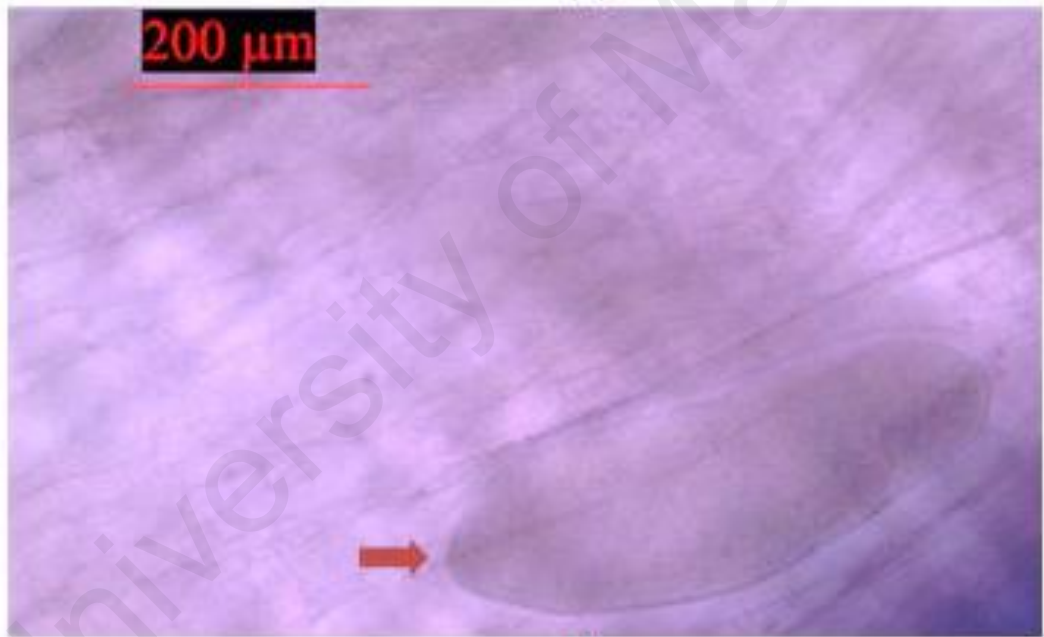
Figure 4.3: Comparison of methylene blue stained (A) and unstained (B) tissue samples on the detection of sarcocyst by tissue compression technique

The samples were observed under light compound microscope (10x magnification).

Table 4.3: Sarcocyst detection from unstained, stained and nested PCR from tissue samples of cattle and goats

Methods	Total number (n = 245)	Detection rate (%) (95% CI)
Nested PCR		
Positive (+ve)	200	81.6
Negative (-ve)	45	(76.7-86.5)
Unstained		
Positive (+ve)	65	26.5
Negative (-ve)	180	(21.0-32.0)
Stained		
Positive (+ve)	187	76.3
Negative (-ve)	58	(71.0-81.6)

n = host tissue sample size

CI = Confidence intervals
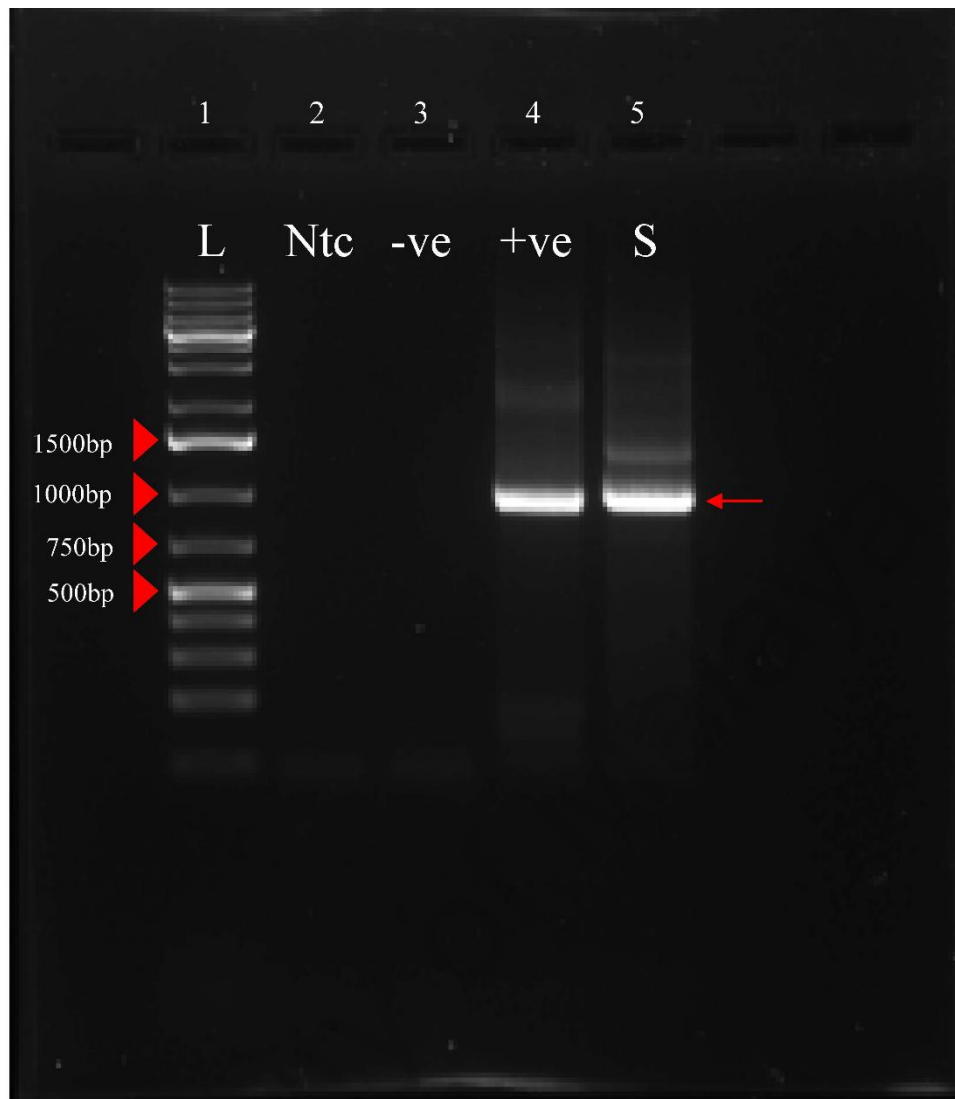
Figure 4.4: Molecular detection of sarcocystosis through nested PCR

The approximate amplicon size (930bp), is indicated by a red arrow (right). Lane 1 = DNA ladder (O'GeneRuler™ 1kb plus DNA Ladder); lane 2 = No template control; lane 3 = Negative control (no sarcocyst muscle tissue); lane 4 = Positive control (+ve), *S. cruzi* (GenBank accession no.: KJ917910); lane 5 = Sample (S).

4.4.3 Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV)

Out of 200 positive samples detected via nested PCR, the examination of the unstained and stained specimens by microscopy recorded the sensitivities of 25.0% (95% CI: 19.0–31.0) and 85.5% (95% CI: 80.6–90.4), respectively. The specificities, on the other hand, were 66.7% (95% CI: 52.9–80.5) and 64.4% (95% CI: 50.4–78.4), respectively. Furthermore, the positive predictive values (PPV) and negative predictive values (NPV) of the unstained method were 34.3% (95% CI: 22.8–45.8) and 56.1% (95% CI: 48.9–63.3), whereas the PPV and NPV of the stained technique were 62.5% (95% CI: 55.6–69.4) and 86.5% (95% CI: 77.7–95.3), respectively.

4.4.4 Statistical calculations

The Cohen's kappa values were estimated according to Cohen's kappa concordance index (Devito *et al*., 2013). Comparatively, sarcocysts detection rate of the stained technique was in moderate agreement with those of the nested PCR, with a Cohen's kappa of 0.449. In contrast, poor agreements were recorded between the unstained technique and the nested PCR, and between the unstained and stained techniques. Both comparisons resulted in negative kappa values, at -0.039 and -0.152, respectively. Apart from that, Mc Nemar's test showed no significant difference between the stained and nested PCR techniques in sarcocyst detection, with $\chi^2 = 3.200$, $p = 0.074$. In contrast, the unstained technique detected significantly fewer sarcocysts when compared with stained technique and nested PCR assay, with $\chi^2 = 83.188$ and 108.824, $p < 0.01$, respectively. All statistical calculations were summarised in Table 4.4.

Table 4.4: Comparative methods in sarcocyst detection (nested PCR (gold standard), unstained and stained microscopy)

Comparison	True positive	True negative	False negative	False positive	χ^2	p	κ	Concordance
Nested PCR / unstained	50	30	150	15	108.824	< 0.01*	-0.039	Poor
Nested PCR / stained	171	29	29	16	3.200	0.074 [ns]	0.449	Moderate
Unstained / stained	38	31	27	149	83.188	< 0.01*	-0.152	Poor

+ = positive, - = negative

χ^2 = McNemar test

ns = not significant

* Significant difference (p < 0.01)

κ = Cohen kappa

4.5   Polymerase Chain Reaction (PCR) on 18S rDNA of *Sarcocystis* spp. and cloning

4.5.1   18S rDNA sequence analysis

Representatives of sarcocysts from 12 cattle and 14 goat samples were selected for DNA extraction. Their 18S rDNA gene were cloned, of these, two to four clones per sample were selected for sequencing. In total, 66 sequences were obtained (Figure 4.5). The sequences were deposited into GenBank (Appendix G and H). BLAST analysis revealed that 31 sequences from cattle were identified as *S. cruzi ssu* rDNA (99-100% similarity with *S. cruzi* JX679468). Eleven of the sequences from goat samples were 99-100% similar to *S. capracanis* (GenBank no. L76472). The remaining 24 sequences were 97-99% similar with *S. tenella* (GenBank no. KC209737).

4.5.2   Phylogenetic tree analysis

Nine representative sequences that have highest identity with respective reference gene through BLAST analysis, were included with 32 sequences from GenBank in maximum likelihood phylogenetic tree analysis. The tree showed four clades of *Sarcocystis*, each corresponding to the respective hosts: Clades A and B, mammals (mostly ruminants); clade C, snakes; and clade D, birds (Figure 4.6). Nine sequences were placed in the ruminant clade B (Figure 4.7). Sequences of sarcocysts from cattle (C11H11, C14H4 and C13D14) were clustered together with *S. cruzi* (GenBank no. AF017120). Interestingly, *S. cruzi* reference gene (GenBank no. JX679468), was distinctly separated from the group. Sequences obtained from goat samples (G6D3, G6E2 and G19H3) clustered together with *S. capracanis* (GenBank no. L76472). Oddly, G24M1, G24M4 and G10M4 which showed high similarity with *S. tenella* in the BLAST analysis formed a separate group from *S. tenella* (GenBank no. KC209734, 35 and 37) and *S. capracanis* (GenBank no. L76472).
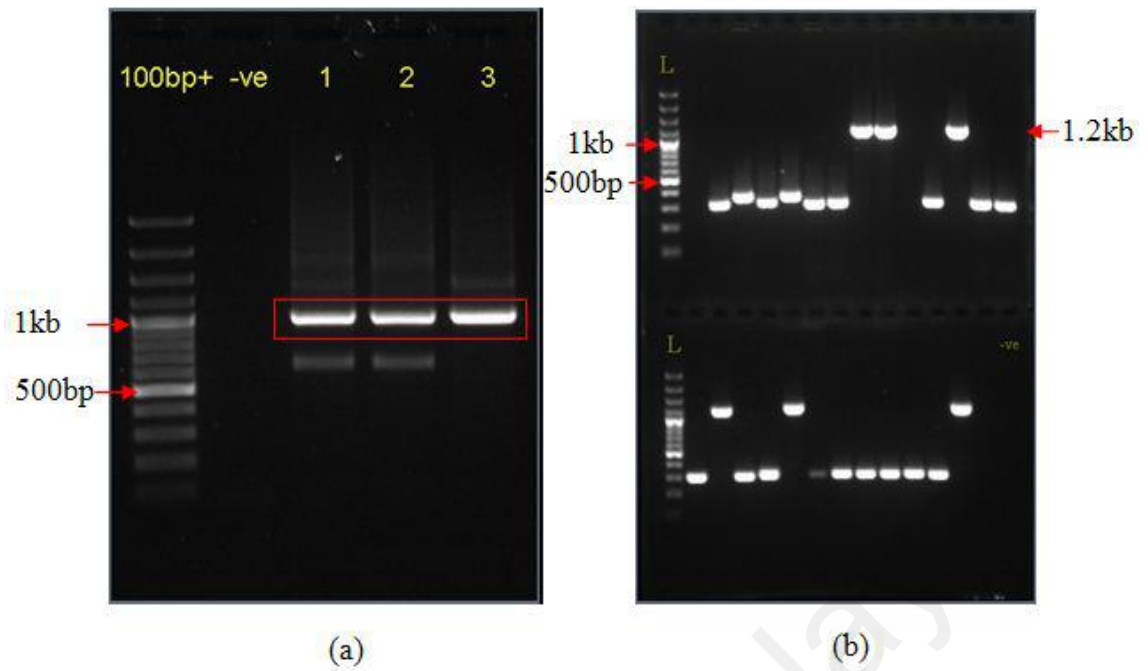
Figure 4.5: Gel electrophoresis images of PCR products of *Sarcocystis* spp.

(a) PCR of 18S rDNA using genomic DNA of *Sarcocystis* spp. Lane 1, 2 and 3 in (a) display positive amplification (framed in red), lane 3 amplifies single band whereas lane 1 and 2, respectively contain extra bands at approximately 650 bp. (b) PCR of *E. coli* clones for positive recombinant selection. Clones that contain positive insertion were amplified about 1.2k bp. L and 100 bp+ represent DNA ladder and −ve indicates non template control in both gel images.
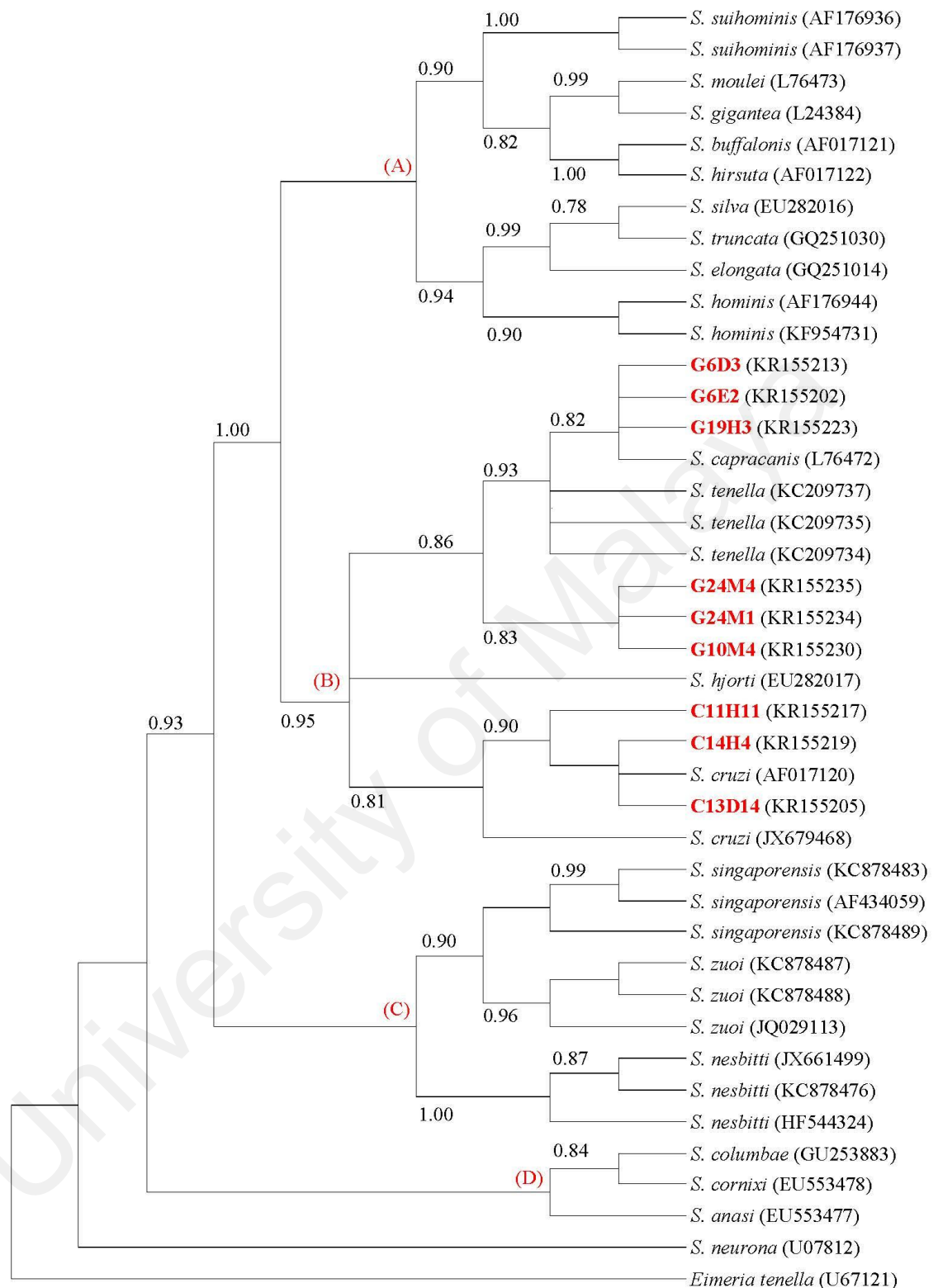
Figure 4.6: Phylogenetic tree displays the relationships of multiple *Sarcocystis* spp. according to 18S rDNA

The tree is constructed using the maximum likelihood, GTR model and the numbers next to each branch are based on branch support (aLRT). This tree is generated using SeaView V4 (Gouy *et al.*, 2010).
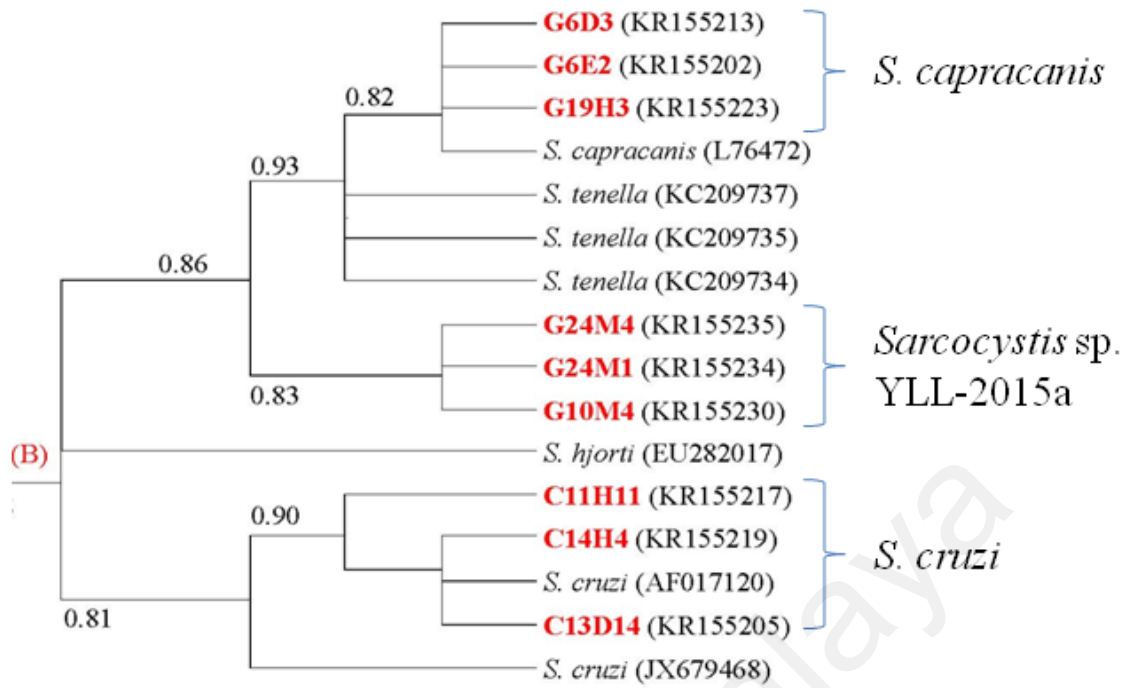
Figure 4.7: Phylogenetic tree of clade B

Sarcocystis spp. in this clade utilise cattle, goats, and sheep as intermediate hosts. All *Sarcocystis* spp. in clade B share the same group of definitive hosts from the family of Canidae.

CHAPTER 5: DISCUSSION

5.1   Efficacy of methylene blue for the detection of sarcocyst in tissue samples of meat producing animals

Sarcocystis spp. are commonly found in livestock such as cattle and goats, with *S. cruzi* and *S. capracanis* pathogenic to cattle and goats (Dubey *et al*., 1989c&d). Infection can contribute to huge economic losses in agriculture, globally. However, this disease has often been neglected probably due to the difficulty in detection within the deep muscle tissues of the infected hosts (Lee *et al*., 2014). Unfortunately, current available methods for species identification are still inconclusive and relatively expensive (e.g. transmission electron microscopy study), therefore not practical to be applied for massive screening. Therefore, the present study was conducted to compare three methods utilising light microscopy micrometric measurements and molecular approach for its effectiveness and cost saving in order to characterise *Sarcocystis* sarcocysts isolated from cattle and goats.

In this study, the methylene blue staining method detected approximately more than three folds of sarcocysts compared to the unstained method and is able to reduce time to examine the samples, as stained sarcocysts are easily detected. Methylene blue in parasitology also has been proven to be useful by Ferreira *et al.* (2006). Commercially available methylene is also used to stain *Trypanosoma cruzi* instead of using fluorochromes, that allows observation using a simple compound microscope rather than fluorescent microscope. Besides, methylene blue is also capable of staining *T. cruzi* more rapidly than the fluorescence dyes. In malaria blood smear diagnosis, methylene blue stains the gametocytes dark blue at the same time producing a pale background, whereas Giemsa stain produces significantly darker background and this method requires additional de-staining steps (time-consuming) (Mohapatra *et al.*, 2014).

Low specificity was noted between unstained and stained method compared to the nested PCR assay, of which 15 unstained and 16 stained gave false positive results. Some samples may have been misinterpreted as positive under microscopy, due to the difficulty of distinguishing between the sarcocysts and sarcocyst-like artefacts. Connective tissue collagen fibres and muscle Purkinje cells are stained by methylene blue and can also be misinterpreted as sarcocysts. In addition, tissue sections with inconsistent thickness, particularly thick sections with low numbers of sarcocysts, make microscopic interpretation more difficult and error-prone, thereby reducing sensitivity. Furthermore, the sensitivity of detection is dependent on experience as experienced microscopists are more likely to detect cysts in unstained slides, thereby narrowing the sensitivity gap between the two techniques. All microscopists in the current study were experienced with multiple observers analysing all samples to reduce bias. The detection rate of sarcocyst by the staining technique and nested PCR were similar. However, the nested PCR was used as the standard in the present study because of its high sensitivity in detecting *Sarcocystis* spp. (Kutty *et al.*, 2015).

Although results from the nested PCR had the highest detection rate, however, there are several disadvantages. First, nested PCR is time-consuming and required a total of 6 h, therefore cannot be applied as a routine diagnostic method. Secondly, improper or mishandling during preparation of PCR steps may potentially produce false results due to cross contamination. Lastly, this method is extremely costly as it requires expensive thermal cycler and PCR reagents.

Acetic acid was omitted in the methylene blue staining protocol as it is harmful on extensive inhalation. Although the staining technique is rapid, user-friendly and cost-effective, several precautionary steps are required when using this method for diagnostic purposes. The dye needs to be checked frequently since debris accumulates in unused solution, resulting in artefacts and, in turn, false-positives. Also, frequent dye usage

increases the probability of introducing contaminants, which can be overcome by filtering or by utilising a simple chromatography test for quality control purposes (Cook, 2006). It is therefore, advisable to change the working solution at frequent intervals to prevent contamination. Finally, the pH of the dye should be monitored regularly since pH changes affect the dye quality by altering its stability (Cook, 2006). The factors contributing to dye instability are not identified, although one possibility could be the dissociation of water molecules into hydronium (H_3O^+) and hydroxide ions (OH^-). As a result, these hydroxide ions actively bind to methylene blue and hinder DNA binding.

The use of methylene blue without acetic acid in the muscle compression technique is rapid, cheap and less toxic than other standard methods for detecting sarcocysts in meat samples. This technique would be a useful in abattoirs and meat inspection centres, where large numbers of meat samples need regular examination.

5.2   Morphometric characterisation of *Sarcocystis* spp.

Measurements of sarcocysts in the cattle in this study was similar to *S. cruzi* found in cattle reported by Obijiaku *et al.* (2013), while the size of sarcocysts infecting goats was within the range of reported *S. capracanis* and *S. hircicanis*, 1000 μm and 2500 μm respectively (Dubey *et al.*, 1989d). However, sarcocysts isolated in goats in this study were smaller than those isolated in Bangalore, India (Dafedar *et al.*, 2008).

Although sarcocysts are frequently used for *Sarcocystis* spp. identification, the measurements need to be interpreted with care (Dubey *et al.*, 1989b). Maturity of sarcocyst vary depended on the time intervals of infection in the hosts. Immature cysts, developing and mature sarcocysts of the same species have different sizes. Furthermore, the size can be affected by various physiological conditions of the muscles (muscle contraction and relaxation) in the hosts (Dubey *et al.*, 1989b). Apart from these factors, the size may also differ depending on the organ or tissue where the sarcocyst is found.

Result from this study is in agreement with Obijiaku *et al.* (2013) which found smaller sarcocysts in the heart muscles. This is possibly due to the more active and stronger contraction and relaxation of the heart muscles. Consequently, the stronger contraction in tightly packed muscle tissues restricts the sarcocysts size.

Current study found only *S. cruzi* in the cattle samples, the species that is most prevalent and pathogenic to the cattle. It affects the cattle by causing severe damage to the meat as well as reduces milk production among female cattle. On the other hand, two *Sarcocystis* spp. are found among feral goats, notably *S. capracanis* and *S. hircicanis*.

5.3   Molecular characterisation of *Sarcocystis* spp.

Sequence analysis of 18S rDNA showed that the imported cattle were infected with *S. cruzi*, and goats with *S. capracanis* and possibly also *S. tenella*. The evolution of *Sarcocystis* spp. causes notable changes in the variable sites of the 18S rDNA (Holmdahl *et al.*, 1999). Interestingly, the high frequency of gaps that have been observed in current sequence alignment was found to be consistent throughout the entire sequence alignment when corroborated with the reference genes. Indeed, the two nested primers, notably 3L (forward) and 2H (reverse), were designed to flank the fragment at about 950 bp where the V4 region is involved (Figure 5.1). The V4 region is known as the most variable sites of all eukaryotes and therefore is useful for assessing their biodiversity (Hadziavdic *et al.*, 2014). Sliding window plot for both multiple *Sarcocystis* spp. (Figure 5.1A) and *Sarcocystis* sequences in the current study in clade B (Figure 5.1B) displayed the highest variability. This suggests the V4 region in current study, is the species determining region of 18S rDNA.
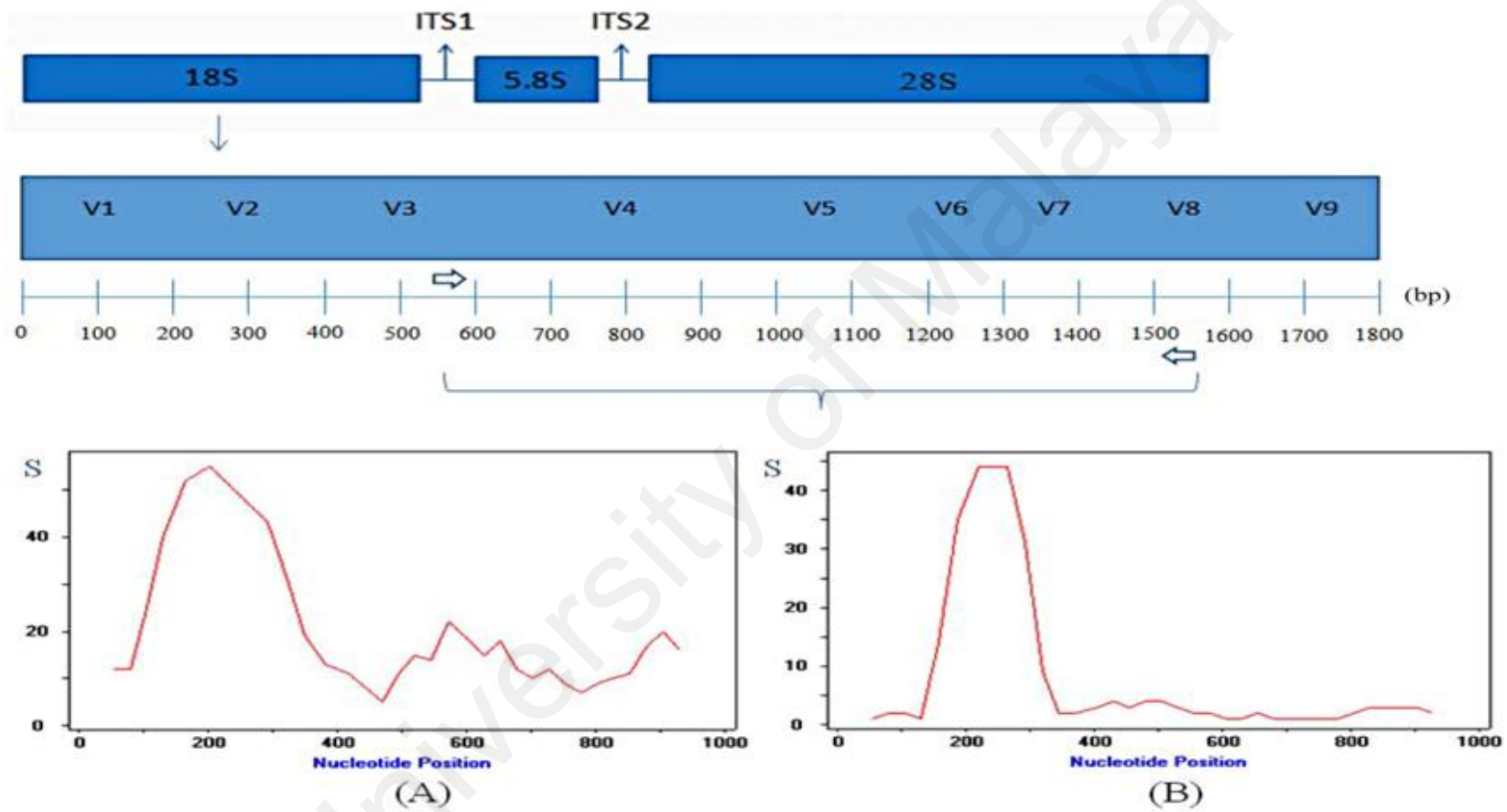
Figure 5.1: Multiple variable regions of 18S rDNA

Forward and reverse arrows indicate the regions which amplified at about 950 bp in current study. Sliding window plots indicate the number of segregating sites (S) corresponding to the nucleotide position. (A): Comparison of all *Sarcocystis* spp. in phylogenetic tree analysis (Figure 4.6), whereas (B): Comparison of *Sarcocystis* spp. in clade B only (Figure 4.7).

Besides, sequence variations among *S. cruzi* may also be due to the presence of microheterogeneity within a sarcocyst which consists of more than one copy of 18S rDNA within the parasite genome. This phenomenon had also been detected by Vangeel *et al*. (2013), where the sequence divergence of *S. hominis* was up to 5.5%. However, the occurrence of microheterogeneity in *Sarcocystis* spp. still remains ambiguous because host tissues are often harbouring more than one sarcocysts. Therefore, the gene variants may have arisen from different sarcocysts rather than a single sarcocyst (Gjerde *et al*., 2015).

The prevalence of sarcocystosis caused by *S. cruzi* among Droughtmaster cattle is approximately two folds compared to the previous Sarcosystis prevalence study done in Malaysia (Latif *et al.*, 2013), which this study used the enhanced technique by using methylene blue stain. High prevalence of cattle muscular sarcocystosis has also been reported elsewhere apart from Malaysia. In Italy, 80% prevalence rates in cattle were caused by *S. cruzi* (Bucca *et al.*, 2011; Chiesa *et al.*, 2013; Domenis *et al.*, 2011; Meistro *et al.*, 2015). In Iran, all cattle (100%) examined were infected with microscopic *Sarcocystis* spp. (Fard *et al.*, 2009; Hamidinejat *et al.*, 2010). In Australia, the prevalence of cattle sarcocystosis was reported to be in between 52% to 90% (Munday, 1975; Savini *et al.*, 1992; Savini *et al.*, 1994). It is noted that the prevalence of sarcocystosis among imported cattle from Australia, is notably lower compared to those from other countries and this is in line with the outcome of the current study. The Droughmaster breed produces more meat, has faster growth rate, and most importantly, is known to be resistant to certain parasitic infections (Donaldson, 2003). Therefore, the breeds of the cattle may be factor that contributes to the differences of sarcocystosis status in different countries. However, many *Sarcocystis* studies did not highlight the cattle breeds in their studies, therefore it is not possible to carry out a comparative study in terms of susceptibility of different breeds of cattle to sarcocystosis.

Surprisingly, in the current study *Theileria spp.* (tick-borne blood parasite) was detected in the tongue and oesophagus of cattle through PCR, which can be presumably attributed to contamination of tissues by infected blood. Current study did not detect *S. hominis* or *S. hirsuta* in its samples. It is noted that *S. hominis* requires primates to complete its life cycle. Human (farmers) faecal contamination is thought to be rare as most farmers are well equipped with hygienic facilities such as toilets and washing stations in all farmlands. Likewise, *S. hirsuta* is transmitted by cats, but cats tend to bury their faeces and seldom to be found in cattle farms, therefore the transmission of *S. hirsuta* is rarely detected in cattle (Latif *et al.*, 2013).

The dynamic of disease transmission is believed to be closely associated with management and hygiene levels of the herds. One possible reason for the detection of *S. cruzi* rather than *S. hominis* or *S. hirsuta* in cattle is the presence of dogs. Dogs are the natural definitive hosts of *S. cruzi* and they excrete *S. cruzi* oocysts in their faeces. Dog faeces containing *S. cruzi* may contaminate the grass that cattle eat, thereby transmitting *S. cruzi* to cattle. Additionally, in Australia farmlands, dogs are used to monitor cattle. Therefore, the source of infection was most likely dog faeces which contaminate the grass and soil of the farms. The lack of proper farm management, especially in terms of cleanliness, encourages effective disease transmission between two hosts. Additionally, flies have been reported to be mechanical vectors in the transmission of oocysts from dog faeces to cattle feeds (Markus, 1980).

On the other hand, feral goats (*C. hircus*) are susceptible to *S. capracanis* infection due to interaction with the definitive hosts, dogs. In Australia, dogs are often used to help shepherd large groups of goats. This increases the interaction between the dogs and feral goats thereby allowing effective transmission of *S. capracanis* between the hosts. Interestingly, in the current study, the 18S rDNA of *Sarcocystis* sp. found in the goats have a high sequence similarity (97-99%) with that of *S. tenella*, which

naturally infects sheep. There is a possibility that *S. tenella* can also infect goats because both sheep and goats belong to the sub-family of Caprinae. Recently, Kolenda *et al.* (2015) reported the finding of *S. tenella* in Tatra chamois (*Rupicapra rupicapra tatrica*), a goat-antelope of the same sub-family. The ability of a *Sarcocystis* spp. to infect multiple hosts is not uncommon. For example, *S. cruzi* has also been shown to infect water buffaloes (*B. bulbalis*) and cattle (*B. taurus*) (Li *et al.*, 2002). Alternatively, the *Sarcocystis* sp. in the goats might be *S. hircicanis*, a species that naturally infects goats (Dafedar *et al.*, 2008). Phylogenetic analysis showed that the 18S rDNA of the *Sarcocystis* sp. formed a separate group from *S. tenella* and *S. capracanis* (GenBank no. L76472). Similar to *S. capracanis*, *S. hircicanis* causes microcyst in goats and is transmitted by dogs as final hosts. Insufficient or lack of the gene information on *S. hircicanis* in the GenBank explains the failure of BLAST to match the 18S rDNA of the *Sarcocystis* sp. with *S. hircicanis*.

5.4   Limitations

This study has encountered many challenges in measuring the sarcocysts walls due to the surrounding host muscle cells, which impeded the microscopic observations. To overcome this problem, one can isolate the cysts either by using a needle (Odening *et al.*, 1996), or by tissues homogenisation, followed by filtration using cheesecloth and separation of sarcocysts from the host muscle tissues using a Percoll gradient technique (Eggleston *et al.*, 2008). By doing this, the isolated sarcocysts from the muscle tissues can be studied more precisely and effectively.

Additionally, the current morphometric approach relies on two parameters notably length and width, which were not able to resolve the species differentiation. This is especially true for *Sarcocystis* spp. in goats, where the sarcocysts have similar sizes despite molecular data showing that there are two species existing within the same

host. Therefore, additional taxonomical data on the cyst wall thickness is needed to corroborate the morphometry study to identify *Sarcocystis* spp. effectively, especially for those with similar cyst size.

Even though the molecular technique by genotyping of 18S rDNA is a solid tool in describing *Sarcocystis* spp., it may still encounter several complications. An insufficient or lack of gene information corresponding to the unknown or new described species can be literally misleading, as can be seen in the current scenario where there is high possibility of *S. hircicanis* in goat being identified as *S. tenella* under BLAST analysis. Therefore, this implies that the sequencing results obtained, particularly for those undiscovered species need to be handled with care. Alternatively, further studies using additional genetic markers, such as cytochrome oxidase subunit 1 (COX 1) and internal transcribed spacer 1 (ITS-1) are needed to confirm the genetic diversity of *Sarcocystis* spp. in Malaysia. COX 1 indeed has been proven to be useful in phylogenetic studies and is able to resolve *Sarcocystis* spp. in terms of clade formation (Gjerde, 2013). This can further support the current discovery of possible *S. hircicanis* or an entire new *Sarcocystis* sp. in goat based on the 18S rDNA genotyping.

CHAPTER 6: CONCLUSION

In summary, the use of methylene blue in the tissue compression technique without acetic acid is rapid, cheap and less toxic than other standard methods for detecting sarcocysts in meat samples. This technique would be a useful tool in abattoirs and meat inspection centres, where large numbers of meat samples need regular examination. Apart from its usefulness to rapidly aid in meat samples screening, it also retains the morphological structure of sarcocyst for downstream characterisation purposes.

Morphological description and morphometric measurements of sarcocysts among *Sarcocystis* spp. can only determine the size of microcysts and macrocysts effectively, but it is still not sufficient for species identification, while molecular and phylogenetic analysis has enabled to accurately identify to species level.

Further genotyping on other genetic markers and increase sample size can provide better understanding about the genetic diversity of *Sarcocystis* spp. in Malaysian livestock.

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71

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LIST OF PUBLICATIONS

Publication from this research project

Ng, Y. H., Subramaniam, V., & Lau, Y. L. (2015). Modified use of methylene blue in the tissue compression technique to detect sarcocysts in meat-producing animals. *Veterinary Parasitology*, *214*(1-2), 200-203. doi:10.1016/j.vetpar.2015.09.032

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Ng, Y. H., Fong, M. Y., Subramaniam, V., Shahari, S., & Lau, Y. L. (2015). Short communication: Genetic variants of *Sarcocystis cruzi* in infected Malaysian cattle based on 18S rDNA. *Research in Veterinary Science*, *103*, 201-204. doi:10.1016/j.rvsc.2015.10.009

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Veterinary Parasitology

Short communication

Modified use of methylene blue in the tissue compression technique to detect sarcocysts in meat-producing animals

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ARTICLE INFO

ABSTRACT

Sarcocystosis in meat-producing animals is a major cause of reduced productivity in many countries, especially those that rely on agriculture. Although several diagnostic methods are available to detect sarcocystosis, many are too time-consuming for routine use in abattoirs and meat inspection centers, where large numbers of samples need to be tested. This study aimed to compare the sensitivity of the methylene blue tissue preparation, unstained tissue preparation and nested PCR in the detection of sarcocysts in tissue samples. Approximately three-fold more sarcocysts were detected in methylene blue-stained tissue compared to unstained controls (McNemar's test: $P < 0.01$). Test sensitivity was comparable to that of the gold standard for sarcocyst detection, nested polymerase chain reaction. These results suggest that methylene blue can be used in tissue compression as a rapid, safe, and inexpensive technique for the detection of ruminant sarcocystosis in abattoirs.

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1. Introduction

Sarcocystis species infect a wide range of intermediate hosts, notably livestock, to cause muscular sarcocystosis. The global prevalence of sarcocystosis in meat-producing animals is high, especially in cattle. Approximately 60–80% of cattle in Europe (Domenis et al., 2011), and 41% of cattle in Malaysia (Latif et al., 2013), have been infected with *Sarcocystis* species. Sarcocystosis causes deterioration in the well being of infected animals and results in reduced milk production, spontaneous abortions occur and death of heavily infected animals (Fayer, 2004). Infected carcasses can transmit the disease to other hosts via the fecal–oral route, including to humans, who may suffer from nausea, vomiting, and enteritis (acute, chronic and severe) (Fayer et al., 2015).

Several methods are commonly used to directly detect sarcocysts in tissue including macroscopic inspection, histological examination, the meat digestion technique, and the unstained tissue compression technique (Dubey et al., 1989). Gross inspection, although relatively quick and inexpensive, is the least sensitive method since only macroscopic sarcocysts can be detected because they are readily observable as rice grain-like cysts. Pepsin digestion of muscle tissue with subsequent light microscopy examination

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has also been used to detect bradyzoites and sarcocysts (Latif et al., 1999). However, the digestion method tends to be time-consuming precluding widespread application. In contrast, the muscle compression method is considered to be a rapid direct method for detecting sarcocysts, but there are concerns that its sensitivity is lower than the pepsin digestion technique (Latif et al., 1999).

Molecular detection of sarcocysts by the polymerase chain reaction (PCR) has improved the efficiency of diagnosis and allowed for accurate species identification (Yang and Zuo, 2000). In addition, the sensitivity and specificity can be further enhanced using nested PCR, in which a second round of amplification is used to detect target within the first product using a second set of primers (Pereira et al., 2008). Nested PCR has been successfully used to detect and identify *Sarcocystis* species in snakes and ruminants by partial 18S rRNA gene genotyping (Tenter et al., 1994; Heckeroth and Tenter, 1999; Lau et al., 2013).

Methylene blue (3,7-bis(dimethylamino)-phenothiazin-5-ium chloride) is a common dye used in parasitology (Malakauskas and Grikienienė, 2002; Pyziel and Demiaszkiewicz, 2009; Ferreira et al., 2006; Prakas et al., 2011; Mohapatra et al., 2014). Although methylene blue has previously been used to detect sarcocysts in the tissue compression method (Malakauskas and Grikienienė, 2002; Pyziel and Demiaszkiewicz, 2009; Prakas et al., 2011), there is a paucity of statistical data with respect to sensitivity and efficacy.

Therefore, we evaluated the efficacy of the methylene blue tissue compression technique compared to unstained tissue

Short communication: Genetic variants of *Sarcocystis cruzi* in infected Malaysian cattle based on 18S rDNA

CrossMark

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ARTICLE INFO

ABSTRACT

Sarcocystis species are pathogenic parasites that infect a wide range of animals, including cattle. A high prevalence of cattle sarcocystosis has been reported worldwide, but its status is unknown in Malaysia. This study focused on utilizing 18S rDNA to identify *Sarcocystis* species in Malaysian cattle and to determine their genetic variants. In this study, only *Sarcocystis cruzi* was detected in Malaysian cattle. The intra-species *S. cruzi* phylogenetic tree analysis and principal coordinate analysis (PCoA), respectively displayed two minor groups among the parasite isolates. This finding was supported by high Wright FST value (FST = 0.647). The definitive hosts (dogs) may play a fundamental role in the development of *S. cruzi* genetic variants. Additionally, the existence of microheterogeneity within the *S. cruzi* merozoites and/or distinct genetic variants arisen from independent merozoites in mature sarcocysts, possibly contributed to the existence of intra-species variations within the population.

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Sarcocystis is a genus of protozoa belonging to the family Sarcocystidae. Like *Toxoplasma gondii* and *Neospora caninum*, *Sarcocystis* species are coccidian parasites with heterogeneous life cycles that require both intermediate and definitive hosts. Briefly, definitive hosts (predators) become infected when they consume infected intermediate hosts. In return, preys get infected when they consume the silages contaminated with the faeces containing oocysts of *Sarcocystis* spp. (Fayer et al., 2015). Livestock, including cattle, goats and sheep, are susceptible to sarcocystosis (Latif et al., 1999), and symptoms include fever, haemorrhages, low meat and milk yields, abortion, encephalomyelitis, and death in severe cases (Fayer et al., 2015).

Sarcocystis infections of muscle tissues (muscular sarcocystosis) are usually found in tongue, oesophagus, diaphragm, heart and skeletal muscles (Prakas and Butkauskas, 2012). The prevalence of cattle sarcocystosis is high in countries like Argentina (90%), (More et al., 2011) and Iran (98%) (Latif et al., 1999). However, the status of cattle sarcocystosis in Malaysia is unknown. There was only one report on the prevalence (40.8%) of sarcocystosis in Malaysian cattle and water buffaloes (Latif et al., 2013). There are multiple cattle breeds available in Malaysia including the indigenous Kedah-Kelantan (KK) breed, while the main imported cattle breeds, such as Brahman and Droughtmaster breeds, are from Australia. Although 85% of the total cattle population in Malaysia are KK breed (Johari and Jasmi, 2009), they are not preferred as meat producing resources due to small body masses and slower growth rate compared with imported cattle.

Currently, identification of *Sarcocystis* species relies on molecular phylogenetic analysis focusing on 18S rDNA (Yang et al., 2001; Rosenthal et al., 2008). 18S rDNA is ideal for phylogenetic studies because it is highly conserved, and analysis of its variable regions allows for effective speciation of organisms within a genus (Maidak et al., 1997). The aims of this study were to identify *Sarcocystis* species in Malaysian cattle via phylogenetic analysis of 18S rDNA sequences and to evaluate their genetic variants.

Muscle tissues of 11 local Malaysian cattle (*Bos indicus*) including the tongue, oesophagus, diaphragm, heart, and skeletal muscle, were collected from Abattoir Shah Alam, Selangor, Malaysia. Tissues were cut into about 2 cm^2 in area, and approximately 2 mm in thickness and stained with methylene blue for 10 min. For each sample, three pieces of tissues were cut at different spots in an attempt to increase the sensitivity for detecting sarcocysts. The stained samples were then compressed between two glass slides and were observed under a light microscope. Samples with sarcocysts were subjected to DNA extraction using a QIAGEN DNeasy Blood and Tissues Kit (QIAGEN, Valencia, CA, USA).

Two sets of primers notably 1L, 1H; and 3L, 2H, were used for the amplification of 18S rDNA via nested Polymerase Chain Reaction (PCR) (Yang et al., 2001). Amplicons were cloned into a pGEM-T Vector System (Promega, Madison, WI, USA) and transformed into TOP 10 competent cells. Positive clones were confirmed with insertions by colony PCR and were sequenced using M13 universal primers. All sequences were analysed and trimmed using Bioedit software (www.mbio.ncsu.edu/bioedit/bioedit.html) before undergoing BLAST analysis for species identification (MegaBlast). Three sequences corresponding to the same sample were further aligned to form a consensus sequence. These sequences were then subjected to multiple sequence alignment

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APPENDIX A: Tools used in tissue compression technique

Scalpel blade

This tool is used to sample tissue sample from both cattle and goats in the slaughter house. Besides, it also used to cut sampled tissues into smaller pieces to be involved in tissue compression technique pre-screening phase.

Forceps

Forceps works on pair with scalpel blade to hold the tissue samples.
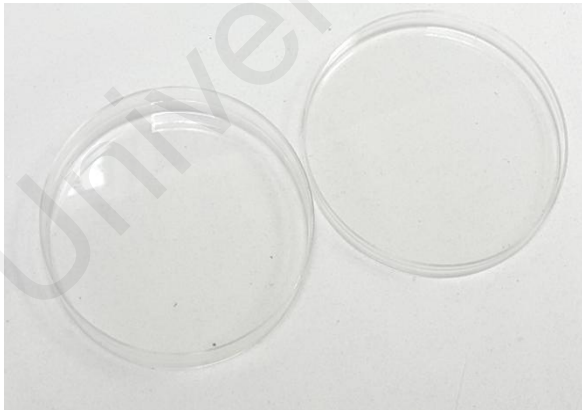
Petri dish

This apparatus serves as a dissecting board for the processing of tissue samples.

APPENDIX B: pGEM®-T Vector Map

T7 Transcription Start

```
5′...TGTAA TACGA CTCAC TATAG GGCGA ATTGG GCCCG ACGTC GCATG CTCCC GGCCG
3′...ACATT ATGCT GAGTG ATATC CCGCT TAACC CGGGC TGCAG CGTAC GAGGG CCGGC
             └──────────────────────┘              └───┘ └───┘  └───┘  └───┘
                    T7 Promoter                      ApaI  AatII  SphI   BstZI
```

```
CCATG GCCGC GGGATT3′(cloned insert)3′T     ATCAC TAGTG CGGCC GCCTG CAGGT CGACC ATATG
GGTAC CGGCG CCCTA        )3′TTAGTG  ATCAC GCCGG CGGAC GTCCA GCTGG TATAC
 └───┘ └─────┘                            └───┘ └─────┘ └───┘  └───┘  └───┘
  NcoI   SacII                             SpeI  NotI   PstI   SalI   NdeI
                                                 BstZI
```

SP6 Transcription Start

```
GGAGA GCTCC CAACG CGTTG GATGC ATAGC TTGAG TATTC TATAG TGTCA CCTAA AT...3′
CCTCT CGAGG GTTGC GCAAC CTACG TATCG AACTC ATAAG ATATC ACAGT GGATT TA...5′
      └───┘ └──────────┘ └───┘         └──────────────────────────┘
       SacI    BstXI       NsiI                 SP6 Promoter
```


pGEM®-T Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–113
SP6 RNA polymerase promoter (−17 to +3)	124–143
SP6 RNA polymerase transcription initiation site	126
pUC/M13 Reverse Sequencing Primer binding site	161–177
lacZ start codon	165
lac operator	185–201
β-lactamase coding region	1322–2182
phage f1 region	2365–2820
lac operon sequences	2821–2981, 151–380
pUC/M13 Forward Sequencing Primer binding site	2941–2957
T7 RNA polymerase promoter (−17 to +3)	2984–3

APPENDIX C: Formulae used for the calculations of sensitivity, specificity, positive predictive value and negative predictive value

$$\% \text{ Sensitivity} = \frac{\text{number of true positives}}{\text{number of true positives} + \text{number of false negatives}} \text{ X } 100\%$$

$$\% \text{ Specificity} = \frac{\text{number of true negatives}}{\text{number of true negatives} + \text{number of false positives}} \text{ X } 100\%$$

$$\% \text{ PPV} = \frac{\text{sensitivity X prevalence}}{\text{sensitivity X prevalence} + (1 - \text{specificity}) \text{ X } (1 - \text{prevalence})} \text{ X } 100\%$$

$$\% \text{ NPV} = \frac{\text{specificity X } (1 - \text{prevalence})}{(1 - \text{sensitivity}) \text{ X prevalence} + \text{specificity X } (1 - \text{prevalence})} \text{ X } 100\%$$

APPENDIX D: The total number of cattle (n=90) sampled in the Shah Alam Abattoir

Sample code	Result	Sample code	Result	Sample code	Result	Sample code	Result	Sample code	Result
C1	0	C21	1	C41	0	C61	1	C81	0
C2	0	C22	1	C42	1	C62	1	C82	1
C3	1	C23	1	C43	1	C63	0	C83	1
C4	1	C24	1	C44	1	C64	0	C84	1
C5	1	C25	0	C45	1	C65	0	C85	1
C6	1	C26	1	C46	1	C66	1	C86	1
C7	0	C27	1	C47	1	C67	0	C87	1
C8	0	C28	1	C48	1	C68	0	C88	1
C9	0	C29	0	C49	1	C69	1	C89	1
C10	0	C30	1	C50	1	C70	1	C90	1
C11	1	C31	1	C51	1	C71	1		
C12	0	C32	1	C52	1	C72	1		
C13	1	C33	1	C53	1	C73	0		
C14	1	C34	1	C54	1	C74	1		
C15	1	C35	1	C55	1	C75	0		
C16	1	C36	1	C56	1	C76	1		
C17	1	C37	1	C57	1	C77	1		
C18	1	C38	1	C58	1	C78	0		
C19	1	C39	1	C59	1	C79	1		
C20	1	C40	1	C60	1	C80	0		

0: Negative; 1: Positive.

APPENDIX E: The total number of feral goats (n=55) sampled in the Shah Alam Abattoir

Sample code	Result	Sample code	Result	Sample code	Result
G1	1	G21	1	G41	0
G2	1	G22	1	G42	0
G3	1	G23	1	G43	0
G4	1	G24	1	G44	1
G5	0	G25	1	G45	1
G6	1	G26	0	G46	0
G7	1	G27	1	G47	1
G8	1	G28	1	G48	0
G9	1	G29	1	G49	0
G10	1	G30	1	G50	1
G11	1	G31	0	G51	1
G12	1	G32	1	G52	1
G13	1	G33	0	G53	0
G14	1	G34	1	G54	0
G15	0	G35	1	G55	1
G16	1	G36	1		
G17	0	G37	0		
G18	1	G38	1		
G19	1	G39	0		
G20	1	G40	1		

0: Negative; 1: Positive.

APPENDIX F: Cohen's kappa concordance index (Devito *et al.*, 2013)

Kappa coefficient	
$\kappa < 0.00$	no concordance
$0.00 \leq \kappa \leq 0.20$	very mild concordance
$0.21 \leq \kappa \leq 0.40$	mild concordance
$0.41 \leq \kappa \leq 0.60$	moderate concordance
$0.61 \leq \kappa \leq 0.80$	substantial concordance
$0.81 \leq \kappa \leq 1.00$	concordance almost perfect

κ = Kappa value
$<$ = Less than
$\leq$ = Less than or equal

APPENDIX G: DNA clone sequences corresponded to tissue samples in cattle

No.	Code	Species identity	Reference number
1	C11H7	*S. cruzi*	KT964001
2	C11H10	*S. cruzi*	KT964002
3	C11H11	*S. cruzi*	KR155217
4	C13D12	*S. cruzi*	KT964003
5	C13D14	*S. cruzi*	KR155205
6	C13D15	*S. cruzi*	KT964004
7	C13H8	*S. cruzi*	KT964005
8	C13H12	*S. cruzi*	KR155218
9	C13M7	*S. cruzi*	KT964006
10	C13M10	*S. cruzi*	KT964007
11	C13M12	*S. cruzi*	KR155227
12	C14D4	*S. cruzi*	KT964008
13	C14D5	*S. cruzi*	KR155206
14	C14D6	*S. cruzi*	KT964009
15	C14H3	*S. cruzi*	KT964010
16	C14H4	*S. cruzi*	KR155219
17	C14H7	*S. cruzi*	KT964011
18	C15H1	*S. cruzi*	KT964012
19	C15H3	*S. cruzi*	KT964013
20	C15H4	*S. cruzi*	KT964014
21	C15H6	*S. cruzi*	KT964015
22	C15E1	*S. cruzi*	KT964016
23	C15E2	*S. cruzi*	KT964017
24	C15T7	*S. cruzi*	KT964018
25	C16H1	*S. cruzi*	KT964019
26	C16H2	*S. cruzi*	KT964020
27	C16H4	*S. cruzi*	KT964021
28	C23D2	*S. cruzi*	KR155207
29	C23T1	*S. cruzi*	KT964023
30	C23T3	*S. cruzi*	KT964024
31	C23T8	*S. cruzi*	KT964025

APPENDIX H: DNA clone sequences corresponded to tissue samples in goats

No.	Code	Species identity	Reference number
1	G3E3	*Sarcocystis* YLL-2015a	KT964026
2	G3E6	*Sarcocystis* YLL-2015a	KT964027
3	G3E9	*Sarcocystis* YLL-2015a	KR155200
4	G3D5	*Sarcocystis* YLL-2015a	KR155208
5	G3D8	*Sarcocystis* YLL-2015a	KR155209
6	G3D9	*Sarcocystis* YLL-2015a	KR155210
7	G3D12	*Sarcocystis* YLL-2015a	KR155211
8	G6D1	*Sarcocystis* YLL-2015a	KR155212
9	G6D3	*S. capracanis*	KR155213
10	G6E1	*Sarcocystis* YLL-2015a	KR155201
11	G6E2	*S. capracanis*	KR155202
12	G7T1	*S. capracanis*	KR155191
13	G7T2	*Sarcocystis* YLL-2015a	KR155192
14	G9D1	*S. capracanis*	KR155214
15	G9D4	*S. capracanis*	KR155215
16	G9D5	*Sarcocystis* YLL-2015a	KR155216
17	G10M1	*Sarcocystis* YLL-2015a	KR155228
18	G10M2	*Sarcocystis* YLL-2015a	KR155229
19	G10M4	*Sarcocystis* YLL-2015a	KR155230
20	G17M1	*S. capracanis*	KR155231
21	G17M2	*S. capracanis*	KR155232
22	G17M5	*S. capracanis*	KR155233
23	G19H1	*Sarcocystis* YLL-2015a	KR155222
24	G19H3	*S. capracanis*	KR155223
25	G19H5	*Sarcocystis* YLL-2015a	KR155224
26	G20E1	*Sarcocystis* YLL-2015a	KR155203
27	G20E3	*Sarcocystis* YLL-2015a	KR155204
28	G24M1	*Sarcocystis* YLL-2015a	KR155234
29	G24M4	*Sarcocystis* YLL-2015a	KR155235
30	G28H1	*Sarcocystis* YLL-2015a	KR155225
31	G28H3	*Sarcocystis* YLL-2015a	KR155226
32	G28T4	*Sarcocystis* YLL-2015a	KR155193
33	G51T1	*Sarcocystis* YLL-2015a	KR155194
34	G51T3	*S. capracanis*	KR155195
35	G51T6	*S. capracanis*	KR155196