STUDIES ON THE PREVALENCE AND BIOLOGY OF Blastocystis spp. ISOLATED FROM ZOONOTIC RESERVOIRS IN MALAYSIA

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FACULTY OF SCIENCE UNIVERSITY OF MALAYA KUALA LUMPUR

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ABSTRACK

Blastocystis is a highly prevalent protozoan parasite of the intestinal tract of a wide range of animal hosts, including humans. To date, very little information is available of parasite in zoonotic potential reservoirs namely, companion animals, household pests and poultry population. The suggestion that the intimate association between humans and animals could facilitate transmission led us to investigate Blastocystis in several animal hosts in the domestic environment such as cats, dogs, rodents, cockroaches, house geckos and chickens as no data is available to date. From October 2012 until April 2015, a total of 938 intestinal contents and/or faecal samples from different hosts were collected from three states of Peninsular Malaysia namely; Kuala Lumpur, Selangor and Perak. The prevalence of *Blastocystis* infection was investigated by screening and in vitro cultivation method using Jones medium supplemented with 10% horse serum. A total of 26.3% (47/179) chicken faecal samples screened were positive for *Blastocystis* infection with high prevalence in free-range species compared to barnreared chicken. Results from this first epidemiological study showed positive infection in broiler chicken despite reared in farming method least prone to contamination. Intestinal infections were equally high 45.4% (133/293) in wild rats and cockroaches 40.4% (61/151) particularly the nymph stage. All infections were observed asymptomatic. Surprisingly, house geckos were free from infection. Light microscopy examination between the animal isolates was almost similar in morphology to B. *hominis* with the exception for their considerable size variations (chicken isolates: 10 to 100 µm; wild rat isolate: 4 to 45 µm; cockroach isolate 9 to 15 µm in diameter). Furthermore, ultrastructure examination demonstrated surface coat thickness and electron density also varied between different isolates. Close to half of the chicken isolates were completely electron-lucent when examined under the transmission electron micrographs whereas electron dense areas were observed in the central vacuole

of the wild rat and cockroach isolate which indicated lipid accumulation. Surface coat were present on all isolates with the cockroach isolates the thickest between 276.17 to 336.67 nm followed by chicken isolates (239.39 to 169.27 nm) and the least in wild rat isolates (135.51 to 196.82 nm). Using the sequenced-tagged site (STS) primers and DNA barcoding method, four subtypes were detected from chicken isolates namely, ST1, ST6, ST7 and ST8. Meanwhile, four subtypes were detected from wild rats with ST1, ST4, ST5 and ST7. In cockroach population, two cockroach isolates were identified as ST3 and one isolate was closely related to allele 114 which is most likely to be the new subtype. Although cultivation was unsuccessful from all cat and dog samples, 12 cat samples were found positive for *Blastocystis* sp. ST1. The finding of this study adds to our understanding of the biology, transmission as well as distribution of this organism in animals living in close association to humans and highlights their zoonotic potential.

ABSTRAK

Blastocystis adalah sejenis parasit protozoa saluran usus yang prevalen secara meluas dalam pelbagai haiwan perumah, termasuk manusia. Pada masa kini, teramat sedikit maklumat mengenai parasit ini dalam reservoir berpotensi zoonotik seperti haiwan peliharaan, makhluk perosak rumah dan populasi ayam. Cadangan bahawa terdapat hubungkait rapat antara manusia dan haiwan bagi memudahkan lagi penyebaran telah mendorong kami untuk menyiasat *Blastocystis* haiwan dalam persekitaran domestik seperti kucing, anjing, tikus, lipas, cicak dan ayam disebabkan pada masa kini tiada terdapat maklumat mengenainya. Dari bulan Oktober 2012 hingga April 2015, sejumlah 938 kandungan usus dan/atau sampel tinja dari perumah yang berbeza telah dikumpulkan dari tiga negeri di Semenanjung Malaysia iaitu; Kuala Lumpur, Selangor dan Perak. Prevalen Blastocystis telah dikaji melalui pemeriksaan dan kaedah pengkulturan in vitro menggunakan medium Jones ditambah dengan 10% serum kuda. Sebanyak 26.3% (47/179) sampel tinja ayam diperiksa adalah positif bagi *Blastocystis* dengan penyebaran meluas dalam spesies ayam yang hidup melata berbanding ayam yang dipelihara dalam sangkar. Hasil dari kajian epidemiologi pertama protozoa menunjukkan jangkitan positif dalam ayam pedaging walaupun dipelihara melalui kaedah yang kurang menjurus kepada pencemaran. Jangkitan usus adalah lebih kurang sama dengan 45.4% (133/293) sampel usus tikus liar dan 40.4% (61/151) lipas khususnya peringkat nimfa. Didapati semua jangkitan adalah asimptomatik. Keputusan tidak dijangka apabila didapati populasi cicak bebas dari sebarang jangkitan. Pemeriksaan mikroskop cahaya keatas kesemua pencilan menunjukkan morfologi adalah sama dengan B. hominis kecuali wujudnya variasi saiz yang ketara (diameter pencilan ayam: 10 hingga 100 µm; pencilan tikus liar; 4 hingga 45 µm; pencilan lipas 9 hingga 15 µm). Tambahan pula, pemeriksaan ultrastructural menunjukkan ketebalan kot permukaan dan kepadatan elektron juga berbeza-beza antara pencilan yang berbeza.

Hampir separuh pencilan ayam adalah bebas elektron apabila diperhatikan dibawah mikrograf elektron transmisi manakala kawasan padat elektron diperhatikan pada vakuol pusat pencilan tikus liar dan lipas menunjukkan berlakunya pengumpulan lipid. Didapati bahawa kot permukaan hadir pada semua pencilan dengan ketebalan pencilan lipas antara 276.17 hingga 336.67 nm diikuti oleh pencilan ayam (239.39 hingga 169.27 nm) dan kot permukaan yang nipis pula adalah pencilan tikus liar (135.51 hingga 196.82 nm). Dengan menggunakan primer tapak tag jujukan (STS) dan kaedah kod bar DNA, empat subjenis telah dikenalpasti daripada pencilan ayam iaitu ST1, ST6, ST7 dan ST8. Manakala, empat subjenis telah dikenalpasti daripada pencilan tikus liar iaitu ST1, ST4, ST5 dan ST7. Dalam populasi lipas, dua pencilan lipas telah dikenalpasti sebagai ST3 dan satu pencilan lipas adalah berkait rapat dengan alel 114 yang berkemungkinan merupakan subjenis baru. Walaupun kaedah pengkulturan tidak berjaya bagi sampel kucing dan anjing, 12 sampel kucing telah didapati positif bagi Blastocystis sp. dengan ST1. Hasil kajian ini telah menambahbaik kefahaman kita tentang biologi, penyebaran disamping taburan organisma ini dalam haiwan yang hidup saling berhubung rapat dengan manusia dan menumpu imej tentang potensi zoonotik penyebaran Blastocystis spp.

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TABLE OF CONTENTS

ABSTRACT	iii
ABSTRAK	V
ACKNOWLEDGEMENT	viii
TABLE OF CONTENTS	ix
LIST OF FIGURES	xiii
LIST OF TABLES	xvii
LIST OF SYMBOLS AND ABBREVIATION	xviii
LIST OF APPENDICES	XX
CHAPTER 1: INTRODUCTION	1
1.1 Research background	1
1.2 Justification of the study	4
CHAPTER 2: LITERATURE REVIEW	8
2.1 Classification of <i>Blastocystis</i>	8
2.1.1 Taxonomic status	8
2.1.2 Speciation	9
2.2 Terminology of Blastocystis	10
2.2.1 Standardization of terminology	10
2.2.2 Current subtypes	12
2.3 Biology of Blastocystis	14
2.3.1 Morphology	14
2.3.1.1 Vacuolar form	14
2.3.1.2 Granular form	14
2.3.1.3 Amoeboid form	15
2.3.1.4 Cyst form	15
2.3.2 Life cycle and transmission of <i>Blastocystis</i>	17
2.3.3 Reproductive mode of <i>Blastocystis</i>	20
2.3.3.1 Binary fission	20
2.3.3.2 Budding	20
2.3.3.3 Schizogony	20
2.4 Detection of <i>Blastocystis</i>	22

2.4.1 Microscopy techniques	22
2.4.1.1 Direct faecal smear	22
2.4.1.2 Staining	23
2.4.2 Concentration techniques	23
2.4.3 In Vitro culture techniques.	24
2.4.4 Molecular techniques	26
2.4.4.1 Sequence-tagged site (STS)	26
2.4.4.2 DNA barcoding	27
2.4.5 Serological techniques	28
2.5 Blastocystis in animals	29
2.5.1 Birds	29
2.5.2 Swine	31
2.5.3 Companion animals	33
2.5.4 Rodent	34
2.5.5 Artiodactyls	35
2.5.6 Wildlife	36
2.6 Blastocystis of animals in Malaysia	37
CHAPTER 3: STUDY ON <i>Blastocystis</i> spp. IN POULTRY	39
POPULATIONS	•
3.1 Introduction	39
3.2 Materials and methods	41
3.2.1 Ethical approval	41
3.2.2 Sampling sites	41
3.2.3 Study population	41
3.2.3.1 Free-range or backyard chicken sample	41
3.2.3.2 Barn-reared chicken sample	42
3.2.4 <i>In vitro</i> cultivation	42
3.2.5 Microscopy examination	43
3.2.5.1 Giemsa staining	43

3.2.5.2 Sudan Black B staining	43
3.2.6 Cytochemical staining	43
3.2.6.1 Fluorescein isothiocyanate (FITC)-labeled Con A	43
(Canavalia ensiformis)	

3.2.6.2 Acridine orange staining	44
3.2.7 Ficoll-Paque concentration method	44
3.2.8 Electron microscopy	44
3.2.8.1 Scanning electron microscopy (SEM)	45
3.2.8.2 Transmission electron microscopy (TEM)	45
3.2.9 Subtyping	46
3.2.9.1 Genomic DNA preparation	46
3.2.9.2 Polymerase chain reaction (PCR) using subtype subtype-	46
specific sequence-tagged-site (STS) primers	
3.2.9.3 DNA barcoding	48
3.2.10 Statistical analysis	48
3.3 Results	49
3.3.1 Prevalence of <i>Blastocystis</i> in chickens	49
3.3.2 Morphological form	52
3.3.3 Mode of reproduction	55
3.3.4 Surface structure	55
3.3.5.Ultrastructure	60
3.3.5.1 Village chicken cultured isolates	60
3.3.5.2 Village chicken faecal sample	60
3.3.5.3 Village chicken caecum sample	60
3.3.5.4 Broiler chicken cultured isolates	60
3.3.6 Cytochemical studies	61
3.3.7 Subtype identification	68
3.4 Discussion	70
3.5 Conclusion	74
CHAPTER 4: STUDY ON Blastocystis spp. IN WILD RATS	83
4.1 Introduction	83
4.2 Materials and methods	85
4.2.1 Ethical approval	85
4.2.2 Sampling	85
4.2.3 Dissection	85
4.2.4 In vitro cultivation	85
4.2.5 Microscopy examination	85

4.2.6 Cytochemical staining	86
4.2.7 Ficoll-Paque concentration method	86
4.2.8 Electron microscopy	86
4.2.9 In vitro growth profile	86
4.2.10 Subtyping	87
4.2.11 Statistical analysis	87
4.3 Results	88
4.3.1 Prevalence of <i>Blastocystis</i> in wild rats	88
4.3.2 Morphological forms	89
4.3.3 Mode of reproduction	89
4.3.4 Surface structure	94
4.3.5 Ultrastructure	94
4.3.6 Cytochemical studies	98
4.3.7 Growth characteristics	102
4.3.8 Subtype identification	105
4.4 Discussion	107
4.5 Conclusion	111
CHAPTER 5: STUDY ON Blastocystis spp. IN COCKROACHES AND	115
HOUSE GECKOS	
5.1 Introduction	115
5.2 Materials and methods	117
5.2.1 Study population	117
5.2.2 Dissection	117
5.2.3 In vitro cultivation	118
5.2.4 Microscopy examination	118
5.2.5 Cytochemical staining	118
5.2.6 Electron microscopy	118
5.2.7 Subtyping	118
5.2.8 Statistical analysis	118
5.3 Results	119
5.3.1 Prevalence of <i>Blastocystis</i> in cockroaches and house geckos	119
5.3.2 Morphological forms	121
5.3.3 Surface structure	121

5.3.4 Ultrastructure	121
5.3.5 Cytochemical studies	127
5.3.6 Subtype identification	130
5.4 Discussion	131
5.5 Conclusion	136

CHAPTER 6: STUDY ON Blastocystis spp. IN COMPANION	138
ANIMALS	
6.1 Introduction	138
6.2 Materials and methods	140
6.2.1 Ethical approval	140
6.2.2 Sampling sites	140
6.2.3 Study population	140
6.2.3.1 Stray cats and dogs	140
6.2.3.2 Sheltered cats and dogs	141
6.2.4 In vitro cultivation	141
6.2.5 Subtyping	141
6.2.6 Viability experiment	141
6.3 Results	143
6.3.1 Prevalence of <i>Blastocystis</i> in cat and dog populations	143
6.3.2 Viability of <i>Blastocystis</i> in acidic conditions	143
6.4 Discussion	146
6.5 Conclusion	153
CHAPTER 7: FINAL DISCUSSION AND CONCLUSION	154
7.1 Final discussion	154
7.2 Final conclusion	165
REFERENCES	168
COMPILATIONS OF PUBLISHED PAPERS	182
APPENDIX	187

LIST OF FIGURES

Figure 2.1	Blastocystis subtypes (STs 1-17) with host specificities	13					
	(Wawrzyniak et al., 2013).						
Figure 2.2	Morphological form of <i>Blastocystis</i> (a) Vacuolar (arrowheads) and cyst forms (arrows) from in vitro culture (b) Granular form with distinct granular inclusions (arrowheads). (c) Amoeboid form showing pseudo-like cytoplasmic extensions (arrow). Scale bar, 10µm (Tan, 2008).						
Figure 2.3	A current view of <i>Blastocystis</i> life cycle (Robert et al., 2014).	19					
Figure 2.4	Various reproductive modes of <i>Blastocystis</i> (a) Binary fission (b) Budding form (c) Schizogony-like organism (arrows). Scale bar, 10µm (Mehlhorn <i>et al.</i> , 2012).	21					
Figure 3.1	The prevalence of <i>Blastocystis</i> infection in the two different chicken groups.	51					
Figure 3.2	<i>Blastocystis</i> isolated from (a) village chicken and (b) broiler chicken. V: vacuolar form. G: granular form						
Figure 3.3	Gigantic form of Blastocystis from chicken.	54					
Figure 3.4	Cyst form of Blastocystis from chicken (circle).	54					
Figure 3.5	Mode of reproduction of <i>Blastocystis</i> stained with Giemsa (a) Binary fission (b) Budding (circle) (c) Schizogony. P; progeny.	57					
Figure 3.6	Surface structure of village chicken <i>Blastocystis</i> from (a) culture and (b) fresh caecum. Bacteria (circle) were often seen adherent to the surface of <i>Blastocystis</i> .	58					
Figure 3.7	Surface structure of broiler chicken <i>Blastocystis</i> from culture.	59					
Figure 3.8	Electron micrographs of <i>Blastocystis</i> isolated from in village chicken (a) Central vacuole filled with electron-lucent materials (b) Central vacuole with electron-dense materials. Nu; nucleus, m; mitochondria, CV; central vacuole, SC; surface coat.	62					
Figure 3.9	Electron micrographs of village chicken <i>Blastocystis</i> from faecal sample. Nu; nucleus, m; mitochondria, CV; central vacuole, SC; surface coat.	63					

xiii

- Figure 3.10 Electron micrographs of village chicken *Blastocystis* from 63 ceacum samples. Nu; nucleus, m; mitochondria, CV; central vacuole, SC; surface coat.
- Figure 3.11 Electron micrographs of broiler chicken *Blastocystis* from 64 culture (a) Central vacuole with flocculent content (b) Central vacuole with electron-dense material. Nu; nucleus, m; mitochondria, CV; central vacuole, SC; surface coat.
- Figure 3.12 *Blastocystis* from chicken stained with Sudan Black B showing 65 dark stain in the central vacuole indicating the presence of neutral lipid under 1000× magnification. Note: dark droplets (arrow).
- Figure 3.13 Binding affinities of *Blastocystis* stained with FITC-labelled 66
 Con A (a) Light microscopic images of chicken Blastocystis (b)
 A same organism stained with FITC-labeled Concanavalin A (ConA) assay, AFU (1+): weak intensity. V; vacuolar form, G; granular form.
- Figure 3.14 Epifluorescence image of *Blastocystis* stained with acridine 67 orange. (a) Light microscopic images of chicken *Blastocystis*(b) The same organism stained with acridine orange. N; nucleus.
- Figure 3.15 PCR amplification reaction of *Blastocystis* from chicken 69 isolates using the sequenced-tagged site (STS) primers SB332 (lane 1, 2, 3 and 4; 338bp) indicating ST6.
- Figure 4.1 Day 1 *Blastocystis* isolated from wild rats recovered from (a) 90 caecum and (b) *in vitro* culture under light microscopy. CV; central vacuole.
- Figure 4.2 Blastocystis isolated from wild rats after prolonged cultures. 91
- Figure 4.3 Multivacuolar forms occasionally seen in the *in vitro* culture. 91
- Figure 4.4 Mode of reproduction of *Blastocystis* from wild rat isolates (a) 93
 binary fission (arrow) (b) budding (arrow) (c) schizogony-like
 organisms. BF; binary fission, B; budding, P; progeny.
- Figure 4.5 Surface structure of *Blastocystis* isolated from wild rats isolated 96 from (a) fresh caecum form (b) cultured form (c) cyst form.

xiv

- Figure 4.6 Electron micrograph of *Blastocystis* isolated from wild rats. (a) 97
 Vacuolar form. (b) Cyst form. N; nucleus, m; mitochondria-like organelle, CV; central vacuole, SC, surface coat, CM; cell surface membrane, CVM; central vacuole membrane, nu; nucleolus, G; glycogen.
- Figure 4.7 *Blastocystis* isolated from wild rats stained with Sudan Black B 99 showing dark stain in the central vacuole indicating the presence of neutral lipid under 1000× magnification. Note: dark droplets (arrow).
- Figure 4.8 Binding affinities of *Blastocystis* stained with FITC-labelled 100
 Con A (a) Light microscopic images *Blastocystis* of wild rat isolate (b) A same organism stained with FITC-labeled Concanavalin A (ConA) assay, AFU (1+): weak intensity. V; vacuolar form, G; granular form.
- Figure 4.9 Epifluorescence image of *Blastocystis* stained with acridine 101 orange (a) Light microscopic images *Blastocystis* of wild rat isolate (b) A same organism stained with acridine orange.
- Figure 4.10 Growth profiles of total number of parasites. 103
- Figure 4.11 Percentages of the presence of vacuolar forms. 103
- Figure 4.12 Percentages of the presence of granular forms. 104
- Figure 4.13 PCR amplification reaction of *Blastocystis* from wild rat 106 isolates using the sequenced-tagged site (STS) primers SB337 (lane *I* and *2*; 487bp) indicating ST4.
- Figure 5.1 The prevalence of infected cockroaches with *Blastocystis* 120 relative to host-stage.
- Figure 5.2 Light micrograph of *Blastocystis* in cockroach under 400× 122 magnification. (a) Vacuolar form (b) Granular form.
- Figure 5.3 *Blastocystis* in cockroach incubated at different temperature 123 under 400× magnification. (a) 25°C (b) 37°C.
- Figure 5.4 Surface structure of vacuolar form of *Blastocystis* from 124 cockroach (a) smooth surface (b) coarse and folded surface.
- Figure 5.5 Electron micrograph of *Blastocystis* from cockroach (a) 125 Vacuolar form (b) Granular form. N; nucleus, V; vacuole, CV;

XV

central vacuole, SC; surface coat, CM; cell surface membrane, CVM; central vacuole membrane.

- Figure 5.6 Measurement of the surface coat from cockroach isolates (a) 126 Vacuolar form (b) Granular form.
- Figure 5.7 Binding affinities of *Blastocystis* stained with FITC-labelled 128
 Con A. (a) Light microscopic images *Blastocystis* of cockroach isolate. (b) A same organism stained with FITC-labeled Concanavalin A (ConA) assay. V; vacuolar form, G; granular form.
- Figure 5.8 Epifluorescence image of *Blastocystis* stained with acridine 129 orange (a) Light microscopic images *Blastocystis* of cockroach isolate (b) A same organism stained with acridine orange.
- Figure 6.1 Total number of viable cells from (a) avian and (b) human 144 isolates after 24 hours incubation.
- Figure 6.2 *Blastocystis* cells of (a) avian and (b) human isolates cultured in 145 a pH range of 1 4 showing the rounded structures of non-viable cells (arrow) whereas cells of (c) avian and (d) human isolates in pH4 showing non-viable vacuolar forms with a wrinkled or shrunk appearance (arrow) compared to the human isolates showing smaller viable vacuolar forms with low parasite count. Meanwhile, *Blastocystis* cells of (e) avian and (f) human isolates in a pH range of 5 7 showing the typical rounded vacuolar form (arrow).
- Figure 7.1 Subtypes classification characterised from animal *Blastocystis* 161 isolates.

LIST OF TABLES

Table 2.1	Correlation of <i>Blastocystis</i> subtype designations and suggestion					
	for consensus terminology (Stensvold et al., 2007b).					
Table 3.1	The STS primer sets used in this study.	47				
Table 3.2	Blastocystis infection in two types of chicken population.	50				
Table 3.3	Blastocystis infection in two study areas.	50				
Table 3.4	Subtype of <i>Blastocystis</i> from chicken isolates obtained <i>Blastocystis</i> Sequence Typing Databases.	69				
Table 3.5	List of previous publication on <i>Blastocystis</i> in birds.	75				
Table 4.1	Prevalence of <i>Blastocystis</i> in wild rats.	88				
Table 4.2	Subtype of <i>Blastocystis</i> from wild rat isolates obtained <i>Blastocystis</i> Sequence Typing Databases.	106				
Table 4.3	List of previous publication on <i>Blastocystis</i> in rodents.	112				
Table 5.1	Prevalence of <i>Blastocystis</i> in cockroaches relative to sampling sites.					
Table 5.2	List of previous publication on <i>Blastocystis</i> in cockroaches and house geckos.	135				
Table 6.1	List of previous publication on <i>Blastocystis</i> in cats and dogs.	150				
Table 7.1	Summary on comparison <i>Blastocystis</i> isolates from human and study animals in terms of morphological, morphometry and genotype characteristics.	162				

LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
0	:	Degree
μl	:	Microliter
μm	:	Micrometer
AFU	:	Affinity fluorescence unit
bp	:	Base pair
CBA	:	Cytometric bead array
DNA	:	Deoxyribonucleic acid
g	:	gram
ICR	:	Ihara's cataract rat
IBS	:	Irritable bowel syndrome
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
pН	:	Power of hydrogen
PVG	:	Piebald Virol Glaxo
RNA	:	Ribonucleic acid
rpm	:	Revolutions per minute
sec	:	Second
SEM	:	Scanning electron microscopy
SHR	:	Spontenously hypertensive rat
sp.	:	Species
SPSS	:	Statistical package for the social science
SSU-rRNA	:	Small sub-unit ribosomal ribonucleic acid
ST	:	Subtype

- STS : Sequence-tagged-site
- Taq : Thernus aquaticus
- TEM : Transmission electron microscopy

LIST OF APPENDICES

Appendix A	Modified Jones' Medium					
Appendix B	Posterior abdominal segments of cockroaches					
Appendix C	Summary of morphological characteristics of Blastocystis	189				
	isolated from village chicken (A3) and peacock (M2) grown in					
	Jones' medium with different pH					
Appendix D	Summary of morphological characteristics of Blastocystis	190				
	isolated from human (H1, H2 and H3) grown in Jones'					
	medium with different pH					

CHAPTER 1:

INTRODUCTION

1.1 Research background

Blastocystis is a stramenopile (Silberman *et al.*, 1996) inhabits the gastrointestinal tracts of humans and many animals (Stenzel and Boreham, 1996 and Abe *et al.*, 2002). There are four described forms namely; vacuolar, granular, amoeboid and cyst form (Tan, 2008). Faecal-oral transmission is the most common pathway with the cystic form as the infective form transmitted through food and waterborne usually via untreated water or poor sanitary conditions (Waikagul *et al.*, 2002; Ithoi *et al.*, 2011). The mode of transmission is believed to occur by animal-to-animal, human-to-human, animal-to-human and possibly, human-to-animal routes (Noël *et al.*, 2005).

Recent molecular studies indicate that this parasite is not a single species, but composed of genetically distinct but morphologically identical genotypes (Clark, 1997; Yoshikawa *et al.*, 1998, 2000). Due to its low species specificity, previous species-naming conventions have become less favored. Identifying nomenclature of the parasite has resulted into a consolidated system of consensus terminology (Stensvold *et al.*, 2007b), which classifies all known *Blastocystis* spp. isolates into subtypes based on sequence similarities in small-subunit ribosomal RNA. The protozoan is currently classified into 17 distinct subtypes (ST1-ST17) isolated from a wide range of hosts i.e., human, mammalian and avian hosts (Noel *et al.*, 2005; Parkar *et al.*, 2010; Alfellani *et al.*, 2013a, b, c). Commonly, ST1-ST9 are found in humans (Wawrzyniak *et al.*, 2013), ST10, ST13 and ST15 in primates (Stensvold *et al.*, 2009; Alfellani *et al.*, 2013a) whereas, ST11 and ST12 in elephants and giraffes, respectively (Parkar *et al.*, 2010).

The presence of this organism has been found in a wide range of animals from domestic birds; chickens (Yamada et al., 1987; Belova and Kostenko, 1990; Bergamo do Bomfim and Machado do Couto, 2013), ostriches (Ponce et al., 2002; Hemalatha et al., 2014 and Chandrasekaran et al., 2014), geese (Belova, 1992a), turkeys (Belova, 1992b), ducks (Belova, 1991; Stenzel et al., 1994), Japanese quails (Bergamo do Bomfim and Machado do Couto, 2013), pheasants (Abe et al., 2003a), partridge (Abe et al., 2003a), primates (McClure et al., 1980; Pakandl, 1991; Abe et al., 2002; Rivera, 2008; Stensvold et al., 2009), swine (Burden, 1976; Pakandl, 1991; Quilez et al., 1995a; Arisue et al., 2003; Navarro et al., 2008), reptiles; snakes, crocodiles, lizards, tortoise (Teow et al., 1991; Noël et al., 2005), insects; cockroaches (Zaman et al., 1993), companion animals; cats and dogs (Knowles and Gupta, 1924; Duda et al., 1998; Abe et al., 2002; Parkar et al., 2007; Jon Shaw, 2012), cattle (Stenzel et al., 1993; Fayer et al., 2012), horse (Thathaisong et al., 2003), amphibians (Yoshikawa et al., 2004b), rodents (Alexeieff, 1911; Knowles and Das Gupta, 1924; Lavier, 1952; Chen et al., 1997a, b), circus animals; ungulates and lion (Stenzel et al., 1993) and zoo animals; elephants, giraffes and quokkas (Parkar et al., 2010). So far, no correlation has been established between high numbers of Blastocystis spp. seen in the faeces with clinical signs in infected hosts (Duda et al., 1998). However, previous studies have shown that infection in pigs and monkeys with diarrhea (Burden et al., 1978; McClure et al., 1980; Pakandl, 1991).

This organism has been extensively studied in Malaysia (Suresh *et al.*, 1997; Rajah *et al.*, 1999; Tan and Suresh, 2006a, b; Chandramathi *et al.*, 2010; Ithoi *et al.*, 2011; Tan *et al.*, 2013) particularly infections in humans. Although, Suresh *et al.* (1996) reported this parasite in a range of host including; laboratory animals, sheep, rabbits, monkeys, dogs

and cats however, the study was limited to a small number of samples. More recently, Tan *et al.* (2013) reported that 30.9% (73/236) goats examined positive for *Blastocystis*.

The close proximity between animals and human could be a potential source for zoonotic infection (Abe *et al.*, 2002). Rajah *et al.* (1999) illustrated that people working intimately with animals were at higher risk of getting *Blastocystis* infection highlighting occupation such as animal handlers were most likely to gained this infection from the animals through the faecal-oral route. Understanding the presence of this organism in the environment is therefore crucial which prompted this study to determine the prevalence of this organism in companion animals, household pests and poultry population as information in Malaysia is scarce. There is also a need to investigate the genetic diversity of *Blastocystis* which would enable to increase the understanding of the zoonotic potential of this organism.

1.2 Justification of the study

Blastocystis infections is common in birds and have been extensively reported in chickens (Belova and Kostenko, 1990), ostriches (Yamada *et al.*, 1987), ducks (Pakandl and Pecka, 1992), geese (Belova, 1992a) and turkeys (Lee, 1970; Belova 1992b). Presently, there are no known studies from the poultry population in Malaysia. This is the first attempt to study the occurrence of *Blastocystis* spp. in poultry and to compare infection between free-range and commercially barn-reared chicken as well as to observe unique chicken isolate features.

Previous prevalence studies of *Blastocystis* sp. in rodents highlighted the presence of human subtype (ST4) in one guinea pig isolate (Yoshikawa et al., 1998) as well as three isolates (two Wistar rats and one Sprague Dawley rat) from Singapore (Noël et al., 2005). Apart from that, Yoshikawa et al. (1998) also showed that isolates from guinea pigs exhibited restriction fragment length polymorphism (RFLP) profiles or random amplified polymorphic DNA (RAPD) patterns similar to those observed in some B.hominis. ST4 was the predominant human subtype with 63% in France, 84% in Nepal and 94.1% in Spain (Roberts et al., 2014). Therefore, it remains to be established whether close contact with rodents poses risk of transmission to humans. The availability information such as prevalence and the subtypes found in rodents would be of interest in understanding the significance of Blastocystis infection in human. In the present study, there is a need to study the epidermiology, phenotypic, subtyping, growth characteristic and the ultrastructural features of Blastocystis sp. in wild rats especially the common wild rats i.e brown rats and house shrew as this has not yet been established before in Malaysia. The information will lead to a better understanding of the present status and the characteristic of this enigmatic intestinal parasite in wild rats in Malaysia.

4

Cockroaches are among the notorious insects inhabitating dwelling, food handling establishment and septic tanks. The suggestion that close association between humans and household pests could also facilitate the transmission sparked to include cockroaches in the present study. Despite reported prevalence and the morphological features of *Blastocystis* in cockroaches previously, however, there have been no further attempts to subtype characterization of *Blastocystis* sp. isolated from cockroaches. Therefore, this study aims to re-assess the current prevalence and to include the phenotype and subtype of this parasite from cockroach population caught from several Malaysian cockroach populations.

Only one study carried out by Suresh *et al.* (1997) elucidated the occurrence as well as the ultrastructural features of the parasite in common household geckos. It is clear that there is still much information needed regarding to *Blastocystis* infection in this reptilian. Hence, the present study aimed to provide a better understanding on *Blastocystis* in the house geckos (*Hemidactylus frenatus*).

Companion animals especially cats and dogs are prone to several protozoan gastrointestinal infections such as *Giardia* (Traub *et al.*, 2004). Recently, increasing interest in *Blastocystis* spp. as a potential cause of gastrointestinal disease in human is increasing. Duda *et al.* (1998) reported high prevalence in dogs and cats in Australia, with infections as high as 70% in both animals using light microscopy on faecal wet mounts while Nagel *et al.* (2012) showed pet dogs/cats of eleven symptomatic *Blastocystis* infected patients harboured at least one *Blastocystis* subtype in common with the patient. This raised the possibility that animals as natural hosts for *Blastocystis* and potential sources of zoonotic transmission to humans. Hence, the present study

aimed to elucidate the nature of *Blastocystis* spp. in cats and dogs from two different environments namely stray and the sheltered.

Hence, the study examines the prevalence and biology of *Blastocystis* spp. in animal hosts closely associated with human dwellings as possible vectors to human transmission. The specific aims for this study are;

1. To assess the prevalence of *Blastocystis* spp. in free-range chickens and commercially barn-reared chickens, household pest i.e wild rats, cockroaches and house gecko as well as companion animals namely cats and dogs obtained from stray and shelter-housed animals.

2. To establish phenotypic characteristics on *Blastocystis* spp. isolated from companion animals, household pests and chickens based on staining characteristics of *Blastocystis* spp. from different isolates using Giemsa stain, Fluorescein isothiocyanate (FITC)-labelled Con A (*Canavalia ensiformis*), Acridine Orange stain, Sudan Black B stain, as well as surface characteristics and ultrastructure of *Blastocystis* spp. using scanning and transmission electron microscopy.

3. To study the life cycle stages of *Blastocystis* spp. isolated from companion animal, household pest and chicken isolates.

4. To assess the subtypes of isolates from companion animals, household pests and chicken through the application of molecular tools by detecting the presence of zoonotic genotypes of *B. hominis* using polymerase chain reaction (PCR) with subtype specific sequence-tagged-site (STS) diagnostic primers as well as investigating the presence of novel subtypes using a vital new tool; DNA barcoding methods.

CHAPTER 2:

LITERATURE REVIEWS

2.1 Classification of *Blastocystis*

2.1.1 Taxonomic status

The classification of *Blastocystis* remains unresolved for a period of time. It was initially identified as a new species and classified as harmless yeast (Alexeieff, 1911; Brumpt, 1912). However, there is not a single characteristic that strongly associates *Blastocystis* as yeast.

This organism was subsequently reclassified as a protist based on a number of protistan features (Zierdt *et al.*, 1967) that include the presence of one or more nuclei, smooth and rough endoplasmic reticulum, Golgi complex, and mitochondrion-like organelles. However, the organism was failed to grow on fungal media and was resistant to antifungal drugs but showed some antiprotozoal drugs activity. Following this, it was then classified as a sporozoan based on morphology, cultural characteristics, mode of division but was later reclassified into the subphylum Sarcodina (Zierdt, 1991).

However, a molecular phylogenetic analysis based on the comparison of smallsubunit ribosomal RNA gene (SSU-rRNA) sequences showed that *Blastocystis* was not monophyletic to yeasts (Saccharomyces), fungi (Neurospora), sporozoans (*Sarcocystis* and *Toxoplasma*) or sarcodines (*Naegleria*, *Acanthamoeba*, and *Dictyostelium*) and only recently classified within the Stramenopiles (Silberman *et al.*, 1996) also called Heterokonta, a diverse group of mostly unicellular or multicellular eukaryotes which includes diatoms, brown algae, slime nets and water moulds. One important characteristic of Stramenopiles is the presence of at least one flagellum permitting motility. However, *Blastocystis* do not possess any flagella or tubular hairs and is non-motile. The link was made using phylogenetic analysis of small subunit ribosomal RNA gene (SSU-rRNA) sequences and has been confirms using other gene sequences (Arisue *et al.*, 2002). Within the stramenopiles, *Blastocystis* was resolved as a sister group to the order Slopalinida and most closely related to *Proteromonas lacertae*, a parasite of reptiles (Pérez-Brocal *et al.*, 2010; Kostka *et al.*, 2004). Because no organelles in organisms specifically and closely related to *Blastocystis* have been studied so far, the answer to the question of its uniqueness remained unclear.

2.1.2 Speciation

Isolates from human is designated as *Blastocystis hominis*, whereas isolates found in a variety of animals are known as *Blastocystis* sp. However, a small number of specific names have been published for isolates from specific host especially rat and reptilian *Blastocystis* species. Rat isolates poses a distinct karyotypic pattern compared to *B. hominis*. Therefore, Chen *et al.* (1997b) concluded that the rat *Blastocystis* is a distinct species, and *B. ratti* was proposed.

Nevertheless, some of the isolates have shown singular phenotypic characteristics that differentiated them from human and other homeothermic animals, such as; seasnakes (*B. lapemi*), reticulated python (*B. phythoni*), rhino iguana (*B. cycluri*) and redfooted tortoise (*B. geocheloni*) (Teow *et al.*, 1991; Singh *et al.*, 1996).

In addition, several new *Blastocystis* species of bird origin are reported on the basis of morphological and host differences. These include *B. galli* from chickens (Belova

and Kostenko, 1990), *B. anatis* from ducks (Belova, 1991) and *B. anseri* from geese (Belova, 1992a). Since *Blastocystis* is known to be polymorphic in its morphology, the morphological features are not adequate for species designation. However, Tanizaki *et al.* (2005) did not accept the criteria of speciation of *Blastocystis* isolates from birds such as quails, geese and chickens.

2.2 Terminology of *Blastocystis*

There is an agreement that at least seven major isolate clades exist in mammals and birds (Yoshikawa *et al.*, 2004a; Noël *et al.*, 2005); however, varying terminologies have been used in the past to designate the subsets of *Blastocystis* isolates which made corroboration, comparison or criticism of published studies difficult.

2.2.1 Standardization of terminology

Human isolates are designated as *Blastocystis hominis*, whereas isolates from other animals are known as *Blastocystis* sp. The extensive genetic diversity of this organism, even among isolates from one host, makes the host-specific naming of species misleading. In bacteria and certain eukaryotic groups such as *Naegleria* (De Jonckheere, 1994), the degree of genetic divergence between the major clades seen in *Blastocystis* would be considered sufficient on its own to justify separate species names for each. However, the more appropriate nomenclature proposed by Stensvold *et al.* (2007b) described isolates as *Blastocystis* sp. subtype *n* where *n* is a number from 1 to 9 (Table 2.1). The reason for using *Blastocystis* sp., rather than *B. hominis*, is that some reptilian and amphibian species fall within the range of variation covered by the mammalian and avian clades (Yoshikawa *et al.*, 2004a, c; Noël *et al.*, 2005).

Clade ^a	Subtype ^b	Group and	Subtype ^d	Ribodeme ^{e,f}	Subgroup ^g	Cluster ^h	Subtype ⁱ	Consensus
		subtype ^c						
Ι	Ι	I/1	1	1, 8 ^j	III	Е	1, 1 variant	Blastocystis sp. subtype 1
II	II	II/5	5	6	V	C,D	_ ^k	Blastocystis sp. subtype 2
-	Х	I+II/1+5 outlier	-	-	-	-	-	Chimaeric sequence
III	III	III/3	3	2, 7, 4? ¹ , 5?	I, II	А	3	Blastocystis sp. subtype 3
IV	IV	IV/7	7	3	IV	В	-	Blastocystis sp. subtype 4
-	IVa	IV/7 outliers	-	-	-	-	-	Blastocystis sp. subtype 8
V	V	V/6	6	-	-	-	-	Blastocystis sp. subtype 5
VI	VI	VI/4	4	9 ^j	-	-	4	Blastocystis sp. subtype 6
-	VIa	VI/4 outliers	-	-	-	-	-	Blastocystis sp. subtype 9
VII	V11	VII/2	2	10	VI^m	-	2	Blastocystis sp. subtype 7
-	VII	VII/2 outliers	-	-	-	-	-	Blastocystis sp. subtype 7

Table 2.1: Correlation of *Blastocystis* subtype designations and suggestion for consensus terminology (Stensvold *et al.*, 2007b).

^aClades described by Arisue et al. (2003) and Yoshikawa et al. (2004d).

^bSubtypes described by Scicluna et al. (2006).

^cGroups and subtypes described by Noel et al. (2005).

^dSubtypes described by Yoshikawa et al. (2000, 1998)

^eRibodemes are groups that share the same SSU-rDNA PCR-RFLP patterns and are described by Clark (1997) and Yoshikawa *et al.* (2000).

^fRibodemes in bold are those originally described by Clark (1997).

^gSubgroups described by Bohm-Gloning et al. (1997) on the basis of PCR-RFLP analysis and partial SSU-rDNA sequences.

^hClusters described by Stensvold *et al.* (2006) on the basis of PCR and sequencing analysis of partial SSU-rDNA sequences.

ⁱSubtypes described by Yoshikawa et al. (2000) using PCR-STS.

^jRibodemes 8 and 9 described by Yoshikawa et al. (2000) differ from those described by Kaneda et al. (2001).

^{k'-'}symbols indicate no equivalent described.

¹Question mark indicates that the subtype equivalence is probable but not proven.

^mSubgroup VI described by Thathaisong et al. (2003) equals ribodeme 10 described by Yoshikawa et al.(2000).

2.2.2 Current subtypes

Recent epidemiological studies revealed the extensive diversity of *Blastocystis* subtypes. At present, the number of subtypes found in humans has remained constant; with nine subtypes (ST1 through ST9) (Parkar *et al.*, 2010) with some primarily found in human (ST3 and ST9). While ST1, 2, 5 and 8 are found both in human and mammalian isolates (primate, pig, human, cattle and pig), while ST4 present among rodents, and ST6, 7 and 8 among avian hosts.

Meanwhile, some subtypes are exclusively found in animals (ST10-17). ST10 and 15 are present among *Artiodactyla* and non-human primates, ST11 among *Proboscidea*, ST12 among *Artiodactyla* and marsupials, ST13 among non-human primates and marsupials, ST14 among *Artiodactyla*, ST16 among marsupials and ST17 among rodents (Stensvold *et al.*, 2009; Parkar *et al.*, 2010; Alfellani *et al.*, 2013a, c; Roberts *et al.*, 2014) (Figure 2.1). To date, a limited number of mammalian species have been screened, making it likely that undiscovered subtypes may exist.



Figure 2.1: *Blastocystis* subtypes (STs 1-17) with host specificities (Wawrzyniak *et al.*, 2013).

2.3 Biology of *Blastocystis*

2.3.1 Morphology

Blastocystis exists in various morphotypes such as vacuolar, multivacuolar, avacuolar, granular, amoeboid and cystic forms (Suresh *et al.*, 2009). These forms have been described in *Blastocystis* from different hosts (Stenzel and Boreham, 1996; Tan and Suresh, 2006b). According to Zierdt (1991), cell physiology or the external environment may be involved for this morphological variety.

2.3.1.1 Vacuolar form

The vacuolar form is considered to be the typical *Blastocystis* cell form *in vitro* culture and stool (Zierdt, 1991; Stenzel and Boreham, 1996). It is generally rounded to ovoid in outline with a single central vacuole which occupies most of the cell space, limiting the cytoplasm and other intracellular components to a thin peripheral rim (Figure 2.2a). Occasionally, irregularly shaped were observed with extensive variations in size ranging from 3 to 120 μ m in diameter. The vacuole contains unevenly distributed flocculent or fine granular material made up of carbohydrates and lipids (Yoshikawa *et al.*, 1995b, c).

2.3.1.2 Granular form

The granular forms are rarely seen in stools, but are found in the *in vitro* cultures. It is commonly observed in old and non-axenized cultures (Tan, 2004). Granular forms structurally resemble the vacuolar form except for the presence of granules in the central body and the cytoplasm (Figure 2.2b). Studies have identified three types of granules namely; metabolic, reproductory and lipid granules (Tan and Zierdt, 1973; Tan and Stenzel, 2003).

2.3.1.3 Amoeboid form

The amoeboid forms are reported present in higher prevalence in the *in vitro* cultures of symptomatic isolates compared to asymptomatic isolates (Tan and Suresh, 2006). Previously, amoebic forms were observed in colonoscopic lavage and patients with acute diarrheal syndrome (Stenzel *et al.*, 1991; Lanuza *et al.*, 1997). The morphological descriptions of the amoeboid form are conflicting (Tan *et al.*, 2002). It is less frequently recovered than vacuolar and granular forms. They are irregular in shape and usually measure around 10 μ m in size. Although the possession of one or two pseudopodia is a characteristic feature, this form is non-motile with a single large vacuole similar to the central body or has multiple smaller vacuoles (Tan and Suresh, 2006b) (Figure 2.2c).

2.3.1.4 Cyst form

The cyst form is smaller than the typical cultured forms ranging from 2 to 5 μ m (Tan, 2008) which maybe confused as faecal debris (Figure 2.2a). The cyst is protected by a thick wall and condensed cytoplasm similar to other protozoan cysts (Mehlhorn, 1988). It is mostly ovoid or spherical in shape, resistant and more commonly found in fresh faeces, long-term cultures (Stenzel and Boreham, 1991) and in stored faecal specimens, suggesting that this form possess a mechanism for survival in the external environment. Zaman *et al.* (1995) have provided a method for concentrating this form from faecal material by repeated washing in distilled water and centrifugation on Ficoll-paque solution.


Figure 2.2: Morphological form of *Blastocystis* (a) Vacuolar (arrowheads) and cyst forms (arrows) in the *in vitro* culture (b) Granular form with distinct granular inclusions (arrowheads) (c) Amoeboid forms showing pseudo-like cytoplasmic extensions (arrow). Scale bar, 10µm (Tan, 2008).

2.3.2 Life cycle and transmission of *Blastocystis*

Upon ingestion of cysts, encystation occurs during the passage along the large intestines to liberate the vacuolar form. The vacuolar forms can transform into any of the other three forms. However, frequent observations of the amoeboid, vacuolar and multivacuolar forms in diarrheal stool suggest that these forms play a role in the pathogenesis. Encystation may also occur as it moves along the colon before cysts is excreted out through the faeces (Tan, 2008) (Figure 2.3).

It is believed that *Blastocystis* is transmitted via the faecal-oral route, although routes of transmission such as waterborne, foodborne, and person-to-person have been speculated (Li *et al.*, 2007a; Leelayoova *et al.*, 2008; Ithoi *et al.*, 2011).

Yoshikawa *et al.* (2004e) reported that different cyst concentration have resulted in different infectivity ranging from 10 to 100%, suggesting that contaminated water and food with just a few number of cysts can establish infection. Besides, presence of viable cysts in sewage samples and surface water supports the plausible transmission by drinking contaminated water still remains in question (Suresh *et al.*, 2005; Ithoi *et al.*, 2011). Other than that, consumption of unboiled or raw water plants could be a source of this infection (Taamasri *et al.*, 2000; Leelayoova *et al.*, 2004; Li *et al.*, 2007a). It was also observed that the cyst forms can survive for up to 19 days or 1 month in water at room temperature (25°C) and for 2 months at 4°C (Moe *et al.*, 1997) proving only this form transmits infection via direct or indirect faecal-oral route.

According to Rajah *et al.* (1999), higher risk of infection was found associated to people with close proximity with animals. Noël *et al.* (2003) provided additional proof by showing similar identity between *Blastocystis* sp. isolated from humans and pigs. In addition, Thathaithong *et al.* (2003) showed similar *B. hominis* isolates band patterns to isolates from the horse and the pig with 92 to 94% identity suggesting that *B. hominis* evolved from domestic animals isolates (*Blastocystis* sp.) such as pigs and horses.



Figure 2.3: A current view of *Blastocystis* life cycle (Roberts et al., 2014).

2.3.3 Reproductive mode of *Blastocystis*

Various authors have observed different modes of reproduction such as binary fission, budding and schizogony (Zhang *et al.*, 2007). However, the current only accepted mode of reproduction is binary fission (Mehlhorn *et al.*, 2012).

2.3.3.1 Binary fission

Binary fission is the most common and well established mode of reproduction. It is characterized by the partition of the cytoplasm of the mother cell resulting two equal sized and shaped daughter cells (Figure 2.4a) (Tan, 2008).

2.3.3.2 Budding

Recent studies confirmed budding or plasmotomy is another reproduction mechanism (Zhang *et al.*, 2012) and is characterized by the cutting off one or more progeny from the roughly circular extensions of the cell (Figure 2.4b).

2.3.3.3 Schizogony

Schizogony occurs within the central body cells that later ruptures, releasing the progeny or daughter vacuolar forms to the environment (Figure 2.4c). Schizogony-like organisms was observed in the *in vitro* culture but rarely in human faecal samples. However, it was only confirmed via light microscopic observations (Zierdt, 1991) but not clearly determined with electron microscopy. Therefore, further research on this reproductive mode needs further clarification whether this mode truly exists.



Figure 2.4: Various reproductive modes of *Blastocystis* (a) Binary fission (b) Budding form (c) Schizogony-like organism (arrows). Scale bar, 10µm (Mehlhorn *et al.*, 2012).

2.4 Detection of *Blastocystis*

Most of the methods to detect the organism in infected faecal samples include direct microscopy, concentration technique, staining, culturing and extraction of DNA followed by polymerase chain reaction (PCR) technique. However, may differ remarkably in terms of diagnostic sensitivity.

2.4.1 Microscopy techniques

2.4.1.1 Direct faecal smear

Direct faecal smear is one of the most common methods used for the detection of *Blastocystis* sp. as it takes less time and resources compared to other methods. However, morphology-based diagnosis has several disadvantages, including the challenge posed by the diversity in cellular forms of *Blastocystis*. The classical spherical vacuolar form may appear smaller while the rare cystic, amoeboid, multivacuolar and avacuolar forms may predominate (Stenzel *et al.*, 1991). In addition, smears may often be mistaken associated to vegetative stages of the parasite as lipid globules or other contaminants (Suresh and Smith, 2004).

Besides, the sensitivity of direct faecal smear is greatly affected by the cell count in the specimens. A very low cell count may lead to a false negative result (Leelayoova *et al.*, 2002). In a clinical setting, a direct consequence a false negative will be the mismanagement of the infection, especially if only method of detection available is direct faecal microscopy.

2.4.1.2 Staining

Trichome staining for permanent smears is the recommended method for the diagnosis of this parasite, although many other stains such as Giemsa, iron hematoxylin, Gram, Wrigh's and Fields stains may also be used. Roberts *et al.* (2014) reported that permanent stains using a modified iron hematoxylin stain was the least effective in detecting *Blastocystis* with more than 50% infections usually get missed. The low sensitivity of this method may be the result of low parasite numbers in the feacal sample.

2.4.2 Concentration techniques

Concentration methods used for other protozoan parasites generally appear unsuitable for *Blastocystis* as this method may cause disruption to the vacuolar, multivacuolar and granular forms. A previous study showed *Blastocystis* could be detected in stained smears of faecal material but not in the concentrated specimens from the same faecal sample (Miller and Minshew, 1988). However, several authors noted concentration method is also effective for *Blastocystis* sp. Ishak *et al.* (2008) reported that formalin ethyl acetate concentration technique was the most sensitive technique with 60.9% prevalence of *Blastocystis* sp. detection compared to 43.5% with direct saline wet mount and 34.8% with trichrome staining despite being time consuming. It is also used widely for the diagnosis of cysts, ova and larvae and applied in numerous *Blastocystis* sp. prevalence studies (Truant *et al.*, 1981; Ishak *et al.*, 2008).

2.4.3 *In vitro* culture techniques

The culture technique is the more sensitive method for the detection for this organism. Leelayoova *et al.* (2002) showed that xenic *in vitro* culture (XIVC) was significantly more sensitive for the detection of *Blastocystis* compared to the concentration technique or direct smear. Likewise, Termmathurapoj *et al.* (2004) preferred XIVC to direct smear and staining method for the detection and molecular study of *Blastocystis* in stool specimens.

The success of the culture depends on the media content that is essential to the natural growth of the organisms and must imitate as close as possible to the natural environment. For free-living protozoa, the optimum condition is not difficult to attain, but in the case of parasites, the media preparation is also a criteria when selecting the best culture medium.

A successful culture allows the organisms to reproduce and increase in numbers for a time period until the nutrients in the medium is exhausted or charged with substances preventing continued growth. The transfer of parasites to fresh medium will allow renewed multiplication. Repeated subculture enables maintenance of the organisms indefinitely.

When a culture is commenced, a varying quantity of material containing the organism is introduced into the culture medium and kept at the requisite temperature. As reported previously, *Blastocystis* isolated from homoiothermal hosts' cultures are usually incubated at 37°C. While, poikilothermal isolates are kept in lower temperature for example isolates recovered from a reptile sea-snake (*B. lapemi*) showed optimal growth at 24°C and fails to survive at 37°C (Teow *et al.*, 1991). Meanwhile, Zaman *et*

al. (1993) demonstarted that *Blastocystis* sp. isolated from household cockroaches collected from Singapore sewers grew at both 25°C and 37°C.

Interestingly, isolates from poikilothermal hosts may originate from homoiothermal hosts. For example, isolate from a rhino iguana (AY266475) and isolate from a toad (AFJ96-H1) could survive at 37°C and 34°C, respectively, while another isolate from the same host iguana species could not (Yoshikawa *et al.*, 2004b) suggests that some isolates in poikilothermal hosts may have originated from homoiothermal hosts, allowing the isolates to grow at higher temperature rather than room temperature.

Various media are available for cultivating *Blastocystis* namely, Locke-egg medium, Iscove's modified Dulbecco's medium, Robinson's medium, TYSGM-9 and Jones' medium. However, the preferred media is the modified Jones' medium (Appendix A) as the medium of choice for xenic culture of *Blastocystis* (Leelayoova *et al.*, 2002; Suresh and Smith, 2004; Stensvold *et al.*, 2007a; Parkar *et al.*, 2007) as the medium is composed of the simplest constituents and can be stored for a longer time in a refrigerator if sterilized. In addition, it is also cost-effective and do not require any specific technique and equipment.

2.4.4 Molecular techniques

Over the years, the advancement of molecular techniques has allowed the detection and classification of this parasite to cope with the expanding number of subtypes of this parasite. The advantage of this technique is the time saving factor (approximately three hours) instead of the 3 to 4 days required for *in vitro* culture. However this technique is costly due the special equipment and expensive consumables in addition to the need for special training required.

Subtype characterization is determined primarily by one of two ways: (1) PCR with subtype specific sequence-tagged-site (STS) diagnostic primers (Yoshikawa *et al.*, 2003) and (2) sequencing of small subunit rRNA gene (SSU-rDNA) PCR products (barcode region) (Scicluna *et al.*, 2006). However, both methods have different advantages and limitations.

2.4.4.1 Sequence-tagged-site (STS) diagnostic primers

The advantages for STS method is it allows straight-forward detection of mixed subtypes carriage and therefore the need for sequencing PCR products can be circumvented. Moreover, STS primers pairs are targeted to Subtypes 1 to 7 (Yoshikawa *et al.*, 2003). The sensitivity is moderate, and therefore postulated that some infections may go undetected (Stensvold, 2013). Besides, some subtypes, for instance, Subtype 3 exhibit substantial intrasubtype genetic diversity (Stensvold *et al.*, 2012).

2.4.4.2 DNA barcoding

DNA barcoding is a new and powerful basic research tool with exceptional potential for the incorporation of new technologies and for future applications. Barcoding appears robust for genetic characterization combining the use of a forward primer of broad, eukaryotic specificity (RD5) (Clark, 1997) and a genus-specific reverse primer (BhRDr) (Scicluna et al., 2006). For sequencing, several regions in the SSU-rDNA have been targeted; however the "barcode region" has been used extensively. This region encompasses the 0.6 5'-most kbp is known to be a valid proxy for complete SSUrDNAs and is a region for which many sequences are available in both GenBank and Blastocystis Subtype (18S) and Sequence Typing (MLST) Databases the (www.pubmlst.org/blastocystis), identified not only to ST level but to 18S allele level which offers higher resolution than subtyping alone (Stensvold, 2012; 2013). However, it should be noted that barcoding PCR should not be used on faecal DNA template as a strictly diagnostic tool since the RD5/BhRDr primer pair typically amplifies common fungal DNA in the absence of *Blastocystis* with no obvious difference in PCR product size (Clark et al., 2013).

2.4.5 Serological technique

Blastocystis infections in human can lead to raised immunoglobin G (IgG) and IgA responses, as detected by indirect fluorescent antibody (IFA) and enzyme-linked immunosorbent assay (ELISA). Mahmoud and Saleh (2003) investigated the secretory IgA, serum IgA, and serum IgG levels in Blastocystis-positive asymptomatic and symptomatic individuals using ELISA technique. The findings showed higher Blastocystis-reactive secretory IgA, serum IgA, and serum IgG levels from symptomatic patients, compared to asymptomatic carriers and healthy controls. Monoclonal antibodies against Blastocystis have been described (Yoshikawa et al., 1995a; Tan et al., 1996) with the majority of antibodies were IgM and localized to surface coat antigens. These antibodies exhibited limited cross-reactivity against different genotypes, indicating antigenic diversity among Blastocystis isolates. Although currently unavailable, monoclonal antibodies specific for human-infective genotypes would be useful for antigen detection studies, as was previously described for Entamoeba histolytica/E. dispar. Considering the limited knowledge of the host immune response to Blastocystis and the apparent antigenic diversity of the parasite, it is not practical to include serology in the routine laboratory diagnosis of *Blastocystis*, and it should be limited to epidemiological and serological studies.

2.5 *Blastocystis* in animals

2.5.1 Birds

Blastocystis infections is common in birds and have been reported in chickens (Yamada *et al.*, 1987; Belova and Kostenko, 1990; Stenzel *et al.*, 1994), ostriches (Yamada *et al.*, 1987; Stenzel *et al.*, 1994; Chandrasekaran *et al.*, 2014; Hemalatha *et al.*, 2014), domestic ducks (Belova, 1991; Pakandl and Pecka, 1992; Stenzel *et al.*, 1994), domestic geese (Belova, 1992a) and turkeys (Lee, 1970; Belova, 1992b). A number of new species of *Blastocystis* have been proposed based on the differences in the host origins, morphology, *in vitro* characteristics and/or karyotype (Belova and Kostenko, 1990; Belova 1991, 1992; Teow *et al.*, 1991; Singh *et al.*, 1996; Chen *et al.*, 1997; Krylov and Belova, 1997).

Three new *Blastocystis* species from chickens, ducks and geese have been proposed. *B. galli* has been described from domestic chickens and turkeys (Belova and Kostenko, 1990; Belova, 1992b); *B. anatis* from ducks (Belova, 1991); and *B. anseri* from geese (Belova, 1992a). The major criterion used to distinguish species was the size, but given the enormous variation seen in cell size within each sample, this is not sufficient to delineate new species.

However, limited genomic analysis on bird isolates has been performed. Based on previous studies, birds are generally infected with multiple genotypes including zoonotic genotypes. Yoshikawa *et al.* (1996) reported the presence of a zoonotic genotype Subtype 1 in chicken. Meanwhile, Abe *et al.* (2003a) genotyped seven *Blastocystis* isolates via polymerase chain reaction (PCR) using diagnostic primers, and the homology among isolates was then confirmed by restriction fragment length polymorphism (RFLP) analysis of the small subunit ribosomal RNA gene. Their study found three isolates from a Chinese bamboo partridge, a vulturine guineafowl, and a Japanese green pheasant amplified with SB332 (Subtype 4), but the RFLP profiles differed from Subtype 4 reported from a human. However, the other four isolates from a Palawan peacook-pheasant, a satyr tragopan, a Himalayan monal pheasant and a great argus pheasant did not amplify to any subtype-specific primer sets.

The transmission of *Blastocystis* infection can occur easily between the same or different bird species. Tanizaki *et al.* (2005) reported positive infection of *Blastocystis* isolates from quails and geese in chickens indicated that host specificity is not acceptable for the criteria of speciation of *Blastocystis* isolates from birds. Therefore, the proposal of new *Blastocystis* species on the basis of different avian host species is problematic.

Evidence to date suggests that although *Blastocystis* is found in diarrhoeic faeces from a range of hosts, there is little evidence to suggest a direct pathogenic effect. Whether *Blastocystis* is a pathogen, commensal or an opportunistic organism in birds is not known. However, experimental infections on red-legged partridges and chicken revealed that no clinical signs were observed (Taylor *et al.*, 1996).

In recent years, molecular studies have produced a growing body of data on STs of *Blastocystis* isolated from various animal hosts. Yoshikawa *et al.* (2004a) reported the STs isolated from birds included ST1 (three chicken isolates, one pheasant isolate), ST2 (one chicken isolate), ST6 (one chicken isolate, five isolates from quails and three from pheasant) and ST7 (one chicken isolate, five isolates from quails and one pheasant). Recently, ST4 was identified in ostriches (Roberts *et al.*, 2013; Chandrasekaran *et al.*, 2014) whereas Guinea fowls were identified as ST7.

2.5.2 Swine

Blastocystis is highly prevalent in pigs (Burden *et al.*, 1978; Pakandl, 1991; Quilez *et al.*, 1995a; Abe *et al.*, 2002), and is capable of surviving in pig manure slurry (Snell-Castro *et al.*, 2004). Burden (1976) first published on *Blastocystis* sp. infection in pigs. Burden *et al.* (1978) found the organism on each of the five pig farms visited and reported a high prevalence (60%), recording usually low numbers of *Blastocystis* sp. cells from the stool samples. Burden *et al.* (1978) also reported high numbers in pigs suffering from diarrhoea, but concluded no evidence to suggest the pathogenic role of *Blastocystis*.

Meanwhile, Pakandl (1991) reported the occurrence of this parasite in suckling pigs from five farms in Southern Bohemia from the age of three days with relatively high prevalence. The ultrastructure study conducted on the pig isolates showed no difference from the human form. This study detected infections with high density, and intensity of infection up to 8 cells per $100 \times$ field failed to show correlation between intensity and the occurrence of diarrhoea.

Quilez *et al.* (1995a) reported the presence of *Blastocystis* sp. in pigs in Spain. The results indicated low overall prevalence (7.5%) but higher in 1-2 month old weaned piglets (18.4%) and 2-6 month old fattening pigs (15.4%) compared with older pigs (2.8%). Diarrhoea was present in two weaned pigs 6 weeks of age, with an intensity of infection of over 5 cells per 40 × field concluding that *Blastocystis* sp. was not a cause of diarrhoea.

Fayer *et al.* (2014) highlighted the presences of *Blastocystis* in a specific locations within the swine gastrointestinal tract i.e., the lumen contents, cecum, jejunum, proximal and distal colon and missing in the duodenum or ileum. In tissue sections,

Blastocystis was found primarily in the lumen usually associated with digested food debris, sometimes in close proximity or appearing to adhere to the epithelium, none were found to penetrate the epithelium or the lamina propria.

Genotypes from pigs are found grouped together with *Blastocystis* genotypes from humans in the same cladogram (Navarro *et al.*, 2008). Parkar *et al.* (2007) has proved that a single *Blastocystis* isolate from a Thai human (Th522H1) belonged to Clade V (*Blastocystis* sp. Subtype 5), which was considered to be specific to pig and cattle isolates (Noël *et al.*, 2005). Meanwhile, a study by Yan *et al.* (2007) indicated that all of the isolates from pigs were identified as *Blastocystis* sp. Subtype 5 (ST2) using the seven kinds of STS primers and the RFLP patterns of all of the isolates from humans except for the mixed one were identical or quite similar to those of the 16 pig isolates with both *Hin*fI and *Rsa*I enzymes, showing zoonotic potential of *Blastocystis* sp. Subtype 5 (ST2).

To date, *Blastocystis* subtypes 1, 2, 3, and 5 have been detected in faeces from Japan, Spain, France, Thailand, and Denmark (Stensvold *et al.* 2009; Fayer *et al.*, 2014). In addition, ST5 was found in six pigs in the USA (Santin *et al.*, 2011).

2.5.3 Companion animals

There are very few studies from cats and dogs worldwide (Knowles and Gupta, 1924; Duda *et al.*, 1998; and Abe *et al.*, 2002). High prevalence was detected in dogs and cats in Australia, with infections as high as 70% (Duda *et al.*, 1998) and similarly, high in dogs from Iran (Daryani *et al.*, 2008) via light microscope in 14 (28%) of 50 faecal samples from two months to four years old dogs with no correlation between infection and host-age and sex.

Wang *et al.* (2013) on the other hand reported low infection in stray dogs from Brisbane (2.5%), Cambodia (1.3%) and India (24%) using 'barcoding' of the small subunit rRNA gene. In the US Pacific Northwest region, 10/103 (9.7%) shelter-resident canines and 12/103 (11.6%) shelter-resident felines were positive whereas, however only two very faint bands observed from two separate client-owned animals (Ruaux and Stang, 2014). In contrast, all dogs and cats were free from infection in Malaysia (Chuong *et al.*, 1996), Japan (Abe *et al.*, 2002) and Germany (König and Müller, 1997). It was unclear why infections varied greatly between the different studies, however one explanation given was the condition in which the animals were housed.

2.5.4 Rodent

There is very little information in the literature on *Blastocystis* in rodents. Knowles and Das Gupta (1924) reported the presence of the parasite in the rat and Lavier (1952) reported from mice. Pakandl (1992) observed no *Blastocystis* infection in 300 laboratory mice. However, Chen *et al.* (1996a) found that 60% of the laboratory rats of different strain were positive for *Blastocystis*. Among the different strains, Wistar and Sprague-Dawley were infected highest. However, mice, rabbits, hamsters, and gerbils were free from infection. Therefore, it was suggested laboratory rats were good models for studies on pathological aspects of the infection due to their susceptibility to *Blastocystis* infections (Tan *et al.*, 2002). Using the pulsed-field gel electrophoresis (PFGE) technique, Chen *et al.* (1997b) noted a distinct karyotypic pattern of the rat *Blastocystis* is a distinct species, and the name *B. ratti* sp. nov. is proposed:

Parasite: *Blastocystis ratti* sp. nov. Host: *Rattus norvegicus* (Wistar strain) Habitat: Large intestine Locality: Singapore

2.5.5 Artiodactyls

There are few reports on *Blastocystis* infection involved artiodactyls. In USA, Fayer *et al.* (2012) found 19.15% purebred Holstein dairy cattle positive for *Blastocystis* sp. while prevalence of 71% was reported in cattle in Japan (Abe *et al.*, 2002) and 1.8% in Aragon, Northeastern Spain (Quilez *et al.*, 1995b). In Malaysia, Lim *et al.* (2008) reported that none of the hoofed animals from Zoo Negara, Kuala Lumpur were infected whereas Tan *et al.* (2013) reported 30.9% goats examined in five different farms in Serdang, Selangor were infected with *Blastocystis* sp. Meanwhile, Hemalatha *et al.* (2014) reported moderate infection in the livestock group with 34.5% in cattle; 28.6% in deers; 30% in gaurs; 65% in goats and 57.9% in sheeps from various government and private establishments in Perak.

2.5.6 Wildlife

Limited number of studies screened wild animals for Blastocystis with high prevalences was in primate species. Stenzel et al. (1993) reported for the first time the presence of *Blastocystis* sp. from 1 of 2 camels (*Cumelus dromedarius*), 1 of 3 llamas (Llama gluma), the only highland bull (Bos taurus) and a lion (Panthera leo) in a travelling circus, although only low numbers were detected by light microscopy. Meanwhile, Lim et al. (2008) reported the occurrence of Blastocystis among the wild animals in captivity particularly the feline, hoofed mammals and primates at Zoo Negara, Kuala Lumpur, Malaysia with low infections in primates (2.1%). Blastocystis isolates were also reported from zoo animals at the National Veterinary Institute, Technical University of Denmark (Stenzvold et al., 2009). A novel subtype from ringtailed lemur (Lemur catta) was identified and designated as Blastocystis sp. ST10. Besides, it was also found that *Blastocystis* from primates belong mainly to ST4, ST5 and ST8. Parkar et al. (2010) reported on the occurrence of the protozoan amongst wild animals from the Perth Zoo, Australia with 50% in primate; 33% in nonprimates; first to report *Blastocystis* isolated in elephants (57%) and giraffes (82%), quokka and western grey kangaroo. Recently, Roberts et al. (2013) screened different animal species from seven locations within New South Wales, Australia. Blastocystis sp. was identified in gorilla, chimpanzee, orang-utan, Francois languar and Macaca sp. This was the first report on the presence of Blastocystis in the eastern grey kangaroo, red kangaroo and eastern wallaroo and ostrich and in addition to the Asian elephant and guinea fowl.

2.6 Blastocystis of animals in Malaysia

A number of studies on *Blastocystis* have been carried out in Malaysia with focus on humans infections (Tan and Suresh, 2006a; Chandramathi *et al.*, 2010; Ragavan *et al.*, 2014; Kumarasamy *et al.*, 2014) while a handful studies involving animals. Suresh *et al.* (1996) worked on small numbers of different laboratory bred animals using *in vitro* cultivation in Jones' medium. They noted all Wistar, SHR and Sprague Dawly rat strains positive with *Blastocystis* sp., while, two of five PVG rats and Dark Agoti rats were positive. In contrast, all laboratory mice (ICR, CBA rat, Balb/c and C3HCJ) were free from infection. In addition, only 1 sheep positive of five, while a guinea pig and a hamster examined were negative. All ten *Macaca fasicularis*, 2 of 10 rabbits, and none of the two dogs and two cats were positive.

Lim *et al.* (2008) investigated the prevalence of intestinal parasites from different groups of mammals housed in a zoological garden in Malaysia. A total of 197 faecal samples were collected randomly from various primates (99), hoofed mammals (70) and feline (28). The animals were found infected with a diversity of intestinal parasitic infection. However, only the pig-tailed macaque and Sumatra Orang Utan were infected with *Blastocystis* (2.1%).

Tan *et al.* (2013) successfully for the first time attempted to determine the occurrence as well as the genetic diversity of *Blastocystis* spp. in goats obtained from five different farms in Peninsular Malaysia. *Blastocystis* spp. infected 30.9% of goats primarily with subtype was ST1, followed by ST7, ST6 and ST3.

Meanwhile, Hemalatha *et al.* (2014) screened a large number of animals including poultry, ruminants, mammals, swine, primates, companion animals, wild animals, and

laboratory animals for the presence of *Blastocystis*. A total of 104 out of 302 animals (34.4%) were infected with this anaerobic parasite. Moderate infection were observed in the ruminant livestock group (34.5% in cattle; 28.6% in deers, 30% in gaurs; 65% in goats and 57.9% in sheeps) whereas in mammals, the prevalence rate varied around 50% of orang utan and 100% of pigs while in horses and chimpanzee, the organism was undetectable. All the ostriches were positive however, companion animals (i.e cats and dogs), laboratory animals (i.e mice, rats, guinea pigs and rabbits) and all wildlife specimens (i.e black panther, lion, tiger, elephants, tapir, camel, terrapins and wild birds) were completely free from the infection.

More recently, Chandrasekaran *et al.* (2014) for the first time demonstrated *Blastocystis* sp. subtypes 6 or ST5 based on the terminology consensus by Stensvold *et al.* (2007b) do infect ostriches (*Struthio camelus*). However, all ostriches were asymptomatic. It was also reported that most of the cells show high lipid storage in the vacuoles of the parasites. The study further provided evidence for potential zoonotic transmission in ostrich farms as *Blastocystis* subtype 6 or ST5 can infect rats and the same subtype have been previously reported in humans.

To date, there are gaps of information on the status, morphology as well as genetic diversity of *Blastocystis* sp. from livestock, domestic animals and wildlife in this country. The hypothesis that intimate association between human and animals could facilitate transmission, led us to investigate the occurrence of *Blastocystis* especially in animals in the domestic environment.

CHAPTER 3:

STUDY ON Blastocystis spp. IN POULTRY POPULATIONS

3.1 Introduction

Blastocystis infections is common in avian hosts and have been reported in chickens (Yamada *et al.*, 1987; Belova and Kostenko, 1990; Stenzel *et al.*, 1994), ostriches (Yamada *et al.*, 1987; Stenzel *et al.*, 1994; Chandrasekaran *et al.*, 2014), domestic ducks (Belova, 1991; Pakandl and Pecka, 1992; Stenzel *et al.*, 1994), domestic geese (Belova, 1992a) and turkeys (Lee, 1970; Belova, 1992b).

Three new species of *Blastocystis* from chickens, ducks and geese have been proposed. *B. galli* was described from domestic chickens and turkeys (Belova and Kostenko, 1990; Belova, 1992b); *B. anatis* from ducks (Belova, 1991); and *B. anseri* from geese (Belova, 1992a). The major criterion used to distinguish species was the organism's size, but given the enormous variation in cell size within each sample, this was not sufficient to delineate new species.

Several studies showed phenotypic characteristics in *Blastocystis* isolated from chickens, duck, geese and ostriches. It was concluded that these isolates could not be distinguished morphologically from the human isolates, except for variation in size as well as the contents seen in the central body of the parasite (Yamada *et al.*, 1987; Stenzel *et al.*, 1994; Chandrasekaran *et al.*, 2014).

To date, six subtypes were identified in birds include ST1, ST2, ST4, ST6, ST7 and ST8. Among the six subtypes, ST6 and ST7 was predominantly in the avian hosts

(Stensvold *et al.* 2009), demonstrating a much stronger association between these subtypes and the avian hosts.

A large number of studies in Malaysia which focused mainly on human infections (Tan, 2008, Tan *et al.*, 2008 and Ragavan *et al.*, 2014) with no known studies in the poultry population bearing in mind that consumption of chicken meat has increased over the years. Just over the last one decade as per capita consumption has gone up from 36 kgs to 39 kgs (Jayaraman *et al.*, 2013). Therefore, the aim of this study is to determine the prevalence, elucidate biological information and determine the subtypes on *Blastocystis* spp. isolated from the poultry population.

3.2 Materials and methods

3.2.1 Ethical approval

All animals used in this study were handled according to Institutional Animal Care and Use Committee (IACUC), University Malaya (No.: ISB/31/01/2013/SNMZ (R)). Permission was sought from the Ipoh City Council and Kuala Lumpur City Hall after obtaining approval from the Ethics Committee from the Faculty of Science, University Malaya. The sampling sites were not privately-owned or protected. Written permission was obtained to carry out the study from the relevant authorities.

3.2.2 Sampling sites

This study involved two states namely Perak and Selangor in the Peninsular Malaysia. Both states are located in the west coast of Peninsular Malaysia with Perak situated on the northen border of Selangor state. Sites were chosen based on types of poultry rearing. Kinta District, Perak is a district in central Perak, Malaysia that covers both rural and urban areas whereas semi-rural areas in Semenyih and Kuala Selangor are districts in Selangor known for communal crop-livestock farming.

3.2.3 Study population

A total of 179 chickens consisted of free-range and commercially barn-reared chicken were screened for *Blastocystis* sp.

3.2.3.1 Free-range or backyard chicken

The free-range or backyard chickens were commonly seen in rural settings where traditional poultry production was practiced (or backyard chicken). The chickens were released daily, allowed to scavenge for food freely and return periodically to the homestead for water and kitchen refuse. In this study, faecal samples were collected from three breed of chicken namely; village chicken, jungle fowl and white silkie chicken commonly reared by the villages.

3.2.3.2 Barn-reared chicken

The barn-reared chickens consisted of commercial broilers were reared specifically for meat. The birds were kept indoors, isolated and were maintained with regulated temperature as well as with right ventilation either on deep litter (e.g. wood shavings) or floor systems with slatted floor. The farms were located in palm oil estates and used tap water while others used pond water pumped directly into the water tanks. The chickens were conventionally reared by integrated poultry companies or small-scale nonintegrated poultry companies and sold to the public in wet markets around the cities. In Peninsular Malaysia, most of the companies used the locally bred Cobbs and Ross.

3.2.4 *In vitro* cultivation

A pea size amount of each faecal sample was inoculated into a sterile screw-top containing 3 ml of Jones' medium supplemented with 10% heat-activated horse serum (Jones, 1946; Suresh and Smith, 2004). Each sample was incubated vertically at 37° C for 48 to 72 hours before examination. A drop of the sediment was examined at $400\times$ magnification for the detection of *Blastocystis*. The parasites were maintained subsequently after isolation, by sub-culturing once every 3 to 4 days. The parasites were then subjected for phenotypic and genotypic analysis. However, when no growth was detected the sediment would be further re-suspended in fresh culture medium and maintained for another additional 48 hours at 37° C incubation. The samples were considered negative if *Blastocystis* sp. forms were absent.

3.2.5 Microscopy examination

3.2.5.1 Giemsa staining

The positive faecal smears were fixed in methanol and stained with 10% Giemsa for the observation of detailed morphology at $400 \times$ and $1000 \times$ magnification using light microscopy.

3.2.5.2 Sudan Black B staining

Isolates obtained from day 3 cultures were smeared on a clean glass slide and immediately dried with a hair dryer at room temperature. This was followed by fixation in glutaraldehyde solution for 1 minute at 2-8°C with gentle agitation followed by thorough rinsing in deionized water. Cells were immersed in Sudan Black B reagent for 5 minutes with intermittent agitation followed by rinsing in 70% ethanol (3 to 5 times) and distilled water. The slides were counterstained in haematoxylin solution for 5 minutes and given a final rinse under tap water. Slides were then examined under 1000× magnification for black droplets in the central vacuole as indication for positive reaction.

3.2.6 Cytochemical staining

3.2.6.1 Fluorescein isothiocyanate (FITC)-labeled Con A (*Canavalia ensiformis*)

Approximately 10 μ l of culture sediment containing parasites were mixed with 10 μ l of 0.1% FITC in an eppendroff tube and incubated at 37°C for 20 minutes. 10 μ l of the prepared sample was placed on a clean glass slide and excess liquid was removed from the slide by placing an absorbent tissue at the edge of the coverslip. The sample was viewed using a fluorescence microscope (Leitz Wetzler, Germany) with appropriate filters at 400× magnification. The results were determined by using affinity fluorescence unit (AFU) (scale of brightness of 1+, 2+, 3+ and 4+).

3.2.6.2 Acridine orange staining

Acridine orange is used to stain the DNA of the nucleus, mucus and RNA as bright green, dull green and flaming red-orange, respectively (Humason, 1972). 5 ml of 0.1% acridine orange stock solution was diluted with 45 ml of phosphate buffered saline (PBS) pH 7.4 before use. Approximately, 5 μ l of culture sediment containing parasites were mixed thoroughly on a clean glass slide with 5 μ l of diluted acridine orange. The sample was viewed using a fluorescence microscope (Leitz Wetzler, Germany) with appropriate filters at 400× mgnification.

3.2.7 Ficoll-Paque concentration method

Ficoll-Paque density gradient centrifugation method was carried out in order to isolate *Blastocystis* cyst from the fresh faecal sample. Briefly, the faecal concentrate resuspended in PBS was layered on 5 ml of Ficoll-Paque and centrifuged at 3,500 rpm for 20 minutes. *Blastocystis* cyst layer which was formed after centrifugation was removed into another tube and re-suspended in 1 ml PBS and observed under microscope for the detection of *Blastocystis* cyst (Suresh and Smith, 2004; Kumarasamy *et al.*, 2014).

3.2.8 Electron microscopy

Day-3 positive culture samples and *Blastocystis* cyst from the fresh faecal sample were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) and sent to the Electron Microscopy Unit in Institute for Medical Research, Kuala Lumpur for processing.

3.2.8.1 Scanning electron microscopy (SEM)

The contents were washed three times with phosphate buffered saline (PBS) pH 7.4. The samples were centrifuged at 3000 rpm for 5 minutes. The pelleted cells were fixed with 2.5% glutaraldehyde and post-fixed with 1% osmium tetroxide. The specimens were mounted on polycarbonate membrane (Nuclipore, Agar Scientific, USA) and dehydrated in increasing concentration of ethanol (30%, 50%, 70%, 80%, 90% and 100%). The specimens were critical–point dried with carbon dioxide coated with gold, and examined with a scanning electron microscope ((FEI-Quanta 200 FESEM, USA) (Ragavan *et al.*, 2014).

3.2.8.2 Transmission electron microscopy (TEM)

The contents were washed three times using phosphate buffered saline (PBS) pH 7.4. The samples were centrifuged at 3000 rpm for 5 minutes. The pelleted cells were resuspended overnight in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3 at 4°C, washed thoroughly with cacodylate buffer and post fixed for 30 min in 1% osmium tetroxide in cacodylate buffer. The fixed cells were dehydrated for 5 minutes in ascending series of ethanols (30%, 50%, 70%, 80%, 90% and 100%) and embedded in epoxy resin. Semithin sections were stained with toluidine blue. Ultra thin sections were cut using and ultramicrotome, contrasted with uranyl acetate and lead citrate and viewed using a transmission electron microscope (LEO Libra120) (Tan & Suresh, 2006a).

3.2.9 Subtyping

3.2.9.1 Genomic DNA preparation

Positive samples were subsequently maintained by sub-culturing in Jones' medium every 3 to 4 days and stored at -20°C for molecular characterization. Genomic DNA was extracted using Qiagen Stool extraction kit according to the manufacturer's protocol. The elution step was carried out using 100 μ l instead of 200 μ l in order to increase the concentration of total DNA.

3.2.9.2 Polymerase chain reaction (PCR) with sequenced-tagged site (STS) primers

All positives *Blastocystis* sp. isolates were subjected to PCR with STS primer using ten primer sets as previously described by Yoshikawa *et al.* (2003) to successfully to classify *B. hominis* populations into seven subtypes (1–7) based on genotypic homology (Table 3.1).

Amplification of 1 µl genomic DNA was carried out in 20 µl reaction containing 0.5mM of the dNTPs, 0.5mM of each primer, 1 x PCR buffer (75mM Tris-HCL, pH 8.8, 20mM (NH₄)₂SO₄ and 0.01% Tween 20), 2.5mMm MgCl₂ and 1 U Taq DNA Polymerase (recombinant) (FERMENTAS, USA). PCR conditions consist of 1 cycle of initial denaturing at 94°C for 3 minutes, followed by 30 cycles including denaturing at 94°C for 30 s, annealing at 57°C for 30s and extending at 72°C for 1 minute, and an additional cycle with a 10 minutes chain elongation at 72°C (Thermo Cycler-Bio-Rad, USA).

The amplification products were then electrophoresed in 1.5% agarose gels (PROMEGA USA) and Tris-Borate-EDTA buffer. Gels were stained with Gelred nucleic acid gel stain and photographed using ultra-violet gel documentation system (Uvitec, United Kingdom). While the primers of Yoshikawa *et al.* (2003) were used, the results were reported in ST format, the consensus terminology for *Blastocystis* subtypes (Stensvold *et al.*, 2007b).

Subtype	STS	Product	Sequence of forward (F) and reverse (R)	Genebank	ST
	primer	size (bp)	primer (5' – 3')	accession no.	format
1	SB83	351	F-GAAGGACTCTCTGACGATGA	AF166086	1
			R -GTCCAAATGAAAGGCAGC		
2	SB155	650	F-ATCAGCCTACAATCTCCTC	AF166087	7
			R -ATCGCCACTTCTCCAAT		
3	SB227	526	F-TAGGATTTGGTGTTTGGAGA	AF166088	3
			R -TTAGAAGTGAAGGAGATGGAAG		
4	SB332	338	F-GCATCCAGACTACTATCAACATT	AF166091	6
			R -CCATTTTCAGACAACCACTTA		
5	SB340	704	F-TGTTCTTGTGTCTTCTCAGCTC	AY048752	2
			R -TTCTTTCACACTCCCGTCAT		
6	SB336	317	F-GTGGGTAGAGGAAGGAAAACA	AY048751	5
			R -GAACAAGTCGATGAAGTGAGAT		
7	SB337	487	F-GTCTTTCCCTGTCTATTCTGCA	AY048750	4
			R -AATTCGGTCTGCTTCTTCTG		

Table 3.1: The STS primer sets used in this study.

3.2.9.3 DNA barcoding

Blastocystis-specific primer, BhRDr (GAGCTTTTTTAACTGCAACAACG; Α paired with eukaryote-specific primer, Scicluna et al., 2006) was RD5 (ATCTGGTTGATCCTGCCAGT; Clark, 1997) and used, in a single step PCR reaction, to amplify a 600 bp region of 18S rRNA. The PCR was performed in a 25 µl volume containing 1.0 mM of dNTPs, 0.5 mM of each primer, 1 x PCR buffer, 2.5 mM MgCl₂,1 U Taq DNA Polymerase (recombinant) (FERMENTAS, USA) and 5 µl of genomic DNA. PCR conditions consisted of an initial denaturing step of 94°C for 1 minute, followed by 30 cycles of 94°C for 1 minute, 59°C for 1 minute and 72°C for 1 minute, followed by a final elongation step of 72°C at 2 minutes (Thermo Cycler Bio-Rad, USA). PCR products were visualized on a 1.5% agarose gel prior to purification and cycle sequencing by a local commercial company. Sequencing data were checked using Seq Scanner 2 software (Applied Biosystems) for quality and subsequently were edited to remove low quality bases and primer sequences using BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Next, the edited sequences were queried against the **Blastocystis** 18S rRNA database (http://www.publmst.org/blastocystis) (Roberts et al., 2013).

3.2.10 Statistical analysis

Statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS) 21.0 software package. Chi-square analysis carried out to determine whether infections were associated to either extrinsic or intrinsic factors. A probability value of less than 0.05 was considered statistically significant.

3.3 Results

3.3.1 Prevalence of *Blastocystis* in chicken population

Out of 179 chickens, a total of 47 (26.3%) chicken faecal samples screened were positive for *Blastocystis* sp. (Table 3.2). None of the birds showed any clinical signs of infection such as diarrhea. The prevalence among barn-reared chicken and free-ranged chicken were 14.9% and 34.3%, respectively (Figure 3.1). There was a statistically significant association between the types of chicken and *Blastocystis* infection ($\chi 2 = 8.455$, [df] = 1, P = 0.004) recorded in this study. The prevalence of *Blastocystis* infections between the two study locations is shown in Table 3.3. The prevalence in both Perak and Selangor did not show marked variation with the prevalence of 29.4% in Perak and 20% in Selangor. There was no statistical significant association between the prevalence of *Blastocystis* sp. between the study locations ($\chi 2 = 1.825$, [df] = 1, P = 0.177).

Study animals	No. faecal samples	No. chicken infected (%)
Barn-reared chickens		
Broiler chicken	74	11 (14.9)
Free-range chickens		
Jungle fowl	1	1 (100)
Village chicken	102	33 (32.4)
White silkie chicken	2	2 (100)
Total	179	47 (26.3)

Table 3.2: Blastocystis infection in two types of chicken population.

Table 3.3: *Blastocystis* infection in two study areas.

	Perak		Selangor	
Study animals	No. faecal samples	No. chicken infected (%)	No. faecal samples	No. chicken infected (%)
Cage-reared chickens				
Broiler chicken	44	9 (20.5)	30	2 (6.7)
Free-range chickens				
Jungle fowl	1	1 (100)	-	-
Village chicken	72	23 (31.9)	30	10 (33.3)
White silkie chicken	2	2 (100)	-	-
Total	119	35 (29.4)	60	12 (20.0)



Figure 3.1: The prevalence of *Blastocystis* infection in the two different chicken groups.
3.3.2 Morphological forms

Blastocystis obtained from free-ranged (Figure 3.2a) and barn-reared chickens (Figure 3.2b) were similar to *B. hominis*, except for the variation of its size. The organisms most commonly seen as vacuolated forms, were always large, spherically shaped cells measuring approximately 10 μ m to 100 μ m in diameter with the average diameter of cells between 20 to 30 μ m. Vacuolar forms of *B. hominis* also vary greatly in size ranging from 2 μ m (Van Saanen-Ciurea and El Achachi, 1985) to more than 200 μ m (Zierdt and Tan, 1976) in diameter with the average diameter usually exhibited smaller values between 4 and 15 μ m (Zierdt, 1991).

Apart from that, the granular forms were similar to the vacuolar forms except that numerous granules are present within the central vacuole or within the thin band of peripheral cytoplasm of the organism. The size ranges from 10 to 30 μ m in diameter. Gigantic granular form (Figure 3.3) with the presence of inclusion bodies or granules in the central vacuole was occasionally seen in chicken isolates ranging from 40 to 90 μ m in diameter.

Meanwhile, the cysts (Figure 3.4) from chicken were generally smaller and rounded to ovoid in shape where the size range of cysts form exhibited smaller values of 3 to 5 μ m in diameter which was consistent with descriptions of *B. hominis* cysts were reported ranging between 5 to 10 μ m (Stenzel and Boreham, 1991) and 3 to 6 μ m (Zaman *et al.*, 1995) in diameter.



Figure 3.2: *Blastocystis* isolated from (a) village chicken and (b) broiler chicken. V; vacuolar form, G; granular form.



Figure 3.3: Gigantic form of *Blastocystis* from chicken.



Figure 3.4: Cyst form of *Blastocystis* from chicken (circle).

3.3.3 Mode of reproduction

Three types of reproduction were noted namely; binary fission, budding and schizogony (Figure 3.5). The most common mode of reproduction observed in the *in vitro* culture of chicken isolates was binary fission (Figure 3.5a) and budding (Figure 3.5b). However, schizogony-like organisms were present in a jungle fowl isolate. The vacuoles had a distinct membrane and appeared as sac-like pouches, showing the developing *Blastocystis* progeny within the sacs (Figure 3.5c).

3.3.4 Surface structure

Scanning electron micrograph showed the surface structure of village chicken *Blastocystis* from culture and fresh caecum possessed smooth and undulating cell surface. Besides, bacteria were also seen attached to the surface of the cell obtained from the fresh caecum (Figure 3.6a and b). Meanwhile, the cell surface of broiler chicken *Blastocystis* from culture showed slightly rough surface coat with indentations and deep grooves (Figure 3.7).







Figure 3.5: Mode of reproduction of *Blastocystis* stained with Giemsa (a) Binary fission (b) Budding (circle) (c) Schizogony. P; progeny.





Figure 3.6: Surface structure of village chicken *Blastocystis* from (a) culture and (b) fresh caecum. Bacteria (circle) were often seen adherent to the surface of *Blastocystis*.



Figure 3.7: Surface structure of broiler chicken *Blastocystis* from culture.

3.3.5 Ultrastructure

3.3.5.1 Village chicken *Blastocystis* from culture

Transmission electron microscopic examination revealed two types of vacuoles forms in the culture form of village chicken isolates; one containing a large central vacuole completely electron-lucent (Figure 3.8a), and the other contained an electron-opaque, in fully distended vacuoles (Figure 3.8b). Apart from that, the organisms possessed a thin wispy surface coat that resembles a slight ruffled appearance of the surface observed under the scanning electron microscope.

3.3.5.2 Village chicken *Blastocystis* from faecal sample

A thick, compact surface coat enveloped the organism (Figure 3.9) measuring between 237.74 to 342.63 nm. The central vacuole contained tiny electron-dense particles, which were unevenly distributed when compared to the parasites from the *in vitro* cultures.

3.3.5.3 Village chicken *Blastocystis* from caecum sample

Transmission electron micrographs showed more prominent protrusion of cytoplasmic materials into the central body of *Blastocystis* from village chicken caecum sample and are surrounded by a thin, electron-lucent membrane layer with measurements ranging between 169.27 to 239.39 nm (Figure 3.10).

3.3.5.4 Broiler chicken Blastocystis from culture

The surface coat was absent in the cells. Occasionally electron-lucent areas within flocculent content were observed (Figure 3.11a). However, in some instances the contents were extremely electron-dense and granular (Figure 3.11b). Besides, the

number of mitochondria-like organelle (MLO) was unusually high with some as high as nine MLO seen in an individual organism.

3.3.6 Cytochemical studies

The lipid dye Sudan Black B, as generally used to demonstrate lipids in the interior of the cell nucleus was studied with regard to its staining properties for *Blastocystis* isolated from chicken. The binding of Sudan Black B with phospholipids enclosed in the form of liposomes on day 3 isolates showed positive reactions are seen as dark droplets in the central vacuole of granular forms (Figure 3.12).

Blastocystis sp. in day 2 culture showed faint green florescence (AFU =1+) seen on the membrane of both vacuolar and granular forms. This indicates lesser binding affinity with FITC-labeled ConA (Figure 3.13). The fluorescence intensity and the percentage of the reactive forms of chicken isolate in FITC-labelled Con A stain range was (1+; 90-100%),

The occurrence of DNA within the nucleus was confirmed by the use of acridine orange showing two nuclei at the opposite end of the cell and in some organisms the nucleus was seen to be located in the central part of the organism. The respective epifluorescence image showed that the central vacuole was stained dull green while the nucleus was stained bright green (Figure 3.14). However, the colour changes as it become cystic.



Figure 3.8: Electron micrographs of *Blastocystis* isolated from in village chicken (a) Central vacuole filled with electron-lucent materials (b) Central vacuole with electrondense materials. Nu; nucleus, m; mitochondria, CV; central vacuole, SC; surface coat.



Figure 3.9: Electron micrographs of village chicken *Blastocystis* from faecal sample. Nu; nucleus, m; mitochondria, CV; central vacuole, SC; surface coat.



Figure 3.10: Electron micrographs of village chicken *Blastocystis* from ceacum samples. Nu; nucleus, m; mitochondria, CV; central vacuole, SC; surface coat.



Figure 3.11: Electron micrographs of broiler chicken *Blastocystis* from culture (a) Central vacuole with flocculent content (b) Central vacuole with electron-dense material. Nu; nucleus, m; mitochondria, CV; central vacuole, SC; surface coat.



Figure 3.12: *Blastocystis* from chicken stained with Sudan Black B showing dark stain in the central vacuole indicating the presence of neutral lipid under $1000 \times$ magnification. Note: dark droplets (arrow).



Figure 3.13: Binding affinities of *Blastocystis* stained with FITC-labelled Con A (a) Light microscopic images of chicken *Blastocystis* (b) A same organism stained with FITC-labeled Concanavalin A (ConA) assay, AFU (1+): weak intensity. V; vacuolar form, G; granular form.



Figure 3.14: Epifluorescence image of *Blastocystis* stained with acridine orange. (a) Light microscopic images of chicken *Blastocystis* (b) The same organism stained with acridine orange. N; nucleus.

3.3.7 Subtype identification

Subtype analysis using PCR with STS primers showed the occurrence of *Blastocystis* sp. ST6 in four broiler chicken isolates (C15, C16, C19 and C23) obtained from a wet market in Perak (Figure 3.15). Meanwhile, based on the DNA barcoding method, four different subtypes were detected in parasites isolated from village chicken found in a mini zoological garden in Perak. These were identified as ST1 in one village chicken isolate (A16), ST8 in two village chicken isolates (A17 and A38), ST6 in two broiler chicken isolates (A20 and A26) from a wet market in Perak and five isolates were identified as ST7 from four village chickens (A2, A4, A6 and A18) and one broiler chicken (A28). Subtype information using DNA barcoding method was obtained from the *Blastocystis* Sequence Typing Database (http://www.publmst.org/blastocystis) (Table 3.4).



Figure 3.15: PCR amplification reaction of *Blastocystis* from chicken isolates using the sequenced-tagged site (STS) primers SB332 (lane *1*, *2*, *3* and *4*; 338bp) indicating ST6.

Table 3.4: Subtype of *Blastocystis* from chicken isolates obtained *Blastocystis*Sequence Typing Databases.

Blastocystis subtype	No. of Sequence-positive isolates	
ST1	1 (A16)	
ST6	2 (A20, A26)	
ST7	5 (A2, A4, A6, A18, A28)	
ST8	2 (A17, A38)	

3.4 Discussion

Blastocystis infection was previously shown to be widespread in the poultry population. To date, several reports on avian *Blastocystis* were recorded. The first description was by Lee (1970) isolating the parasite from the caecum of turkeys. It was found that domestic fowls and ostriches showed 100% infection in Japan (Yamada *et al.*, 1987) whereas in Russia, domestic hen showed 80 - 100% infection (Belova and Kostenko, 1990) and domestic ducks 80% infection (Pakandl and Pecka, 1992). In England, Taylor *et al.* (1996) recorded the presence of this parasite in red-legged partridge. Similar studies were also conducted in chickens, ducks and Japanese quails from two municipal markets in Rio de Janeiro, Brazil (Bergamo do Bomfim and Machado do Couto, 2013) with the prevalence of 32.9%, 46.6% and 23.9%, respectively. A recent study by Chandrasekaran *et al.* (2014) showed a high prevalence of *Blastocystis* infection in the ostrich population from a local farm in Malaysia. However, there is no known study to assess the prevalence of *Blastocystis* in chickens in Malaysia. Table 3.5 summaries the previous research studies conducted on *Blastocystis* sp. in poultry worldwide.

The overall prevalence of *Blastocystis* was moderately higher in the free-range than barn-reared chickens. Free-range chickens were likely to be more prone to *Blastocystis*, owing to their scavenging habits. Good hygiene and sanitation are crucial to prevent the chickens from being contaminated from the environment via the faecal-oral transmission (Lee and Stenzel, 1999).

The present study is the first epidemiological survey carried out on broiler chickens. Despite reared in an intensive barn system in addition to treatment with antibiotic to assure minimal contamination, *Blastocystis* was still present. The source of infection in the birds remains unknown however one plausible explanation is contamination of the water and food provided. Lee and Stenzel (1999) noted no infection in an establishment with high-quality hygiene and sanitary conditions suggesting that good hygiene practices contributed to better health maintenance of the birds.

Many reproductive modes for *Blastocystis* have been suggested (Tan and Stenzel, 2003; Windsor *et al.*, 2003). However, to date only binary fission is the only accepted mode (Stenzel and Boreham, 1996). In the present study, in addition to binary fission, budding was also a common mode of reproduction observed in the *in vitro* cultures of chicken isolates. In addition, schizogony-like organism was seen with many progeny or daughter vacuolar form in the central body of jungle fowl isolate.

Blastocystis is polymorphic and exist in many forms. In this study, the vacuolar form was the predominant *in vitro* cultures, which concurred with Tan (2004). Under light microscopic examination, the isolates from chickens were morphologically similar to human isolates (Matsumoto *et al.*, 1987) with the exception of variation in size as also concurred with Yamada *et al.* (1987).

Ultrastructural descriptions of *Blastocystis* isolated from the chickens have been described previously (Stenzel *et al.* 1994; Cassidy *et al.* 1994). The transmission electron micrographs showed the organism possessed an electron-lucent material within central body when examined. However, some forms also showed finely granular or flocculent content seen distributed evenly within the central body. The electron dense material reacted to staining with Sudan Black B indicating lipid content. Chandrasekaran *et al.* (2014) demonstrated that *Blastocystis* isolated from ostriches showed high lipid storage in the central body of the vacuolar forms. However, Zierdt

and Williams (1974) postulated that the highly electron dense material were granules to form the granular form and suggested that the central body probably acts as a form of energy storage for cell growth. Notably, the granular form was similar ultrastructurally to the vacuolar form, apart from the nature of its central vacuolar contents (Dunn *et al.*, 1989).

Blastocystis isolated from chicken showed smooth undulating cell surface although some organisms showed indentation with deep grooves which may influence the adherence of *Blastocystis*. Besides, chicken isolates showed an outer coat without surface coat projection or fibrillar structure as seen in the human diarrheic faecal sample (Zaman *et al.*, 1999). This finding contradicted with a study by Cassidy *et al.* (1994) who described small surface projections on some of their chicken isolates. Apart from that, chicken isolates have similar surface characteristic to asymptomatic human isolate (Ragavan *et al.*, 2014) with lesser binding affinity to FITC-Con A whereby less pathogenic isolate owing to compromised adhesion.

There are limited studies conducted on the genomic analysis of *Blastocystis* spp. isolated from chickens. Results from this study showed chickens were infected with multiple genotypes including zoonotic ones. A total of six subtypes were identified in chickens including ST1, ST6, ST7 and ST8 (Abe *et al.*, 2003a; Arisue *et al.*, 2003; Noël *et al.*, 2003). This study also highlighted two subtypes; ST6 and ST7 as prevalent in the chicken population (Stensvold *et al.*, 2009).

In this study, ST6 was recovered from six broiler chickens obtained from a wet market in Perak. Based on the PCR amplification using the DNA barcoding primer, isolates from two broilers (A20 and A26) were identified as ST6 while the other four isolates (C15, C16, C19 and C23) were analysed using the sequenced-tagged site (STS) primers. The finding of *Blastocystis* infection in broiler chickens was unexpected especially because these birds were reared in an intensive barn system, treated with antibiotic and antihelminthics. The same zoonotic subtype was also recovered from three isolates from Chinese bamboo partridge, vulturine guineafowl and Japanese green pheasant at a zoo in Japan (Abe *et al.*, 2003a). ST6 is uncommon in humans with the prevalence as low as 1% in the Netherlands (Bart *et al.*, 2013) and 3.6% in Thailand (Jantermtor *et al.*, 2013). This subtype was recently observed in the Indian street dogs (Wang *et al.* 2013).

ST7 was also identified in four village chickens (A2, A4, A6, and A18) and one broiler chicken (A28). ST7 was previously isolated from chickens, quails, geese and birds. However, Tan *et al.* (2013) also reported the presence of ST7 in goats in Selangor. Since this subtype was previously reported in human particular the Malaysian populations (Tan *et al.* 2009), domestic animals namely chicken and goat may serve as reservoir hosts for transmission to humans.

Meanwhile, ST1 was detected in village chicken isolates (A16) from a mini zoological garden in Perak. This subtype was similarly reported in chicken (Yoshikawa *et al.*, 1996), dogs (Wang *et al.*, 2013; Ruaux *et al.*, 2014), pigs (Navarro *et al.*, 2008), chimpanzees, and gorillas (Roberts *et al.*, 2013). It is one of the most common pathogenic human subtypes (Moosavi *et al.*, 2012). A study in Thailand showed nearly 80% of the schoolchildren were infected with *Blastocystis* ST1 (Jantermtor *et al.*, 2013).

ST8 was previously isolated from non-human primates, as well as humans and chickens (Stensvold *et al.*, 2009). In the present study, ST8 was detected in two village

chickens (A17 and A38) from a mini zoo in Perak. Due to its low host specificity, transmission between humans and poultry warrant serious attention particularly among animal handlers such as chicken farmers and those working in slaughter houses or wet markets. Additionally, none of the chicken examined showed any symptoms of infection unlike the consequence of human infection (Yan *et al.*, 2006).

3.5 Conclusion

The present study is the first to elucidate the prevalence, phenotypic characteristic, ultrastructural and subtype of *Blastocystis* spp. isolated from chickens in Peninsular Malaysia. Furthermore, this study also justifies that chickens reared locally showed multiple subtypes. This is the first study to show the presence of *Blastocystis* spp. in boiler chickens.

Host	Reference/Origin	Prevalence (%)	Morphological characteristics			Genotypes
			Light microscope	SEM	TEM	
Chicken	Yamada <i>et al.</i> (1987)/Japan	- 10/10 (100)	 Although polymorphic forms were rarely found in the lumen of the chicken's caecum, these forms were frequently found shortly after <i>in vitro</i> cultivation. The central vacuole usually did not stain with iodine, but was sometimes lightly to heavily stain. Size: 9-32 µm in the lumen contents of fowl caeca. 	-	_	-
	Belova and Kostenko (1990) : Hen	- 80-100%	 The parasite form varies from round to ellipsoid. There were 1 to 4 nuclei Cytoplasm contains a great number of ribosomes and mitochondria with cristae resembling in their shape oval or round small sacs. Nucleus contains nucleolus. Chromatin mass is concentrated on one of the poles of the nucleus as individual bodies. Sizes: 7.5-35.0 x 6.25-30.0 (18.67 x 17.05) µm. 	_		-

Table 3.5:	List of	previous	publication	on Blastocystis	in birds.
		1	1	-	

Host	Reference/Origin	Prevalence (%)	Morphological characteristics			
	_		Light microscope	SEM	TEM	
Chicken	Stenzel <i>et al.</i> (1994)/Australia	- 14/18 (77.8)	 The cells were always large, irregularly shaped cells. Size: 15-20 μm in diameter. Larger cells (> 30 μm in diameter) 	- The cells were surrounded by a thick, fibrillar surface coat.	 Vacuolar contents were varied, ranging from a few clumps of flocculent material to homogeneous electron-opaque contents. Clumps of condensed chromatin were present in the nucleus. The nuclei were more elongated. Multinucleated cells were frequently seen. 	-
	Yoshikawa <i>et al.</i> (1996)/Japan	-	-	-	-	ST1
	Yoshikawa <i>et al.</i> (2003)/Japan	-	-	-	-	ST1 and ST6
	Arisue et al. (2003)/Japan	-	-	-	-	ST6
	Noël et al. (2003)/France	-	-	-	-	ST7
	Abe <i>et al.</i> (2004) /Japan	-3/6 (50)	-	-	-	ST1 and ST7
	Alfellani <i>et al.</i> (2013c)/Libya	-1/3 (33.3)	-	-	-	ST7

I I C valence (70)	Morp	nological characterist	ICS	Genotypes
	Light microscope	SEM	TEM	
- 23/70 (32.9)	Light microscope Vacuolar - The cells were rounded in shape and contain a central body resembling a large vacuole. - Size: 10.9 - 32.1 μm. Granular - The granular form showed a different quantity of granules in their interior. - Size: 9.0 to 28.3 μm <u>Amoeboid</u> - It was found in small amounts in the stained fecal smears. - Size: 13.4 to 45.5 μm. <u>Cyst</u> - The cells were characterized as rounded or ovoid, with one or two internal nuclei.			
	- Size: 2.1 to 5.5 µm.			
	- 23/70 (32.9)	Light microscope - 23/70 (32.9) Vacuolar - The cells were rounded in shape and contain a central body resembling a large vacuole. - The cells were rounded in shape and contain a central body resembling a large vacuole. - Size: 10.9 - 32.1 µm. - Size: 10.9 - 32.1 µm. - The granular form showed a different quantity of granules in their interior. - Size: 9.0 to 28.3 µm - Size: 9.0 to 28.3 µm - It was found in small amounts in the stained fecal smears. - Size: 13.4 to 45.5 µm. - Size: 13.4 to 45.5 µm. - The cells were characterized as rounded or ovoid, with one or two internal nuclei. - Size: 2.1 to 5.5 µm.	Light microscope SEM - 23/70 (32.9) Vacuolar - - The cells were rounded in shape and contain a central body resembling a large vacuole. - - - Size: 10.9 - 32.1 μm. Granular - - The granular form showed a different quantity of granules in their interior. - - - Size: 9.0 to 28.3 μm Moreboid - - It was found in small amounts in the stained fecal smears. - - - Size: 13.4 to 45.5 μm. Cyst - - The cells were characterized as rounded or ovoid, with one or two internal nuclei. - -	Itight microscope SEM TEM - 23/70 (32.9) Vacuolar - - - The cells were rounded in shape and contain a central body resembling a large vacuole. - - - Size: 10.9 - 32.1 µm. Granular - - - The granular form showed a different quantity of granules in their interior. - - - - Size: 9.0 to 28.3 µm Amoeboid - - - - It was found in small amounts in the stained fecal smears. - - - - Size: 13.4 to 45.5 µm. Evst - - - The cells were characterized as rounded or ovoid, with one or two internal nuclei. - -

Host	Reference/Origin	Prevalence (%)	Morphological characteristics			
			Light microscope	SEM	TEM	
Chicken	Present study :Free-ranged chicken	- 36/105 (34.3) - 11/74 (14.9)	<u>Vacuolar</u> - The cells always large, spherically shaped cells - Size: 10 μm to 100 μm in diameter <u>Granular</u> - Size: Granular (20 to 40 μm in diameter) Gigantic granular form (40 to 90 μm in diameter). <u>Cyst</u> - The cells were generally smaller and rounded to ovoid in shape. - Size: 3 to 5 μm.	 The cell surface was generally rounded to spherical in shape and had a smooth surface coat with undulating cell surface whereas some organisms showed indentations or deep grooves with infoldings. Bacteria were seen attached to the surface of the cell. 	 Two types of vacuoles forms in the culture form of village chicken isolates; one containing a large central vacuole completely electron- lucent, and the other which contains electron-opaque, in fully distended vacuoles. The organisms possess a thin wispy surface coat which resembles a slight ruffled. 	ST1, ST7 and ST8
Duck	Belova (1991)	-	 The cells appeared rounded to ellipsoid with one to four nuclei. Size: ~18 μm in diameter. 	-	-	-
	Pakandl and Pecka (1992)	 80% in adults 25% in ducks month old 	-	-	- The ultrastructure of <i>Blastocystis</i> from ducks was similar to that of the human parasite.	-

Host	Reference/Origin	Prevalence (%)	e (%) Morphological characteristics			Genotypes
	_		Light microscope	SEM	TEM	
Duck	Stenzel <i>et al.</i> (1994)/Australia	- 3/4 (75)	 The cells were generally ovoid or rounded, being only occasionally slightly irregular in outline. Size: 10-15 µm in diameter. 	 The surface coat approximately 0.5 μm thick was present on all cells, although this often appeared to be separating from the cell in one of the samples. Other samples showed the surface coat to be closely associated with the cell membrane. 	 Vacuolar contents contain opaque concentration of flocculent material. Nuclei showed a crescent of condensed chromatin. Very rarely was an elliptical band of chromatin seen with commonly a single nucleus was present. 	-
	Noël et al. (2003)/France	-	-	-	-	ST7
	Bergamo do Bomfim and Machado do Couto (2013)/Brazil	- 34/73 (46.6)	- The general morphology of <i>Blastocystis</i> for duck was similar to that of the chicken parasite (above).	-	-	-
Goose	Belova (1992a)	-	- Size: 7.5-46.2 x 7.5-46.2 μm.	-	-	-
	Stenzel <i>et al.</i> (1994)/Australia	6/7 (85.7)	 The cells were rounded to elongate, occasionally slightly irregular in outline. Size: 10-15 μm in diameter. 	- The surface coat was thick (0.5 μm), compact and fibrillar.	 Central vacuole contents were consistently dense, flocculent material. The nuclei were rounded with a crescent of condensed chromatin being observed. 	-

Host	Reference/Origin	Prevalence (%)	(%) Morphological characteristics			
			Light microscope	SEM	TEM	
Ostrich	Yamada <i>et al.</i> (1987)/Japan	- 2/2 (100)	-	-	-	-
	Stenzel <i>et al.</i> (1994)/Australia	9/9 (100)	 The cells were rounded to ovoid. -Size: 6-10 μm in diameter. 	- The surface coat was thick $(0.5 \ \mu\text{m})$ and fibrillar, and no cell was seen without a surface coat.	 Vacuolar contents were clumped, fibrous materials, frequently exhibiting a dense fibrous inclusion. A rounded nucleus showing an elliptical concentration of condensed chromatin. 	-
	Roberts et al. (2013)	6/10 (60)	-	-	-	ST4
	Chandrasekaran <i>et al.</i> (2014)/Malaysia	37/37 (100)	 The vacoular and granular forms are generally smaller. Size: 15 to 30 μm (vacuolar) and 5 to 20 μm (granular). 	- Surface strcture revealed spherical and smooth surface with tiny pores.	 High electron dense area was observed in the central vacuole of most of the cells. Membrane thickness of 235.48 to 345.22 nm. 	ST5
Turkey	Lee (1970)	-	 The cells from the caecal contents appeared as around or oval in shape. Size: 5 to 10 μm. 	-	 The fine structure showed it to have mucilaginous coat, a central vacuole, several mitochondria, and one or more nuclei. The central vacuole contained finely granular material and occasionally a ctystalline inclusion. 	-

Host	Reference/Origin	Prevalence (%)	Moi	phological characteristics		Genotypes
			Light microscope	SEM	TEM	
Turkey	Belova (1992b)	-	 The shape varies from round and oval to ellipsoid and amoeboid. Size: 2.555.1 x 2.551.3 μm. 	-	-	-
	Noël et al. (2003)/France	-	_	-	-	ST6
Quail	Arisue <i>et al.</i> (2003) /Japan	-	-	-	-	ST6
	Yoshikawa <i>et al.</i> (2003)/Japan	-	-	-	-	ST6 and ST7
	Abe et al. (2004)/Japan	2/9 (22.2)	-	-	-	ST6 and ST7
	Bergamo do Bomfim and Machado do Couto (2013)/Brazil	- 17/71 (23.9)	- The general morphology of <i>Blastocystis</i> for quail was similar to that of the chicken parasite (above).	-	-	-
Pheasant	Abe <i>et al.</i> (2002)/Japan	-	-	-	-	ST6

Host	Reference/Origin	Prevalence (%)		Genotypes		
			Light microscope	SEM	TEM	
Pheasant	Abe et al. (2003a)/Japan	-	-	-	-	ST8
	Abe et al. (2004)/Japan	3/9 (33.3)	-	-	-	ST1 and ST7
Partridge	Taylor <i>et al.</i> (1996)/England : Red-legged patridge	-	- The cells were rounded.	-	-	-
	Abe <i>et al.</i> (2002)/Japan : Chinese bamboo partridge	-	-	-	-	ST6
	Abe et al. (2003a)/Japan	-	-	-	-	ST6
Guinea fowl	Abe et al. (2002)/Japan	-	-	-	-	ST6
	Abe <i>et al.</i> (2003a) /Japan	-	-	-	-	ST7

CHAPTER 4: STUDY

STUDY ON Blastocystis spp. IN WILD RATS

4.1 Introduction

Wild rats have increasingly become a nuisance with not only causing the destruction of foodstuff, gnawing of electrical wiring in buildings, contaminate the surroundings with excreta resulting, in significant economic losses but also hosts to transmit a wide range of pathogens, such as *Leptospira* sp., *Toxoplasma gondii*, *Cryptosporidia* and *Blastocystis*.

The reports of *Blastocystis* in rodents are limited with the earliest findings in rats (Knowles and Das Gupta, 1924) and mice (Lavier, 1952). Later studies highlighted its presence in laboratory bred rats with high infection (60%) in Wistar and Sprague-Dawley rats (Chen *et al.*, 1997a). While other laboratory bred animals such as rabbits, hamsters, gerbils and laboratory mice were free from infection (Pakandl, 1992).

Isolates from rats are known as *B. ratti* based on the differences in karyotypic patterns (Chen *et al.*, 1997b). Although ST4 have been reported among rodents, but the total number of samples and host species studied thus far has been small with only five rat samples (Noël *et al.*, 2003, 2005; Yoshikawa *et al.*, 1998) and two guinea pigs (Yoshikawa *et al.*, 1998). Alfellani *et al.* (2013c) suggested rodent infections of this subtype were species related. Ramírez *et al.* (2014) recorded ST2 from *Rattus rattus* in Colombia. Both subtypes are common in Europe in which ST2 prevalent in patients with diarrhea and healthy individuals whereas ST4 linked to diarrhea and/or irritable bowel syndrome (IBS) (Alfellani *et al.*, 2013b; Ramírez *et al.*, 2014).

Despite these reports, many still remains to be understood about *Blastocystis* in rodents and little attention were given to the commensal urban rat populations. The brown rat or Norway rat is one of the principal commensal pests in urban cities in Malaysia commonly found close to human inhabitants (Mohd Zain *et al.*, 2012). Since the landscape of cities and towns in Malaysia will be typically reflective that of any other Asian metropolitan setting, a survey of *Blastocystis* in rodents obtained from the streets and local restaurants in town centers from Malaysia may provide important insights on the potential transmission possibility of *Blastocystis* sp. to humans in this region.

4.2 Materials and methods

4.2.1 Ethical approval

The ethics approval has been obtained as described in greater detail in Chapter 3.2.1.

4.2.2 Sampling

From June 2013 until August 2014, 293 urban wild rats namely; the brown rat (*Rattus norvegicus*) and house shrew (*Suncus murinus*) were captured from the vicinity of public spaces with food surplus and abundant shelter such as human dwellings, restaurants, lifestock pens and wet markets in the two urban cities namely Ipoh, in the state of Perak and Kuala Lumpur, the capital city of Malaysia by adopting a convenient sampling method using the wire-box traps with selected baits. On trapping, all rats were examined. These rats appeared normal devoid of any clinical symptoms of disease.

4.2.3 Dissection

All rats were humanly euthanized, dissected and the caecum was removed. The contents were collected and stored in a container prior to their contents being subjected for *in vitro* culture.

4.2.4 In vitro cultivation

All samples were subjected to *in vitro* cultivation as described in Chapter 3.2.4.

4.2.5 Microscopy examination

The positive faecal smears were stained with 10% Giemsa and Sudan Black B stain according to procedures highlighted in Chapter 3.2.5.

4.2.6 Cytochemical staining

Cytochemical staining was conducted on selected isolates according to procedures highlighted in Chapter 3.2.6.

4.2.7 Ficoll-Paque concentration method

In order to collect the cyst form of *Blastocystis*, Ficoll-Paque concentration method was subjected to selected faecal samples as in the procedure highlighted in Chapter 3.2.7.

4.2.8 Electron microscopy

Selected day-3 positive culture samples and *Blastocystis* cyst were subjected to ultrastructural studies using scanning and transmission electron microscopy as described in the procedures highlighted in Chapter 3.2.8.

4.2.9 In vitro growth profile

The parasites of each isolates were pooled together from day 2 cultures to make a final concentration of 1×10^5 cells per ml in 3 ml of Jones' medium containing 10% horse serum. All cultures were kept in airtight tubes and incubated at 37°C for up 10 days. All experiments were done triplicate. The parasite count was done using haemacytometer chamber (Improved Neubauer, Hausser Scientific) with 0.5% Tryphan blue solution as viability indicator. The parasite count was determined daily for up to 10 days. Only viable cells that did not take up Tryphan blue stain were counted. 50 parasites were randomly selected for size measurement using eyepiece graticule precalibrated with stage micrometer on light microscope (Olympus). The number of the vacuolar form, granular form and amoeboid form was calculated daily. On day 10 of the

growth profile, the sediment containing the parasites of each isolates was sub-cultured to determine the viability of the parasites.

4.2.10 Subtyping

All positives isolates were then subjected to sequence tagged site (STS) primerpolymerase chain reaction (PCR) and DNA barcoding according to Chapter 3.2.9.

4.2.11 Statistical analysis

Statistical analyses were described in Chapter 3.2.10.
4.3 Results

4.3.1 Prevalence of *Blastocystis* in wild rats

A total of 133 (45.4%) (95% confidence interval [CI] = 39.3-51.2%) brown rats were positive for *Blastocystis* while not one of the house shrews caught were infected. Male infections (38.5%) were lower than females (43.6%) respectively. There was no association observed between infection with host-sex ($\chi 2 = 0.424$, [df] = 1, P = 0.515) as well as the geographical location ($\chi 2 = 0.042$, [df] = 1, P = 0.839). Meanwhile, prevalence in Perak (46.3%) was slightly higher than in Kuala Lumpur (45%) (Table 4.1).

Infection	Samplin	Sampling sites (%)Total (%)		
	Perak	Kuala Lumpur		
Positive	38 (46.3)	95 (45.0)	133 (45.4)	
Negative	44 (53.7)	116 (55.0)	160 (54.6)	
Total no. of wild rat	82 (28.0)	211 (72.0)	293	

Table 4.1: Prevalence of *Blastocystis* in wild rats.

4.3.2 Morphological forms

The morphological forms of parasites isolated from wild rats were examined in the fresh caecum specimens as well as in cultured form. The fresh caecum specimens contained elliptical vacuolar forms (Figure 4.1a) similar to those seen in day 1 cultured forms (Figure 4.1b). However, after prolonged cultures, rounded vacuolar forms were predominantly seen with measurements ranging from 4 μ m to 45 μ m in diameter (Figure 4.2). The cells often possess one or two nuclei, and occasionally, quadrinucleate cell. Multivacuolar forms (Figure 4.3) were also seen in the isolates. The cyst of wild rat isolate is smaller than vacuolar form and ovoid in shape which ranged from 2 to 10 μ m in diameter.

4.3.3 Modes of reproduction

Binary fission and plasmotomy (budding) (Figure 4.4a) of the vacuolar forms is the most commonly observed mode of reproduction. However, schizogony-like organisms, which possess many progeny or daughter cell structures in the central body were less frequently observed in *in vitro* cultures of wild rat isolates (Figure 4.4b).



Figure 4.1: Day 1 *Blastocystis* isolated from wild rats recovered from (a) caecum and (b) *in vitro* culture under light microscopy. CV; central vacuole.



Figure 4.2: *Blastocystis* isolated from wild rats after prolonged cultures.



Figure 4.3: Multivacuolar forms occasionally seen in the *in vitro* culture.







Figure 4.4: Mode of reproduction of *Blastocystis* from wild rat isolates (a) binary fission (arrow) (b) budding (arrow) (c) schizogony-like organisms. BF; binary fission, B; budding, P; progeny.

4.3.4 Surface structure

Scanning electron micrograph showed the fresh caecum form with slightly rough surface (Figure 4.5a). Meanwhile, the surface coat of the cultured form and cyst form appeared spherical in shape with smooth surface (Figure 4.5b and c).

4.3.5 Ultrastructure

Transmission electron micrographs examination of *Blastocystis* from wild rat isolates revealed an irregular form of vacuolar form in which the organelles were present in a thin rim of cytoplasm surrounding a large central vacuole (Figure 4.6a). The cell had a thin membrane layer with the measurement ranging from 135.51 to 196.82 nm. The mitochondria-like organelle varied in shape from round to oblong, but the Golgi complex was not seen. The nucleus was slightly elongated which was bound by double membrane in addition to a cresentic band of electron opaque material which is also known as nucleolus. Meanwhile, a low electron dense area was observed in the central vacuole. The cyst form is relatively smaller in size with a thicker layer of membrane surrounding the parasites compared to a relatively thin layer of membrane surrounding the vacuolar form (Figure 4.6b).







Figure 4.5: Surface structure of *Blastocystis* isolated from wild rats isolated from (a) fresh caecum form (b) cultured form (c) cyst form.



Figure 4.6: Electron micrograph of *Blastocystis* isolated from wild rats. (a) Vacuolar form. (b) Cyst form. N; nucleus, m; mitochondria-like organelle, CV; central vacuole, SC, surface coat, CM; cell surface membrane, CVM; central vacuole membrane, nu; nucleolus, G; glycogen.

4.3.6 Cytochemical studies

Light microscopic images *Blastocystis* isolated from day 3 culture of wild rat stained with Sudan Black B showed positive reactions are seen as dark droplets in the central vacuole of the cells (Figure 4.7a).

There was a faded light green florescence with Fluorescein isothiocyanate (FITC)labeled Con A (*Canavalia ensiformis*) binding on the membrane of both vacuolar and granular forms (Figure 4.8) of *Blastocystis* sp. from day 2 culture. The fluorescence intensity and the percentage of the reactive forms of wild rat isolate in FITC-labelled Con A stain range was (1+; 88-100%).

Acridine orange is a cell-permeant nucleic acid binding dye that emits green fluorescence when bound to double stranded DNA and red fluorescence when bound to single stranded DNA or RNA. The respective epifluorescence image showed that the vacuolar form was stained dull green while the granular form was stained bright green (Figure 4.9).



Figure 4.7: *Blastocystis* isolated from wild rats stained with Sudan Black B showing dark stain in the central vacuole indicating the presence of neutral lipid under 1000× magnification. Note: dark droplets (arrow).



Figure 4.8: Binding affinities of *Blastocystis* stained with FITC-labelled Con A (a) Light microscopic images *Blastocystis* of wild rat isolate (b) A same organism stained with FITC-labeled Concanavalin A (ConA) assay, AFU (1+): weak intensity. V; vacuolar form, G; granular form.





Figure 4.9: Epifluorescence image of *Blastocystis* stained with acridine orange (a) Light microscopic images *Blastocystis* of wild rat isolate (b) A same organism stained with acridine orange.

4.3.7 Growth characteristics

Over a 10-day growth period of *Blasocystis* cultured in Jones' medium supplemented with 10% horse serum, two randomly chosen isolates (T9 and T21) showed different parasite growth rates. The parasite counts peak on day 7 for isolate T9 whereas on day 5 for isolate T21. The isolates started initially with a parasite count of 1 x 10^6 cells/ml and peaked at 14.3 x 10^6 cells/ml (Isolate T9) and 25.4 x 10^6 cells/ml (Isolate T21). Meanwhile, the numbers dropped dramatically on day 8 for both isolates to 13.1 x 10^6 cells/ml (Isolate T9) and 8.1 x 10^6 cells/ml (Isolate T21) (Figure 4.10).

The vacuolar forms were predominant in cultures on day 1 until day 4 showed a mean diameter values of 2 to 14 μ m, 3 to 10 μ m, 2 to 15 μ m and 3 to 16 μ m respectively. Vacuolar forms counts peaked on day 3 for both isolates with a percentage ranged from 92.4% (Isolate T21) to 63.9% (Isolate T9) however, dropped drastically by day 7 for both isolates (Figure 4.11).

The granular forms appeared on day 1 but dropped by day 4 for both isolates. The average size range of granular forms on day 1 until day 4 was 3 to 8 μ m, 3 to 7 μ m, 3 to 11 μ m and 3 to 6 μ m respectively in diameter. The percentage of granular forms in both isolates increased gradually from day 5 to day 7 with a percentage ranging from 44.2% (Isolate T9) to 78.7% (Isolate T21) (Figure 4.12). However, both isolates dropped drastically as it approaches day 8. In addition, the growth profile was similar for both isolates where the numbers of granular forms was able to multiply and increase once again on day 9 onwards.



Figure 4.10: Growth profiles of total number of parasites.



Figure 4.11: Percentages of the presence of vacuolar forms.



Figure 4.12: Percentages of the presence of granular forms.

4.3.8 Subtype identification

Using the sequenced-tagged site (STS) primers, two of the wild rat isolates (WR11 and WR12) were confirmed to be ST4 (Figure 4.13). Meanwhile, using the DNA barcoding method on additional 45 isolates revealed four distinct subtypes. They comprised two isolates of ST1, 41 isolates of ST4, and each one isolate of ST5 and ST7 (Table 4.2).



Figure 4.13: PCR amplification reaction of *Blastocystis* sp. from wild rat isolates using the sequenced-tagged site (STS) primers SB337 (lane *1* and *2*; 487bp) indicating ST4.

 Table 4.2: Subtype of *Blastocystis* from wild rat isolates obtained *Blastocystis*

 Sequence Typing Databases.

Blastocystis sp. subtype	Sequence-positive isolates
ST1	2
ST4	41
ST5	1
ST7	1

4.4 Discussion

Information on *Blastocystis* isolated from wild rodents is scarce whilst most previous descriptions and prevalence data were primarily from laboratory rats. This parasite was first described in rats (Knowles and Das Gupta, 1924), mice (Lavier, 1952) and the Wistar and Sprague-Dawley rats (Chen *et al.*, 1997a). This study records for the first time infections in urban wild rat population particularly the brown rats (*Rattus norvengicus*). Table 4.3 summaries the previous studies on *Blastocystis* spp. in rodents. Results from this study provide clearer evidence that high prevalence of *Blastocystis* sp. in brown rats. Unfortunately, its presence in the house shrew was not detected possibly due to the small sample capture. The finding is the first to demonstrate the parasite in a large scale survey and the health implications since these animals live in close association with humans and its role in tranmsitting to other hosts including humans through contaminating the environment, food and water sources with *Blastocystis* spp.

Amidst the towering buildings that make up Ipoh and Kuala Lumpur iconic skylines, these cities are plagued by an ever-increasing population of rats. Rats are drawn to places with surplus food, abundant shelter and poor sanitation. Market places mainly the illegal dumping sites, the cooking areas of roadside stalls and unhygienic restaurants are ideals places for rats to thrive.

Chen *et al.* (1997b) described the morphology of *Blastocystis* from Wistar rats and concluded that the morphology of rat *Blastocystis* isolates was rather similar with the various forms of *B. hominis*. This study also reports the first description of *Blastocystis* from the caecum of wild rats and *in vitro* culture. The findings showed a distinct morphological form observed in fresh caecum isolate and when cultured on day 1. The vacoular forms were elongated but reverted to become rounded after prolonged cultures

which concurred with Stenzel *et al.* (1994) findings that culture conditions may alter the actual morphological. Furthermore, the surface structure of vacoular forms isolated from fresh caecum samples had a smoother cell surface compared with the cultured forms. The cell membrane in the present study was thinner compared to human isolates as showned by Ragavan *et al.* (2014). To date, no ultrastructural description is available on *Blastocystis* sp. ST4 from wild rats.

Growth profile was used previously to demonstrate phenotypic differences (Tan *et al.*, 2008). In the present study, the growth profile of *Blastocystis* sp. ST4 from wild rat isolates (T9 and T21) concurred with the growth profile of *Blastocystis* isolated from asymptomatic individual (Tan *et al.*, 2008). Besides, the results also revealed that the viable granular forms from day 5 to 10 was able to multiply and remained viable up to 3 days when sub-cultured on day 10 which suggests the ability of viable granular forms to produce new progeny of *Blastocystis* after sub-culturing.

This study represents the first successful attempt to determine the genetic diversity of *Blastocystis* spp. from wild rat population in Malaysia. The results revealed that 37 brown rats were identified as ST4. The genotypic characterization in this study was consistent with those described by Noël *et al.* (2003; 2005) and Yoshikawa *et al.* (1998) to which this same subtype was recovered from rat isolates from Singapore and Japan. Although ST4 appears to be restricted to rodents, a greater host range was present in kangaroo, ostriches, Snow Leopard and non-human primates (Roberts *et al.*, 2013; Alfellani *et al.*, 2013c). In most studies, ST4 was the fourth most common subtype found in humans. This subtype is commonly found across Europe, UK and Sweden but rare in other countries (Alfellani *et al.*, 2013b; Forsell *et al.*, 2012). Recent studies indicated that this subtype appears to be linked to diarrhea and/or IBS (Alfellani *et al.*, 215).

2013b). It is notable that ST4 is rare or absent in the Asian countries especially in Malaysia. The present study reported that ST4 were dominant in the urban wild rats in Peninsular Malaysia.

It was recently argued that methodology used may influence the results. Based on Stensvold (2013), it appears that ST4 accounts for approximately 1% and 17% of *Blastocystis* carriage detected by the sequence-tagged site (STS) primers method and non-STS methods, respectively. Although potentially coincidental, it should be noted that ST4 is rare or absent mainly in those countries where surveys have been conducted using the STS primers method, typically Asian and Middle Eastern countries. Therefore, the use of DNA barcoding in these countries is especially needed to validate the absence of ST4 in these particular regions.

ST1 could be identified in both humans and a wide rage of animals. In human, it is the most dominant subtype in Thailand (Tan, 2008) with the prevalence of 77.9% in schoolchildren (Leelayoova *et al.*, 2008) and 21.4% in patients from Srinagarind Hospital (Jantermtor *et al.*, 2013). Apart from that, previous study reported that it is the second most common variant in China (24.5%) (Li *et al.*, 2007b), Germany (21%) (Böhm-Gloning *et al.*, 1997), Greece (20%) (Menounos *et al.*, 2008) and Singapore (22%) (Wong *et al.*, 2008). Recently, Alfellani *et al.* (2013b) reported that this subtype was detected in the IBS group. In addition, Kumarasamy *et al.* (2014) reported the occurrence of ST1 in patients with colorectal cancer from University of Malaya Medical Centre (UMMC), Malaysia. It is suggested that this subtype may be related to pathogenicity with a higher subtype-symptom relationship being noted (Yan *et al.*, 2006). As mentioned above, a wide range of animals could be a reservoir of *Blastocystis* particularly monkeys, chimpanzees, cattles, pigs, horse, ostriches, dogs, goats and

chickens (pheasant) (Alfellani *et al.*, 2013c; Yoshikawa *et al.*, 2003; Navarro *et al.*, 2008; Tan *et al.*, 2013; Wang *et al.*, 2013; Ruaux *et al.*, 2014). Others suggested that this subtype appears to be linked to zoonotic transmission from farm animals (Noël *et al.*, 2003; Tan, 2008). In Malaysia, Tan *et al.* (2013) reported that this subtype is the most predominant *Blastcystis* subtype harboured by locally reared goats from different farms in Selangor. Remarkably, this study revealed new sights on *Blastocystis* reservoirs. The subtypes analyses demonstrate that two brown rat obtained from wet market in Kuala Lumpur, Malaysia were identified as ST1. Therefore, the study raises the possibility of zoonotic transmission of *Blastocystis* from household pests living in a community based environment is feasible via the faecal-oral route.

A recent study by Wang *et al.* (2014) reported a high prevalence of *Blastocystis* carriage in pigs (76.7%) with all pigs harbouring ST5. Based on these results, pigs are likely to be a natural host of *Blastocystis* with ST5 being the host adapted subtype. This subtype was also commonly found in Libyan domestic animals i.e cattle yet was not found in humans which means either that those hosts do not contribute to human infections or that humans are not susceptible to infection with this subtype (Alfellani *et al.,* 2013b). Interestingly, this study is the first time that wild rat isolate has been assigned to this subtype. It is therefore very likely that continued sampling will uncover additional new hosts for existing subtypes.

ST7 was also been identified in a wild rat obtained from a market in Kuala Lumpur (R22). Previous studies have reported this subtype in chickens, quails, geese and birds. However, Tan *et al.* (2013) recently reported that *Blastocystis* sp. ST7 in the goats in Selangor, Malaysia. Since this subtype was previously reported in human infection in particular the Malaysian populations (Tan *et al.*, 2008; Tan *et al.*, 2009), domestic

animals namely chicken, goat and also wild rat may serve as reservoir hosts for transmission to humans.

4.5 Conclusion

The present study represents the first study to elucidate the epidermiology, phenotypic, ultrastructural and subtype characteristics of *Blastocystis* isolated from wild rats in Malaysia in order to determine the true pathogenicity of this zoonotic parasite. The importance of understanding *Blastocystis* in the environment is crucial because rodents are in close contact with human and a source of human infection. Therefore, rodent control and eradication measures must be carried out by the local municipals in order to prevent rodent borne diseases.

Host	Reference/Origin	rence/Origin Prevalence (%)	Morphological characteristics			Genotypes
			Light microscope	SEM	TEM	
Rat	Knowles and Das Gupta, (1924)	- Infected with Blastocystis (Not mentioned)	-	-	-	-
	Chen <i>et al.</i> (1997a) /Singapore	- 60% : Laboratory rats	-	-	-	-
	Chen <i>et al.</i> (1997b) /Singapore	-	Axenic culture - The cells were generally smaller, mostly typical vacuolar forms.	-	-	-
			 Size: 6±15 μm in diameter. Ameboid forms were rare. Cyst forms were also found. 			
			Axenic culture - About 90% of the cells were vacuolar forms. - Multivacuolar forms were also found together with the vacuolar forms. - In one of the vacuolar forms, about tap vasiala like			
			 about ten vesicle-like structures were seen. The giant cells measuring 40 μm were occasionally seen. Ameboid forms were frequently found. 			

Table 4.3: List of previous publication on *Blastocystis* in rodents.

Host	Reference/Origin	Prevalence (%)	Morphological characteristics			
			Light microscope	SEM	TEM	
Rats	Arisue <i>et al.</i> (2003) /Japan	-	-	-	-	ST4
	Noël <i>et al.</i> (2005) /Singapore	-	-	-	-	ST4
	Present study	- 133/293 (45.4) : Brown rats	 This contained elliptical vacuolar forms similar to those seen in day 1 cultured forms. After prolonged cultures, rounded vacuolar forms were predominantly Size: 4 μm to 45 μm in diameter. The cells often possess one or two nuclei, and occasionally, quadrinucleate cell. Multivacuolar forms were also seen in the isolates. The cyst of wild rat isolate is smaller than vacuolar form and ovoid in shape. Size: 2 to 10 μm in diameter. 	 The surface of fresh caecum form showed slightly rough surface. The surface coat of the cultured form and cyst form appeared spherical in shape with smooth surface. 	 The cells revealed an irregular form of vacuolar form. The cell had a thin membrane layer with the measurement ranging from 135.51 to 196.82 nm. The mitochondria-like organelle were round to oblong in shape. The Golgi complex was not seen. The nucleus was slightly elongated. A low electron dense area was observed in the central vacuole. The cyst form is relatively smaller in size with a thicker layer of membrane. 	ST1, ST4, ST5 and ST7.
Mice	Lavier (1952) /France	- Infected with Blastocystis (Not mentioned)	-	-	-	-

Host	Reference/Origin	Prevalence (%)	Mo	Genotypes		
	_		Light microscope	SEM	TEM	
Mice	Pakandl (1992) /Czechoslavakia	- 0 : Laboratory mice	-	-	-	-
Shrew	Present study	- 0 : House shrew	-	-	-	-
Rabbit	Chen <i>et al.</i> (1997a) /Singapore	- 0/37 (0)	-	-	-	-
Hamster	Chen <i>et al.</i> (1997a) /Singapore	- 0/37 (0)	-	-	-	-
Gerbil	Pakandl (1992) /Czechoslavakia	- 0	-	-	-	-
Guinea pig	Silberman <i>et al.</i> (1996) /USA	-	-	-	-	ST4

CHAPTER 5:

STUDY ON Blastocystis spp. IN COCKROACHES AND HOUSE GECKOS

5.1 Introduction

Cockroaches and house geckos are common house pest throughout the tropics. Cockroaches are notorious mechanical vectors to many diseases and have worldwide distribution, infesting many types of dwelling. It is believed that cockroaches may be a reservoir for a range of bacteria including *Staphylococcus* and *Streptococcus*. Meanwhile, house geckos faeces are often considered to be a nuisance and can even cause *Salmonella* infection when ingested (Callaway *et al.*, 2011).

The occurrence of *Blastocystis* in the American cockroach (*Periplaneta americana*) was reported in Singapore with 80% of the cockroaches caught from sewage tanks were positive for *Blastocystis* (Zaman *et al.*, 1993). To date, only one Malaysian study reported of the low occurrence of *Blastocystis* in cockroaches from human dwellings (10%) (Suresh *et al.*, 1997). Both studies described the isolates were similar to *B. hominis*. However, little was known of those isolates as descriptions were based on morphological criteria only (Zaman *et al.*, 1993; Suresh *et al.*, 1997) and no systematic studies on subtype characterization of this parasite.

Meanwhile, the only published report on *Blastocystis* sp. infection in house geckos was conducted by Suresh *et al.* (1997). It was found that 7% out of 30 captured house geckos (*Hemidactylus platyurus*) were positive for *Blastocystis* with most of the cells seen were granular form.

Blastocystis is widespread in nature and infecting a wide range of hosts. However, there has been no conclusive evidence of the presence of this parasite in cockroaches as well as house geckos as they are commonly found in human dwellings and may play a role as a possible vector. Thus, prompted this study to determine the current prevalence, biological features as well as subtype characterization of *Blastocystis* isolated from cockroaches and house geckos as well as factors associated to infection.

5.2 Materials and methods

5.2.1 Study population

Cockroaches were captured in according to the types of urban dwellings and structures (drainage system, residential homes and grocery stores) from two states in Malaysia, namely in Ipoh, Perak and Puchong, Selangor. The cockroaches were trapped using sticky traps and barehanded technique. Each specimen was transferred into a sterile container, transported to the laboratory and anaesthetised at 4°C for 10 min. The host-stage for each specimen was noted prior to dissection (Appendix B).

Meanwhile, house geckos (*Hemidactylus platyurus*) were caught at night in randomly selected households within the study area in their hiding places. They were placed into transparent plastic container with old newspaper to provide a near similar environment of their hideout in which the lid of the bucket was finely perforated for ventilation and taken to the laboratory for *Blastocystis* screening.

5.2.2 Dissection

Dissection was carried out under sterile conditions. For cockroaches, the legs and wings were removed. The sides of the abdomen were cut on either side of the anus, and the complete gut were removed posteriorly. The contents were then removed and stored in a container prior to the *in vitro* culture method.

As for house geckos, the animals were anesthetized and a ventral midline incision was made to remove the entire intestine and rectum. The intestinal walls and rectum were scraped off using a blunt knife to remove the contents and placed in a sterile container prior to the cultivation method.

5.2.3 In vitro cultivation

All samples were subjected to in vitro cultivation as described in Chapter 3.2.4.

5.2.4 Microscopy examination

The positive faecal smears were stained with 10% Giemsa and Sudan Black B stain according to procedures highlighted in Chapter 3.2.5.

5.2.5 Cytochemical staining

Cytochemical staining was conducted on selected isolates according to Chapter 3.2.6.

5.2.6 Electron microscopy

Selected day-3 positive culture samples and *Blastocystis* cyst were subjected to ultrastructural studies using scanning and transmission electron microscope as described in Chapter 3.2.7.

5.2.7 Subtyping

All positives isolates were then subjected to DNA barcoding according to Chapter 3.2.8

5.2.8 Statistical analysis

Statistical analyses were described in Chapter 3.2.10.

5.3 Results

5.3.1 Prevalence of *Blastocystis* in cockroaches and house geckos

Out of 151 cockroaches, 61 (40.4%) were positive for *Blastocystis* infection. More adult (79) were captured compared to nymph (72) stages of which, higher infection 58.2% (46/79) were observed in the nymphs compared to 20.8% (15/72) the adult stage with significant association between the parasite infection with host-stage ($\chi 2 = 21.877$, [df] = 1, P = 0.000). *Blastocystis* infection was also associated to types of dwellings ($\chi 2 = 22.987$, [df] = 3, P = 0.000) where infection was highest in cockroaches from grocery stores (62.9%) compared to residential homes (30.6%) and drainage system (20.8%). In addition, infections were all asymptomatic.

However, this study failed to detect any *Blastocystis* positive sample out of 45 house geckos using the *in vitro* cultivation method since no organisms was not present up to day 3 in culture.



Figure 5.1: The prevalence of infected cockroaches with *Blastocystis* relative to host-stage.

	Total (%)		
Drainage system	Dwelling	Grocery store	
11 (20.8)	11 (30.6)	39 (62.9)	61 (40.4)
42 (79.2)	25 (69.4)	116 (37.1)	90 (59.6)
53 (28.5)	36 (23.8)	62 (41.1)	151
	Drainage system 11 (20.8) 42 (79.2) 53 (28.5)	Drainage system Dwelling 11 (20.8) 11 (30.6) 42 (79.2) 25 (69.4) 53 (28.5) 36 (23.8)	Drainage system Dwelling Grocery store 11 (20.8) 11 (30.6) 39 (62.9) 42 (79.2) 25 (69.4) 116 (37.1) 53 (28.5) 36 (23.8) 62 (41.1)

Table 5.1: Prevalence of *Blastocystis* in cockroaches relative to sampling sites.

5.3.2 Morphological forms

The *in vitro* cultures of cockroach isolates contained mostly vacuolar forms ranging in size from 7 to 14 μ m (Figure 5.2a). On occasion, granular forms were also observed measuring between 9 to 20 μ m (Figure 5.2b) in which the granules were prominent and refractile. It was also noted that the isolates grew at both 25°C and 37°C. However, cell growth was optimal at room temperature (25°C) with higher number of cells recovered (Figure 5.3a) compared to isolates incubated at 37°C (Figure 5.3b).

5.3.3 Surface structure

Scanning electron images showed that vacuolar form isolated from cockroach possessed a smooth surface (Figure 5.4a) and some showed a more coarse and folded surface (Figure 5.4b).

5.3.4 Ultrastructural

Transmission electron micrographs of *Blastocystis* isolated from cockroach showed spherical in shape with multiple vacuolar present in the cytoplasm (Figure 5.5a). High electron dense material was observed in the central vacuole of the granular from compare to the vacuolar form with two nucleuses was seen in the *Blastocystis* cells of cockroach isolates (Figure 5.5b). Meanwhile, the cell membrane of *Blastocystis* in vacuolar form was slightly thicker as compared to granular form with the measurement of 214.31 to 248.03 nm (Figure 5.6a) and 185.88 to 222.04 nm (Figure 5.6b), respectively.



Figure 5.2: Light micrograph of *Blastocystis* in cockroach under 400× magnification.(a) Vacuolar form (b) Granular form.



Figure 5.3: *Blastocystis* in cockroach incubated at different temperature under $400 \times$ magnification (a) 25°C (b) 37°C.




Figure 5.4: Surface structure of vacuolar form of *Blastocystis* from cockroach (a) smooth surface (b) coarse and folded surface.



Figure 5.5: Electron micrograph of *Blastocystis* from cockroach (a) Vacuolar form (b) Granular form. N; nucleus, V; vacuole, CV; central vacuole, SC; surface coat, CM; cell surface membrane, CVM; central vacuole membrane.



Figure 5.6: Measurement of the surface coat from cockroach isolates (a) Vacuolar form(b) Granular form.

5.3.5 Cytochemical studies

There was a lower FITC-labeled ConA binding observed in the membrane of both vacuolar and granular forms (Figure 5.7) of *Blastocystis* on day 2 of L82 culture isolate as evidenced by a faded green florescence. The fluorescence intensity and the percentage of the reactive forms of L82 isolate in FITC-labelled Con A stain range was (1+; 90-100%).

Acridine orange is a cell-permeant nucleic acid binding dye that emits green fluorescence when bound to double stranded DNA and red fluorescence when bound to single stranded DNA or RNA. The respective epifluorescence image showed that the vacuolar form was stained dull green while the nucleus was stained bright green (Figure 5.8).





Figure 5.7: Binding affinities of *Blastocystis* stained with FITC-labelled Con A. (a) Light microscopic images *Blastocystis* of cockroach isolate. (b) A same organism stained with FITC-labeled Concanavalin A (ConA) assay. V; vacuolar form, G; granular form.



Figure 5.8: Epifluorescence image of *Blastocystis* stained with acridine orange (a) Light microscopic images *Blastocystis* of cockroach isolate (b) A same organism stained with acridine orange.

5.3.6 Subtype identification

Using the DNA barcoding method, a total of eight DNA sequences covering the first 500 base pairs (5'-end) of the *Blastocystis* small subunit (SSU) rRNA gene were obtained. It was found that two isolates were identified as ST3, one isolate was closely related to allele 114 which is most likely to be the new subtype and one isolate showed low/poor signal-to-noise ratio which probably due to too little DNA template added to sequencing reaction or primer binding to the template was not very efficient. Another four sequences were found to be fungi.

5.4 Discussion

Information on *Blastocystis* in cockroaches and house geckos is very limited. Table 5.2 summaries the previous descriptions and prevalence data on this parasite in cockroaches and house geckos.

The flat-tailed house geckos or *Hemidactylus platyurus* caught in this study showed negative result of *Blastocystis* infection. However, the only published report on *Blastocystis* in house geckos by Suresh *et al.* (1997) found that 7% infection house geckos caught from human dwellings in Kuala Lumpur, Malaysia. There were several factors that could affect *Blastocystis* infection such as habitat and diet. House geckos have less exposure to contamination since the habitat was relatively clean compared to cockroaches commonly inhabit sewers and poorer sanitation areas. Meanwhile, gecko's diets are composed mainly of insects from the order of Hemiptera, Hymenoptera and Coleoptera (Diaz Perez *et al.*, 2012) also show no evidence of *Blastocystis* in those insects. Therefore, infection via the oral route is uncommon. However, the low prevalence of *Blastocystis* infection reported by Suresh *et al.* (1997) suggest that house geckos may be transiently and opportunistically infected by whichever *Blastocystis* is present in their environment, be it from a human or non-human source. Thus far, house geckos are unlikely to act as natural host for *Blastocystis*.

As compared to the previous study, a moderate *Blastocystis* infection was recorded (40.4%) in the cockroach in this study. This is the only study with a large number of cockroaches (n=151) caught from drainage system, grocery store and dwelling.

Blastocystis infections were found associated to types of habitat. High infections were noted for cockroaches captured from grocery stores compared to residential homes

and drainage system. The abundance of starchy foods, sweet substances and meat product makes this type of habitat ideal for this nocturnal arthropod to come out from hiding such as sewers and toilets to forage. Zaman *et al.* (1993) reported infection incidences up to 80% in cockroaches and attributed the infections to type of habitats i.e. sewage tanks. Therefore, control of cockroaches can substantially minimize the spread of this parasite contaminating the environment.

In addition, significant association was observed between *Blastocystis* infection with host-stage with higher infections in nymphs compared to adults. Typically, nymphs are similar to adults, except for the absence of the wings, genitalia and also body colouring. Nymphs also differ in their feeding behaviour. According to Richter and Barwolf (1994), nymphs of *P. americana* took larger meals during the first three days postmoult. This behaviour suggests that nymphs were most highly exposed to infection while foraging for food.

The light microscopy observations exhibited general morphology similar to *Blastocystis* from cockroaches collected from sewers in Singapore (Zaman *et al.*, 1993). The cultures contained mostly vacuolar and granular forms. However, cyst-like stage and amoeboid forms were absent in this study.

The isolates grew in culture at both room temperature (24°C) and 37°C as reported previously by Zaman *et al.* (1993) however optimal growth was observed for isolates at room temperature (24°C) with an increasing number of viable cells compared to those incubated at 37°C. As previously described, some *Blastocystis* organisms in poikilothermal animals may have originated from homoiothermal hosts; *Blastocystis cycluri* isolated from an iguana and AFJ96-H1 isolated from a toad could survive at 37

°C and 34 °C, respectively, while another isolate from the same host iguana species could not (Yoshikawa *et al.*, 2004b). These lines of evidence suggested that some isolates in poikilothermal animals were originated from homoiothermal hosts, and thus these isolates were positioned within the isolates of homoiothermal hosts. Therefore, phylogenetic analysis showed that the cockroach isolates are specifically related to herptiles (reptile and amphibian) and insect *Blastocystis*.

Scanning electron microscopy showed that *Blastocystis* isolated from cockroach possess a smooth surface similar to asymptomatic human isolates as well as coarse and folded surface as seen in IBS isolates (Ragavan *et al.*, 2014).

The ultrastructural features of *Blastocystis* were observed as previously described by Yoshikawa et al. (2007) with multiple vacuolar present in the cytoplasm. However, general morphological vacuolar form was seen in Blastocystis isolated from cockroach in this study whereas the granular form contained a highly electron dense material within the central body. Besides, its surface coat seen more prominently thicker by TEM studies. It is high likely that this surface could be sticky and may influence in the adherence of *Blastocystis*. In human, the thicker surface coat shown by the ultrastructural study IBS isolates could in influence cytopathic effect of Blastocystis towards the intestinal lining of the gut (Ragavan et al., 2014).

There has been one report of the SSU rRNA sequences of *Blastocystis* isolates from cockroaches, which demonstrates that four isolates from cockroaches form a new clade that branches early within the *Blastocystis* lineage. Based on the previous study, amphibian and reptilian *Blastocystis* isolates were separately located at different

positions with an amphibian/reptilian clade (clade VIII) emerging immediately after the divergence of the cockroach clade (clade X) (Yoshikawa *et al.*, 2007).

We then employed DNA barcoding to characterize the isolates of *Blastocystis* from cockroaches. Using this method, two isolates were identified as ST3 which had not been reported in this species. ST3 is the most common subtype in humans exclusively in patients with IBS (Alfellani *et al.*, 2013; Ramírez *et al.*, 2014). Besides, it was also been reported in other animal hosts such as non-human primates (Pertráŝová *et al.*, 2011), giraffe (Alfellani *et al.*, 2013), goats (Tan *et al.*, 2013). With representation among humans, non-primates, insects and artiodactyls, ST3 clearly has a very wide host range. Meanwhile, another cockroach isolate was found to be most likely the new subtype which was closely related to allele 114.

Host	Reference/Origin	Prevalence (%)	nce (%) Morphological characteristics			
	_	/habitat	Light microscope	SEM	TEM	
Cockroach	Zaman <i>et al.</i> (1993) /Singapore	- 8/10 (80) /sewage tanks	 The morphology of all of the isolates was similar to that of <i>B</i>. <i>hominis</i>. The cultures contained mostly vacuolated forms. Size: 10 to 20 μm. Large forms measuring up to 50 gm as well as granular and amoeboid forms were also seen. 	-	<u>Cyst</u> - Rounded or ovoid and had a distinct electron-dense cyst wall; a fuzzy layer surrounded the cyst. - Its appeared to be present within a mother cell which surrounded the cyst as a filamentous layer. - Nuclei contained rounded cristae-like structures. - Several vacuoles were seen in the cytoplasm. - Endoplasmic reticulum-like structures were observed around the nuclei. - Mitochondria were not present.	-
	Suresh <i>et al</i> . (1997) /Malaysia	- 3/30 (10)/dwelling	-	-	-	-
	Present study	- 11/53 (20.8) /drainage system - 11/36 (30.6) /dwelling -39/62 (62.9) /grocery store	 Mostly vacuolar forms ranging in size from 7 to 14 μm. The granular forms were also observed measuring between 9 to 20 μm in which the granules were prominent and refractile. 	 The vacuolar form isolated from cockroach possessed a smooth surface The granular form showed a coarser and folded surface. 	 The vacuolar showed a spherical in shape with multiple vacuolar present in the cytoplasm. The cell membrane in vacuolar form was slightly thicker than granular form (214.31 to 248.03 nm). 	ST3 (allele 56) and new subtype (allele 114)

Table 5.2: List of previous publication on *Blastocystis* in cockroaches and house geckos.

Host	Reference/Origin	Prevalence (%) /habitat	Morphological characteristics			
			Light microscope	SEM	TEM	
House gecko	Suresh <i>et al</i> . (1997) /Malaysia	- 2/30 (7)	 The cells found in cultures from the intestinal contents were rounded, numerous in number and varied in size with typical peripheral nuclei characteristic. Most of the cells were granular forms. Cyst-like forms were seen with one of the cells being enclosed within an outer membrane and another displaying a highly refractile, thickened cytoplasm. 	-	 TEM studies revealed the multivacuolar from with rounded mitochondria that showed prominent cristae. One of the cells contained three large vacuoles which were bordered by a distinct membrane and had the appearance of sac-like pouches enclosing rounded granules. One of the vacuoles showed a rupture of the membrane, releasing granules to the outer central body of the parasite. 	-
	Present study	- 0	one of the cells being enclosed within an outer membrane and another displaying a highly refractile, thickened cytoplasm.		appearance of sac-like pouches enclosing rounded granules. - One of the vacuoles showed a rupture of the membrane, releasing granules to the outer central body of the parasite.	

5.5 Conclusion

Zero prevalence of *Blastocystis* infection in house geckos caught in this study may be attributed to low risk of food and water contamination as well as minimum exposure in their environment to faecal material from human and non-human hosts. Considering this prevalence, they are unlikely to be natural hosts of *Blastocystis*. Besides, this study presents the findings of *Blastocystis* infections in the common household cockroaches in Malaysia with a large number of samples and a first to characterize the subtypes infecting cockroaches in order to shed light on the transmission and distribution as well as, better understanding of the evolution and polymorphism of this organism.

CHAPTER 6:

STUDY ON Blastocystis spp. IN COMPANION ANIMALS

6.1 Introduction

Several *Blastocystis* human subtypes were isolated from companion animals (Lee *et al.*, 2012; Leelayoova *et al.*, 2013) especially cats and dogs were proposed as a potential source of *Blastocystis* infection to humans (Nagel *et al.*, 2012). Knowles and Das Gupta (1924) first reported the presence of *Blastocystis* in domestic cats in Australia and more recently high prevalence of *Blastocystis* was reported in domestic cats (67.3%) and dogs (70.8%) (Duda *et al.*, 1998). In contrast, Wang *et al.* (2013) reported low prevalence in stray dog populations in Australia (2.5%), and in Cambodia (1.3%) and India (24%). Ruaux and Stang (2014) recorded the prevalence of *Blastocystis* in dogs in shelters in the US Pacific Northwest as between 9.7-11.7%. Chuong *et al.* (1996) and Suresh *et al.* (1996) failed to detect *Blastocystis* in cat and dogs samples in Malaysia. A generally low prevalence in cats and dogs across the previous studies suggest these domestic animals are not a significant reservoir. Nevertheless, according to Parkar *et al.* (2010), *Blastocystis* sp. ST1 through to ST9 has been reported from both human isolates and non-human animals.

In culture, *Blastocystis* grow optimally at neutral pH (Stenzel and Boreham, 1996). Zhang *et al.* (2012) reported no significant changes to the reproduction capability of B. hominis under pH conditions between 7 and 8, while the short-term and in vitro culture method is reported to be optimal at pH values between 7 and 7.5. The low prevalence of *Blastocystis* in cat and dog populations is possibly due to the low gastric pH and high enzymes secretion in cat and dog gastrointestinal tracts making the environmentunsuitable for *Blastocystis* reproduction (Sutton, 2004). High acidity in the gastrointestinal system facilitates protein breakdown and kill the pathogenic bacteria often abundant in flesh foods. Both cats and dogs have the ability to secrete concentrated hydrochloric acid (HCl) (pH 1 to 2) in the presence of food. Following a meal, the average feline stomach has a pH of 2.5 (Brosey *et al.*, 2000) while the average canine stomach has a pH between 2.0 and 2.5 (Sjaastad *et al.*, 2010).

Therefore, the objectives of the present study was to determine the transmission potential of *Blastocystis* to humans via cat and dog reservoirs, specifically to determine the prevalence and subtypes of Blastocystis in cat and dog populations in Peninsular Malaysia and to assess changes in the morphology and viability of *Blastocystis* isolates when subjected to pH changes.

6.2 Materials and methods

6.2.1 Ethical approval

The ethics approval for all non-human animal subjects in this study has been obtained as described in greater detail in Chapter 3.2.1. Meanwhile, human ethical approval for this study was obtained in accordance with University Malaya Medical Centre research policy with Reference. No: 20154-1218.

6.2.2 Sampling sites

This study sites involved three selected states in Peninsular Malaysia namely; Ipoh, Perak, Penang Island and Federal Territory of Kuala Lumpur, in the west coast of Peninsular Malaysia. In Perak, sampling was carried out in Ipoh situated at Kinta District, a district in central Perak governed under the Ipoh City Council whereas in Penang Islands, sampling was carried out in Jelutong, a suburb of Georgetown and Teluk Bahang, a fishing village in the northwest corner of Penang. In Federal Territory of Kuala Lumpur, sampling was carried out in Ampang and Setapak, two metropolitan areas governed under the Kuala Lumpur City Hall. The three sites were randomly selected as the landscapes were typical in terms of geographical location and where there are presence of both stray and sheltered dogs and cats.

6.2.3 Study population

6.2.3.1 Stray cats and dog

Stray animals refer to street, alley or semi-dependent cats and dogs that may possibly receive food directly from humans also through scavenging scraps from rubbish bins, dump sites or from slaughter remains on farms. No attempt was made to house these animals and normally inhabit man-made structures such as buildings or abandoned vehicles. In this study, 10 and 38 stray cats and dogs respectively were screened for *Blastocystis*.

6.2.3.2 Sheltered cats and dogs

Sheltered animals refer to cats and dogs that are housed, fed and generally cared for by human or owner. During certain periods in the day and night they are kept free within a confined space, usually well fed and looked after. A total of 178 and 44 domestic cats and dogs from animal shelter such as SPCA (Society for the Prevention of Cruelty to Animals), Cat Beach Sanctuary, private pet shops, government and private veterinary clinics were randomly selected for *Blastocystis* screening.

6.2.4 In vitro cultivation

All samples were subjected to *in vitro* cultivation as described in Chapter 3.2.4.

6.2.5 Subtyping

All positives isolates were then subjected to DNA barcoding according to Chapter 3.2.9.

6.2.6 Viability experiment

Blastocystis isolates were obtained from fresh faecal samples of a village chicken (*Gallus gallus*) (A3) and a peafowl (*Pavo cristatus*) (M2) from a mini zoo in Perak. Three *Blastocystis* isolates (H1, H2 and H3) were obtained from patients at University of Malaya Medical Centre (UMMC). Human ethical approval for this study was obtained in accordance with University Malaya Medical Centre research policy with Reference. No: 20154-1218.

Six media (Jones' medium supplemented with 10% horse serum) with a range of acidities (pH 6, 5, 4, 3, 2, 1), adjusted by adding 01M HCl, were prepared to simulate gastrointestinal pH. A control media (pH 7) was also prepared. Approximately 1 x 10⁶ cells of day-3 culture of avian isolates (A3 and M2) and 1x 10⁵ cells of human isolates (H1, H2 and H3) were inoculated into 3 ml of the seven media. Culture tubes were prepared in triplicate for each media and subsequently incubated at 37°C. After 24 hours, the cells were counted using a haemocytometer chamber (Improved Neubauer, Hausser Scientific) with 0.5% Tryphan blue solution. The cells were evaluated for distinct morphological details, including size, shape and staining properties (indicative of viability).

6.3 Results

6.3.1 Prevalence of *Blastocystis* in cat and dog populations

Faecal samples from 188 cats (*Felis catus*) and 82 dogs (*Canis lupus*) obtained from stray and shelter-housed animals showed no presence of *Blastocystis* sp. in the *in vitro* culture medium. However, DNA barcoding of twelve cat samples (20%) were positive for *Blastocystis* with sequences assigned to ST1. Additionally, another five faint PCR bands were observed from five separate samples from cats which were considered negative or indicative of a very low parasite burden.

6.3.2 Viability of *Blastocystis* in acidic conditions

The number of viable cells observed after 24 hours was highly correlated with pH, with higher pH media (less acidic) having a greater number of viable cells (Figure 6.1). *Blastocystis* isolates of avian and human origin showed the typical round vacuolar form when cultured in media at pH 5 to pH 7 (Figure 6.2e and f). When cultured in media at pH 4, the vacuolar forms of the avian isolate *Blastocystis* exhibited a wrinkled, shrunken morphology (Figure 6.2c dan d); in contrast, the human *Blastocystis* isolates showed typical morphology at pH 4. In media at pH 3, 2 and 1 the growth of *Blastocystis* of both avian and human origin was suppressed and no viable cells were observed (Figure 6.2 a and b).



Figure 6.1: Total number of viable cells from (a) avian and (b) human isolates after 24 hours incubation.



Figure 6.2: *Blastocystis* cells of (a) avian and (b) human isolates cultured in a pH range of 1 - 4 showing the rounded structures of non-viable cells (arrow) whereas cells of (c) avian and (d) human isolates in pH4 showing non-viable vacuolar forms with a wrinkled or shrunk appearance (arrow) compared to the human isolates showing smaller viable vacuolar forms with low parasite count. Meanwhile, *Blastocystis* cells of (e)

avian and (f) human isolates in a pH range of 5 - 7 showing the typical rounded vacuolar form (arrow).

6.4 Discussion

The prevalence of *Blastocystis* infections in companion animals varies greatly worldwide, according to the available reports. High prevalence was reported in Australia (Duda *et al.*, 1998) and Iran (Daryani *et al.*, 2008), while no infections were detected in cats and dogs in Germany (König and Müller, 1997), Malaysia (Chuong *et al.*, 1996) and Japan (Abe *et al.*, 2002). Wang *et al.* (2013) suggested geographical and subtype variation in the *Blastocystis* infecting dog populations. Higher prevalence was reported in stray dog populations in India, with a greater subtype diversity, compared to Australian and Cambodian dogs (Wang *et al.*, 2013). Ruaux and Stang (2014) reported high prevalence of subtype ST10 in shelter-resident dogs in the United States indicating low transmission potential, particularly to animal handlers and owners.

The variation in the detection results for *Blastocystis* across studies reflects the sensitivity of the adopted methods (Stensvold *et al.* 2007a). Roberts *et al.* (2011) reported that light microscopy had the lowest sensitivity compared to PCR-based detection. In contrast, Wang *et al.* (2013) suggested that light microscopy may be prone to false positive detections as *Blastocystis* is a pleomorphic organism with extensive variation in morphology which may lead to misinterpretation. Leelayoova *et al.* (2002) demonstrated that xenic in vitro culture was a significantly more sensitive detection method compared to the concentration or direct smear/light microscopy technique. Duda *et al.* (1998) successfully cultured *Blastocystis* from two dog samples with inspissated egg slant medium over a short growth period but failed to culture *Blastocystis* from cat faecal samples. Presently, the medium of choice for xenic culture

of *Blastocystis* is Jones' medium supplemented with 10% horse serum (Leelayoova *et al.*, 2002; Suresh and Smith, 2004). None of the cat or dog samples obtained as part of this study was successfully cultured in this medium. A DNA barcoding method (sequencing a species specific DNA fragment) was adopted instead and successfully detected the presence of *Blastocystis* in cat samples. Despite being costly and time-consuming, this method was most sensitive compared to light microscopy and cultivation (Stensvold *et al.*, 2007a) and proved to be a useful proxy for intra-subtype diversity. Nevertheless, concerns were raised that the template for PCR amplification may have been DNA derived from dead *Blastocystis* were successfully cultured in growth medium.

Overall, the result of our field study together with those from the literature review reporting the generally low prevalence of *Blastocystis* in cats and dogs suggest these companion animals do not represent a significant reservoir capable of shedding potentially zoonotic subtypes (ST1, ST2, ST3, ST4, ST5, ST6 and ST10). Both cats and dogs were possibly transiently or opportunistically infected with diverse subtypes of *Blastocystis* through coprophagia of other hosts faeces or through drinking contaminated water.

In this study, we detected the *Blastosytis* subtype ST1 in isolates from 12 shelterhoused cats. *Blastocystis* ST1 is the most common human subtype in Europe, Thailand, Libya, Nigeria and Peninsular Malaysia (Alfellani *et al.*, 2013; Leelayoova *et al.*, 2008; Nithyamathi *et al.* 2016) and is associated with irritable bowel symptom (IBS) in patients (Alfellani *et al.*, 2013), and is also zoonotic, having been isolated from farm animals (Tan, 2008). Subtype ST1 was also the subtype predominantly detected from dogs in Australia, Cambodia and India (Wang *et al.*, 2013) and also from a cat from the United States (KJ872776) (Ruaux and Stang, 2014).

Physiologically, *Blastocystis* requires a neutral pH to grow (Stenzel and Boreham, 1996) and results from this study supported the view that pH values between 7 and 5 facilitate optimal growth of avian *Blastocystis* isolates. However, *Blastocystis* isolated from human appear to have a slightly broader optimal pH range (pH 7 to 4). In this study, a smaller number of smaller sized, yet still viable *Blastocystis* cells were observed at pH 4. These results suggest that human *Blastocystis* isolates may be more tolerant to mild acidic condition compared to *Blastocystis* isolated from birds, due to the low pH levels in human digestive tract (Kararli, 1995).

In this study, in vitro cultivation using Jones' medium, the standard and universal medium for *Blastocystis* culture, failed to culture *Blastocystis* from cat and dog samples. It is postulated that viable cells or cysts of *Blastocystis* were not present in the faecal samples of the cat and dogs due to the extreme acidic (pH 1, 2 and 3) condition of the cat and dog gastrointestinal tract, which suppressed the growth of the parasite. This hypothesis is supported by the results of our viability experiment described above.

The low pH in the stomach of cats and dogs is attributable to gastric juice, the digestive fluid produced by parietal cells (also called oxyntic cells). Gastric juice comprises concentrated hydrochloric acid (HCl) as high as 0.1 M, potassium chloride (KCl) and sodium chloride (NaCl). This fluid is responsible for the initial steps of protein digestion; protein denaturation and the activation of the first proteolytic enzyme, pepsin, as well as inactivation of ingested microorganisms such as bacteria (Persson, 2008).

In contrast, a bird's stomach is well differentiated with the gastric acid secretion occurring only in the proventriculus and is responsible for protein denaturation and the activation of pepsinogen to pepsin. According to Rynsburger (2009), there is a linearly decline from 5.20 - 3.37 and 3.49 - 3.27 in pH respectively, in the proventriculus and gizzard of an ageing broiler chicken. However, it is also noted that poultry feed (i.e. oilseed meals, soybean and wheat) may also effect the pH on both organs.

Host	Reference/Origin	Origin Prevalence (%)	Morphological characteristics			Genotypes
			Light microscope	SEM	TEM	_
Canine	Chuong <i>et al.</i> (1996) /Malaysia	- 0 : Dog	-	-	-	-
	König and Müller (1997) /Germany	- 0 : Dog	-	-	-	-
	Duda <i>et al.</i> (1998) /Australia	- 51/72 (70.8) : Dog	 The cells were irregular in shape. Size: 3 to 10 μm in diameter (average 4.5 μm). The cells appeared as the vacuolar form: a thin outer rim of cytoplasm, containing barely discernible organelles, surrounded a central vacuole of varying morphology. 	-	 Blastocystis sp. from canine isolates appeared morphologically similar to cultured isolates of <i>B. hominis</i>. In the shorter term cultures, the cells had a thick surface coat up to 0.3 μm, often with adherent bacteria. The nuclei were rounded or slightly elongated and were approximately 1 to 2 μm in diameter. The nuclei commonly contained a crescentic band of electron opaque material and, sometimes, an additional 'spot' of electron opaque material. 	-
	Abe et al. (2002)/Japan	- 0/54 (0) : Pet dog	-	-	-	

Table 6.1: List of previous publication on *Blastocystis* in cats and dogs.

Host	Reference/Origin	Prevalence (%)	Morphological characteristics			Genotypes
			Light microscope	SEM	TEM	
Canine	Daryani <i>et al.</i> (2008) /Iran	- 14/50 (28) : Dog	-	-	-	-
	Wang <i>et al.</i> (2013) /Australia	- 2/80 (2.5) : Pet and pound dog (Australia)	-	-	-	ST1
		- 1/80 (1.3) : Semi- domesticated dog (Cambodia)	-	-	-	ST2
		- 19/80 (24) : Stray dog (India)	-	-	-	ST 1, ST4, ST5 and ST6
	Ruaux and Stang (2014) /USA	- 10/103 (9.7) : Shelter-resident canines	-	-	-	ST1 and ST10
	Present study	- 0/71 (0) : Dogs from the sheltered and stray population	-	-	-	-
Feline	Knowles and Gupta (1924) /India	- Infected with Blastocystis (Not mentioned)	-	-	-	-

Host	Reference/Origin	Prevalence (%)	Morphological characteristics			Genotypes
			Light microscope	SEM	TEM	
Feline	Duda <i>et al.</i> (1998) /Australia	- (67.3) : Cat				
	Ruaux and Stang (2014) /USA	- 12/103 (11.65) : shelter-resident felines	-	-	-	ST1, ST3 and ST10
	Present study	- 0/130 (0) : Cats from the sheltered and stray population.	-	-	-	-

6.5 Conclusion

The viability of *Blastocystis* isolates in culture is pH-dependent with suppression of growth at low pH while a further increase in pH towards neutral conditions significantly increases the number of viable cells. It is proposed that both cats and dogs in Peninsular Malaysia were clear of *Blastocystis* infection, and the low prevalence found in cats and dogs in global reports, is attributable to the high acidity of cat and dog gastrointestinal tracts making them uninhabitable for the growth of *Blastocystis*. Therefore, they are unlikely to be natural hosts of *Blastocystis*.

CHAPTER 7:

FINAL DISCUSSION AND CONCLUSION

7.1 Final Discussion

Numerous studies on *Blastocystis* have been carried out in Malaysia however, mostly on human infections (Tan and Suresh, 2006a, b; Chandramathi *et al.*, 2010; Ithoi *et al.*, 2011). There are limited studies in a range of animal hosts including laboratory animals, sheep, rabbits, monkeys, dogs and cats however, only in a small numbers (Suresh *et al.*, 1996). Therefore, in order to better understand the zoonotic nature of this pathogen in animals a study was conducted to determine the prevalence, phenotypic, ultrastructural, and molecular studies of *Blastocystis* in animals hosts that were in close association to humans and their surroundings particularly; companion animals (cats and dogs), household pests (rats, house gecko and cockroaches) and poultry population as no data is available. Epidemiological studies on *Blastocystis* sp. among these animal hosts are important as it will provide a better understanding on the role of these animals as possible potential for zoonotic transmission.

The findings of this study now provide proof that chickens, wild rats and cockroaches were the natural hosts of this parasite as the infections remained asymptomatic in these animal hosts in addition to be infected with the human subtypes. The possibility of these animal hosts contaminating the environment, food and water sources with *Blastocystis* spp. posed a public health risk as these animals live in close association with humans.

Results of the first epidemiological survey of *Blastocystis* in broiler chickens showed surprisingly high infections despite being raised in an intensive barn system known for clean and hygienic environment which was less exposed to contamination. It was unclear the sources of contamination, however one plausible explanation could be the conditions the animals were housed and sources of food and water. Lee and Stenzel (1999) investigated an establishment with high-quality hygiene and sanitary conditions resulted with no positive birds for this protozoan parasite indicating good hygiene practices contributed to better health maintenance of the birds.

Cats and dogs were not the natural host for this organism. No presence of infection was detected from faeces and *in vitro* cultivation, in both the local sheltered and stray population. However, DNA barcoding of twelve cat samples (20%) were positive for *Blastocystis* with sequences assigned to ST1. The result prompted a pH medium study to understand the nature of gastric pH on the influenced *Blastocystis* infection in gastrointestinal tract of carnivorous animals. The viability of *Blastocystis* isolates in culture is pH-dependent with suppression of growth at low pH while a further increase in pH towards neutral conditions significantly increases the number of viable cells. It is proposed that both cats and dogs in Peninsular Malaysia were clear of *Blastocystis* infection, and the low prevalence found in cats and dogs in global reports, is attributable to the high acidity of cat and dog gastrointestinal tracts making them uninhabitable for the growth of *Blastocystis*. Both cats and dogs were possibly transiently or opportunistically infected with subtypes of *Blastocystis* through coprophagia of other hosts faeces or through drinking contaminated water.

Similarly, no *Blastocystis* infection was found from *in vitro* cultivation and faeces of house geckos. This could be attributed to low risk of food and water contamination as

well as minimum exposure in their environment. Therefore, they are unlikely to be natural host of *Blastocystis* However, further molecular and larger scale studies should be carried out to obtain clearer epidemiological information on *Blastocystis* in these reptilians.

Microscopic examination revealed vacuolar and granular forms were most commonly observed with the multi-vacuolar rarely seen. While, the amoeboid forms were absent in the animal culture medium. Vacuolar forms in cultures from chicken isolates were the largest ranging between 10 to 100 μ m, followed by isolates from wild rats (4 to 45 μ m) and cockroaches (9 to 15 μ m) in diameter (Table 7.1). Otherwise, the general morphology appeared similar to *B. hominis*. As these morphological characteristics were in accord with the general features of *Blastocystis*, it was difficult to differentiate the newly isolated *Blastocystis* organisms from human and animals.

This study also reports the first comparative description of *Blastocystis* directly from the infected caecum of wild rats and with forms *in vitro* culture. The findings showed distinct morphological differences with the elongated vacoular forms reverted to become more rounded after prolonged cultures which concurred with Stenzel *et al.* (1994) that culture conditions may alter the actual morphology of this organism. However, this unique morphology was not observed in other animal isolates apart from rats.

Furthermore, the surface analysis of vacoular forms isolated from chickens, wild rats and cockroaches when cultured showed similarity to the surface structure of human isolates with a smooth and undulating cell surface with some organisms showing a coarser and folded surface without the small projections seen in the fresh forms. According to Zaman *et al.* (1999), the surface coat enables the parasite to adhere to the intestinal epithelial of the gut. Also, it has been suggested that the surface coat acted in protection against osmotic shock (Mehlhorn, 1988).

Ultrastructure description of *Blastocystis* isolated from animal hosts is scarce compared to human isolates. Therefore, this study was carried out to elucidate the ultrastructure of *Blastocystis* isolated from chicken, wild rat and cockroach. Among the interesting findings were the variation in surface coat thickness and electron density varied between host's isolates. In addition, the central vacuole showed considerable variation in its contents, ranging from completely electron-lucent to electron-opaque with fully distended vacuoles. Occasionally high electron dense areas were observed in the parasites isolated from the chicken, wild rat and cockroach samples. The electron dense material responded to the dark staining by Sudan Black B indicating the presence of lipids. Zierdt and Williams (1974) postulated that the highly electron dense material were granules to form the granular form and suggested the central body probably acted as an energy storage for cell growth. A surface coat was present on all isolates with thickness varying in sizes. The surface coat was thickest in the cockroach isolate followed by chicken isolate. Only a thin surface coat surrounded the parasite isolated from wild rat. Although the results suggested that basic morphology of Blastocystis organisms isolated from different animal host were similar however, several morphological variations were observed within those isolates.

The animal host isolates also differed in the optimal growth temperature and was indicative of their origins. However, the cockroach isolates grew in culture at both room temperature (24°C) and 37°C as a result of the origin of this organism in poikilothermal animals was originally from homoiothermal hosts. *Blastocystis cycluri* isolated from an

iguana and AFJ96-H1 isolated from a toad survived both at 37 °C and 34 °C, respectively, while another isolate from the same host iguana species failed (Yoshikawa *et al.*, 2004b). These lines of evidence suggested that some isolates in poikilothermal animals could have originated from homoiothermal hosts, which eventually adapted to homoiothermal hosts. Therefore, phylogenetic analysis showed that the cockroach isolates are specifically related to herptiles (reptile and amphibian) and insect *Blastocystis*.

Using molecular identification, high diversity of *Blastocystis* subtypes was recorded in the local poultry population. The presence of five different subtypes were noted namely; ST1, ST6, ST7 and ST8. ST6 and 7 all of which were considered as avian subtypes (Stensvold *et al.*, 2009) however, was also found for the first time in humans in Thailand (Jantermtor *et al.*, 2013). However, these subtypes were rarely reported in Asia.

This study also highlighted two zoonotic subtypes from wild rat population with ST4 predominantly circulating in the population followed by ST1. As previously described, rodents are known reservoir host of ST4 (Noël *et al.*, 2003, 2005; Silberman *et al.*, 1996; Yoshikawa *et al.*, 1998). Interestingly, the findings of this study also reported for the first time the occurrence of ST5 and ST7 in wild rat isolates in Peninsular Malaysia. It is therefore very likely that continued sampling will uncover additional new hosts for existing subtypes.

There has been one report of the SSU rRNA sequences of *Blastocystis* isolates from cockroaches, which demonstrates that four isolates from cockroaches form a new clade that branches early within the *Blastocystis* lineage (Yoshikawa *et al.*, 2007). Based on

the previous study, amphibian and reptilian *Blastocystis* isolates were separately located at different positions with an amphibian/reptilian clade (clade VIII) emerging immediately after the divergence of the cockroach clade (clade X). However, the present study demonstrated for the first time that cockroaches shared the same subtype as those usually been in humans which was ST3.

Blastocystis is ubiquitous and known to infect a wide array of animals especially livestock or domestic animals. Figure 7.1 shows variable subtypes distribution of *Blastocystis* subtypes in both humans and animals surrounding the environment. Domesticated mammals such as pigs, cattles, goats, and poultry shared the same subtypes as in humans (ST1 to ST4) (Parkar *et al.*, 2010, Stensvold, 2013) whilst subtypes from cattle and goats (ST10) rarely in humans. This study is the first to provide evidence supporting the zoonotic potential of *Blastocystis* in cats, dogs, wild rats, chickens and cockroaches in Peninsular Malaysia.

Tan *et al.* (2013) revealed the presence of *Blastocystis* ST1, ST3, ST6 and ST7 in the local goat population with similar human subtypes in particularly the local communities (Tan *et al.* 2008, 2009). Notably, ST6 and ST7, avian subtypes (Stensvold *et al.*, 2009) were also found infecting goats and therefore can be postulated that both chickens and goats are reservoir for human infections as *Blastocystis* exhibited low host specificity.

Sequence and phylogenetic analyses of partial ssu rDNA of *Blastocystis* from a human, pig and horse by Thathaisong *et al.* (2003) revealed a common subgroup which demonstrated isolates from the pig and horse were monophyletic and closely related to the *Blastocystis* isolated from humans, with 92 to 94% identity. Therefore, *Blastocystis* isolates from domestic animals are closely linked to humans.
Animals may act as reservoirs for *Blastocystis* and facilitate zoonotic transmission from animals to the community particularly among animal handlers working in zoos/slaughter houses/wet markets or even consumers. Li *et al.* (2007a) reported pig ownership was a risk factor for *Blastocystis* in humans in China similar subtype ST5 infecting 16 pigs, as well three humans living in the same rural area (Yan *et al.*, 2007). However, the potential risks can be minimised with proper farm hygiene as well as pest control and eradication measures carried out by the local municipals. As *Blastocystis* infection is fast becoming a common feature in humans, it is also crucial to screen animals especially poultry and livestocks in order to maintain good hygiene during processing meat and meat products to eliminate the risk of infection to humans.



Figure 7.1: Subtypes classification characterised from animal *Blastocystis* isolates.

^{*} Subtypes described by ¹Tan *et al.* (2013), ²Stensvold *et al.* (2009), ³Abe *et al.* (2003b), ⁴Yoshikawa *et al.* (2004a), ⁵Thathaisong *et al.* (2003), ⁶Parkar *et al.* (2007), ⁷Noël *et al.* (2003), ⁸Arisue *et al.* (2003), ⁹Hess *et al.* (2006), ¹⁰Wang *et al.* (2013), ¹¹Ruaux and Stang, ¹²Present study and ¹³Suresh *et al.* 1997.

Table 7.1: Summary on comparison *Blastocystis* isolates from human and study animals in terms of morphological, morphometry and genotype

characteristics.

	Human (Tan <i>et al.</i> , 2006; 2008)	Chicken	Wild rat Cockroach		House Gecko	Cat and dog
Morphological characteristics	Rounded shape with a large vacuole, immotile	Similar to <i>B. hominis</i> , except for the variation	Eliptical vacuolar forms similar to day 1 cultured forms but after	Similar to <i>B. hominis</i> with small size in		
Vacoular forms	Vacoular forms and surrounded by a thin peripheral band of cytoplasm.		prolonged cultures, rounded vacuolar forms were predominantly seen	ranged.		
Granular forms	Rounded shape with a large vacuole, immotile, surrounded by a thin peripheral band of cytoplasm and presence of inclusion bodies or granules in the central vacuole.	Occasionally seen in chicken isolates, similar to the vacuolar forms except for the present of numerous granules.	Similar to <i>B. hominis</i>	Similar to the vacuolar forms in which the granules were prominent and refractile.		
Ameoboid forms	Irregular shape with prominent nucleus at the central zone and multiple extended pseudopodia at the periphery, immotile.	Not seen.	Similar to <i>B. hominis</i> but rarely seen.	Not seen.		
Size range						
Vacoular form	5 µm - 90 µm	10 μm - 100 μm	4 μm - 45 μm	7 µm - 14 µm		

	Human (Tan <i>et al.</i> , 2006: 2008)	Chicken	Wild rat	Cockroach	House Gecko	Cat and dog
Granular forms	10 μm - 35 μm	10 μm - 30 μm	5 μm - 15 μm	9μm - 20 μm		
Ameoboid forms	5 µm - 65µm	Not seen.	10 µm - 20 µm	Not seen.		
Biochemical analysis Acridine orange staining : vacuolar forms	Nucleus: bright green fluorescence	Nucleus: bright green fluorescence Central body: dull green	Nucleus: bright green fluorescence Central body: dull green	Nucleus: bright green fluorescence Central body: dull green		
: granular forms	Nucleus: yellow to orange Central body: yellow to orange	Nucleus: bright green fluorescence Central body: bright green fluorescence	Nucleus: bright green fluorescence Central body: bright green	Nucleus: bright green fluorescence Central body: bright green		
FITC-labeled ConA : vacoular forms	Nil	Dull green florescence	Dull green florescence	Dull green florescence		
: granular forms	granular forms Nil Dull green florescence		Dull green florescence Dull green florescence			
Surface level analysis Surface structures	nalysis Rounded to spherical in Appeared spherical in Spherical and smooth shape and had a smooth shape with slightly ures surface with tiny pores. surface coat with rough surface. undulating cell surface. undulating cell surface. undulating cell surface. undulating cell surface.		Vacuolar form: smooth surface. Granular form: coarser and folded surface.			

	Human (Tan <i>et al.</i> , 2006; 2008)	Chicken	Wild rat	Cockroach	House Gecko	Cat and dog
Ultrastructural analysis Surface coat thickness	Thin membrane (184.70 to 208.72 nm)	Thick and compact membrane (237.74 to 342.63 nm)	Thin membrane (135.51 to 196.82 nm)	Slightly thicker membrane (214.31 to 248.03 nm)		
Central vacuole	Clear	10% of the cells contain electron-dense material.	Clear	Clear		
Localization of lipid (Sudan stain)	Negative reaction (No staining was observed in the central vacuole)	10% of the cells in portions shows positive reactions (Dark droplets were observed in the central vacuole)	Negative reaction (No staining was observed in the central vacuole)	Negative reaction (No staining was observed in the central vacuole)		
Molecular Genotyping	ST1 – ST9	ST1, 6, 7 and 8	ST1, 4, 5 and 7	ST15, ST17 and <i>Blastocystis</i> C2 isolate (Clade X)		



Nil (Negative for *Blastocystis* sp. infection)

7.2 Final conclusion

1. The present study is the first to elucidate the current status, ultrastructural and subtype of *Blastocystis* sp. isolated from the local poultry population. The finding highlighted zoonotic implication to humans especially among animal handlers in the chicken farming community. Furthermore, this study also recorded multiple *Blastocystis* subtypes in the chicken isolates.

(Farah Haziqah, M.T., Mohd Zain, S.N., Suresh, K., Chandrawathani, P. and Mohd Khalid, M.K.N. (2017). Diversity of *Blastocystis* subtypes in broiler and village chicken population in Peninsular Malaysia. Manuscript in preparation).

2. The present study represents the first study to elucidate the epidermiology, phenotypic, ultrastructural and subtype characteristics of *Blastocystis* sp. in urban wild rodents. The finding has important implications as *Blastocystis* ST4 and pathogenic subtype; ST1 was recorded for the first time from the local wild rat population. The importance of understanding *Blastocystis* sp. in the environment is crucial because rodents found in close contact with humans and source infection to humans. Therefore, rodent control must be carried out by the local municipals in order to prevent rodent borne diseases.

(Farah Haziqah M.T., Mohd Zain S.N., Suresh, K.G., Panchadcharam, C., Bathmanaban P. & Rajamanikam A. (2016). **ST1, 4, 5 and 7 in wild rodents – Possible potential for zoonotic transmission?** *Parasites and Vector*. Submitted)

3. This study presents the findings of *Blastocystis* infections in *P. americana*, the common household cockroach in Malaysia and a first to characterize the subtypes infecting cockroaches namely, the newly discovered subtype, ST15 and ST17. The study shed light on the transmission and distribution as well as, better understanding of the evolution and polymorphism of this organism.

(Farah Haziqah M.T., Mohd Zain S.N., Suresh, K.G. & Mohd Nasri N.A. (2016). **Current status of** *Blastocystis* in cockroaches *Tropical Biomedicine*. Submitted)

- 4. House geckos were free from infection and could be attributed to their diet and low risk of food and water contamination as well as minimum exposure in their environment to faecal material from human and non-human hosts. As a result, house geckos were unlikely to be the animal reservoir of *Blastocystis* sp. However, further molecular and larger scale studies would provide clearer picture of the epidemiology of *Blastocystis* in house geckos.
- 5. Faecal samples from cats and dogs from both shelter-housed and stray populations were screened for *Blastocystis* using culture/microscopy and DNA barcoding. In addition, we investigated the viability of human and avian *Blastocystis* isolates cultured across a range of pH conditions. DNA barcoding detected *Blastocystis* ST1 in 12 feline samples, but cultivation was unsuccessful from all cat and dog samples. The cultures of human and avian isolates showed an increase in the number of viable cells as the pH conditions changed from acidic to neutral. The results provide evidence that the low pH in the intestinal tract of cats and dogs create conditions unsuitable for

Blastocystis and therefore these animals are unlikely to be natural hosts of *Blastocystis*.

(Farah Haziqah Meor Termizi, Chandrawathani Panchadcharam, Benacer Douadi, Suresh Kumar Govind, John-James Wilson, Mohd Khairul Nizam Mohd Khalid, Arutchelvan Rajamanikam, John W. Lewis, Siti Nursheena Mohd Zain. (2017). **Cats and dogs: Viability and predominant of** *Blastocystis* **sp. ST1 in acidic stomachs** *Veterinary Parasitology*. Submitted)

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COMPILATIONS OF PUBLISHED PAPERS

PUBLICATIONS:

- Farah haziqah M.T., Chandrawathani P., Mohd Zain S.N., Suresh Kumar G., Hemalatha C. & Premalaatha B. (2014). A Preliminary Study of *Blastocystis* sp. from Chicken in Perak and Selangor, Malaysia. *Malaysian Journal of Veterinary Research* 5 (1): 21-25.
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- Farah Haziqah M.T., Mohd Zain S.N., Suresh K., Chandrawathani P., Erwanas A.I., & Nanthiney D.R. (19-20 March 2013). A preliminary study of intestinal protozoan parasites of cats and dogs from Kuala Lumpur and Ipoh, Perak. 49th Annual Scientific Seminar Malaysian Society of Parasitology and Tropical Medicine. Grand Seasons Hotel Kuala Lumpur.
- Farah Haziqah M.T., Chandrawathani P., Mohd Zain S.N., Suresh Kumar G., Hemalatha C. & Premaalatha B. (13-15 September 2013). A preliminary study of *Blastocystis* sp. isolated from chicken in Perak and Selangor, Malaysia. 25th Veterinary Association Malaysia (VAM) Congress 2013. Lotus Desaru Beach Resort Kota Tinggi, Johor.
- 3. Farah Haziqah M.T., Chandrawathani P., Mohd Zain S.N., Suresh K., Sabapathy D., Hemalatha C. & Premaalatha B. (5-7 March 2014) A survey of *Blastocystis* sp. in various animals from wildlife park. 6th ASEAN Congress of Tropical Medicine & Parasitology (ACTMP). Intercontinental Kuala Lumpur, Malaysia.
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- 9. Farah Haziqah M.T., Mohd Zain S. N., Suresh Kumar G. & Chandrawathani P. (3rd & 4th March 2015). Electron microscopy study of *Blastocystis* sp. isolates from human, poultry, wild rat and cockroach. 51th Annual Seminar Scientific Conference of the Malaysian Society of Parasitology and Tropical Medicine. Grand Seasons Hotel, Kuala Lumpur.

- 10. Farah Haziqah M.T., Mohd Zain S.N., Suresh Kumar G., Chandrawathani P. & Premaalatha B. (3rd & 4th March 2015). A preliminary study of *Blastocystis* sp. in cockroach population in Malaysia. 51th Annual Seminar Scientific Conference of the Malaysian Society of Parasitology and Tropical Medicine. Grand Seasons Hotel, Kuala Lumpur.
- 11. Farah Haziqah M.T., Mohd Zain S.N., Suresh Kumar G., Chandrawathani P., Premaalatha B., Tan P.S. & Hemalatha, C. (23rd - 27th March 2015). A neglected zoonotic protozoan: *Blastocystis* sp. from wild rat population. 6th Pan Commonwealth Veterinary Conference & 27th VAM Congress 2015. The Royale Chulan Hotel, Kuala Lumpur.

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- Farah Haziqah M.T., Mohd Zain S.N., Suresh Kumar G. & Chandrawathani P. (May 2013). A preliminary study of *Blastocystis* sp. from chicken in Perak and Selangor, Malaysia. Monthly Meeting of Research & Development (R&D). Veterinary Research Institute, Ipoh, Malaysia.
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- Farah Haziqah M.T., Mohd Zain S.N., Suresh Kumar G. & Chandrawathani P. (April 2014). Detection of *Blastocystis* sp. in companion animals. Monthly Meeting of Research & Development (R&D). Veterinary Research Institute, Ipoh, Malaysia.

- Farah Haziqah M.T., Mohd Zain S.N., Suresh Kumar G. & Chandrawathani P. (December 2014). *Blastocystis* sp. in cockroaches (Dictyoptera: Blattidae). Monthly Meeting of Research & Development (R&D). Veterinary Research Institute, Ipoh, Malaysia.
- 5. Farah Haziqah M.T., Mohd Zain S.N., Suresh Kumar G. & Chandrawathani P. (January 2015). Isolation, detection and identification of *Blastocystis* sp. in companion animals, household pests and poultry population. Postgraduate Seminar. Institute of Biological Sciences, Faculty of Science, University of Malaya.

APPENDIX A

Modified Jones' Medium

Stock solution

1.233 g of sodium hydrogen phosphate (Na₂HPO₄)

0.397 g of potassium hydrogen phosphate (KH₂PO₄)

960 ml of distilled water

7.087 g of sodium chloride (NaCl)

- Discard 12.5 ml of the solution
- Add 100 ml of 1% yeast extract (100 ml water + 1g yeast) into the solution
- Autoclave at 121°C for 15 minutes
- The medium was then supplemented with 10% of horse serum (Heat inactivated at 56°C for 30 min)
- Transfer into 3 ml tubes
- Store in the chiller

APPENDIX B



Posterior abdominal segments of cockroaches

- (a) Male dorsal view
- (b) Male ventral view
- (c) Female dorsal view
- (d) Female ventral view

(Source: http://biology4isc.weebly.com)

APPENDIX C

Summary of morphological characteristics of *Blastocystis* isolated from village

chicken (A3) and peacock (M2) grown in Jones' medium with different pH

		Village chi	cken isolates	Peacock isolates		
pН	Morphological	Average	Cells	Average	Cells	
	characteristics	size range	viability	size range	viability	
1	Non–viable vacuolar	5.0µm -		4.0µm -		
	forms with the typical	10.0µm		8.0µm		
2	characteristics seen.	5.0µm -	Not viable	4.0µm -	Not viable	
		10.0µm		8.0µm		
3		4.0µm -		4.0µm -		
		8.0µm		8.0µm		
4	Non-viable vacuolar	4.0µm -		4.0µm -		
	forms appeared shrunk	8.0µm		8.0µm		
	with less perfectly					
	rounded to ovoid in					
	shape.					
5	Smaller vacuolar forms	5.0µm -	17.6 x	5.0µm -	12.8 x	
	with low parasite	10.0µm	10 ⁷ cells/ml	10.0µm	10 ⁷ cells/ml	
	numbers were seen.					
6	Vacuolar forms	5.0µm -	22.0 x	5.0µm -	38.4 x	
	predominated with no	10.0µm	10 ⁷ cells/ml	20.0µm	10 ⁷ cells/ml	
	significant changes					
	noted.					
7	Large rounded	10.0µm -	24.0 x	5.0µm -	41.6 x	
	vacuolar form	20.0µm	10 ⁷ cells/ml	10.0µm	10 ⁷ cells/ml	
	surrounded by a thin					
	peripheral band of					
	cytoplasm.					

APPENDIX D

Summary of morphological characteristics of *Blastocystis* isolated from human

		Isolates H1		Isolates H2		Isolates H3	
pН	Morphological	Average	Cells	Average	Cells	Average	Cells
	characteristics	size	viability	size	viability	size	viability
		range		range		range	
1	Non-viable	10.0µm	Not	10.0µm	Not	20.0µm	Not
	vacuolar forms	-	viable	-	viable	-	viable
	with the typical	50.0µm		20.0µm		30.0µm	
2	characteristics	10.0µm		10.0µm		20.0µm	
	seen.	-		-		-	
		50.0µm		20.0µm		30.0µm	
3		10.0µm		10.0µm		20.0µm	
		-		-		-	
		50.0µm		20.0µm		30.0µm	
4	Smaller vacuolar	20.0µm	3×10^5	20.0µm	$7 \ge 10^5$	20.0µm	2×10^5
	forms with low	-	cells/ml	-	cells/ml	-	cells/ml
	parasite numbers	70.0µm		30.0µm		40.0µm	
	were seen.						
5	Large rounded	30.0µm	5×10^5	20.0µm	13×10^5	20.0µm	4×10^{5}
	and viable	-	cells/ml	-	cells/ml	_	cells/ml
	vacuolar form	80.0µm		30.0µm		40.0µm	
6	surrounded by a	30.0µm	$6 \ge 10^5$	20.0µm	31×10^5	20.0µm	4×10^5
	thin peripheral	-	cells/ml	-	cells/ml	-	cells/ml
	band of	80.0µm		50.0µm		40.0µm	
7	cytoplasm.	30.0µm	7×10^5	20.0µm	94 x 10 ⁵	20.0µm	$1 \ge 10^5$
		-	cells/ml	-	cells/ml	-	cells/ml
		80.0µm		50.0µm		40.0µm	

(H1, H2 and H3) grown in Jones' medium with different pH