UNIVERSITI PUTRA MALAYSIA

MOLECULAR EPIDEMIOLOGY, RISK FACTORS OF ZOONOTIC ENTERIC PROTOZOA AND GENETIC DIVERSITY OF Blastocystis INFECTING CATTLE IN PENINSULAR MALAYSIA

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By

DONEA ABDULRAZAK ABDULLAH

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

November 2017

DEDICATION

I would like to dedicate this thesis to my loving parents. May their soul rest in peace. My lovely husband Khaldoon F. Mahmmod, my children Basma K. Fathi, Nasma K. Fathi, and Yousif K. Fathi, my sisters Layla A. Abdulla, Salwa A. Abdullah have never left my side and are very special to me.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

MOLECULAR EPIDEMIOLOGY, RISK FACTORS OF ZOONOTIC ENTERIC PROTOZOA AND GENETIC DIVERSITY OF *Blastocystis* INFECTING CATTLE IN PENINSULAR MALAYSIA

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Chairman : Reuben Sunil Kumar Sharma, DVM, MVSc, PhD, MRSB, CBiol.
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Enteric protozoa infections are one of the major constraints for profitable dairy and beef industries in tropical and subtropical countries, including Malaysia. Among the various economically important bovine diseases, enteric protozoan infections such as blastocystosis, cryptosporidiosis, giardiasis and buxtonellosis, are recognized as major causes of sub-clinical and clinical illness in cattle. In Malaysia, ruminant livestock farming is an important component of the agricultural sector, however there is a paucity of information on epidemiology and genetic diversity of enteric protozoa infecting cattle, especially those with zoonotic potential. In this study 824 faecal samples were collected from 39 cattle (beef and dairy) farms in different locations throughout Peninsular Malaysia. Genomic DNA was extracted from faecal samples and subjected to molecular detection of *Cryptosporidium*, *Giardia*, *Blastocysti*s and *Buxtonella* using genus-specific primers. A high percentage (37.8%) of the cattle was infected with either one or more intestinal protozoa. *Blastocystis* was the most common (25.4%) enteric protozoa detected in the cattle, followed by *Cryptosporidium* (12.5%), *Giardia* (4.3%) and *Buxtonella* (2.7%). Double entric protozoa species co-infection was the most prevalent (4.7%), followed by triple species co-infection (0.7%). The most common (2.9%), and significantly corellated (r_s=0.994; p<0.01) combination was *Blastocystis* + *Cryptosporidium*. Multivariable logistic regression shows that herd size, management system, production type, deworming frequency, and distance to water body were the risk factors associated with *Blastocystis* infection. For *Cryptosporidium*, deworming frequency, distance to human settlement, and management system were significant risk factors associated with the infection. *Giardia* infection was significantly associated with the cattle age, deworming frequency, zone and distance to human settlement, while risk factors for *Buxtonella* infection include herd size, distance to human settlement and deworming frequency. Positive amplicons of *Blastocystis* were cloned and sequenced to determine the genetic variability of the local *Blastocystis* isolates. Bioinformatics and phylogenetic analysis

revealed the presence of ST1, ST3, ST5, ST10 and ST14 genotypes among the infected cattle. ST10 recorded the highest prevalence of 45.8%, follow by ST5 (37.4%). Of particular concern is the discovery of potentially human infective subtypes, namely ST1, ST3 and ST5. Although various subtypes were dominant among the animal and environmental based epidemiological parameters examined, there was no consistent pattern of prevalence. Similarly, the spatial distribution patterns did not exhibit a consistent pattern, as most of the genotypes were widespread throughout the country. There was a considerably high level of genetic variability among the *Blastocystis* subtypes whereby a total of 117 haplotypes were amplified, with high nucleotide diversity. The present study constitutes the first attempt to apply molecular detection techniques to determine epidemiological risk factors for enteric protozoa infections among cattle in Malaysia. It is also the first to genetically characterize the zoonotic *Blastocystis* protozoa among cattle in the country. The epidemiological and genotyping data obtained from this study will be beneficial for the control and prevention of zoonotic enteric protozoa among humans and ruminant livestock in the region.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

EPIDEMIOLOGI MOLEKULAR, FAKTOR RISIKO PROTOZOA ENTERIK ZOONOSIS DAN KEPELBAGAIAN GENETIK *Blastocystis* YANG MENJANGKITI LEMBU DI SEMENANJUNG MALAYSIA

Oleh

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Jangkitan protozoa usus adalah salah satu daripada kekangan utama dalam industri lembu tenusu dan daging di negara tropika dan subtropika, termasuk Malaysia. Antara penyakit yang berasal daripada jangkitan protozoa usus yang dihidapi oleh lembu adalah blastocystosis, cryptosporidiosis, giardiasis dan buxtonellosis yang diiktiraf sebagai punca utama penyakit separa-kinikal dan klinikal yang teruk dalam lembu. Di Malaysia, ladang ternakan ruminan merupakan salah satu komponen penting bagi sektor pertanian, namun maklumat mengenai kajian epidemiologi dan genetik bagi protozoa usus zoonotik yang menjnagkiti lembu adalah kurang. Sebanyak 824 sampel tinja telah dikumpulkan daripada 39 ladang lembu (daging dan tenusu) dari lokasi yang bebeza di seluruh Semenanjung Malaysia. Melalui teknik molekular, *Cryptosporidium*, *Giardia*, *Blastocystis* dan *Buxtonella* telah dikesan dengan menggunakan 'primer' khusus genus. Sebanyak 37.8% lembu telah dijangkiti oleh satu atau lebih protozoa usus yang dikaji. *Blastocystis* merupakan protozoa yang kerap dikesan dalam lembu (25.4%) diikuti *Cryptosporidium* (12.5%), *Giardia* (4.3%) dan *Buxtonella* (2.7%). Jangkitan dua spesies protozoa usus adalah yang paling kerap (4.7%) yang telah dikesan diikuti oleh jangkitan bersama tiga spesies (0.7%). Jangkitan yang paling ketara (2.9%) adalah gabungan *Cryptosporidium* dengan *Blastocystis* ($rs=0.994$; $p < 0.01$). Kajian logistik regresi menunjukkan bahawa saiz kawanan, pengurusan sistem, jenis pengeluaran dan pemberian ubat cacing dan jarak ladang daripada badan air adalah faktor-faktor risiko yang berkaitan dengan jangkitan *Blastocystis*. Manakala faktor risiko untuk jangkitan dengan *Cryptosporidium* adalah pemberian ubat cacing, jarak penempatan manusia dan sistem pengurusan adalah faktor-faktor risiko yang berkaitan dengan jangkitan. Jangkitan dengan *Giardia* menunjukkan bahawa usia lembu, pemberian ubat cacing, zon dan jarak ke penempatan manusia adalah faktor risiko utama dan factor risiko untuk jangkitan *Buxtonella* adalah saiz kawanan, jarak untuk penempatan manusia dan pemberian ubat cacing. Sampel yang positif dengan *Blastocystis* telah diklon dan dihantar untuk

'sequencing' untuk menentukan diversiti genetik. Bioinformatik dan analisis filogenetik mendedahkan kewujudan genotip ST1, ST3, ST5, ST10 dan ST14 antara lembu dijangkiti. ST10 mencatatkan kekerapan tertinggi sebanyak 45.8%, diikuti dengan ST5 yang mencatatkan 37.4% dan sedikit dikesan pada ST14. Penemuaan ST1, ST3 dan ST5 pada lembu adalah penting kerena subtipe ini berpotensi untuk menjangkiti manusia. Walaupun beberapa subtipe adalah dominan mengikut faktor haiwan dan persekitaran, namun corak yang ketara tidak dapat dikesan. Kebanyakan genotipe *Blastocystis* didapati tersebar di seluruh kawasan yang dikaji di Semanjung Malaysia. Terdapat juga kepelbagaian genetik yang tinggi diantara subtipe *Blastocystis* dimana sejumlah 117 haplotipe telah dapat dikesan dengan diversiti nukleotid yang tinggi. Kajian ini merupakan kajian pertama yang telah dijalankan dengan mengguna cara pengesana molekular untuk menentukan risiko epidemiologi untuk protozoa usus pada lembu di Malaysia. Ia juga merupakan kajian pertama dalam mengesan kepelbagaian genetik *Blastocystis* dalam lembu di negara ini. Data epidemiologi yang diperolehi daripada kajian ini akan memberi manfaat dalam kawalan dan pencegahan protozoa usus zoonotik antara manusia dan ternakan ruminan di rantau ini.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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10×	ten times
1×	one times
2×	Two times
A	Alanine
a	Adenosine
AFLP	Amplified Fragment Length Polymorphism
bp	base pair
C	Cysteine
CDC	Center for Disease Control
CNS	Central Nervou System
ddH_2O	double distilled water
dNTP	deoxynucleatide triphosphate
DVS	Department of Veterinary Services
EDTA	Ethylenediaminatetraacetic acid
FI	Fluorescent
G	Gauge
g	Gram
GIS	Geographical Information System
h	Hour
kb	Kilobase
M	Molar
m	Metre
mg	Milligram
$MgCl_2$	magnesium chloride
min	Minute
mM	Milimolar
ML	Maximum likelihood
N	Number
NJ	Neighbour-Joining
^oC	degree Celsius
OIE	World Organization for Animal Health
RNA	ribonucleic acid
SM	Sungai Merab
sec	Seconds
spp	Species
T	Thymine
TAE	tris-acetic-EDTA
UV	Ultraviolet
V	Voltage

CHAPTER 1

INTRODUCTION

Enteric protozoa comprise a group of diverse microorganisms which inhabit the gastrointestinal tract of both animals and humans. A vast majority of these microbes are commensals or symbionts and may have free living stages in the environment. However, a number of these parasites are known to be pathogenic to their animal host, of which certain genera are known to cause zoonotic diseases in humans (Chalmers *et al*., 2011; Josephine *et al*., 2011; Rimšeliene *et al*., 2011; Lee *et al*., 2014a Li *et al*., 2016). These zoonotic enteric pathogens have been implicated in animal and human diseases associated with diarrhoea, loss of appetite and emaciation (Dhama *et al*., 2013). The illness cause by these organisms can be severely debilitating and even fatal (Kenny and Kelly, 2009; Dixon *et al*., 2011; Dhama *et al*., 2013), especially in immunecompromised hosts (Sterling and Adam, 2004; Kucerova *et al*., 2011; Sokolova *et al*., 2011). In livestock, diseases caused by enteric protozoa are of economic importance, since acute cases may be fatal, while chronic and sub-clinical infections often cause suboptimal productivity leading to significant losses to the industry (Stanley, 2003; Thompson, 2004; Tomley and Shirley, 2009; Torgerson and Macpherson, 2011).

Enteric protozoan can spread to both humans and animals through a number of different routes, including indirect transmission *via* inadequately treated sewage/sewage products, fecal contaminated water and food, and animal handling (Yibeltal and Simenew, 2015). Transmission of these organisms may also be direct between animals, humans, and animals to humans (Karanis *et al*., 2007a; Dixon *et al*., 2011). Zoonotic transmission of enteric protozoa is a major public health concern (Thompson *et al*., 2010; Dixon *et al*., 2011) especially in developing countries where farm biosecurity, hygiene, sewage disposal and waste water treatment are still largely inadequate (Haque *et al*., 2003; Sterling and Adam, 2004; Schuster& Ramirez, 2008; Bakheit *et al*., 2008; Mor and Tzipori, 2008; Escobedo *et al*., 2010; Kumar *et al*., 2016). These zoonotic pathogens have been detected in streams, rivers and recreational water bodies in many

Southeast Asian countries, including Malaysia (Lim *et al*., 1999; Macpherson *et al*., 2000; Farizawati *et al*., 2005; Lim *et al*., 2009; Baldursson and Karanis, 2011; Khanum *et al*., 2012 Noradilah *et al*., 2016). In addition, wild animals may be reservoirs of infection for both livestock and humans, and the sylvatic transmission cycle is of serious concern (Thompson *et al*., 2009; Thompson *et al*., 2010).

Inspite of the serious concern posed by enteric protozoal infections in animals and humans, and the high economic costs associated with the disease burden, the estimation of the disease prevalence is often confounded by the lack of sensitivity of routine diagnostic techniques (Fletcher *et al*., 2012). With the advent of molecular diagnosis targeting specific gene fragments of the pathogens, a clearer picture of the

prevalence has become possible, leading to a more accurate inference of the epidemiology and extent of the infection in animals and humans (Lim *et al*., 2009; Lim *et al*., 2011; Lim *et al*., 2013b). In addition, genetic characterization of isolates has afforded clearer differentiation between the zoonotic and animal specific genotypes of these enteric pathogens (Thompson *et al*., 2000; Appelbee *et al*., 2005; Lim *et al*., 2011). Among the myriad of enteric protozoa, the genus *Cryptosporidium, Giardia, Blastocystis* and *Buxtonella* are established pathogens of ruminants and other vertebrate hosts including humans (Abe *et al*., 2003; Ramirez *et al*., 2004; Lim and Ahmad, 2004;

Lim *et al*., 2005; Lim *et al*., 2007; Lim *et al*., 2008a; Chalmers *et al*., 2011; Torgerson and Macpherson, 2011; Tung *et al*., 2012; Ramírez *et al*., 2014; Ehsan *et al*., 2015; Thomson *et al*., 2016Li *et al*., 2016).

The genus *Cryptosporidium* are widely distributed parasitic protists, which live freely in surface water (Medema *et al*., 2006). This pathogen may infect a wide diversity of vertebrates including reptiles, birds, fish, mammals and even humans (Fayer, 2004; Xiao *et al*., 2004). *Cryptosporidium* transmission is through ingestion of oocyst that are highly resistant, and the parasite usually infects the gastrointestinal tract (Jex *et al*., 2008). The infection causes enteritis and is characterized by severe watery diarrhea and abdominal pain (Kosek *et al*., 2001; Chen *et al*., 2002). Sub-clinical infections are common, usually with mild sign of enteritis. However, immunocompromised hosts may suffer prolonged infections, which can be severe and sometimes fatal (Skerrett and Holland, 2001; Palit *et al*., 2005; Cohen *et al*., 2006; Siwila *et al*., 2007). The transmission of *Cryptosporidium* is believed to be more anthroponotic than zoonotic (Ramirez *et al*., 2004; Xiao and Ryan, 2004; Caccio, 2005).

Giardia is a unicellular flagellate that causes diarrhea in animals and humans. *Giardia* is known to be a major cause of diarrhea all over the world (Yang *et al*., 2005), especially diarrhea outbreaks related to water and foodborne transmission (Cedillo-Rivera *et al*., 1989; Mintz *et al*., 1993; Barwick *et al*., 2000). There is a high prevalence and incidence of *Giardia* in developing countries which may cause long-term growth retardation due to the chronic nature of the infection (Fraser *et al*., 2000).

Blastocystis is a cosmopolitan and pathogenic enteric protozoa that may cause enteritis in both animals and humans (Stenzel and Boreham, 1996; Tan, 2004, 2008), and is among the more common organisms isolated during parasitological surveys (Ok *et al*., 1997; Abou and Negm, 2001; Baldo *et al*., 2004; Aksoy *et al*., 2007). Several genotypes or subtypes of *Blastocystis* exist and recently observations show that human host may acquire zoonotic subtypes which are predominant in animals (Abe, 2004; Noël *et al*., 2005).

Buxtonella is a ciliate that is very similar to a species of organism that is found in humans and pigs called *Balantidium coli* (Tomczuk *et al*., 2005). The pathogenic role of this organism in ruminants is still unequivocal, and many have suggested that

Buxtonella is a normal microflora of the ruminant digestive tract (Al Saffar *et al*., 2010; Tung *et al*., 2012;. Kočiš *et al*., 2014) Nevertheless, improper nutrition of the animal host could lead to an increase in the number of the parasites which later invade the digestive system, resulting in inflammation and metabolic changes which may finally result in clinical disease with manifestation of diarrhea (AlSaffar *et al*., 2010). Neonatal diarrhea as a result of *Buxtonella* could be fatal, which is an important factor to be considered in intensive farming systems (Al-Zubaidi and Al-Mayah, 2011; Kočiš *et al*., 2014).

In Malaysia, a number of surveys have been carried out to detect the presence of these zoonotic protozoa among livestock (Colley and Mullin, 1971; Long, 1974; Lai, 1992; Kamel *et al*., 1994; Kamell *et al*., 1994; Lim *et al*., 1997; Salim *et al*., 1999; Lim and Ahmad, 2001; Lim *et al*., 2005; Ak *et al*., 2006; Lim *et al*., 2007; Lim *et al*., 2008a; Lim *et al*., 2008b; Muhid *et al*., 2011, 2012; Lim *et al*., 2013a; Anuar *et al*., 2013; Lala *et al*., 2015 Hisamuddin *et al*., 2016, Yap *et al*., 2016). These pathogens are also known to be present in various recreational water bodies and river systems (Lim *et al*., 1999;

Lim and Ahmad, 2004; Farizawati *et al*., 2005; Lim *et al*., 2009; Lim *et al*., 2013b; Onichandran *et al*., 2013; Lee *et al*., 2014; Kumar *et al*., 2016). In addition, Farizawati *et al*. (2005) have implicated that cattle farms may contribute towards river contamination with *Giardia* cysts and *Cryptosporidium* oocysts in Selangor, Malaysia. Contamination of water by these pathogens is a major concern among immunosuppressed individuals (Asma *et al*., 2011; Lim *et al*., 2011) and the rural settlers and indigenous people in the country (Al-Mekhlafi *et al*., 2013; Choy *et al*., 2014; Chin *et al*., 2016). While these pathogens are of concern to human health, there remains a paucity of information on the epidemiology and risk factors, livestock hosts range, and hotspots of infection with these zoonotic enteric pathogens over a wide spatial distribution in Malaysia. In addition, the zoogeographical genetic diversity for most of these protozoa have not been determined locally. The present investigation, therefore, was undertaken to determie the molecular epidemiology, spatial distribution, host affinities and genetic diversity of zoonotic enteric protozoa among livestock in peninsular Malaysia. It is envisaged that the data obtained will provide the much needed information on the role of livestock as sources of zoonotic enteric protozoa in the country. This will facilitate effective control programs in order to prevent the spread of these zoonotic protozoa among animals and humans.

1.1 Hypothesis

Research Hypothesis

1. Bovine enteric protozoa (*Blastocystis, Cryptosporidium, Giardia, Buxtonella*) are present in Peninsular Malaysia with no defined pattern of distribution.

2. Numerous risk factors at the farm and animal levels are associated with bovine enteric protozoa infection in Peninsular Malaysia.

3. There is genetic variability of *Blastocystis* infection among cattle in Peninsular . Malaysia in relation to that reported for the parasite in other parts of the world.

1.2 Objectives

The specific objectives of the present study are:

1. To determine the prevalence, spatial distribution and occurrence of co-infection of bovine enteric protozoa in Peninsular Malaysia using molecular detection methods.

2. To determine the epidemiology and risk factors of infection with these parasites in relation to animal and environmental variables.

3. To ascertain the subtype grouping, genetic variability, and phylogenetics of *Blastocystis* isolated from cattle in Peninsular Malaysia in relation to isolates from other parts of the world.

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