



**MOLECULAR EPIDEMIOLOGY OF ENTERIC PROTOZOA AND
GENOTYPING OF *Blastocystis* FROM PORCINE ON THE WEST COAST
OF PENINSULAR MALAYSIA**

RUVINIYIA A/P KALAISELVAM

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By

RUVINIYA A/P KALAISELVAM

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirements for the Degree of Master of Science**

November 2017

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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November 2017

**Chairman : Reuben Sunil Kumar Sharma, DVM, MVSc, PhD, MRSB, CBiol
Faculty : Veterinary Medicine**

Enteric protozoa of animals are of a major public health concern as a number are known to be zoonotic with high rates of transmission *via* contaminated food and water. Pigs are known to be the reservoirs for many zoonotic pathogens worldwide that exert negative impacts to human health and the agricultural economy of many countries. The present study aims at determining the molecular prevalence and risk factors associated with enteric protozoa infection in pigs reared on farms located along the west coast of Peninsular Malaysia, and to genetically characterized and determine the diversity of *Blastocystis* subtypes that are infecting these pigs in the country. Fecal samples were collected from 450 pigs from 15 different farms along the west coast of Peninsular Malaysia, and subjected to nested PCR amplification using genus specific primers. Molecular detection revealed that pigs in the country harbored a relatively high prevalence of *Balantidium* (70.4%), *Blastocystis* (55.6%), *Enterocytozoon* (40.7%), *Giardia* (22.0%) and *Cryptosporidium* (19.3%). A total of 94.2% of the pigs examined were infected with one or more of these enteric protozoa. The highest protozoa co-infection was triple co-infection (37.0%), followed by quadruple co-infection (29.0%). Multiple logistic regression showed that the age of the farm and distance of farm from human settlement were significant risk factors associated with *Giardia* infection. The age of the farm and number of swine on the farms were significantly associated with *Blastocystis* infection. The multivariate model also showed that the age of pigs and the distance of the farms from human settlement were significant risk factors for *Balantidium* and *Enterocytozoon* infection. In addition, the farm production type also played a significant role in the prevalence of the latter. None of the epidemiological factors examined were significantly associated with *Cryptosporidium* infection. 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 four subtypes (ST1, ST3, ST5 and ST10) among the infected swine, of which ST1, ST3 and ST5 are known

to be able to infect humans. The highest prevalence was with ST5 (66.7%), followed by ST10 (24.4%), ST3 (15.6%) and lastly ST1 (8.9%). Analysis of haplotype diversity and prevalence revealed that H029 and H061 of the ST5 group were the most common haplotypes in the pigs. The present study constitutes the first attempt to determine the molecular epidemiology of porcine enteric protozoa in the country, and the genetic composition of *Blastocystis* among swine in Malaysia. It is envisaged that the data obtained from this study will afford a clearer understanding on the epidemiology and genetics of zoonotic enteric protozoa infecting swine in the country, which in turn may facilitate country-wide efforts in the control and prevention of these zoonotic pathogens.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

**EPIDEMIOLOGI MOLEKULAR PROTOZOA USUS DAN GENOTYPING
Blastocystis DARI PORSIN SEPANJANG PANTAI BARAT SEMENANJUNG
MALAYSIA**

Oleh

RUVINIYA A/P KALAISELVAM

November 2017

Pengerusi : Reuben Sunil Kumar Sharma, DVM, MVSc, PhD, MRSB, CBiol
Fakulti : Perubatan Veterinar

Protozoa usus haiwan merupakan masalah kesihatan umum dengan sebahagian daripadanya bersifat zoonotik dengan kadar transmisi yang tinggi melalui makanan dan air yang tercemar. Babi dikenali sebagai haiwan yang mudah dijangkiti oleh patogen zoonotik yang memberi kepada impak negatif terhadap kesihatan manusia dan juga ekonomi pertanian di serata dunia. Tujuan kajian ini adalah untuk menentukan prevalens molekular dan faktor risiko yang mengaitkan jangkitan protozoa usus di babi yang dipelihara di ladang sepanjang pantai barat Semenanjung Malaysia dan juga untuk menentukan kepelbagaian genetik dan mengetahui kepelbagaian 'subtypes' *Blastocystis* yang menjangkiti babi di Malaysia. Sampel najis telah dikumpulkan daripada 450 ekor babi dari 15 buah ladang yang berlainan sepanjang Pantai Barat Semenanjung Malaysia dan PCR bersarang telah dilakukan menggunakan 'primer' spesifik genera. Pengesanan molekular telah menunjukkan bahawa babi di negara ini mempunyai prevalens yang tinggi iaitu *Balantidium* (70.4%), *Blastocystis* (55.6%), *Enterocytozoon* (40.7%), *Giardia* (22.0%) and *Cryptosporidium* (19.3%). Jumlah keseluruhan babi yang telah dijangkiti dengan satu atau lebih protozoa usua adalah 94.2%. Jangkitan protozoa bersama yang tertinggi adalah infeksi tiga protozoa (37.0%) diikuti oleh empat protozoa (29.0%). Kajian logistik regresi berbilang menunjukkan usia ladang dan jarak penempatan manusia daripada ladang adalah faktor risiko yang berkaitan dengan jangkitan *Giardia*. Usia ladang dan jumlah babi dalam ladang adalah faktor risiko untuk jangkitan *Blastocystis*. Modal 'multivariate' menunjukkan usia babi dan jarak penempatan manusia daripada ladang merupakan faktor risiko untuk infeksi *Balantidium* dan *Enterocytozoon*. Selain itu, jenis ladang juga merupakan faktor risiko prevalens yang kedua. Jangkitan *Cryptosporidium* tidak dapat dikaitkan dengan mana-mana faktor risiko yang dikaji. Sampel positif *Blastocystis* telah diklon dan di 'sequenced' untuk menentukan kepelbagaian genetik *Blastocystis*. Analisis bioinformatik dan filogenetik mendedahkan kewujudan empat jenis 'subtype' (ST1, ST3, ST5 and ST10) dalam babi

yang telah dijangkiti dimana ST1, ST3 dan ST5 boleh menjangkiti manusia. Prevalens yang tertinggi adalah ST5 (66.7%), diikuti ST10 (24.4%), ST3 (15.6%) dan akhirnya ST1 (8.9%). Analisis kepelbagaian 'haplotype' mendedahkan bahawa H029 dan H061 dari kumpulan ST5 merupakan 'haplotype' yang paling lazim dalam babi. Kajian ini merupakan kajian yang pertama dijalankan untuk mengetahui epidemiologi molekular protozoa usus babi dan juga komposisi genetik *Blastocystis* dalam babi di Malaysia. Data yang diperolehi daripada kajian ini akan memberi pemahaman yang lebih jelas mengenai epidemiologi dan genetic protozoa usus zoonotik di negara ini yang boleh memudahkan usaha untuk mengawal dan menghalang patogen zoonotik ini.



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Name of Chairman
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Signature: _____
Name of Member
of Supervisory
Committee: Professor Dr. Yvonne Lim Ai Lian

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LIST OF ABBREVIATION AND SYMBOLS

AIDS	Acquired Immunodeficiency Syndrome
bp	Basepair
CD4	Cluster of differentiation 4
CDC	Centre of Disease Control
DNA	Deoxyribonucleic acid
DVS	Department of Veterinary Services
EDTA	Ethylenediaminetetraacetic acid
EF-1alpha	Eukaryotic translation elongation factor 1 alpha
g	gram
HIV	Human Immunodeficiency Virus
ITS	Internal Transcribed Spacer
LB	Lysogeny broth
m	meter
MgCl ₂	Magnesium chloride
ML	Maximum Likelihood
mM	milimolar
mm	milimeter
n	Number
NJ	Neighbor joining
°C	degree celcius
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
rpm	revolutions per minute
sec	seconds

sp	species
SSU-rDNA	small subunit ribosomal deoxyribonucleic acid
SSU-rRNA	small subunit ribosomal ribonucleic acid
ST	Subtype
TAE	Tris- acetic EDTA
UV	Ultraviolet
µg	microgram
µl	microliter
µl	microliter
µm	Micrometer



CHAPTER 1

INTRODUCTION

Protozoa that inhabit the gastrointestinal tract of both animals and humans comprise a diverse group of microorganisms which are predominantly commensals or symbionts. However, a number of genera are known to be pathogenic to animal host, with a number of species having zoonotic potential and may cause diseases in humans (Chalmers *et al.*, 2011; Josephine *et al.*, 2011; Rimšeliene *et al.*, 2011; Lee *et al.*, 2014a; Li *et al.*, 2016). These enteric pathogens have been known to cause animal and human diseases associated with diarrhoea, loss of appetite and emaciation (Dhama *et al.*, 2013), which may be severe and even fatal (Kenny and Kelly, 2009; Dixon *et al.*, 2011; Dhama *et al.*, 2013) especially in immune-compromised host (Sterling and Adam, 2004; Kucerova *et al.*, 2011; Sokolova *et al.*, 2011). In many livestock species, the diseases caused by these organisms have an economic impact on the industry as acute cases may be fatal, while chronic and sub-clinical infections may cause a reduction in performance and productivity (Stanley, 2003; Thompson, 2004; Tomley and Shirley, 2009; Torgerson and Macpherson, 2011).

Parasitic zoonoses possess a significant burden to public health especially in low income communities where basic sanitation is often lacking (Murell, 1991; Torgerson & Macpherson, 2011; Hotez & Alibek, 2011; Fletcher *et al.*, 2012; Devleesschauwer *et al.*, 2014;). In Southeast Asia, zoonotic parasites are of a major public health concern with high rates of transmission *via* contaminated food and water (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 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). The source of infection may be traced back to water contamination either from human waste or from runoffs from livestock farms (Farizawati *et al.*, 2005; Ithoi *et al.*, 2011; Santharasekaran and Husni, 2012; Noradilah *et al.*, 2016). These major enteric pathogens of veterinary and medical concern include *Cryptosporidium*, *Giardia*, *Blastocystis*, *Balantidium* and *Enterocytozoon*.

The genus *Cryptosporidium* infects the gastrointestinal tract of animals and human causing a disease known as cryptosporidiosis. This parasite is a significant waterborne pathogen that is resistant to disinfection techniques and has the ability to pass through physical water treatment process that contributes to the occurrence of these pathogen in the environment (Carey *et al.*, 2004). *Cryptosporidium* causes diarrheal illness in young livestock animals worldwide with their environmentally resistant oocysts that are excreted through faeces (Xiao, 2010; Rimšeliene *et al.*, 2011). Cryptosporidiosis occurs through the fecal oral route and in some cases, indirect transmission may occur through the ingestion of contaminated medium such as water or food (Smith *et al.*, 2006).

Giardia are flagellated protozoan parasites that invades the gastrointestinal tract of human as well as large diversity of animals worldwide through the ingestion of environmental resistant infective cysts (Thompson, 2000; Van der Giessen *et al.*, 2006; Soares *et al.*, 2011). Giardiasis is considered a zoonotic waterborne disease and a major problem to public health in both develop and developing countries (Levine *et al.*, 1990; Thurman *et al.*, 1998; Hoque *et al.*, 2002; Leclerc *et al.*, 2002). *Giardia* has been isolated from various animals including livestock (; Bjorkman *et al.*, 2003; Coklin *et al.*, 2007; Armson *et al.*, 2009), non-human primates (Montrez-Diaz *et al.*, 2011) and also fish (Yang *et al.*, 2010). Animals that are been infected with giardiasis experiences diarrhea and weight loss that result in substantial loss in production as well as mortality (Aloisio *et al.*, 2006). Seven different assemblages been described for *Giardia duodenalis*, whereby assemblage A and B are known to be zoonotic as it infects both humans and mammalian animals (Yaoyu & Xiao, 2011; Thompson & Smith, 2011; Martinez-Diaz *et al.*, 2011; Lebbad, 2010; Franzen *et al.*, 2009; Giangaspero *et al.*, 2007; Thompson, 2004).

Blastocystis is a unicellular, anaerobic emerging pathogen that colonize the gastrointestinal tract of diverse host such as insects, reptiles, birds, mammals as well as humans (Noel *et al.*, 2005). The prevalence of *Blastocystis* infection in human increases with age and it is usually associated with chronic sign and symptoms such as diarrhoea (Parkar *et al.*, 2007; Yoshikawa *et al.*, 2008; Parkar *et al.*, 2010.) *Blastocystis* infection was mostly reported in developing countries compared to developed countries due to poor sanitary infrastructure and environmental hygiene as the transmission of this parasite is through the fecal-oral route (Yoshikawa *et al.*, 2004; Tanizaki *et al.*, 2005; Leelayova *et al.*, 2008). To date, 17 subtypes of *Blastocystis* with varying pathogenicity has been found in both humans and animals (Roberts *et al.*, 2014; Verweij & Rune 2014).

Balantidium is a ciliated protozoan that causes infection in humans and is considered to be a major zoonosis from pigs which are the natural asymptomatic reservoir (Thompson & Smith, 2011). *Balantidium* spreads *via* the fecal-oral route from pigs to human and *vice versa* (Schuster & Visvesvara, 2004). The cysts of *Balantidium* will remain viable in moist environmental conditions and can survive for up to 10 days outside its host (Zaman, 1978). This protozoa is commonly found in tropical and subtropical regions worldwide (Zaman 1998; Farthing *et al.*, 2003; Owen, 2005) and human that are in close contact with pigs have a higher in risk of being infected (Schuster & Ramirez-Avila, 2008). In animals, *Balantidium* is usually harmless, but may sometimes cause severe diarrhoea that can be fatal (Murray, 2006).

Enterocytozoon is an intracellular obligate eukaryotic pathogen that can be found in both human and animal with approximately 1200 named species (Didier & Weiss, 2006; Santin & Fayer, 2011). The transmission and outbreak of this microsporidia is rare worldwide compared to the other enteric protozoan parasites. However, it has been known to cause cases of food and waterborne infection (Cotte, 1999; Decraene *et al.*, 2012). In humans, infection of *E. bineusi* is primarily found in HIV- infected patients. To date, 200 genotypes of *E. bineusi* have been described based on variation

in the Internal Transcribed Spacer (ITS) region, with the capability of infecting a variety of wild and domestic animals, of which several genotypes are known to be zoonotic (; Santin & Fayer, 2016; Li et al., 2017). In pigs alone, 60 genotypes have thus far been described (Fiuza *et al.*, 2015).

The growing livestock industry in Malaysia is faced with many challenges, including production costs, disease transmission and management of farm waste products and contaminated runoffs. Environmental contamination of waste from ruminant and pig farms is a major issue that requires urgent attention. Studies in the past (Farizawati *et al.*, 2005; Santharasekaran and Husni, 2012; Onichandran *et al.*, 2013) have implicated that runoffs from cattle farms have contributed significantly to environmental load with zoonotic pathogens including enteric protozoa. These pathogens have been isolated from drinking water sources and recreational water bodies in the country (Lim *et al.*, 2009; Ithoi *et al.*, 2011; Richard *et al.*, 2016).

Pigs have been known to be the reservoirs for many zoonotic pathogens worldwide, and some of these enteric pathogens even contribute to negative impact to the economy of the respective countries (Morens & Taubenberger, 2010; Zimmerman *et al.*, 2012; Khan *et al.*, 2013). To date there is a paucity of published data on the prevalence of enteric protozoa in pigs in Malaysia, especially so for those that have the potential to cause zoonotic infections like *Cryptosporidium*, *Giardia*, *Blastocystis*, *Balantidium* and *Enterocytozoon*. The present study was therefore undertaken to provide baseline information on the prevalence and epidemiology of these enteric protozoa in pig farms in the country, and to determine the genetic diversity of *Blastocystis* which is considered a major zoonotic pathogen. In addition, this study constitutes the first attempt in the country to determine the prevalence of these zoonotic pathogens using molecular tools. It is envisaged that the data obtained here will provide a clearer understanding on the molecular epidemiology of swine related zoonotic enteric pathogens that will assist in the control and prevention of the infection in both pigs and humans.

The specific objectives of this study are:

1. To determine the molecular prevalence and risk factor associated with zoonotic enteric protozoa infection in pigs reared on farms located along the west coast of Peninsular Malaysia.
2. To genetically characterize and determine the diversity of *Blastocystis* subtypes that are infecting these pigs in the country.

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