

**STUDIES ON PREVALENCE OF *BLASTOCYSTIS* SP.
IN ANIMALS AND EXPERIMENTAL INFECTION *IN-
VIVO***

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ABSTRACT

Blastocystis sp. is an enteric protozoan organism, found in the intestinal tract of a wide range of animals and humans. The parasite is often incriminated to cause symptoms such as diarrhoea, abdominal pain, bloating and flatulence. Many publications have highlighted the high prevalence of *Blastocystis* sp. in various animal hosts, however, only very few studies have been conducted in Malaysia. Therefore, this study aimed to screen a range of animals in Malaysia for *Blastocystis* sp. infection which could be a reservoir for human infection when in close association. High prevalence of *Blastocystis* sp. were seen in the ostriches and pigs with 100% positive respectively and for the first time, subtype 6 *Blastocystis* sp. was seen in ostrich isolates. Previous findings have described on either cysts or vacuolar forms, however, these are confined to limited animals. This study has provided evidence that the parasite's thick cyst wall is primarily responsible for causing the infection in rats and the thickness of the cyst wall corresponded to the number of days taken to cause the infection. This is the first study to establish a schematic drawing which provides a key-like guidance to differentiate the morphology of the vacuolar and cystic forms of *Blastocystis* sp. isolated from animals which can provide information for source tracking. The worldwide distribution and increase in the infection rate demonstrate the zoonotic potential and the parasite's low host-specificity. The present study also suggests that *Blastocystis* sp. exhibits low host specificity and the possibility of human to animal cross-infectivity cannot be ruled out. Despite studies reporting on the ability of *Blastocystis* sp. to be invasive, there have been no studies to assess the histopathological changes in rats infected with *Blastocystis* ST3 isolated from symptomatic and asymptomatic patients. The gross changes in the histopathology and elevated level of

serum amylase in symptomatic rats despite being infected with the same subtype from symptomatic and asymptomatic human isolates further confirm the pathogenic role of *Blastocystis* sp. Very few studies have described the transformational details during the excystation of *Blastocystis* sp. cysts. To date, there has been no comparison on the excystation rates and transformational changes between parasites from symptomatic and asymptomatic isolates. This present study demonstrated marked differences in the excystation process between *Blastocystis* sp. from asymptomatic and symptomatic isolates. The present study has shown that extensive mucosal sloughing occurred in the intestines of rats infected with asymptomatic isolate which revealed that *Blastocystis* sp. excysts and proliferate soon after infection compared to symptomatic group. This finding concurred with previous studies that showed proliferation to be the highest in *in-vitro* cultures of the asymptomatic isolates between days 3 and 6. Results also showed that rapid excystation and proliferation in asymptomatic isolates may cause heightened symptoms immediately to the host once infected.

ABSTRAK

Blastocystis sp. merupakan protozoa yang terdapat dalam saluran usus pelbagai haiwan dan manusia. Parasit ini sering disabitkan menyebabkan gejala seperti cirit-birit, sakit abdomen, kembung dan flatulens. Banyak penerbitan telah menekankan prevalens *Blastocystis* sp. yang tinggi dalam pelbagai haiwan, walaubagaimanapun, hanya sedikit kajian telah dijalankan di Malaysia. Oleh itu, kajian ini bertujuan untuk menyaring jangkitan *Blastocystis* sp. di kalangan pelbagai haiwan di Malaysia yang boleh menjadi sarang jangkitan kepada manusia apabila terdapat hubungan rapat di rantau. Prevalens *Blastocystis* sp. yang tinggi telah dilihat dalam burung unta dan babi dengan masing-masing 100% positif dan buat kali pertama, subjenis 6 *Blastocystis* sp. dilihat dalam burung unta. Penemuan sebelum ini telah menghuraikan tentang bentuk sista atau vacuolar yang hanya terdapat dalam haiwan yang terhad. Kajian ini telah mengemukakan bukti bahawa dinding sista yang tebal terutamanya bertanggungjawab menyebabkan jangkitan pada tikus dan ketebalan dinding sista itu dapat dikaitkan dengan bilangan hari yang diambil untuk menyebabkan jangkitan ini. Ini adalah kajian pertama menunjukkan ilustrasi skematik yang boleh digunakan sebagai panduan utama bagi membezakan morfologi bentuk vakuolar dan sista daripada pelbagai haiwan yang boleh memberi maklumat untuk mengesan sumber. Taburan di seluruh dunia dan peningkatan kadar jangkitan menunjukkan potensi zoonotik dan spesifisiti perumah yang rendah dalam parasit ini. Kajian ini juga menunjukkan bahawa *Blastocystis* sp. mempamerkan spesifisiti perumah yang rendah dan kemungkinan infektiviti silang daripada manusia kepada haiwan tidak boleh diketepikan. Walaupun kajian melaporkan keupayaan *Blastocystis* sp. menjadi invasif, bagaimanapun, tiada penerbitan yang menilai perubahan histopatologi pada tikus yang dijangkiti dengan *Blastocystis* sp. ST3

yang dipencilkan daripada pesakit simptomatik dan asimptomatik. Perubahan kasar dalam histopatologi dan tahap tinggi amilase dalam serum tikus daripada kumpulan simptomatik walaupun dijangkiti dengan subjenis yang sama dari isolat simptomatik dan asimptomatik mengesahkan lagi peranan patogenik *Blastocystis* sp. Hanya sedikit kajian telah menghuraikan transformasi terperinci semasa eksistensi *Blastocystis* sp. ke dalam bentuk vakuloar secara *in-vitro*. Setakat ini, tidak ada perbandingan mengenai kadar eksistensi dan perubahan transformasi antara parasit daripada isolat simptomatik dan asimptomatik. Kajian ini menunjukkan perbezaan yang ketara dalam proses eksistensi *Blastocystis* sp. daripada isolat simptomatik dan asimptomatik. Kajian ini telah menunjukkan bahawa pengeluaran mukosa yang banyak di dalam usus tikus yang telah dijangkiti isolat asimptomatik mendedahkan bahawa sel-sel *Blastocystis* sp. membahagi dengan lebih cepat ke dalam bentuk vakuolar berbanding dengan *Blastocystis* sp. daripada isolat simptomatik. Penemuan ini pernah dilaporkan oleh kajian sebelumnya, yang menunjukkan bahawa proliferasi sel-sel *Blastocystis* sp. daripada isolat asimptomatik adalah yang tertinggi antara hari 3 dan 6 berdasarkan kajian *in-vitro*. Keputusan juga menunjukkan bahawa eksistensi *Blastocystis* sp. yang pantas dan proliferasi dalam isolat asimptomatik boleh menyebabkan simptom awal dilihat dengan lebih segera dalam perumah sebaik dijangkiti.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
°	Degree
μl	Microliter
μm	Micrometer
AIDS	Acquired immune deficiency syndrome
AP-PCR	Arbitrarily-primed PCR
ATP	Adenosine triphosphate
BALB	Bagg Albino
bp	Base pair
C	Celsius
cDNA	Complementary DNA
dH ₂ O	Distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
g	Gram
hr	Hour
kDa	Kilo Dalton
mg	Milligram
min	Minute
ml	Milliliter
MLO	Mitochondrion-like-organelle
mRNA	Messenger ribonucleic acid
MTZ	Metronidazole

nm	Nanometer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RNA	Ribonucleic acid
rpm	Revolutions per minute
sec	Second
SEM	Scanning electron microscopy
sp	Species
SSU_rRNA	Small sub-unit ribosomal ribonucleic acid
ST	Subtype
STS	Sequence-tagged site
Taq	<i>Thermus aquaticus</i>
TEM	Transmission electron microscopy
TMP-SMX	Trimethoprim/sulfamethoxazole
α	Alpha

CHAPTER 1

INTRODUCTION

1.0 Introduction

1.1 Research background

Blastocystis sp. is an anaerobic enteric protozoan which belongs to the phylum stramenophile (Arisue et al., 2002; Ciferri & Redaelli, 1938). This organism is globally reported in humans who can be symptomatic or asymptomatic (Scanlan, 2012) and in a wide range of animals such as mammals (Boreham & Stenzel, 1993), reptiles (Teow et al., 1992), birds (Yoshikawa et al., 2004a) and insects (Zaman et al., 1993). This parasite is also known as a polymorphic organism with four commonly described forms which are the vacuolar, granular, amoeboid and cystic forms (Stenzel & Boreham, 1996). The cyst, being the infective stage, is mainly responsible for causing the infection (Leelayoova et al., 2002). Symptoms which are often associated with *Blastocystis* infection are gastrointestinal symptoms such as diarrhoea, abdominal pain, vomiting, anorexia and flatulence. However, more severe symptoms such as intestinal inflammation, altered bowel habits, lethargy, chronic diarrhoea and death were observed in animals with heavy infection (Boreham & Stenzel, 1993; Stenzel & Boreham, 1996; Tan et al., 2002).

Studies have shown that *Blastocystis* sp. has a worldwide distribution and is found to have a high prevalence in developing countries especially in the tropics and subtropics (Ashford & Atkinson, 1992; Zierdt et al., 1995). Yoshikawa et al., 2004b have emphasised that the high prevalence of this organism among children and adults could be due to low personal hygiene and improper sanitation system especially in lower economic groups. Besides that, consumption of contaminated water and food as well as close contact with animals facilitates the transmission of this parasite. Earlier

studies have suggested a few modes of transmission which includes faecal-oral route, waterborne, food borne and sexual transmission (Nimri & Batchoun., 1994; Casemore, 1990; Garavelli et al., 1989; Llibre et al., 1989). *Blastocystis* sp. was also isolated from various animals including pigs, cows, primates, dogs, cats, birds and cockroaches. The prevalence varies among different animal species and their geographical location (Parkar et al., 2007; Wang et al., 2014; Yoshikawa, 2012a). Meanwhile, in Malaysia, only two studies have shown *Blastocystis* sp. in primates (Lim et al., 2008) and goats (Tan et al., 2012). Thus, there is a need to screen a range of animals in Malaysia for *Blastocystis* sp. infection.

In the past, some authors revealed that cystic forms of *Blastocystis* sp. from non-human hosts are morphologically similar to the human cysts (Mehlhorn, 1988; Stenzel & Boreham 1991; Zaman et al., 1999), nevertheless, some have described the ultrastructural differences among the cystic and vacuolar forms of *Blastocystis* sp. from animal hosts such as pigs (Cassidy et al., 1994), monkeys (Cassidy et al., 1994; Stenzel et al., 1996) and chickens (Cassidy et al., 1994; Stenzel et al., 1996; Lee & Stenzel, 1999). Whilst there have been reports of *Blastocystis* sp. in various animal hosts there has been no consolidated description of these vacuolar and cystic stages isolated from them.

The worldwide distribution and increase in the infection rate demonstrate the zoonotic potential and the parasite's low host-specificity. Numerous studies have described the ubiquitous nature of *Blastocystis* sp. (Parija & Jeremiah, 2013) and that it is one of the most frequently isolated organism from stool examinations carried out worldwide. High prevalence of this parasite in humans especially animal handlers and pet lovers (Horiki et al., 1997; Salim et al., 1999; Tan et al., 2012) and the ability of cyst

from humans to infect experimental rats (Moe et al., 1997; Yoshikawa et al., 2004a; Chandramathi et al., 2012) explains further its zoonotic feature. Till this date, there has been no study on cross infectivity with cysts from a range of animal hosts, therefore it is crucial that we better understand the transmission possibility between humans and animals.

Blastocystis sp. is a genetically diverse parasite with 17 distinct subtypes (ST1-ST17) seen in humans, non-human primates, other mammals and birds (Noel et al., 2005; Stensvold et al., 2007; Scanlan & Pauline, 2012). Recent studies have shown that subtype variability has been reported to play a crucial role in the pathogenicity of *Blastocystis* sp. in which subtype 3 is mainly highlighted to cause more pathogenic effects during the course of infection (Tan et al., 2008; Santos et al., 2014). The pathogenicity of *Blastocystis* sp. still remains a matter of debate with conflicting reports on the pathogenic role of the organism as this parasite is seen to be present in patients with gastrointestinal symptoms as well as healthy individuals (Tan et al., 2002; Stensvold et al., 2012). Another study by Tan et al., (2008) further confirmed the suspicion when *Blastocystis* sp. isolated from asymptomatic individuals and symptomatic patients were found to be phenotypically different despite being infected with the same subtype.

Invasion and inflammation of the intestine due to parasitic infections including *Blastocystis* sp. have been shown previously (Moe et al., 1997; Puthia et al., 2008; Chandramathi et al., 2010). On the other hand, Hussein et al., (2008) have shown moderate to severe degrees of pathological changes in rats infected with symptomatic subtypes while mild degree in asymptomatic subtypes infected rats. Despite studies reporting on the ability of *Blastocystis* sp. to be invasive, however, none have assessed

the histopathological changes in rats infected with *Blastocystis* ST3 isolated from symptomatic and asymptomatic patients. Hence, the question whether *Blastocystis* sp. subtype 3 isolated from symptomatic and asymptomatic persons can influence changes at the biochemical and haematological levels and further cause histological changes in the hosts has yet to be answered.

Suresh et al., (1994) have shown the ultrastructural changes during encystation process. They further suggested that ameobic forms were the intermediary forms between vacuolar and cystic forms. Meanwhile, very few studies have described the transformational details during the excystation of *Blastocystis* sp. cysts (Moe et al., 1999; Chen et al., 1999). To date, there has been no comparison on the excystation rates and transformational changes between parasites from symptomatic and asymptomatic isolates. Therefore, it is salient to describe the development of cystic forms isolated from both symptomatic and asymptomatic patients of subtype 3 into vacuolar forms at several time points.

1.2 Justification of the study

There has been no comprehensive study to assess the prevalence of *Blastocystis* sp. among pets, domestic and zoo animals in Malaysia. This information is vital to assess the consequence of the parasite on the host as well as to establish the possibility of transmission to humans. There is also a need to compare and contrast the distinct ultrastructural characteristics of cystic and vacuolar forms from a wide range of animals. These descriptions will not only be important to provide greater insights on the morphological diversity of the various vacuolar and cystic forms from animal isolates but useful for source tracking especially when these life cycle stages are isolated from aquatic sources. There has been no study to assess if *Blastocystis* sp. cysts from animal hosts can cause experimental infection in rats. This will facilitate the understanding of the transmission patterns of *Blastocystis* sp. between humans and animals.

There has been no study to assess if subtype 3 isolated from symptomatic and asymptomatic patients can exert different influence in infected rats. The gross changes in the histopathology and biochemistry in rats despite being infected with the same subtype will further confirm the pathogenic role of *Blastocystis* sp. The *in-vivo* effects of the parasite cannot be gauged if transformational events between the cyst and the vacuolar forms cannot be ascertained. Hence, there is a need to study the transformational changes during excystation so that we can correlate the histopathological and biochemical events during infections.

1.3 Objectives of the study

- i. To determine the prevalence of *Blastocystis* sp. infection among pets, domestic, and zoo animals.
- ii. To describe distinct ultrastructural characteristics of cystic and vacuolar forms from animal isolates.
- iii. To carry out cross-infectivity studies of *Blastocystis* sp. isolated from animal hosts into rats.
- iv. To carry out *in-vivo* experimental studies using symptomatic and asymptomatic human isolates of subtype 3:
 - a.) by assessing changes at the biochemical, haematological and histopathological levels.
 - b.) by assessing transformational changes during excystation of cysts of *Blastocystis* sp.

1.4 Research hypothesis

- i. Surface coat thickness in the cyst stage of *Blastocystis* sp. does play a role in influencing the prevalence of infection in animals.
- ii. Cross infection and speed of causing experimental infection in rats is dependent on the thickness of the surface coat of the cystic forms that cause the infection.
- iii. *Blastocystis* sp. cysts from various animals have low host specificity.
- iv. There can be gross changes in the histopathology and biochemistry of experimentally infected rats despite being infected with the same subtype from the symptomatic and asymptomatic human isolates.
- v. Rapid excystation and proliferation in asymptomatic isolates causes heightened symptoms immediately to the host once infected.

CHAPTER 2

LITERATURE REVIEW

2.1 Parasitic infection

Parasitic infections, one of the major problems in tropical and sub-tropical regions especially in the regions of Sub-Saharan Africa, Asia and Latin America and the Caribbean, can cause serious risk of morbidity and death (Harhay et al., 2010). Studies have shown that communities from developing and third world countries are more vulnerable to parasitic infections especially the immigrants and travelers who return from endemic countries (Crompton & Savioli, 1993). Intestinal parasites can be largely categorized into two different groups namely helminths and protozoa that reside in the intestines of both humans and animals (Norhayati et al., 2002). In recent years, reports have shown that protozoan parasite have increased and gained equal importance to helminths. These organisms are transmitted via contaminated water or food through the faecal oral route. Generally, intestinal protozoan parasites can cause non-specific gastrointestinal symptoms such as diarrhoea, nausea, abdominal pain, bloating, flatulence, malnutrition and inflammation of intestine. Currently, the common protozoan parasitic infections are giardiasis, caused by *Giardia duodenalis*, amoebiasis, caused by *Entamoeba histolytica* and cryptosporidiosis, caused by *Cryptosporidium parvum*. Other protozoan parasites such as *Blastocystis* sp., *Isospora* sp. and *Dientamoeba fragilis* have also been implicated to cause gastrointestinal diseases in immunocompromised patients and children (Norhayati et al., 2002). Reports have suggested that *Blastocystis* sp. is the most frequently observed parasite in stool examination though its pathogenicity still remains a disputable issue (Scanlan, 2012).

2.2 *Blastocystis* sp.

Blastocystis sp. is known to be a common enteric protozoan parasite of humans and many animals (Stenzel et al., 1996; Tan, 2004). This organism is anaerobic, polymorphic and a genetically heterogeneous protist (Arisue et al., 2002). It exhibits distinct morphological features with extensive genetic variation among isolates from both humans and animals. Since the early 1900s, this unusual protist has been an enigma to taxonomists, microbiologists and clinicians (Brumpt., 1912). The earlier studies only focused more on the morphological aspects of *Blastocystis* sp. while other biological aspects such as taxonomy, lifecycle, mode of reproduction and pathogenicity of this parasite were yet to be established. Over the years, several *in vivo*, *in vitro* and also numerous epidemiological studies incriminated *Blastocystis* sp. to be a pathogenic parasite (Windsor et al., 2002).

2.3 Taxonomy

The taxonomic classification of *Blastocystis* sp. has proven to be challenging and has long been an unsolved puzzle for taxonomists, microbiologists and clinicians as various observations have been made over the years. *Blastocystis* sp. was regarded previously as annular or cholera bodies by Brittan and Swayne while studying the infamous cholera epidemic of London in 1849. In the early 1900s, *Blastocystis* was proposed to be harmless saprophytic yeast of the intestinal tract. The genus name “*Blastocystis*” was coined by Alexeieff while Brumpt provided the species name, “*hominis*” (Alexeieff, 1911; Brumpt, 1912). Subsequently, Zierdt and colleagues reclassified this unicellular organism as a protist based on a number of protistan features such as one or more nuclei, smooth and rough endoplasmic reticulum, Golgi bodies,

mitochondrion-like organelles, failure to grow on fungal media and resistant to antifungal drugs but susceptible to the antiprotozoal drugs such as metronidazole (Flagyl) and emetine (Zierdt., 1991; Zierdt et al., 1988). In 1988, *Blastocystis hominis* was classified in the subphylum Sporozoa, class Blastocysta and order Blastocystida based upon the methods of division, morphological and culture characteristics by Zierdt. Subsequently *B.hominis* was reclassified to the subphylum Sarcodina (Zierdt et al., 1988). In the year 1989, molecular sequencing studies were carried out by Johnson et al., 1989. In this study, a single axenic human isolate (Netsky stock) were carried out by small-subunit rRNA sequencing techniques and the results revealed that *Blastocystis hominis* is not monophyletic with the yeast *Saccharomyces* neither with any of the sarcodines or sporozoans. A study concluded that *Blastocystis hominis* should not be assigned to the subphylum Sarcodina nor placed in the Apicomplexa, but proposed a new classification as follows (Jiang and He, 1993):

Kingdom	:	Protista
Subphylum	:	Blastocysta
Class	:	Blastocystea
Order	:	Blastocystida
Family	:	Blastocystidae
Genus	:	<i>Blastocystis</i>
Species	:	<i>hominis</i>

The subsequent molecular analysis of *Blastocystis* small sub-unit ribosomal RNA (SSU rRNA) and elongation factor 1 α (EF-1 α) gene sequences resulted in distinct

conclusion on its taxonomic and phylogenetic affiliations. Silberman et al., (1996) sequenced the entire *Blastocystis* SSU rRNA gene and showed that *Blastocystis* could be placed within the stramenopiles and closely related to *Proteromonas* (Silberman et al., 1996). The stramenopiles were defined as a complex heterogeneous group that includes both unicellular and multicellular protists whose flagella bear tripartite tubular hairs (Patterson, 1989). The phylum Heterokontophyta commonly called as heterokonts or stramenophiles comprising of algae, diatoms, slime moulds and oomycetes. *Blastocystis* forms the newer member of this complex group of “botanical Protists”. Stramenopiles have flagella surrounded by lateral hair which is absent in *Blastocystis*. Therefore, there is a proposal to revise the current five kingdom classification and place this organism in a separate sixth kingdom named “Chromista”. With the advent of molecular tools, analysis of multiple conserved gene sequences of *Blastocystis* sp. including SSU rRNA, cytosolic-type70-kDa heat shock protein, translation elongation factor 2, and the non-catalytic “B” subunit of vacuolar ATPase clearly demonstrated that *Blastocystis* is a stramenopile (Arisue et al., 2002).

The species name for *Blastocystis* sp. was previously derived based on the host such as *Blastocystis hominis* from humans (Carbajal et al., 1997), *Blastocystis ratti* from rats (Chen et al., 1997a), *B. lemuri* from primates (Krylov & Belova, 1996), *B. galli* from chickens (Belova & Kostenko, 1989), *B. anatis* from domestic ducks (Belova, 1991), and *B. lapemi* from a sea snake (Teow et al., 1992). But it was later deduced that *Blastocystis* was isolated from diverse hosts and also animal-human transmission. So the practice of assigning *Blastocystis* species according to host origin resulted in confusing reports regarding variations in pathogenesis and cell biology, since these differences could be attributed to distinct genotypes. Thus, the host specificity and the pathogenic potential of different isolates were correlated with sequence variations in the

SSU rRNA. Based on these variations, *Blastocystis* was classified into several subtypes (ST), which could possibly be termed as species (Noel et al., 2005). More recently, the small sub-unit rDNA (SSU-rDNA) was found to have a better correlation with ST and 17 STs have been reported till date (Stensvold, 2013). In the present study the nomenclature will be standardized by using STs.

2.4 Biology

2.4.1 Morphology

Blastocystis sp. is a polymorphic organism when observed under light microscopy and exists as vacuolar, granular, amoeboid and cystic forms (Figure 2.1) (Stenzel & Boreham, 1996; Tan, 2008). Similar morphological forms of *Blastocystis* sp. isolated from a wide range of hosts such as humans, mammals, reptiles, birds and insects, continuously steered researchers to use molecular methods in differentiating isolates (Yamada & Yoshikawa, 2012).

2.4.1.1 Vacuolar forms

Vacuolar forms of *Blastocystis* sp. are typical *Blastocystis* cell forms which are commonly found in *in vitro* cultures. Vacuolar forms are spherical in shape with a large central vacuole occupying 90% of the cell's volume with a thin peripheral band of cytoplasm surrounding it. The sizes can vary greatly ranging from 2 to 200 μm in diameter with an average diameter between 4 and 15 μm (Stenzel and Boreham, 1996). These cells are usually surrounded by a slimy layer or capsule which is known as the surface coat (Zierdt & Tan, 1976). Coated pits are also seen on the plasma membrane and appear to have function in endocytosis (Stenzel et al., 1989). The central vacuole

contains fine granular or flocculent material of varying electron density which is reported to be made up of carbohydrate or lipid (Yoshikawa et al., 1995a; Yoshikawa et al., 1995b) and appears to function as storage. Nucleus and organelles such as mitochondria, Golgi apparatus and endoplasmic reticulum reside within the peripheral band of cytoplasm (Boreham and Stenzel 1993). A surface coat of varying thickness and morphology surrounding most vacuolar forms of *Blastocystis* is reported to become thin gradually when grown in *in vitro* culture for a number of days. It was postulated that the possible role of the surface coat was to aid in the adherence of bacteria for nutrition purposes (Stenzel et al., 1991).

2.4.1.2 Granular forms

The granular forms are 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. There are three types of granules identified namely as metabolic, reproductive and lipid granules. The size of the granular forms ranges from 15 to 25 μm and the largest is reported to be 80 μm in diameter (Zierdt, 1973; Zierdt & Williams, 1974). These forms were postulated to arise from the vacuolar forms in some culture conditions such as when there was an increased serum concentrations in the culture medium, axenization of the culture and also addition of some antibiotics (Stenzel and Boreham, 1996). The granules were reported to be myelin-like inclusions, small vesicles, crystalline granules and lipid droplets (Dunn et al., 1989). Reproductive granules have been suggested to play a role in schizogony-like division to produce viable progenies of *Blastocystis* sp. (Suresh et al., 1994b; Zierdt, 1991; Zierdt et al., 1967). However, this suggestion has been disputed by other researchers due to lack of

evidence and the only plausible mode of reproduction continues to be binary fission (Singh et al., 1995; Suresh et al., 1994).

2.4.1.3 Amoeboid forms

Amoeboid forms are rarely seen compared to the vacuolar and granular forms in *in vitro* culture of *Blastocystis* sp. These forms are usually observed in older cultures, cultures treated with antibiotics and occasionally in faecal samples (Zierdt, 1973). It has been suggested that amoeboid forms of *Blastocystis* sp. could be the intermediate form between the cyst and vacuolar form (Singh et al., 1995; Suresh et al., 1994). Most amoeboid forms are irregular in shape with a size range from 10-15µm in diameter (Tan & Zierdt, 1973) and possess two large pseudopods without a cell membrane (Zierdt & Tan, 1976), few Golgi bodies, mitochondria and endoplasmic reticulum (Tan et al., 2001). Several studies have suggested that these forms are commonly observed in symptomatic patients with diarrhoea and associated with protease activities which possess a higher pathogenic potential by further exacerbating the gastrointestinal symptoms in the host (Tan et al., 2006 and Zhang et al., 2012).

2.4.1.4 Cystic forms

Cystic forms are usually found in the fresh faecal and occasionally in laboratory cultures (Stenzel & Boreham., 1991). Cystic stage with a thick cyst wall is believed to be responsible in transmitting this parasite to a wide range of hosts (Abou & Negm, 2001). They are spherical to ovoid in shape surrounded by a thick and multi-layered cyst wall and smaller than the vacuolar and the granular forms. The size of cystic form isolated from humans usually ranges from 3-6µm in diameter while cystic forms

isolated from animal hosts are relatively larger than humans (Stenzel et al., 1996 & Zaman et al., 1995a). These forms contain condensed cytoplasm consisting of many mitochondria and small vacuoles made of glycogen or lipids. The cysts isolated from humans are commonly bi-nucleated; however, the number of nuclei can vary from one to four (Stenzel & Boreham, 1991). The unique characteristic of cystic forms is the presence of a thick multi-layered cyst wall which allows the parasite to withstand non-conducive environment (Moe et al., 1997). The cysts are also reported to be an infective stage, which develop into vacuolar forms (excystation) upon entering a suitable host (Moe et al., 1999 & Yoshikawa et al., 2004). Meanwhile, *in vitro* encystation of *Blastocystis* have been described to produce cystic stage which is able to withstand hypotonic shock (Villar et al., 1997) and cause experimental infection in laboratory rats via oral inoculation (Suresh et al., 1995).

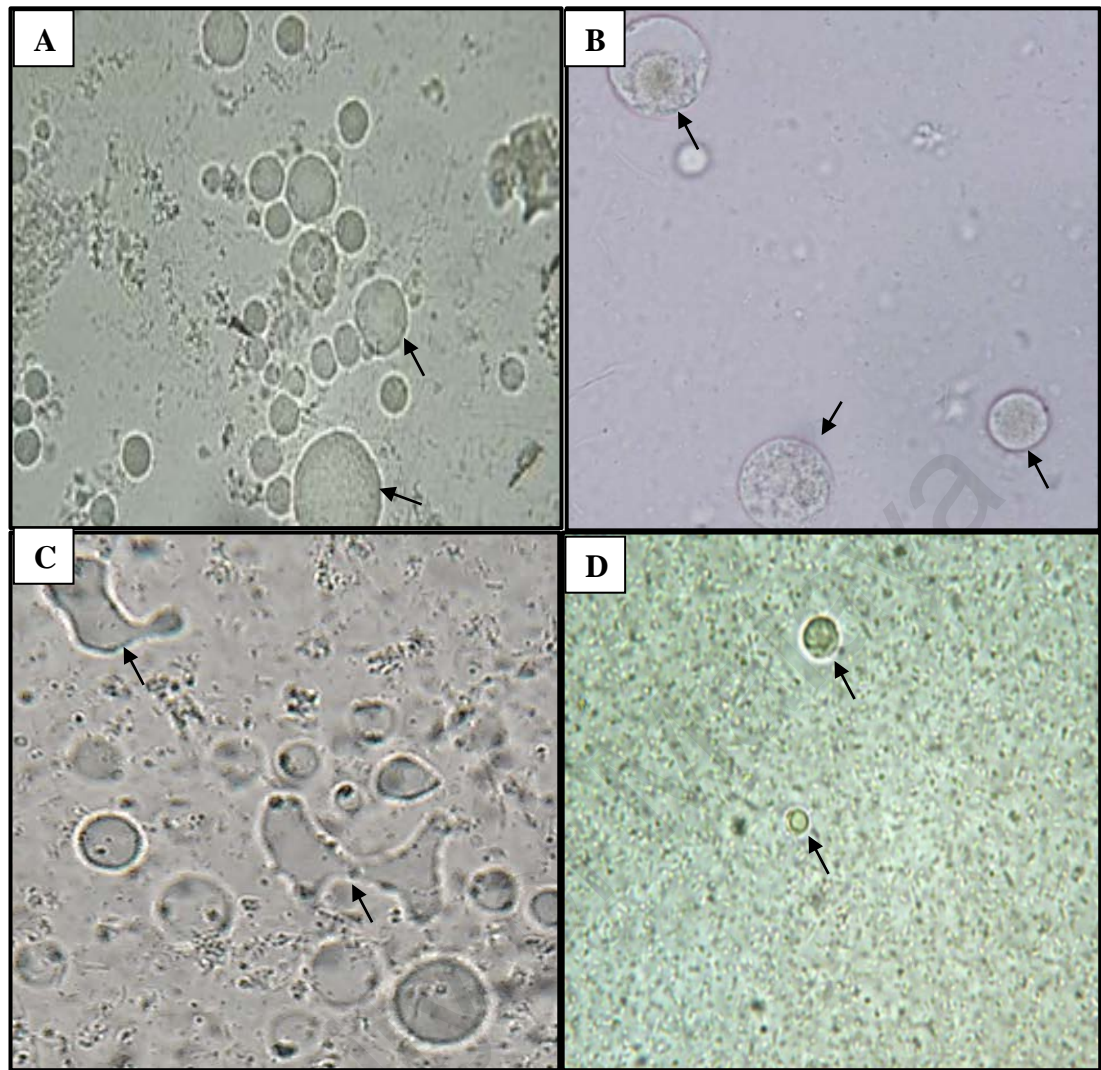


Figure 2.1: Morphologies and life cycle stages of *Blastocystis* sp. Illustration of wet preparation stool culture of *Blastocystis* sp. (A) Vacuolar forms, (B) Granular forms with distinct granular inclusions within the central vacuole (arrows), (C) Amoebic forms with pseudopod-like cytoplasmic extensions (arrows) and (D) Cystic forms with a refractile appearance and loose outer coat of the cysts (arrows) seen under light microscopy at x400.

2.4.2 Life cycle

Numerous studies have proposed various life cycles for *Blastocystis hominis*. However, there are many inconsistencies concerning the life cycle and modes of division of *Blastocystis* sp. due to a lack of a suitable animal model (Tan et al., 2002). The life cycle of *Blastocystis* sp. incorporates all four forms of this parasite which include amoebic, cystic, granular and vacuolar. The first life cycle for *Blastocystis hominis* was reported by Alexeiff (1911) which involves binary fission of a bi-nucleate stage to form primary cyst and autogamy, a sexual phenomenon (Fig 2.2). However, the reliability of this life cycle was questioned by other researchers (Ciferri and Redaelli, 1938). Later in 1973, Zierdt proposed a new life cycle based on light microscopy where the vacuolar form was seen to differentiate into either the granular form, which subsequently released vacuolar daughter cells from the central body, or to the amoeboid form which produced prospective vacuolar daughter cells by plasmotomy process (Fig 2.3).

Twenty three years later, another new life cycle was proposed by Boreham and Stenzel (1993). This life cycle suggested that the predominant form present in the colon of humans appeared to be a small non-vacuolated cell without a surface coat. As the non-vacuolated form passed through the colon, the small vesicles coalesced to form the multi-vacuolar form seen in faeces. This form had a thick surface coat, which was sloughed off once a cyst wall had formed underneath. The resultant cyst was hypothesized to be resistant to external environmental conditions and was the dominant infective stage of the life cycle. Meanwhile, the amoeboid form arose from the non-vacuolated form. The vacuolar form was suggested to arise in culture from the multivacuolar form by coalescence in culture (Fig 2.4). Another life cycle proposed by

Singh et al (1995) was based on ultrastructural changes seen in the parasites during an *in-vivo* encystment. In this study, thick and thin walled cysts were suggested to be responsible for external and internal transmission respectively. This life cycle incorporates both the suggested schizogony-like mode of reproduction as well as the accepted binary fission (Fig 2.5) (Singh et al., 1995).

A revised life cycle proposed by Tan (2004) incorporates the involvement of animals (Moe et al., 1997) and phylogenetic analysis (Arisue et al., 2002; Noel et al., 2003) which implicate *Blastocystis* sp. as a zoonotic organism (Figure 2.6). The mode of transmission of cysts in humans and animals is via faecal-oral route. Faecal-oral route is the most accepted pathway for the transmission of *Blastocystis* sp. in humans and animals. The cysts will excyst into vacuolar form and reproduce through binary fission (Tan, 2004). He further updated the life cycle taking into account zoonotic genotypes of *Blastocystis* sp. (subtypes 1 to 7) with various host specificities (Figure 2.7) (Tan, 2008). The proposed life cycle suggested that human infection can take place when seven or more species (or STs) of *Blastocystis* from animals are transmitted to humans. Most recently, Parija and Jeremiah (2013) proposed a life cycle, incorporating the excystation and encystation of *Blastocystis* (Figure 2.8). In the colon, the cysts will excyst into vacuolar forms (Moe et al., 1997) which will then develop into amoeboid, avacuolar, granular and multi-vacuolar forms. These forms have been suggested to play plausible role in the pathogenesis of the parasite (Zhang et al., 2012). Later, the vacuolar forms in the intestinal lumen encyst to form cysts which are released in the faeces and causing further transmission.

All the proposed life cycles of *Blastocystis* have proven that human and animal infection occurs through faecal cysts via faecal-oral route. There are many ongoing

studies carried out to elucidate the role of amoeboid, avacuolar, granular and multi-vacuolar forms of *Blastocystis* sp. and their association to pathogenicity as well as molecular studies to establish the possibility of zoonotic transmission from animals to humans.

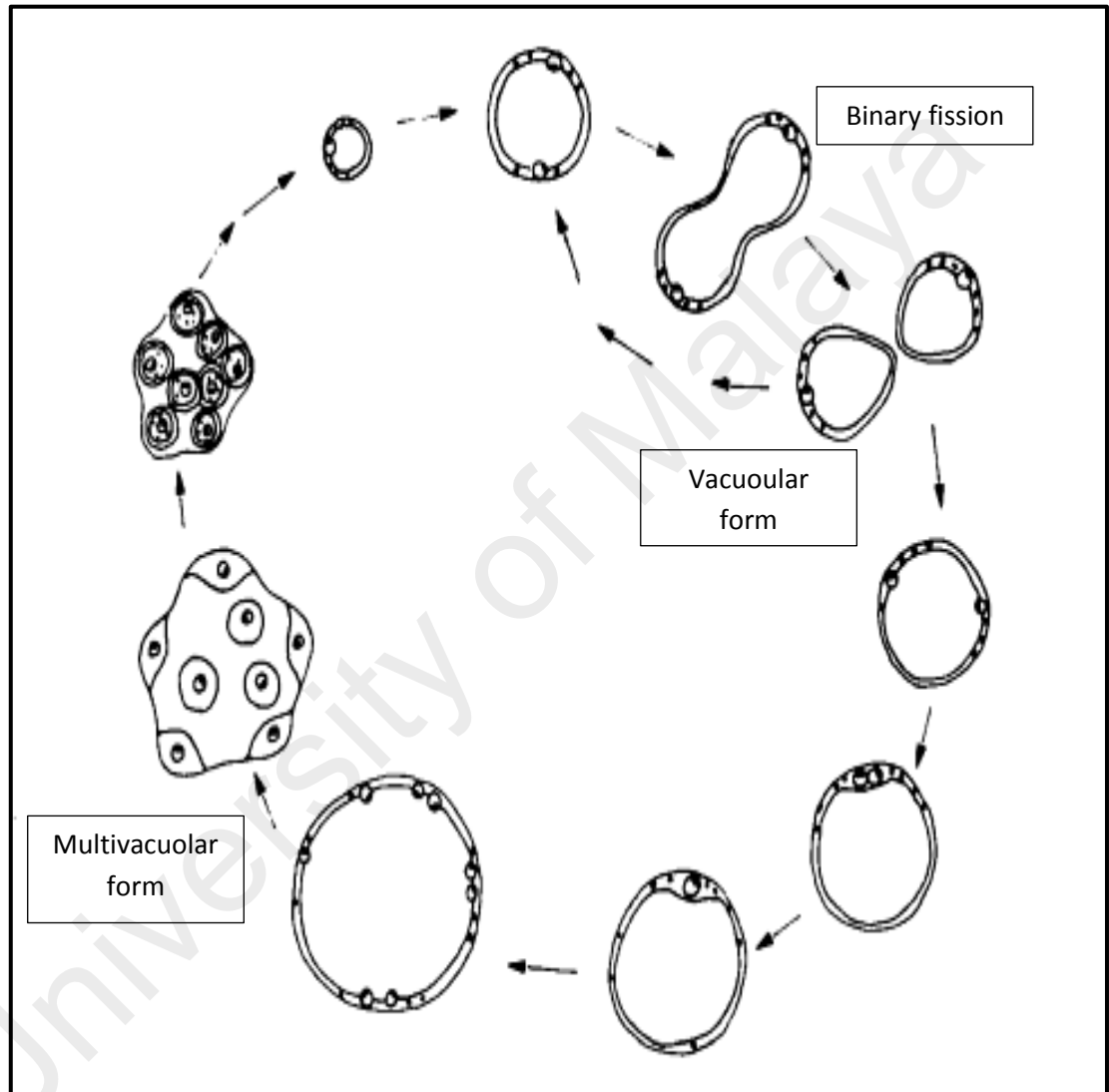


Figure 2.2 Life cycle of *Blastocystis* sp. as proposed by Alexeieff (1911)

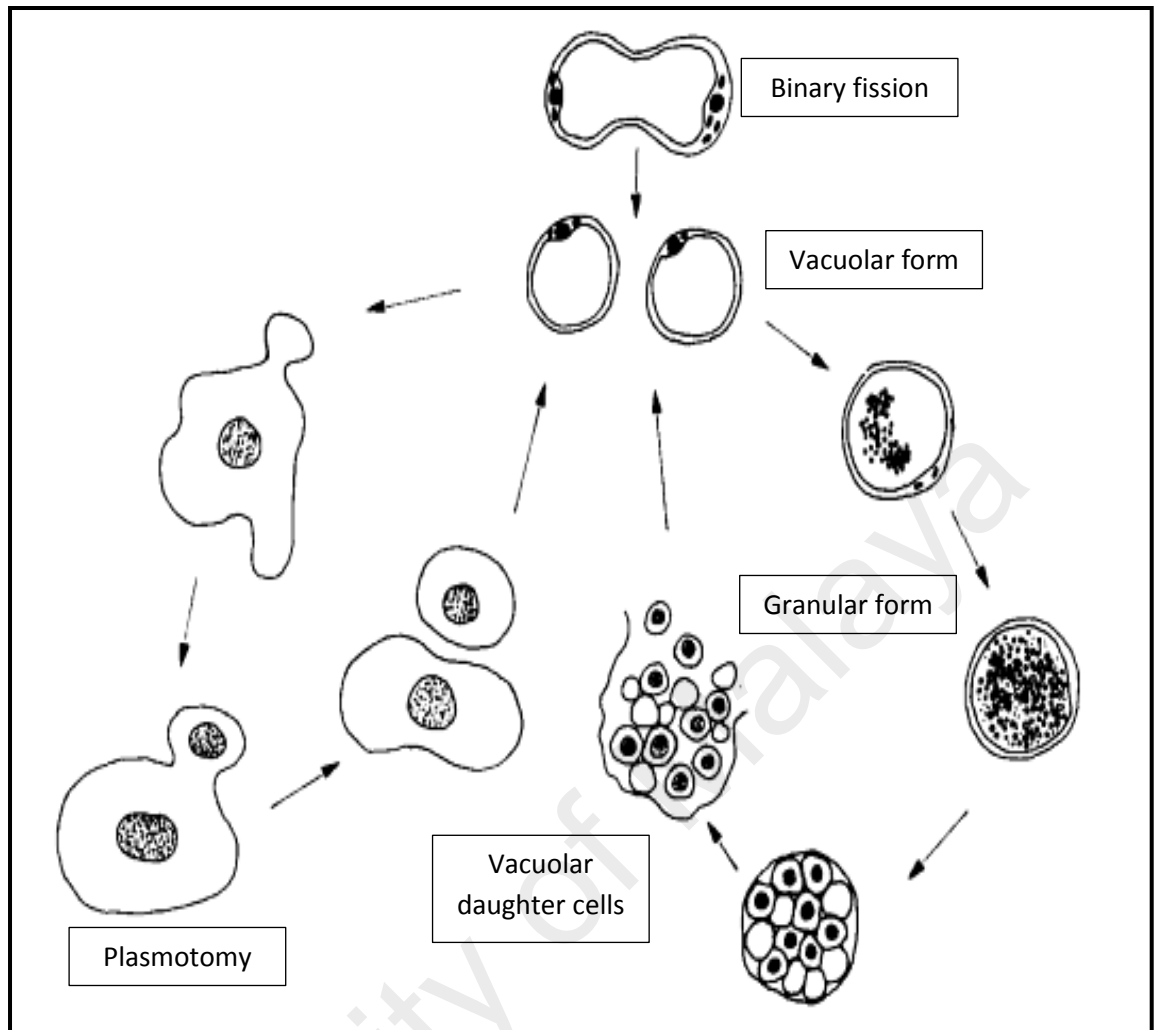


Figure 2.3 Life cycle of *Blastocystis hominis* proposed by Zierdt (1973) [adapted from Boreham and Stenzel, 1993]

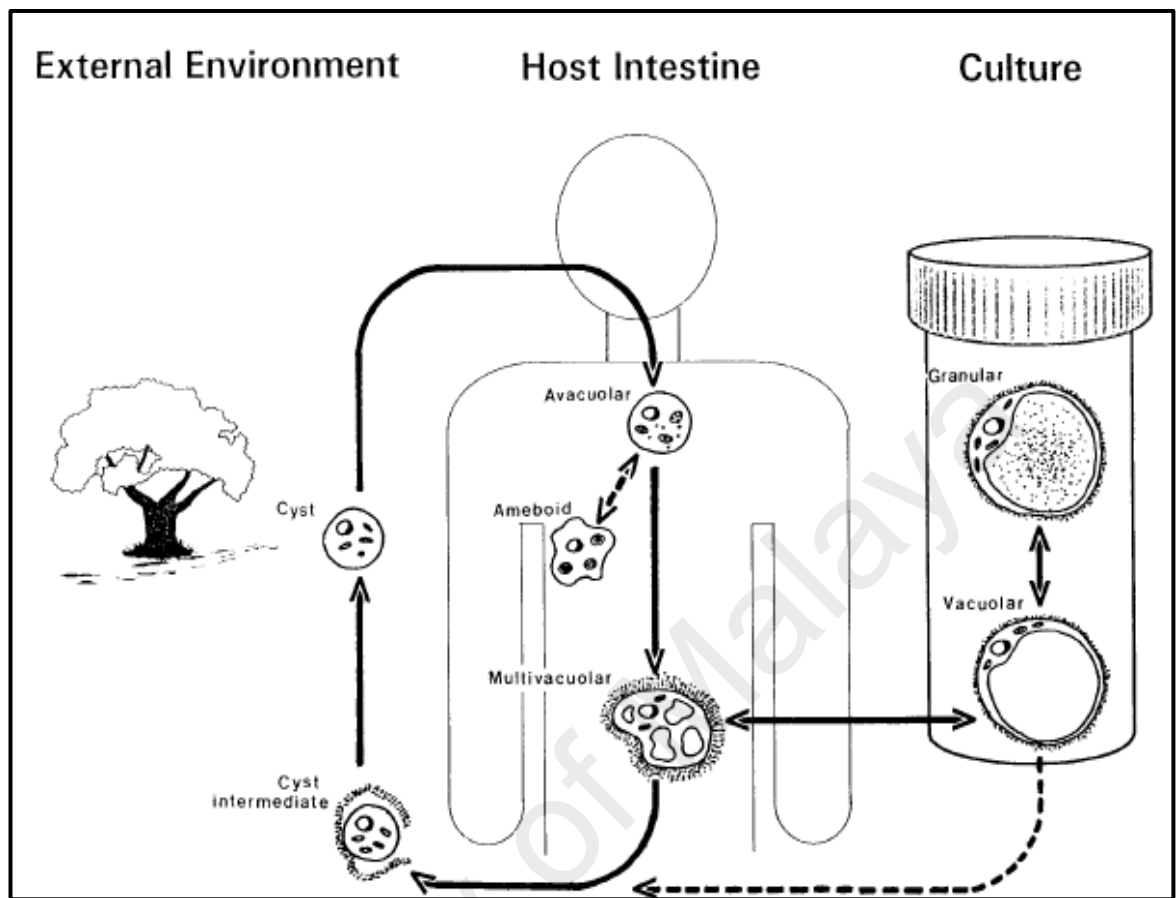


Figure 2.4 Life cycle of *Blastocystis hominis* as proposed by Boreham and Stenzel (1993) [adapted from Stenzel and Boreham, 1996]. The broken lines indicate hypothetical pathways.

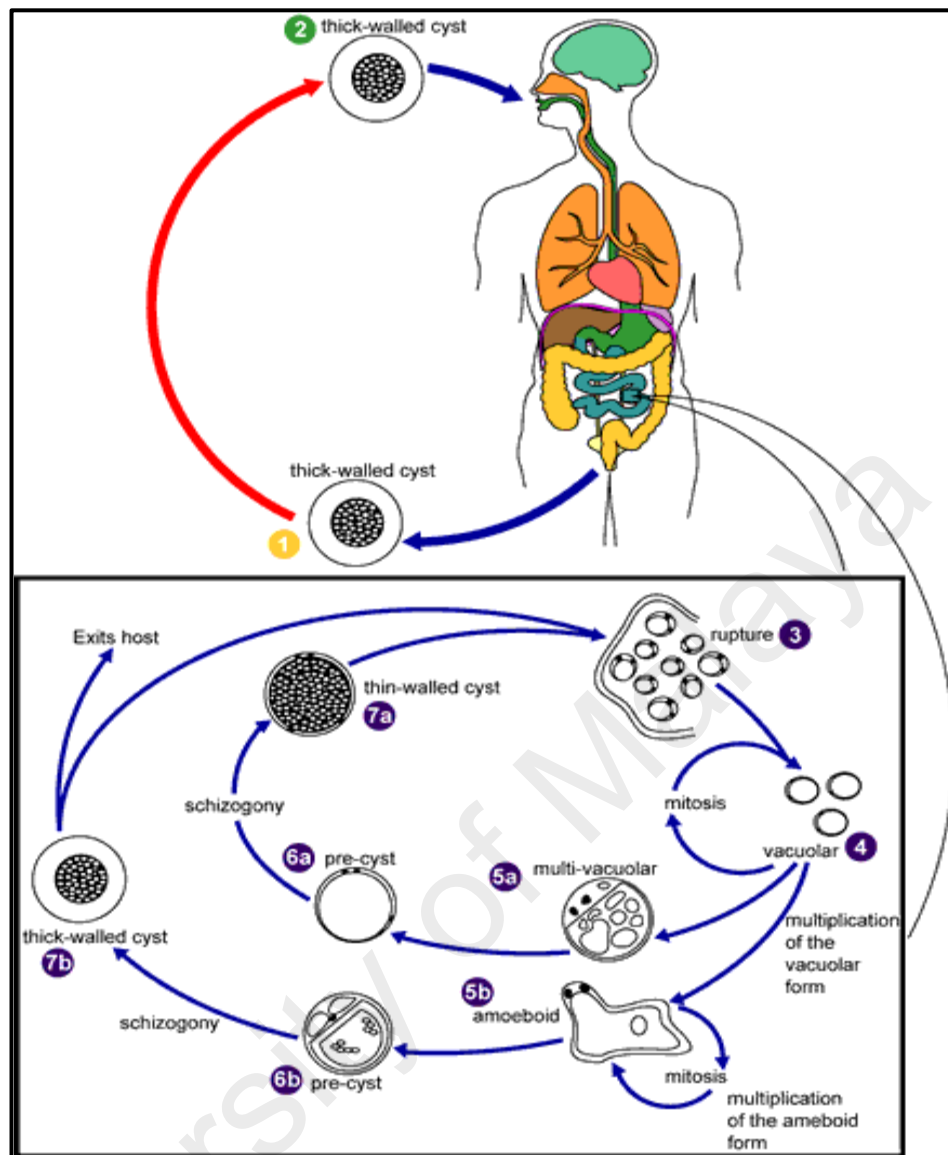


Figure 2.5 The proposed life cycle of *Blastocystis* sp. by Singh et al (2005).

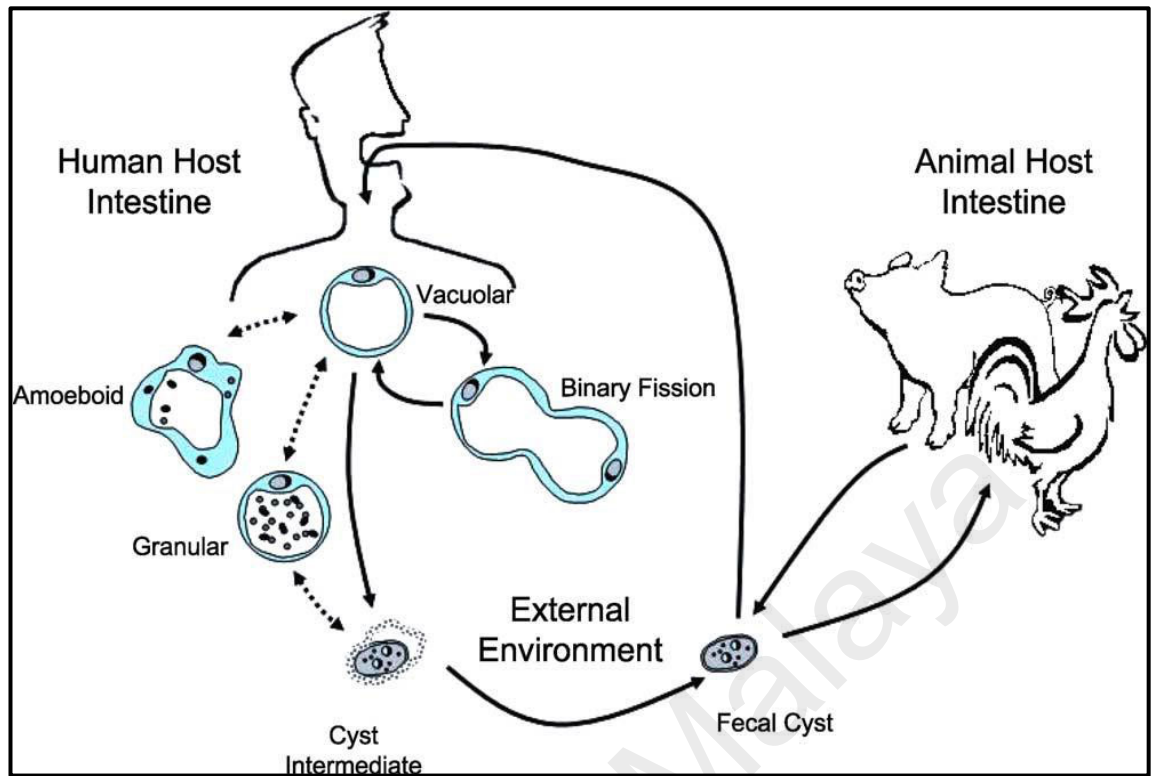


Figure 2.6 Life cycle of *Blastocystis* sp. as proposed by Tan (2004) [adapted from Tan et al., 2004]. Note: The transition of granular and amoeboid with respect to the vacuolar form is less well understood and is represented with dashed lines.

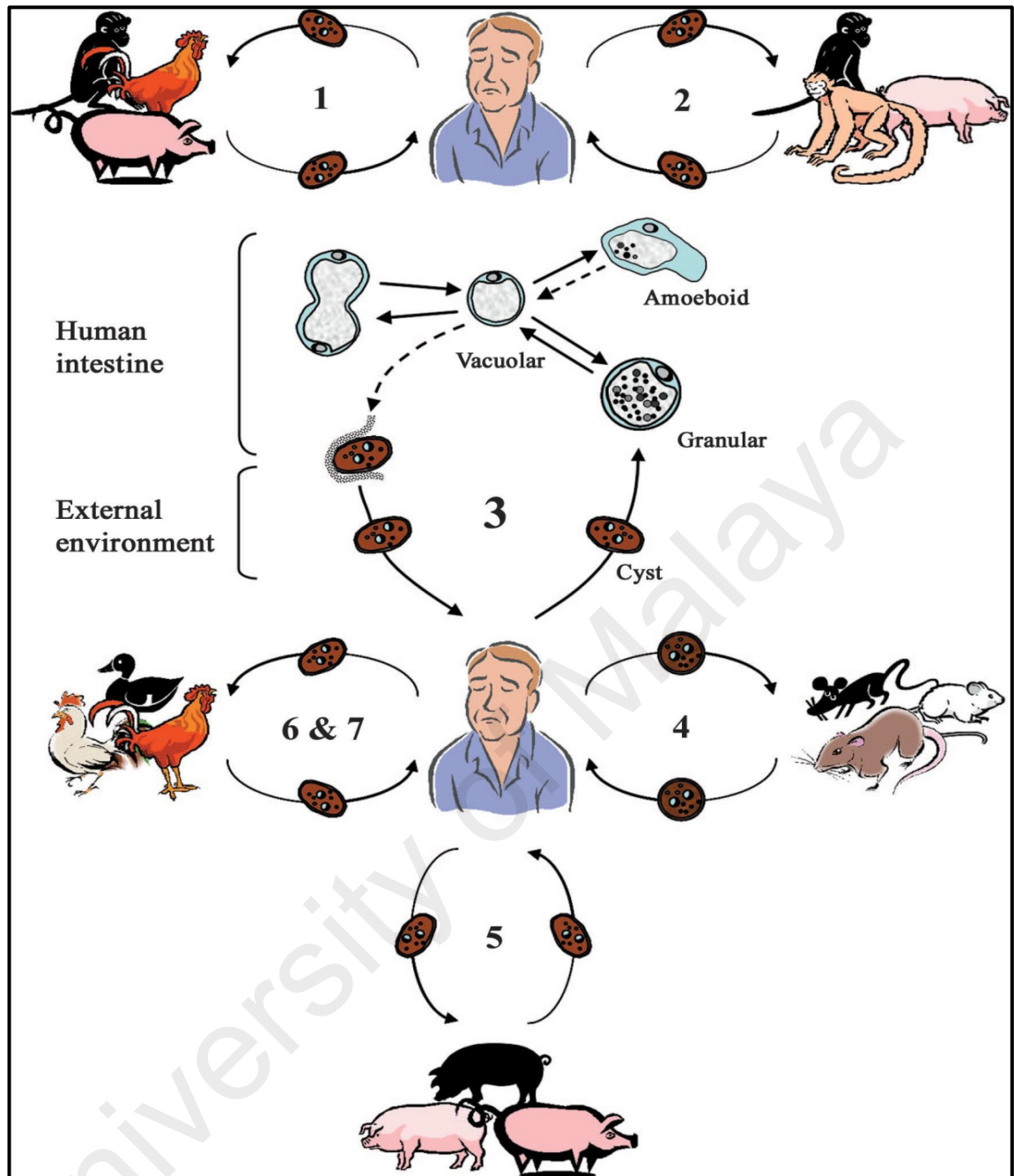


Figure 2.7 Life cycle of *Blastocystis* sp. as proposed by Tan (2008) [adapted from Tan et al., 2008]. Note: Numbers in the figure indicate the subtypes 1-7 of *Blastocystis* sp. Subtype 1 was cross infective among mammalian and avian isolates; subtypes 2, 3, 4, and 5 comprised of primate/pig, human, cattle/pig, and rodent isolates, respectively; and subtypes 6 and 7 included avian isolates.

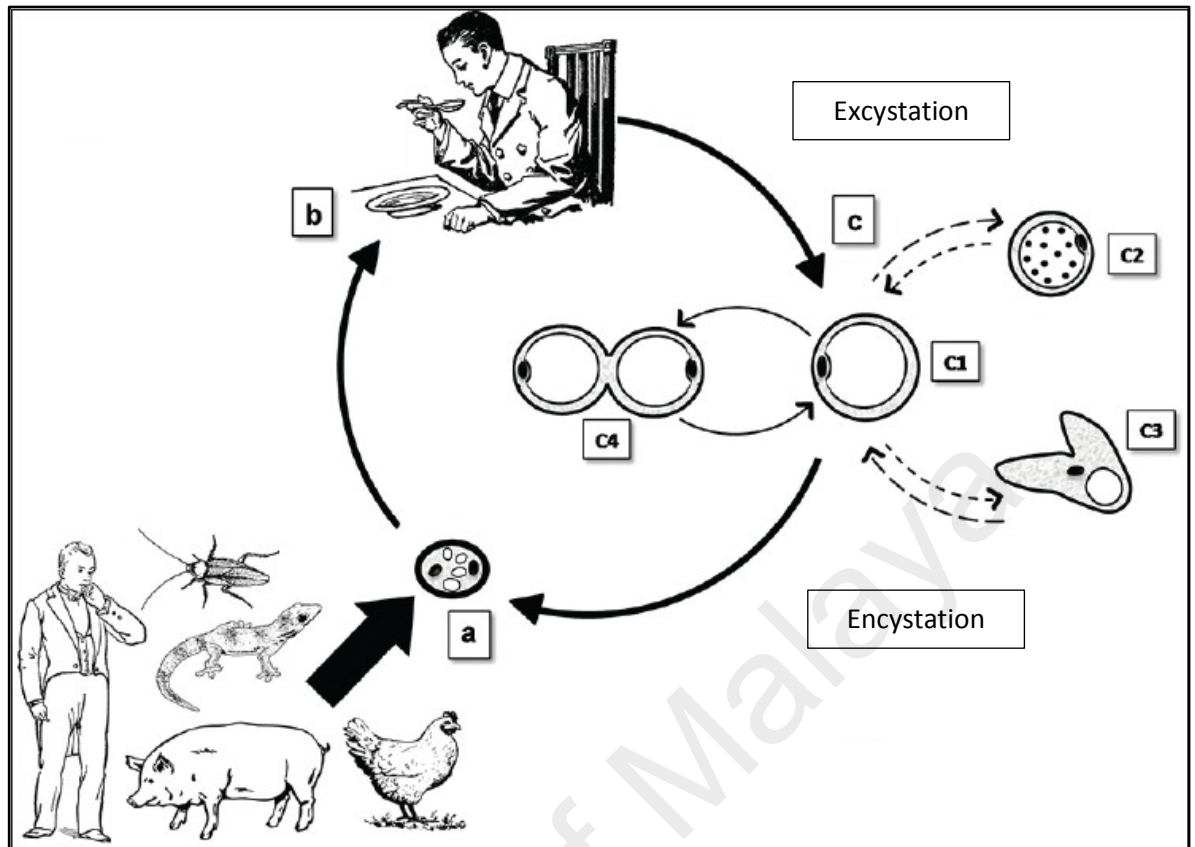


Figure 2.8 Life cycle of *Blastocystis* sp. as proposed by Parija and Jeremiah (2013) [adapted from Parija and Jeremiah, 2013]. Note: (a) Excretion of cyst form via faeces of infected host, (b) Humans acquire infection through consumption of contaminated food or water with faeces containing cyst, (c) Transformation of vacuolar form (c1) transform into granular form (c2) or amoeboid form (c3) and vice versa and the vacuolar forms multiplies by binary fission (c4).

2.5 Mode of transmission

In a manner similar to other gastrointestinal protozoa, faecal-oral route is the suggested route of transmission for *Blastocystis* sp. as reported by the increasing number of epidemiological studies carried out globally (Tan, 2008). Transmission of this parasite have been reported to be human-to-human (Yoshikawa et al., 2000), waterborne (Suresh et al., 2005) and also zoonotic (Salim et al., 1999). Several studies have shown that contamination by infective cyst of *Blastocystis* sp. occurs due to untreated water or poor sanitary conditions (Lee et al., 2012; Nimri and Batchoun, 1994) and food borne transmission (Garavelli et al., 1989). Figure 2.9 shows the possible pathway of *Blastocystis* infection among humans and animals.

Transmission has been reported to be common in occupations involving exposure to animals such as animal handlers and zookeepers (Parkar et al., 2007; Salim et al., 1999), among family members (Guglielmetti et al., 1989), mentally deficient persons in institutions (Hunt et al., 1990), children in day care centres (Guimaraes and Sogayar, 1993) and homosexuals (Moura et al., 1989). Doyle et al., 1990 have reported that patients with histories of exposure to pets or farm animals were more likely to be infected which indicates the zoonotic features of *Blastocystis* sp. In Nepal, a study highlighting the waterborne zoonosis of *Blastocystis* sp. have shown that similar subtypes were found in the stool samples of the villagers, their pet animals and in the nearby rivers (Lee et al., 2012). These findings suggest that poor hygiene is the root cause of *Blastocystis* sp. infection affecting community health.

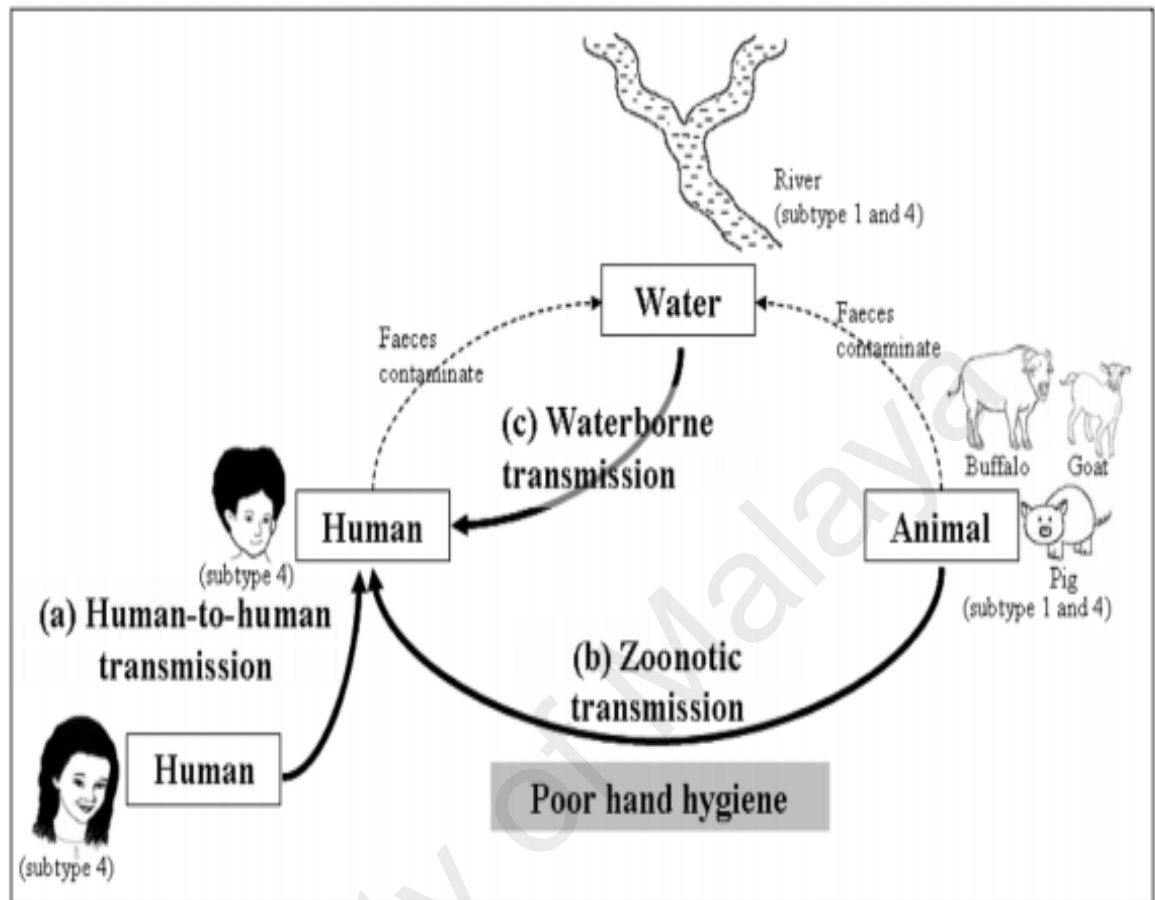


Figure 2.9 Possible human-to-human, zoonotic and waterborne transmission of *Blastocystis* sp. (adapted from Lee et al., 2012).

2.6 Prevalence

Blastocystis sp. is an extremely ubiquitous parasite seen in humans and animals with a worldwide distribution. Infection of *Blastocystis* sp. is observed both in patients with gastrointestinal symptoms and from healthy individuals. Prevalence varies widely across various countries and within various communities of the same country (Stenzel and Boreham, 1996). Several authors have reported that developing countries have higher prevalence of the parasite than developed countries which could be due to poor

hygiene, exposure to animals, and consumption of contaminated food or water. The occurrence of *B. hominis* infection was observed to be higher in immigrants, refugees and adopted children from developing countries compared to adults and children raised from birth in their community (Guglielmetti et al., 1989; Lee, 1991). A recent survey in children from Senegal, Africa showed 100% prevalence with all 93 positive stool samples and this was considered to be the highest prevalence recorded worldwide for *Blastocystis* sp. (El Safadi et al., 2014). However, in developed countries, the prevalence rate of *Blastocystis* sp. ranged from 0.5% in Japan (Horiki et al., 1997) and up to 23% in the United States (Amin, 2002).

Several publications have shown that *Blastocystis* sp. is more prevalent in lower socioeconomic groups who suffer from poor sanitary conditions compared to the higher socioeconomic groups within the same country (Borda et al., 1996; Cirioni et al., 1999; Taamasri et al., 2000). High incidence of *Blastocystis* sp. infection was also seen in immunocompromised patients such as HIV-positive patients (Cirioni et al., 1999; Paboriboune et al., 2014; Prasad et al., 2000), patients who undergo renal transplantation (Rao et al., 2003) and patients with hematological malignancy (Tasova et al., 2000).

After a few decades of research on this parasite, many prevalence studies had been conducted throughout the countries across the world such as Bangladesh (Yoshikawa et al., 2004b), China (Li et al., 2007; Yan et al., 2006), Germany (Bohm-Gloning, 1997; Yoshikawa et al., 2004b), Egypt (Hussein et al., 2008), Denmark (Stensvold et al., 2007; Stensvold et al., 2006), Greece (Menounos et al., 2008), Japan (Kaneda et al., 2001; Yoshikawa et al., 2004b), Pakistan (Yoshikawa et al., 2004), Singapore (Wong et al., 2008), Thailand (Thathaisong et al., 2003) and Turkey

(Dogruman-Al et al., 2008; Ozyurt et al., 2008). The prevalence studies of *Blastocystis* infection infecting humans in different geographical regions have been correlated with subtyping (Table 2.1).

Blastocystis sp. besides being highly prevalent in human, was also found in various animals which implicate the source of zoonotic transmission to humans based on phylogenetic (Arisue et al., 2003; Noel et al., 2003) and other molecular approaches (Abe., 2004; Thathaisong et al., 2003). Many authors have reported on the high prevalence of *Blastocystis* sp. continuously observed in various animal hosts mainly monkeys and fowls (Yamada et al., 1987), sea snakes (Teow et al., 1992), pigs (Quilez et al., 1995a), cattle (Quilez et al., 1995b), ostriches (Ponce et al., 2002), chickens (Lee and Stenzel, 1999), birds (Yoshikawa et al., 2004b), amphibians (Yoshikawa et al., 1998a), rats (Chen et al., 1997a), cockroaches (Zaman et al., 1993). In a recent study by Lim et al., 2008, very low prevalence of *Blastocystis* sp. was seen among the zoo animals in Malaysia. Meanwhile, a study conducted in five different farms from Peninsular Malaysia showed 30.9% prevalence with 73 of 236 goats positive for *Blastocystis* sp. (Tan et al., 2013). Hence, very few studies have been conducted on the prevalence of *Blastocystis* sp. among various animals in Malaysia.

Table 2.1 Distribution of *Blastocystis* subtypes infecting humans in different geographical regions [adapted from Tan (2008)]

TABLE 3. Distribution of *Blastocystis* subtypes infecting humans in different geographic regions

Country/region and type of isolates (no. of infected individuals studied)	Subtype distribution (%) ^a										No. of positive isolates/total no. of isolates (%) ^b
	1	2	3	4	5	6	7	8	9	Unknown/mixed	
Bangladesh (26)	7.7	—	92.3	—	—	—	—	—	—	—	NA
Guangxi, China (35)	37.1	—	40	—	—	—	5.7	—	—	17.2	NA
Yunnan, China (78)	20.5	1.3	70.5	1.3	—	—	—	—	—	6.5	NA
Denmark (29)	3.4	20.7	51.7	24.1	—	—	—	—	—	—	NA
Denmark (28)	17.9	32.1	46.6	3.8	—	—	—	—	—	—	NA
Egypt (44)	18.2	—	54.5	—	—	18.2	9.1	—	—	—	NA
Germany (166)	21	1	66	7	—	—	—	—	—	5	NA
Germany (12)	25	16.7	41.7	16.7	—	—	—	—	—	—	12/67 (17.9)
Greece (45)	20	13.3	60	2.2	—	2.2	2.2	—	—	—	NA
Japan (55)	20	21.8	43.6	10.9	—	—	—	—	—	3.6	NA
Japan (50)	8	—	52	4	—	22	10	—	4	—	50/2,037 (2.45)
Pakistan (10)	20	—	70	—	—	10	—	—	—	—	NA
Singapore (9)	22.2	—	77.8	—	—	—	—	—	—	—	9/276 (3.3)
Thailand (153)	90.2	—	4.6	—	—	—	1.3	—	—	3.9	334/924 (36.1) ^c
Turkey (87)	9.2	13.8	75.9	—	—	—	1.1	—	—	—	NA
Turkey											
Isolates from pediatric patients (51)	21.6	19.6	52.9	—	—	—	—	—	—	5.9	51/161 (31.7)
Isolates from adult patients (41)	14.6	24.4	58.5	—	—	—	—	—	—	2.4	41/125 (32.8)

^a —, subtype not detected.^b NA, not available.^c Three hundred thirty-four *Blastocystis*-positive samples were obtained by in vitro culture of stool specimens. Out of these 334 isolates, only 153 were amenable to PCR amplification.

2.7 Ultrastructural studies on *Blastocystis* sp. isolated from various hosts

Several publications have described the morphological characteristics of cysts and vacuolar forms of *Blastocystis* sp. isolated from humans and non-human hosts based on transmission electron microscopy and scanning electron microscopy observations. Based on an ultrastructural study conducted by Stenzel et al., 1996, cystic forms of *Blastocystis* sp. have shown distinct morphological differences when compared to previous findings where the cystic forms isolated from *Macaca* monkeys were larger in size ($12\pm 15\ \mu\text{m}$ diameter) than the cystic forms from humans ($3\pm 10\ \mu\text{m}$ diameter) and non-human hosts samples such as monkeys, pigs and chickens ($2\pm 5\ \mu\text{m}$ diameter) reported previously. The authors also reported that *Blastocystis* sp. with multilayered cyst wall similar to that previously described for *Blastocystis hominis* were often observed in both the monkeys and domestic chickens (Stenzel et al., 1996). In another study, morphological variations were seen in the surface coat of *Blastocystis* sp. isolated from several host species. However, the researchers were unable to provide evidence on the speciation due to insufficient differences among the *Blastocystis* sp. from humans and animal hosts (Cassidy et al., 1994).

2.8 Genetic diversity and subtyping

Many researchers believed that different subtypes of *Blastocystis* sp. have different reservoir hosts, geographical distributions, and modes of transmission. This raised the research interest in elucidating the genetic diversity of this organism. Till early 2000, a large number of subtypes (ST1-13) have been elucidated by various molecular approaches which show the extensive genetic diversity of *Blastocystis* sp. (Yoshikawa, et al., 2004b; Yoshikawa et al., 2003). Seven major subtypes managed to

be identified by PCR-based molecular methods which belong to human and other mammals (Yoshikawa, et al., 2004b; Noel et al., 2005).

To date, a total of 17 subtypes (STs) were identified based on gene barcoding of SSU rDNA (Scanlan & Stensvold, 2013). These isolates belong to humans, other mammals, birds, reptiles and insects. Reports worldwide have detected 90% of ST1-ST4 to be the STs' that infect humans with variation in the distribution of STs across regions (Alfellani et al., 2013a; Malheiros et al., 2011), while the remaining subtypes consisting of ST5-ST9 were found in non-human hosts except for ST9. Recent findings have discovered some novel non-human subtypes such ST10 to ST17 (Alfellani et al., 2013b).

Subtyping of *Blastocystis* using 10 subtype specific sequence-tagged site (STS) primers have been deployed widely in various laboratories (Yoshikawa, Abe, et al., 2004; Yoshikawa et al., 2000). The STS primers developed from the arbitrarily-primed PCR (AP-PCR) analysis of known strains of *Blastocystis* only amplifies the seven distinct subtypes which correspond to the phylogenetically different clades inferred from the SSU rRNA sequences (Arisue et al., 2003; Yoshikawa et al., 2004b).

Following this, a rapid method known as DNA barcoding used in *Blastocystis* sp. subtyping had been developed which resembles DNA barcoding in animals. This method only requires a single set of primers which were designed to amplify and sequence a 600bp region of the SSU_rRNA of *Blastocystis* to detect the established subtypes (Scicluna et al., 2006). Recently, Poirier et al (2011) established a high throughput real-time quantitative PCR (qPCR) to detect *Blastocystis* in human stool samples. This assay only targets the partial sequence of the *Blastocystis* sp. SSU rRNA

gene by direct sequencing of the qPCR products. The size of the amplified DNA fragments range from 320 to 342 bp depending on the subtypes. In comparison to the conventional direct-light microscopy and *in vitro* culture method, this rapid method has been proven to be highly sensitive. This method has the ability to detect various forms of *Blastocystis* sp. including cysts and to quantify the parasite at the same time. These new discoveries with standardized methods could facilitate in improving epidemiological studies and better categorization of the parasite according to subtypes (Poirier et al., 2011).

2.9 Host specificity and zoonotic potential

Several studies have reported that some human subtypes of *Blastocystis* sp. are found in animals as well which suggests that animal to human or zoonotic transmission of this unicellular organism is possible (Popruk et al., 2015). Each subtype has a specific tendency for specific hosts. Previously, ST 3 was known to be a human strain as it has been predominantly seen only in humans (Roberts et al., 2013), pigs and cattle are the main hosts of ST5 and rodents are the main hosts of ST4 (Tan, 2008). In contrast, Noel et al., 2005 have shown that ST1, ST2, and ST4 are zoonotic as identical isolates of human and animal origin were identified and clustered within these STs. Although ST11, ST12 and ST13 seem to be host specific, further studies of *Blastocystis* sp. isolated from elephants, giraffes and Australian native fauna species are required (Parker et al., 2010) to reveal the zoonotic feature of this parasite.

In another study conducted by Salim et al., (1999), a high prevalence of *Blastocystis* sp. infection was observed amongst zoo-keepers with 12 out of 19 zoo-keepers being positive. Human isolates similar to the primates were also seen in the

study. Thus, these findings indicate that the *Blastocystis* sp. infection was due to close contact between the animals and zoo-keeper (Salim et al., 1999).

2.10 Cross infection studies

Very few studies have been conducted to investigate the cross-infectivity of *Blastocystis* isolates from various animal hosts. Ubiquitous nature of *Blastocystis* sp. raised research interest to elucidate the zoonotic potential and host specificity of *Blastocystis* sp. Tanizaki et al., (2005) have reported that *Blastocystis* sp. infection are transmitted easily between the same or different bird species, thus concluding that a new *Blastocystis* species on the basis of different avian host species cannot and should not be introduced (Tanizaki et al., 2005). Similar findings were also reported by Pakandl, 1991 where the porcine strains of *Blastocystis* sp. were able to infect the laboratory mice which indicate the low host specificity of *Blastocystis* sp. (Pakandl, 1991). However, there has been no study thus far to cross-infect rats with cysts from different animal hosts.

2.11 Biochemical and haematological changes due to *Blastocystis* sp. infection

Several studies have been conducted to investigate the effects of parasitic infection on the biochemical and haematological parameters in humans and animals (Cotte et al., 1993; Ojiako & Onyeze, 2008; Janarthanan et al., 2011). Chen et al., (2003) revealed that no significant changes were observed in the albumin and glucose level in patients infected with *Blastocystis* sp. when compared to healthy individuals (Chen et al., 2003). Similarly, no significant changes were seen in the level of alkaline

phosphatase and serum amylase in HIV patients with multiple intestinal protozoan infections (Cotte et al., 1993).

In another study, serum amylase was observed to be elevated in patients with intestinal obstructions, inflammatory bowel disease and acute pancreatitis (Stephen et al., 2014; Triantafillidis & Merikas, 2010; Winslet et al., 1992; Sugimoto et al., 2015). According to a study (Chen et al., 2003), no significant changes were observed in the haematological parameters comprising of white blood cells, eosinophils, haemoglobin, platelets and erythrocyte sedimentation rate in *Blastocystis* sp., infected patients. Till this date, there are no conclusive findings which correlate the effects of *Blastocystis* sp. infection on the biochemical and haematological parameters in humans and animals.

2.12 Pathogenicity

The issues related to pathogenicity of *Blastocystis* sp. still remains questionable due to the parasite's presence in both symptomatic and asymptomatic individuals with insignificant difference in parasite abundance (Albrecht et al., 1995; Leder et al., 2005; Salim et al., 1999; Udkow & Markell, 1993). The difficulty of excluding all other viral, bacterial or other protozoal infections as a possible cause of symptoms and the lack of suitable experimental animal models have hindered attempts to incriminate the pathogenicity of *Blastocystis hominis*.

The gastrointestinal symptoms such as anorexia, nausea, flatulence, diarrhoea and abdominal pain are common symptoms attributed to *Blastocystis* sp. infection (Boreham & Stenzel., 1993). Several other reports have suggested that *Blastocystis* sp. is associated with a variety of disease which include colitis (Russo et al., 1988), terminal ileitis (Tsang et al., 1989), tropical pulmonary eosinophilia (Enzenauer et al.,

1990), ulcerative colitis (Jeddy & Farrington, 1991), reactive arthritis (Lakhanpal et al., 1991), chronic urticarial (Pasqui et al., 2004), hypoalbuminemia (Nassir et al., 2004) and irritable bowel syndrome (Boorom et al., 2008); Yakoob et al., 2004). This organism has also been associated with diabetes (Sheehan & Ulchaker., 1990), cancer (Chandramathi et al., 2012; Tan et al., 2009) and leukaemia (Ghosh et al., 1998; Yang et al., 1996).

In a case control study (Leder et al., 2005), no correlation was observed between clinical symptoms and *Blastocystis* sp. infection. This study also reported that *Blastocystis* sp. was detected without significant differences between asymptomatic and symptomatic groups in the absence of any enteric pathogen. Previous studies have also suggested that *Blastocystis* sp. may be an opportunistic pathogen in immunosuppressed patients, particularly patients with AIDS (Lebbad et al., 2001; Paboriboune et al., 2014; Rivero Rodriguez et al., 2013). Moe et al., (1997) have previously correlated the histopathological findings with *Blastocystis* sp. infection where infected BALB/c mice revealed intense inflammatory-cell infiltration, oedematous lamina propria and mucosal sloughing in the intestines. The following year, Moe et al., (1998) have shown severe inflammatory reaction and myonecrosis in affected muscle cells of BALB/c mice infected with *Blastocystis hominis* which indicates the pathogenic effect of this parasite.

Another important factor which influences the pathogenicity of *Blastocystis* is its genotypic variability (Souppart et al., 2009). Reports have suggested pathogenic potential in ST 1, ST 2 and ST 4 causing intestinal illness (Hussein et al., 2008; Kaneda et al., 2001; Vogelberg et al., 2010). In contrast, increasing studies have incriminated that *Blastocystis* subtype 3 is the most common subtype isolated from patients with various gastrointestinal disorders (Dogruman-Al et al., 2008; Kaneda et al., 2001;

Souppart et al., 2009). This finding was further supported by another discovery where higher occurrence of *Blastocystis* sp. ST 3 were seen in symptomatic than asymptomatic patients (Doyle et al., 1990; Scanlan & Stensvold, 2013; Souppart et al., 2009), however its pathogenic role has not been clearly explained. Despite few studies which justified that *Blastocystis* is non-pathogenic, however, equal publications have reported on the pathogenicity of this puzzling organism which proposed *Blastocystis* to be an emerging protozoan pathogen.

2.13 Excystation and Encystation of *Blastocystis* sp.

Various morphological forms comprising of vacuolar, granular, amoeboid, and giant cells were observed in the *in-vitro* culture of *Blastocystis hominis* (Zierdt 1991; Suresh et al. 1994). However, the development of the cystic forms into vacuolar and granular forms is not clearly established. More than 10 years ago, Moe et al., (1997) provided ultrastructural evidence for the development of cysts isolated from symptomatic patients to vacuolar forms in *in vitro* culture (Moe et al., 1997). However, there have been no studies carried out thus far comparing the excystation of *Blastocystis* sp. from symptomatic and asymptomatic isolates.

CHAPTER 3

STUDIES TO DETERMINE THE PREVALENCE OF *BLASTOCYSTIS* SP. INFECTION AMONG PETS, DOMESTIC AND ZOO ANIMALS.

3.1 Introduction

Prevalence studies carried out worldwide showed *Blastocystis* sp. infections in humans to vary from 0.5% (Horiki et al., 1997) to 60% (Pegelow et al., 1997). The parasite is also seen in a wide range of animals, such as cattle, pigs, monkeys, chickens, birds and quails with prevalence ranging from 40-60% (Tan et al., 2008; Tan et al., 2012; Yoshikawa et al., 2004a). Many publications have highlighted the high prevalence of *Blastocystis* sp. in various animal hosts (Stenzel & Boreham, 1996; Abe et al., 2002; Roberts et al., 2013; Parkar et al., 2010) including non-human primates (Yamada et al., 1987), mammalian (Quilez et al., 1995a; Quilez et al., 1995b), avian (Yamada et al., 1987; Ponce et al., 2002; Yoshikawa et al., 2004b) and amphibians (Yoshikawa et al., 1998a) and less frequently in rats (Chen et al., 1997a), reptiles (Teow et al., 1992) and insects (Zaman et al., 1993). In Malaysia, (Lim et al., 2008) reported low prevalence of *Blastocystis* sp. among the animals at a local zoo. *Blastocystis* sp infection was recently reported in five different farms in Peninsular Malaysia with 73 (30.9%) of 236 goats examined found to be positive for *Blastocystis* sp. (Tan et al., 2013).

There are 17 distinct subtypes (ST1-ST17) seen in humans, non-human primates, other mammals and birds (Noel et al., 2005; Stensvold et al., 2007; Scanlan & Pauline, 2012; Alfellani et al., 2013). Although only some subtypes are described to be host specific eg subtype 6 which is commonly found in pigs and cattle, however most of the subtypes showed low host specificity (Yoshikawa et al., 2000) which may easily cause cross-transmission between animals and humans especially when humans have close proximity with pets or farm animals (Doyle et al., 1990). Doyle showed that persons who came into close contact with pets or farm animals were more prone to this infection which suggests the possibility of human-animal cross-infectivity. Furthermore,

experimental cross-transmission has been achieved in chickens with *Blastocystis* sp. from domestic chickens, quails and domestic geese (Tanizaki et al., 2005), guinea pigs (Phillips et al., 1976) and rats (Suresh et al., 1995) with *Blastocystis* sp. from human isolates providing evidence of low host specificity.

To date, very limited studies have been carried out to determine the prevalence of *Blastocystis* sp. among various animal hosts in Malaysia. This study therefore, attempts to determine the prevalence of *Blastocystis* sp. in pets, domestic and zoo animals.

3.2 Materials and methods

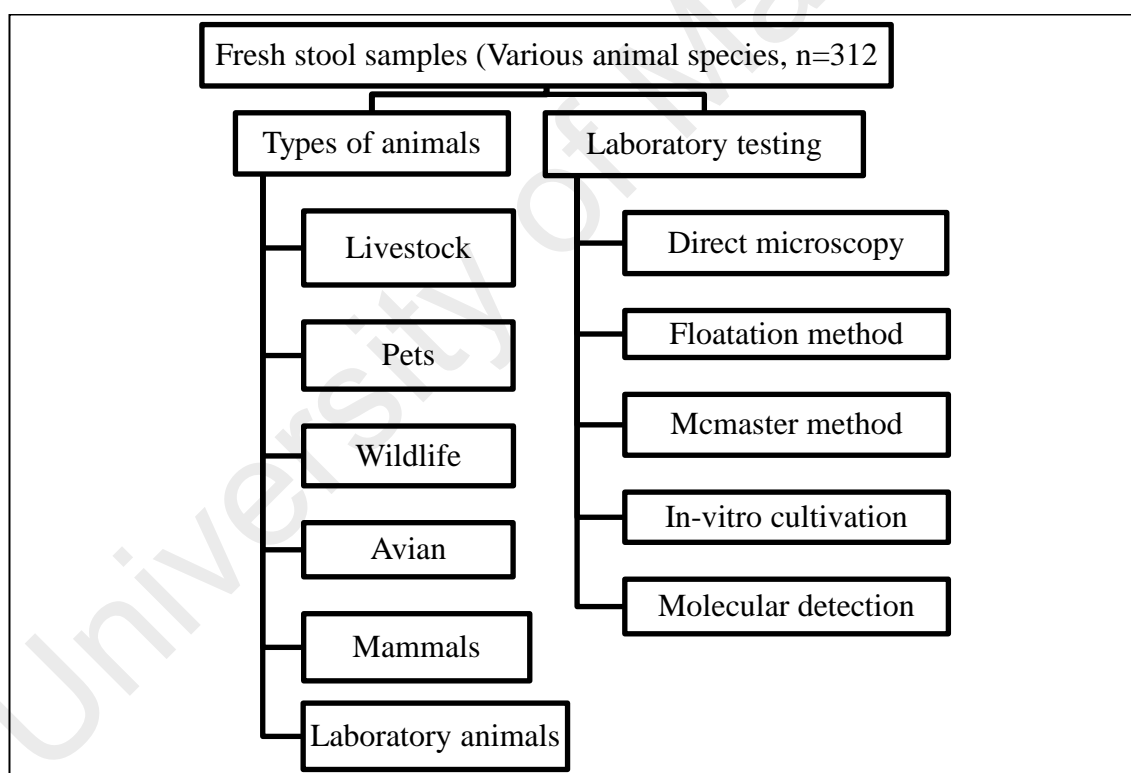


Figure 3.1: Schematic representation of the overall methodology emphasizing on the prevalence of *Blastocystis* sp. in various animals.

3.2.1 Ethical approval

The ethical approval was obtained for this study from Institutional Animal Care and Use Committee (IACUC), University Malaya guidelines with the Reference. No: PAR/29/06/2012/LIL(R).

3.2.2 Experimental animals

A total of three hundred and twelve (n=312) faecal samples were collected from various animal species in Perak. Faecal samples of the animals were collected from various government farms, private farms, veterinary clinics, local zoo, as well as animal house (Table 3.1).

Table 3.1: Location of sampling area

Location	Species	Number of samples collected
Infoternak farm, Sg.Siput	Cow	29
	Goat	20
	Ostrich	37
	Sheep	38
Department of Veterinary Services Ipoh	Cat	24
	Dog	32
Taiping zoo	Black panther	1
	Camel	1
	Chimpanzee	1
	Deer	14
	Elephant	2
	Gaur	10
	Lion	1
	Tapir	1
	Tiger	1
Perak Wildlife Conservation Centre	Terrapin	3
	Wild bird	30
Bukit Merah Conservation Centre	Orang utan	10
	Peacock	10
Private farm, Kampar	Pig	10
Private farm, Tambun	Horse	5
Animal house, VRI Ipoh	Laboratory animals	32
Total		312

3.2.3 Source and Isolation of *Blastocystis* sp.

For avian, livestock, mammals, wild animals and animals bred in the laboratory, sampling were carried out from faecal droppings from the ground housing the respective animals whereas rectal swabs were made directly on the smaller ruminants such as goats and sheep. Rectal swabs were also made from pet animals such as dogs and cats. The fresh faecal samples collected from animals were stored in stool containers and processed as soon as possible after collection.

3.2.4 Laboratory testing

3.2.4.1 *In-vitro* cultivation of *Blastocystis* sp. isolates

The parasites were isolated from the faecal samples of various animals by *in-vitro* cultivation using Jones' medium supplemented with 10% heat-inactivated horse serum at 37°C. Subsequently after isolation, the parasites were maintained in Jones' medium by consecutive sub-cultures every 2 to 3 days for at least one month prior to phenotypic and subtype analysis (Suresh et al., 1994).

3.2.4.2 Floatation method

Approximately one gram of fresh faeces, emulsified with saturated salt solution was filtered through gauze into a centrifuge tube. Saturated salt solution was then later added up to the meniscus of the test tube before placing a coverslip on the top of the culture tube. The coverslip was then lifted and placed on a clean slide. Samples were then observed under 10x objective lens of compound microscope for the presence of helminth eggs (Norakmar et al., 2010).

3.2.4.3 McMaster method for parasite ova or oocyst detection

Approximately three gram of the fresh faeces was placed in a jar with saturated salt solution added up to 45 ml with 1:15 dilution. Contents were then mixed using pestle and mortar. The content was then filtered through a 85 mesh screen and the filtrate were collected into a glass jar. The filtrate was then used to fill the counting chambers of McMaster's slide and observed under 10x objective lens of light microscopy for the presence of helminthes eggs (Norakmar et al., 2010).

3.2.5 Molecular detection

3.2.5.1 Genomic DNA preparation

DNA was extracted from the culture sample of all positive animal isolates using QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol (Yoshikawa et al., 2004b).

3.2.5.2 Subtyping of *Blastocystis* sp. isolates

All parasite isolates were subjected to sequenced-tagged site (STS) primer-polymerase chain reaction using the following ten sets of primers (Yoshikawa et al., 2004b) (Table 3.2). Two to five µl of DNA preparations were used to amplify the genomic sequences in a 20µl reaction containing 0.5mM of the dNTPs, 0.5 mM of each primer, 1 x PCR buffer (75mM Tris-HCL, pH 8.8, 20 mM (NH₄)₂SO₄ and 0.01% Tween 20), 2.5 mM MgCl₂ and 1 U Taq DNA Polymerase (recombinant) (FERMENTAS, USA). PCR conditions consisted 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 30 s and extending at 72°C for 1 minute, and an additional cycle with a 10min chain elongation at 72°C (thermocycler Eppendorf, Germany). The amplification products were electrophoresed in 1.5% agarose gels (PROMEGA USA) and Tris-Borate-EDTA buffer. Gels were stained with ethidium bromide and photographed using ultra-violet gel documentation system (Uvitec, United Kingdom). The PCR amplification for each primer pair was repeated thrice for each isolate (Yoshikawa et al., 2004b). The classification of the subtypes for each *Blastocystis* sp. isolate was based on the standard terminology (Stensvold et al., 2007).

Table 3.2: List of sequenced-tagged site (STS) primers for subtyping of *Blastocystis* sp.

Subtype	STS primer	Product size (bp)	Sequence of forward (F) and reverse (R) primer (5' – 3')	Genebank accession no.
1	SB82	462	F-TCTTGCTTCATCGGAGTC R-CCTTCTCGCAGTTCTTTATC	AF166085
1	SB83	351	F-GAAGGACTCTCTGACGATGA R-GTCCAAATGAAAGGCAGC	AF166086
2	SB155	650	F-ATCAGCCTACAATCTCCTC R-ATCGCCACTTCTCCAAT	AF166087
3	SB227	526	F-TAGGATTTGGTGTGTTGGAGA R-TTAGAAGTGAAGGAGATGGAAG	AF166088
3	SB228	473	F-GACTCCAGAACTCGCAGAC R-TCTTGTTTCCCCAGTTATCC	AF166089
3	SB229	631	F-CACTGTGTCGTCATTGTTTTG R-AGGGCTGCATAATAGAGTGG	AF166090
4	SB332	338	F-GCATCCAGACTACTATCAACATT R-CCATTTTCAGACAACCACTTA	AF166091
5	SB340	704	F-TGTTCTTGTGTCTTCTCAGCTC R-TTCTTTCACACTCCCGTCAT	AY048752
6	SB336	317	F-GTGGGTAGAGGAAGGAAAACA R-GAACAAGTCGATGAAGTGAGAT	AY048751
7	SB337	487	F-GTCTTTCCCTGTCTATTCTGCA R-AATTCGGTCTGCTTCTTCTG	AY048750

3.3 Results

A total of 111 out of 312 animals (35.58%) were found positive for *Blastocystis* sp. from faecal examination (Table 3.3). High prevalence of *Blastocystis* infection were observed in the ruminant livestock group; i.e 34.5% (10/29) cows, 28.6% (4/14) deers, 30% (3/10) gaurs, 65% (13/20) goats, 57.9% (22/38) sheeps and 100% (10/10) pigs. In contrast, all dogs and cats from the veterinary clinic were negative for *Blastocystis* sp. infection. Meanwhile, high incidence of *Blastocystis* sp. infection were seen in the avian host such as domestic ostriches with 100% (37/37) and peacock with 70% (7/10) positive for *Blastocystis* sp. All wildlife specimens including black panther, lion, tiger, elephants, tapir, camel, terrapins and wild birds were completely free from the organism. In mammals, the prevalence rate of the organism varied with 50% (5/10) of orang utans were found positive for *Blastocystis* sp., while in horses and chimpanzee, the organism was undetectable in their respective faecal samples. Specimens from the laboratory animals such as mice, rats, guinea pigs and rabbits were found to be negative.

Binary fission was frequently observed in *Blastocystis* sp. cultures. The size range of the vacuolar forms differ slightly among different animal isolates but is generally smaller than the *Blastocystis* sp. Granular forms were seen more often in older cultures however amoeboid forms were not seen in the cultures at all. Stronglye eggs and coccidian oocyst were seen in some ruminant cultures. Using sequenced-tagged site primer-PCR, only 14 ostrich isolates were confirmed to be subtype 6 (Figure 3.1). Meanwhile, the subtype of remaining 298 isolates when amplified with PCR using sequence-tagged site (STS) revealed no bands when assessed using primers for subtypes 1 to 7.

Table 3.3: Prevalence of *Blastocystis* sp. in various animal hosts

Species	Common name	Infected/examined (%)
Livestock		
<i>Bos taurus</i>	Cow	10/29 (34.5)
<i>Axis axis</i>	Deer	4/14 (28.6)
<i>Bos gaurus hubbacki</i>	Gaur	3/10 (30)
<i>Capra aegagrus</i>	Goat	13/20 (65)
<i>Ovis aries</i>	Sheep	22/38 (57.9)
<i>Sus domesticus</i>	Pig	10/10 (100)
Total		62/121 (51.2)
Pet		
<i>Felis catus</i>	Cat	0/24 (0)
<i>Canis lupus</i>	Dog	0/32 (0)
Total		0/56 (0)
Wildlife		
Total		0/40 (0)
Avian		
<i>Struthio camelus</i>	Ostrich	37/37 (100)
<i>Pavo cristatus</i>	Peacock	7/10 (70)
Total		44/47(93.6)
Other animals (mammals)		
<i>Pan troglodytes</i>	Chimpanzee	0/1 (0)
<i>Equus ferus</i>	Horse	0/5 (0)
<i>Pongo pygmaeus</i>	Orang utan	5/10 (50)
Total		5/16 (31.3)
Laboratory animal		
<i>Cavia porcellus</i>	Guinea pig	0/7 (0)
<i>Mus musculus</i>	Mice	0/10(0)
<i>Oryctolagus cuniculus</i>	Rabbit	0/5 (0)
<i>Rattus norvegicus</i>	Rat	0/10 (0)
Total		0/32 (0)
Total (Overall)		111/312 (35.6)

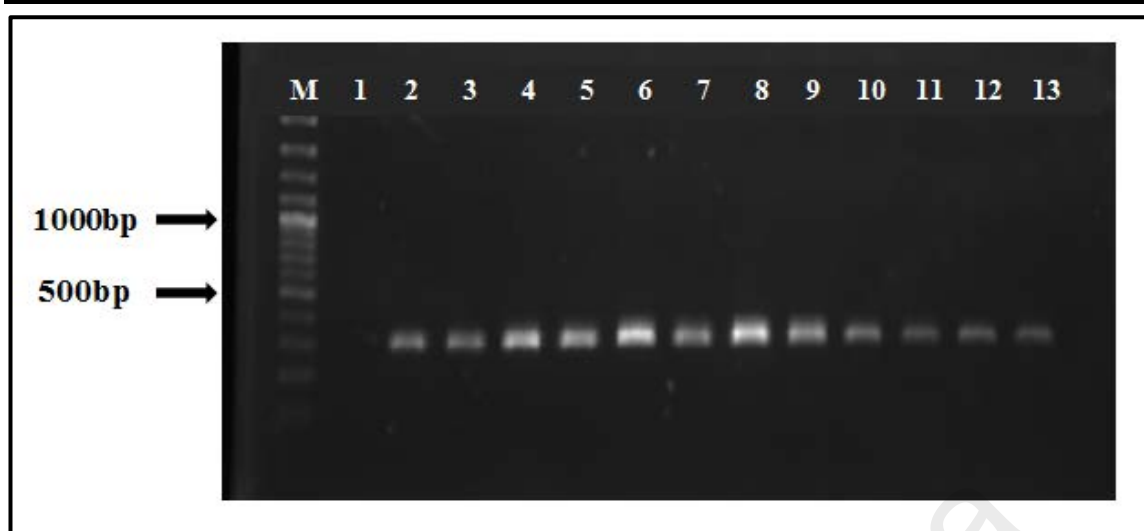


Figure 3.2: Agarose gel image of *Blastocystis* sp. from the ostrich isolate amplified by sequenced-tagged site (STS) primers. Lane M= DNA markers of 100bp plus DNA ladder (Fermentas, USA); lane 1= negative control; lane 2= positive control and lane 3-13= subtype 6. The amplicon size of subtype 6= 317bp.

3.4 Discussion and conclusion

Many epidemiological studies on other intestinal parasites have been carried out in a variety of animals but only two reports on *Blastocystis* sp. infection involving domestic animals such as goats, gaurs, cows, and deers have been carried out in Malaysia (Lim et al., 2008 and Tan et al., 2013). In the present study, moderate infection with a prevalence ranging from 25% to 60% was observed in the ruminant livestock group which includes cows, sheeps, goats, deers and gaurs. Similar findings were also reported in the European countries (Quilez et al., 1995a and Pakandl., 1991) and Japan (Abe et al., 2002). There could be few possible causes for the infection in the livestock group which is due to overcrowding of animals, poor sanitation, animals sharing the same water source and poor management of farms.

Besides that, high prevalence of *Blastocystis* sp. in ostriches might be due to housing condition where ostriches are reared in an open space pens which are easily exposed to other birds and rats that could be naturally infected with *Blastocystis* sp. Similarly, peacocks are housed together in an open cage where they are easily exposed to rats, which might have increased the chances of cross-transmission. Meanwhile, since pig farms are mostly for commercial purpose, overcrowding is often observed in these farms, thus, facilitating the spread of infection among the animals. As for the primates, 50% of the orang utan were positive for *Blastocystis* sp. infection, however the chimpanzee was negative. These occurrence are relatively moderate, while it has been reported to be high among the primates in Japan with 85% of them being positive for this organism (Abe et al., 2002). It is also speculated that *Blastocystis* infection had been transmitted by contact with neighboring primates in the farms since some of the primates are housed together. Meanwhile, the sample from the chimpanzee obtained from a zoo was negative and this could be largely due to the fact that the primates were housed individually.

In the present study, the absence of this organism in companion animals was similar to the finding in dogs and cats carried out in Germany and Japan (Konig and Muller, 1997; and Abe et al., 2002). The cats and dogs sampled were not stray animals but had owners. They were kept in as domesticated pets within house premises. Thus this could explain the absence of *Blastocystis* sp. in these animals. In addition, horses and laboratory animals were also negative for *Blastocystis* infection in this study which concurred with similar findings reported in Australia (Roberts et al., 2013). However, it has been reported previously that 60-70% of the laboratory rats were found to be positive for *Blastocystis* sp. whereas all the mice, rabbits and hamsters were negative (Chen et al., 1997). This could be possibly due to proper animal management in such

animal houses as these are usually bred for laboratory experiments and hence often regular screening to prevent diseases is usually carried out.

All wildlife specimens in the present study including black panther, lion, tiger, elephants, tapir, camel, terrapins and wild birds were completely free from *Blastocystis* infection. These results were consistent with the study carried out in Germany (Konig and Muller, 1997 and Japan (Abe et al., 2002). However, a study carried out among the circus animals in Australia (Stenzel et al., 1993) reported that lion and camel were found to be positive for *Blastocystis* sp. In this study, most of the wildlife animals were housed separately or confined within their respective groups which probably reduced the chances of cross-transmission.

The morphological analysis of *Blastocystis* sp. isolated from the animal isolates has shown that the vacuolar forms were generally smaller than that of human isolates which usually range from 2 to 200 μm (Stenzel & Boreham, 1996; Zierdt, 1991). Otherwise, the general morphology of this organism from this isolate appeared similar to the *Blastocystis hominis*. Therefore, it was found difficult to differentiate animal isolates from the general morphology of human isolates as these characteristics were similar (Tan et al., 2006). The strongyle eggs seen in the ruminant cultures are the ones commonly reported in ruminants. The coccidian oocysts seen in the present study were *Eimeria bovis*, *Eimeria ovinoidalis* and *Eimeria minasensis* which were frequently seen in the faecal samples of cows, sheep and goats respectively. These are opportunistic pathogens which are mostly prevalent due to poor sanitation or overcrowding (Norakmar et al., 2010).

Although subtype 6 was previously seen in livestock animals (Alfellani et al., 2013) especially pigs and cows (Stensvold et al., 2009), this is the first study to demonstrate subtype 6 in ostriches when amplified with polymerase chain reaction using sequence-tagged site (STS) primers. In conclusion, the present study highlighted the importance of monitoring *Blastocystis* infection among domestic, pet and zoo animals which are in close contact with humans as these animals could be a reservoir for human infections.

Further molecular studies need to be carried out in order to have a better understanding on the reservoir hosts and origin of *Blastocystis* infection among animals. As *Blastocystis* infection is prevalent in human and animals, there is a need to screen both humans and animals. There is a need to maintain good hygiene practices during the processing of meat and meat products to eliminate the risk of infection to humans. The present study warrants that animal handlers need to be educated on personal hygiene, such as wearing of a mask and gloves to eliminate the risk of *Blastocystis* infection to humans.

CHAPTER 4

ULTRASTRUCTURAL STUDIES ON THE CYSTIC AND VACUOLAR FORMS OF *BLASTOCYSTIS* SP. FROM ANIMAL ISOLATES

4.1 Introduction

Many authors have reported on the ultrastructural descriptions of vacuolar forms (Mehlhorn., 1988; Zaman et al., 1997a) and cystic forms (Zaman et al., 1995b; Moe et al., 1996; Khalifa., 1999) of human isolates. Zaman et al., (1997b) observed two layers of surface coat surrounding the vacuolar forms. He further described that in some cells, the attachment of the fibrillar material of the surface coat to bacteria may cause cytoplasmic damage to the bacterial cell. In contrast, Cassidy et al., (1994) have suggested that the surface coat protects the parasite against osmotic shock. Most cystic forms are surrounded by an irregular fibrillar layer with a thickness ranging from 50-100 nm with some parasites showing as many as three mitochondria (Zaman et al., 1995a). Besides this, other studies have reported the presence of prominent rough endoplasmic reticulum and nucleus containing chromatoid-like structures in some cystic forms (Moe et al., 1996; Khalifa., 1999).

Only a few studies have described the ultrastructural features of *Blastocystis* sp. in pigs (Cassidy et al., 1994), monkeys (Cassidy et al., 1994; Stenzel et al., 1997), chickens (Cassidy et al., 1994; Stenzel et al., 1997; Lee et al., 1999), ducks and geese (Stenzel et al., 1994), cockroaches (Yoshikawa et al., 2007) and circus animals comprising of camel, llama and highland bull (Stenzel et al., 1993). This could possibly be due to the limited numbers of cysts usually found in the faecal material of animal hosts. Till to date, most descriptions on either cysts or vacuolar forms of *Blastocystis* sp. isolated from animal hosts have been based on a limited range of animals. There has been no comprehensive study thus far to compare and contrast the vacuolar and cystic forms isolated from a wide range of animals within the scope of one study. This would

provide a better basis to compare the morphology of these life cycle stages from a wider range of animal groups. Therefore the present study attempts to describe the distinct ultrastructural characteristics of cystic and vacuolar forms from a range of animals which includes peacock, orang utan, ostrich, pig, cow, goat and sheep. This will help in source tracking when cysts are detected in water sources especially when an unknown *Blastocystis* sp. is found in the future as well as provide insights into the morphological diversity of these life cycle stages.

4.2 Materials and methods

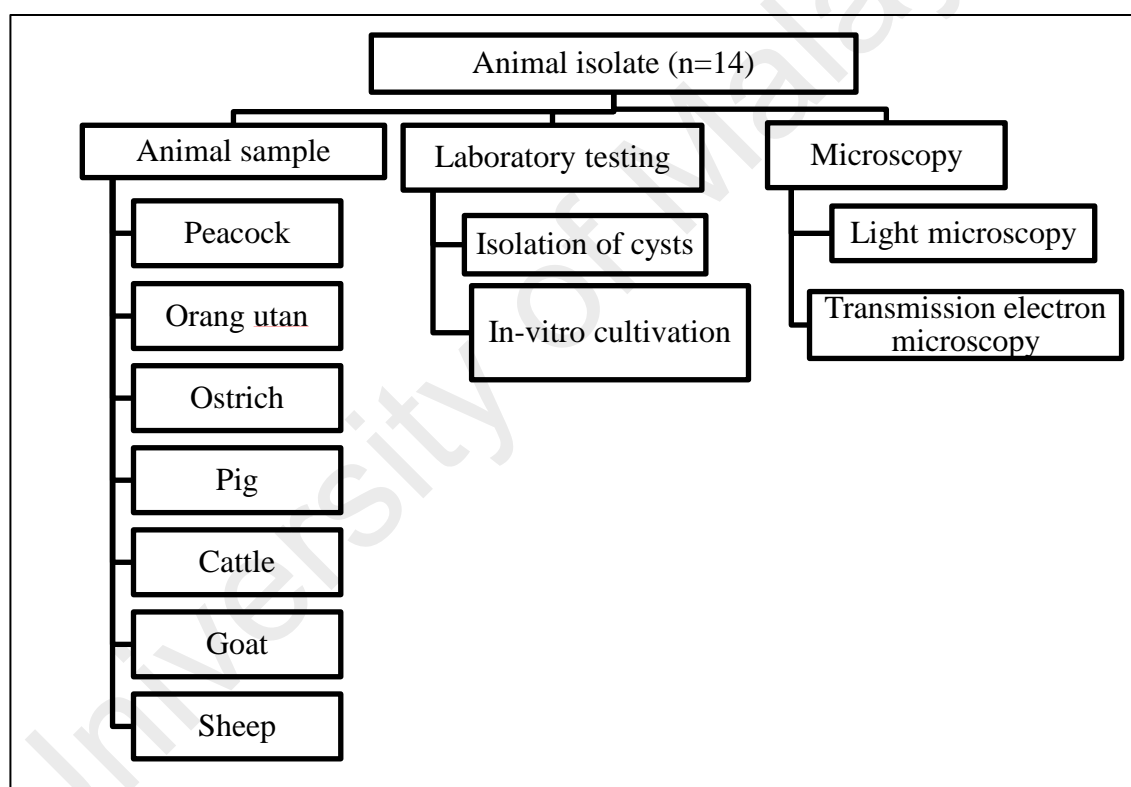


Figure 4.1: Schematic representation of the overall methodology emphasizing on the ultrastructural analysis of *Blastocystis* sp. isolated from animals.

4.2.1 Source of *Blastocystis* sp. cyst

Faecal samples of seven animal species which included ostrich, cow, goat and sheep were collected from Infoternak Sg.Siput farm located at Ipoh, while faecal

samples of orang utan and peacock were collected from Bukit Merah Conservation Centre and pig samples were collected from a private farm in Ipoh. The present study, focused only on the 7 out of 9 animals found positive for *Blastocystis* sp. shown in the previous study (Chapter 3). Due to the limited number of cysts obtained from the fecal samples of gaurs and deer despite several attempts, these two groups of animals were omitted from the present study. For the peacock, orang utan, ostrich, pigs and cows, sampling were carried out from faecal droppings from the ground housing the respective animals whereas rectal swabs were made directly on the smaller ruminant animals such as goats and sheep. The fresh faecal samples from animals were stored in stool containers and processed soon after collection.

4.2.2 Laboratory testing

4.2.2.1 *In-vitro* cultivation of *Blastocystis* sp. isolates

Blastocystis sp. was isolated from the faecal samples of various animals by *in-vitro* cultivation using Jones' medium supplemented with 10% heat-inactivated horse serum at 37°C. Subsequently after isolation, the parasites were maintained in Jones' medium by consecutive sub-cultures every 2 to 3 days for at least one month prior to ultrastructural analysis (Suresh et al., 1994).

4.2.2.2 Isolation of *Blastocystis* sp. cysts

Fresh faecal samples were dissolved in normal saline and sieved into 15ml Falcon culture tubes. The culture tubes were centrifuged at 3000rpm for 10min. The sediment were layered on a 5ml of Ficoll-Paque solution in a culture tube and

centrifuged at 3500rpm for 20 minutes using Beckman Coulter 365303 Spinchron DLX centrifuge machine. The cystic stages, banded between 3 and 4cm located between third and fourth layer in the culture tube (Figure 4.1). These were pipetted into clean 14ml culture tubes. The cysts were washed twice by adding up to 14ml with phosphate buffer solution (PBS). The culture tubes were capped tight, inverted a few times and centrifuged at 3000rpm for 10 minutes for each washing. The supernatant was then discarded completely and 1ml of PBS solution was added into the culture tube containing the sediment. The cysts were fixed in 4% glutaraldehyde fixative and stored at -20°C prior to ultrastructural analysis.

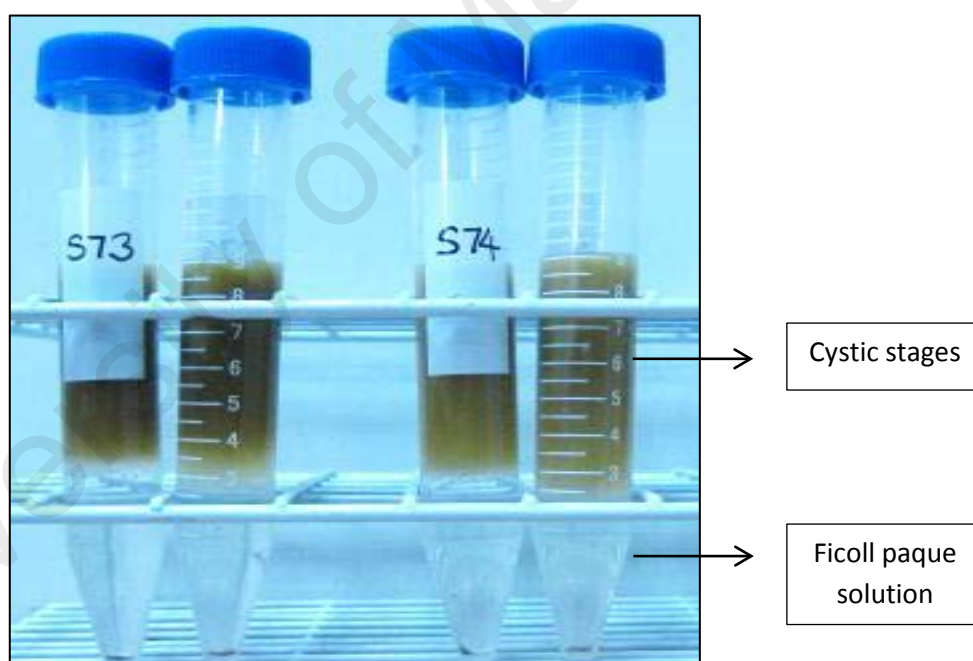


Figure 4.2: Ficoll-Paque concentration of the fecal samples.

4.2.3 Microscopy

4.2.3.1 Light microscopy

All the 14 isolates were examined for *Blastocystis* sp. by wet preparation under light microscopy at 40x magnification. The cells of each isolate were pooled together from day 3 cultures to make a final concentration of 1×10^6 cells/ml in 3 ml screw-capped tubes containing Jones' medium supplemented with 10% horse serum. All cultures were kept in airtight screw-capped tubes and incubated at 37°C. Fifty parasites were randomly chosen from the day 3 culture tube for size measurement. All experiments were done in triplicates.

4.2.3.2 Transmission electron microscopy

The cysts were extracted from the fresh faecal samples of animals using Ficoll paque concentration technique. Subsequently, the parasites were cultured in 3ml Jones' medium supplemented with 10% horse serum at 37°C. Each set consisted of cysts obtained from isolation after Ficoll-paque concentration and day 3 culture were prepared in triplicate. The parasites of each isolate were pooled together after Ficoll-paque concentration to make a final concentration of 1×10^6 parasites/ml. Similarly, the contents of day 3 culture were pooled together to make a final concentration of 1×10^6 parasites/ml. All samples were washed three times using phosphate buffered saline (PBS) pH 7.4 before subjecting them for the transmission electron microscopy analysis. The samples were centrifuged at 3000rpm for 5 minutes. The pelleted cells were re-suspended 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. Semi-thin sections were stained with toluidine blue. Ultrathin sections were cut, contrasted with uranyl acetate and lead citrate and viewed using a transmission electron microscope (LEO Libra120) (Tan et al., 2006).

4.3 Results

4.3.1 Light microscopic observation

4.3.1.1 Vacuolar form

Spherical vacuolar form with various sizes were commonly seen in the faecal culture of all seven animal species comprising of ostrich, pig, goat, orang utan, cow, peacock and sheep (Figure 4.3 A-G). The size of vacuolar form isolated from ostrich, pig, goat, orang utan, cow, peacock and sheep ranged from 9 to 42.9 μm , 4 to 35 μm , 6 to 30 μm , 5 to 25 μm , 10 to 55 μm , 3 to 15 μm and 5 to 28 μm respectively. Vacuolar forms of *Blastocystis* sp. with the largest size range were seen in the isolate from the cow whilst the smallest size range was seen in the peacock isolate (Table 4.1).

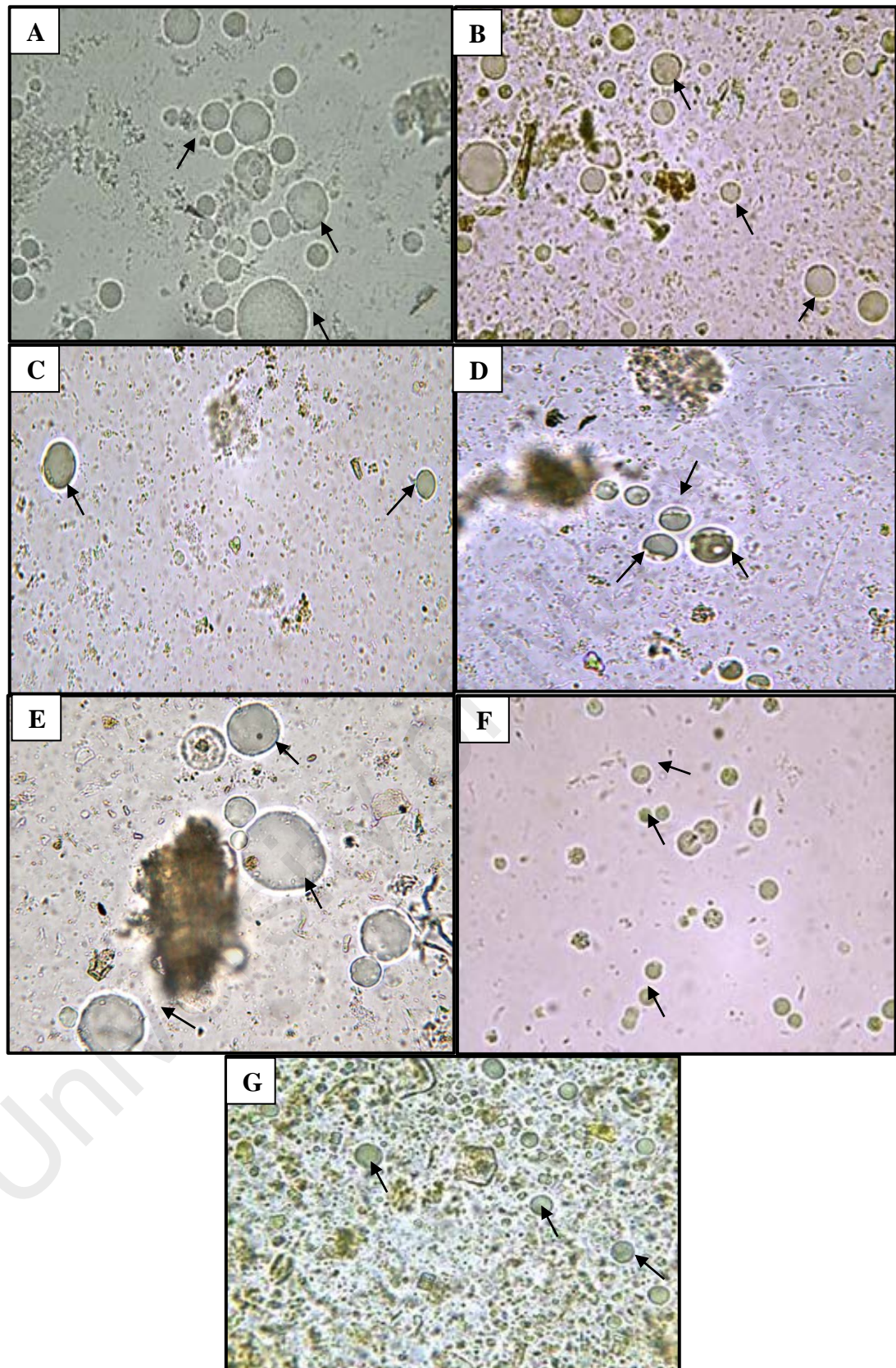


Figure 4.3: Vacuolar form of *Blastocystis* sp. from faecal cultures of A.) ostrich, B.) pig, C.) goat, D.) orang utan, E.) cow, F.) peacock and G.) sheep seen under light

microscopy at 40x magnification. Various sizes of vacuolar forms (*arrow*) were observed.

Table 4.1 Vacuolar forms (VF) of *Blastocystis* sp. from animal isolates (light microscopy)

Animals	Ostrich	Pig	Goat	Orang utan	Cow	Peacock	Sheep
Mean (Size range) (μm)	28.4 (9 - 42.9)	18.6 (4 - 35)	13.9 (6 - 30)	13.2 (5 - 25)	30.2 (10 - 55)	9 (3 - 15)	15.7 (5 - 28)
Shape	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical

4.3.1.2 Cystic form

Rounded cystic form were commonly seen in fresh faecal samples from all seven animal species comprising of ostrich, pig, goat, orang utan, cow, peacock and sheep (Figure 4.4 A-G). The mean diameter of cystic forms isolated from ostrich, pig, goat, orang utan, cow, peacock and sheep ranged from 3.5 to 6.8 μm , 3.3 to 6.3 μm , 2.5 to 5.5 μm , 3.5 to 6.6 μm , 4.5 to 7.1 μm , 2.0 to 4.5 μm and 3.0 to 5.8 μm respectively. Cystic forms of *Blastocystis* sp. with the largest size range were seen in the faecal samples from the cow and smallest size range in the peacock isolate. Table 4.2 shows the description of cystic forms of *Blastocystis* sp. from animal isolates.

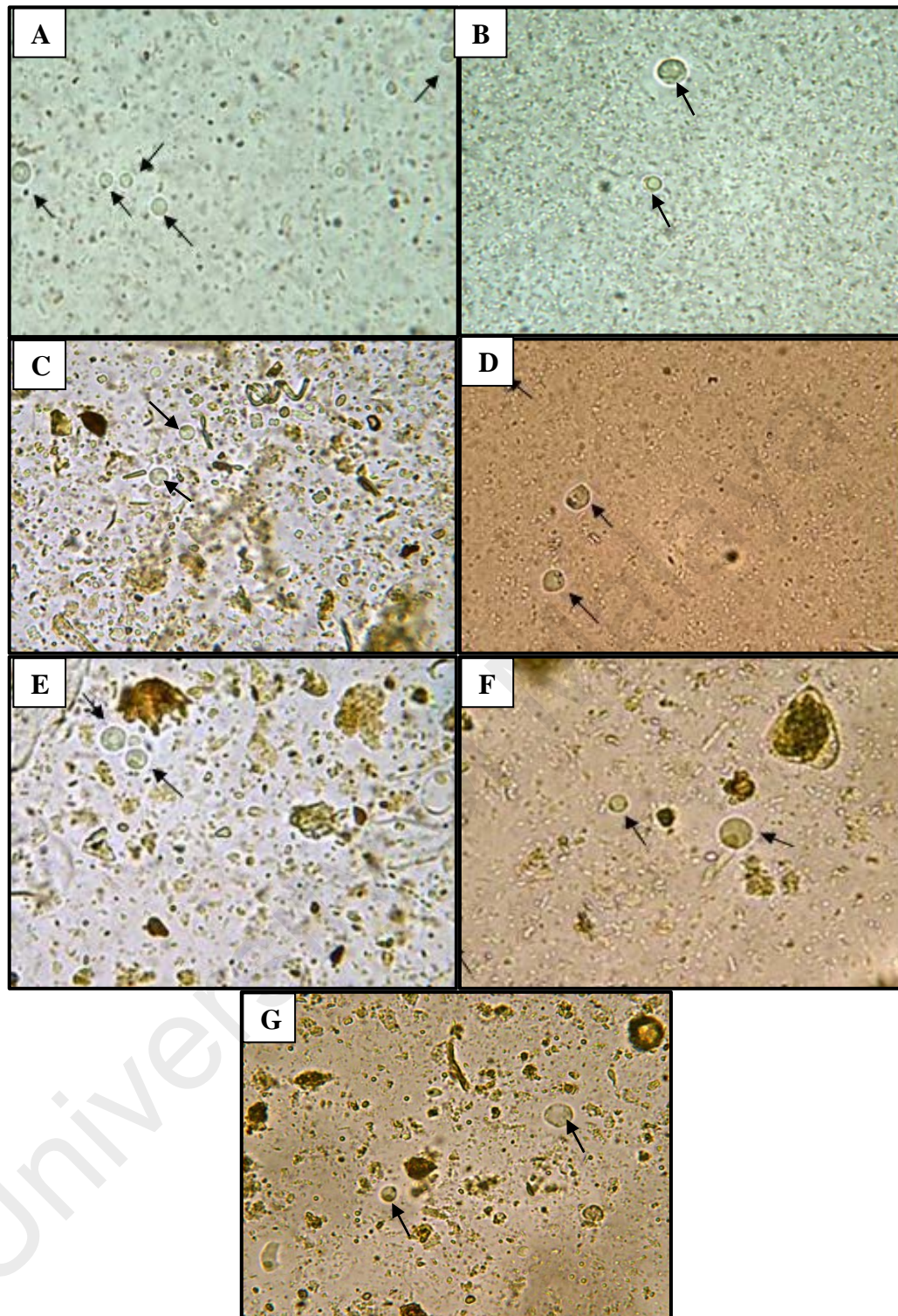


Figure 4.4: Cystic form of *Blastocystis* sp. from fresh faeces of A.) ostrich, B.) pig, C.) goat, D.) orang utan, E.) cow, F.) peacock and G.) sheep seen under light microscopy at 40x magnification. Various sizes of cystic forms (*arrow*) were observed.

Table 4.2: Cystic forms (CF) of *Blastocystis* sp. from animal isolates (light microscopy)

Animals	Ostrich	Pig	Goat	Orang utan	Cow	Peacock	Sheep
Mean (Size range) (µm)	5.1 (3.5-6.8)	5 (3.3-6.3)	4.2 (2.5-5.5)	4.9 (3.5-6.6)	6 (4.5-7.1)	3.4 (2.0-4.5)	4.3 (3.0-5.8)
Shape	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical

4.3.2 Transmission electron microscope analysis

Cysts from the faecal samples of seven animals found positive for *Blastocystis* sp. was extracted from the faecal sample of the respective animals. The TEM figures shown are the representative of what were generally seen in each group of animals. Two animals were selected from each group of animals. TEM study showed distinct ultrastructural characteristics of cystic and vacuolar forms from the seven animals which includes the shape, the presence of prominent nucleus, mitochondrion-like-organelles (MLO), electron dense material and granules as well as the thickness of membrane.

4.3.2.1 Typical characteristics of vacuolar form of *Blastocystis* sp. from animal isolates based on transmission electron microscopic analysis

Vacuolar forms of *Blastocystis* sp. isolated from the ostrich culture were mostly irregular in shape with a high electron dense area observed in the central body. Prominent mitochondrion-like-organelle with cristae and nucleus were seen at the peripheral of cytoplasm with a thick fuzzy coat surrounding the parasite (Figure 4.5A).

Most of the vacuolar forms of *Blastocytis* sp. isolated from the pigs were spherical in shape with the high electron dense material in the central body and a prominent nucleus observed at one end of the peripheral cytoplasm in most of the cells (Figure 4.5B). *Blastocytis* sp. vacuolar forms isolated from the goat were spherical in shape with some showing central body filled with electron-dense granules. Prominent nucleus was observed in the cytoplasm (Figure 4.5C). Vacuolar forms of *Blastocytis* sp. isolated from the orang utan were mostly irregular in shape with less electron dense material in the central body. There was a prominent thick fuzzy coat surrounding the parasite with the presence of MLO showing prominent cristae (Figure 4.5D). Spherical vacuolar forms of *Blastocytis* sp. isolated from the cow show a thin fuzzy coat surrounding the parasite with high electron dense material observed within the central body (Figure 4.5E). Vacuolar forms from the peacock culture were mostly irregular in shape with numerous granules in the central vacuole. Thick and intact fuzzy coat was often seen surrounding the parasite (Figure 4.5F). Most of the vacuolar forms from the sheep isolate were irregular in shape with a prominent thick fuzzy coat surrounding the parasite. High electron dense material were seen to surrounding the central body containing the electron dense granules (Figure 4.5G). The membrane layer surrounding the vacuolar forms isolated from ostrich, pig, goat, orang utan, cow, peacock and sheep ranged from 235.48 to 345.22 nm, 227.99 to 258.22 nm, 214.89 to 232.71 nm, 110.45 to 142.50 nm, 77.18 to 119.33 nm, 118.72 to 167.99 nm and 104.84 to 157.47 nm respectively. The thickest membrane observed was seen in the parasites from the ostrich isolate whilst the thinnest in parasites from the cow isolate. Detailed description of vacuolar forms of animal isolates is shown (Table 4.3).

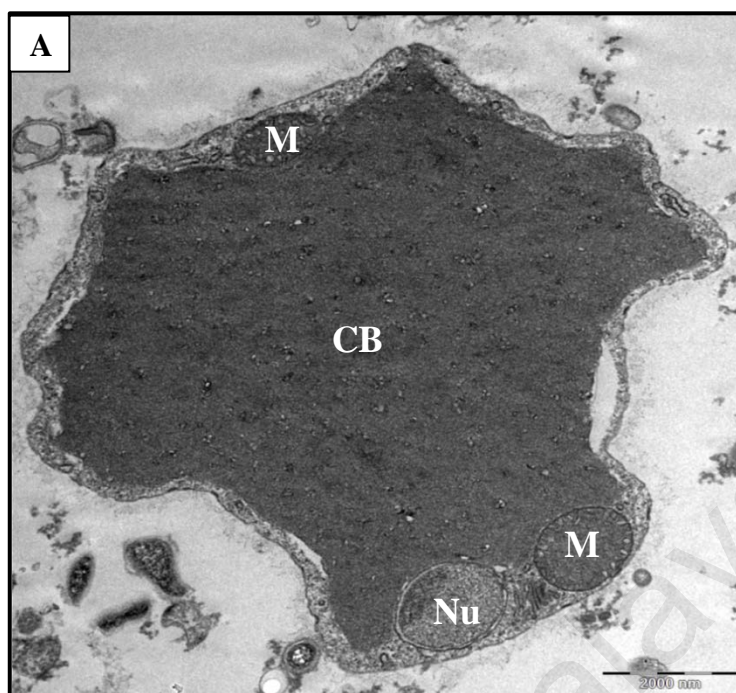


Figure 4.5A Irregular vacuolar form of *Blastocytis* sp. seen in the ostrich isolate based on TEM analysis. Note a thick fuzzy coat surrounding the parasite. A high electron dense area was observed in the central body (CB). Prominent mitochondrion-like-organelle (M) with cristae and nucleus (Nu) were seen at the peripheral of cytoplasm.

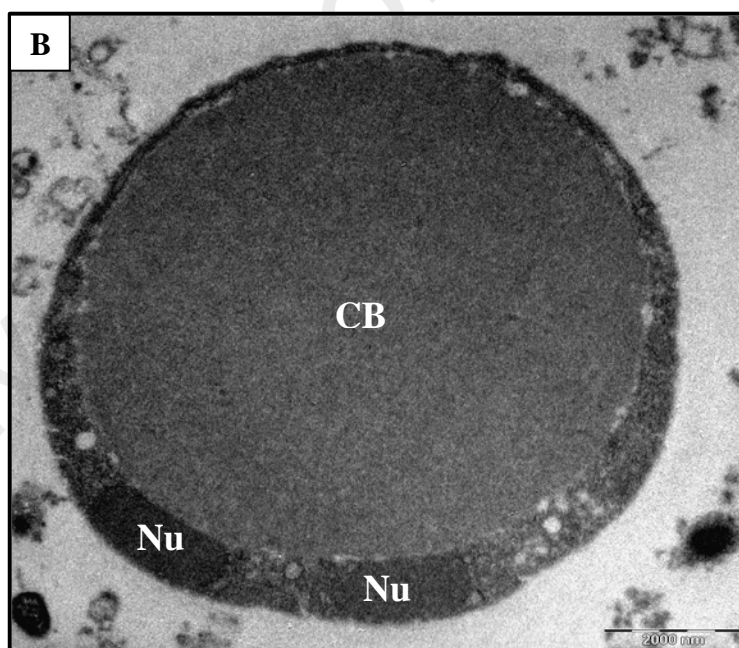


Figure 4.5B Spherical vacuolar form of *Blastocytis* sp. from the pig isolate based on TEM analysis. Central body (CB) contains high electron dense material with prominent nucleus (Nu) at one end of the peripheral cytoplasm.

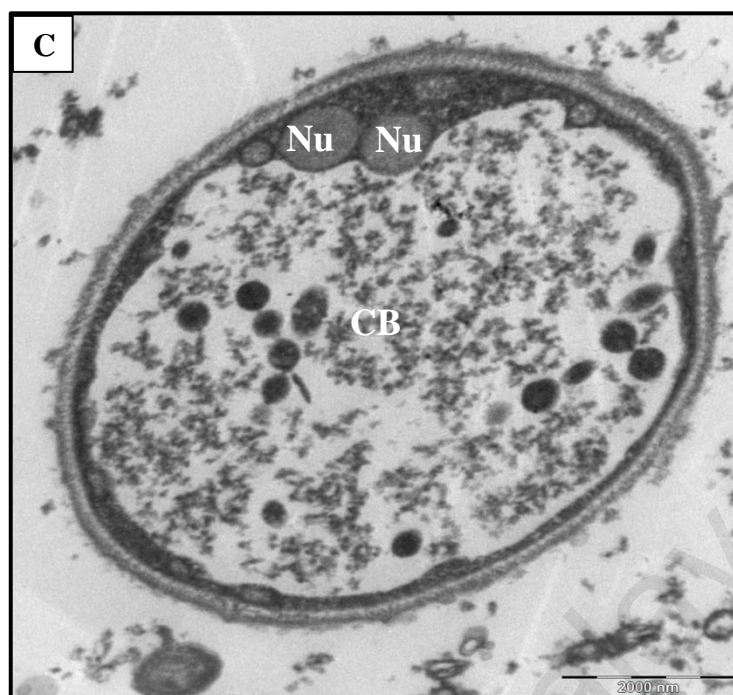


Figure 4.5C Spherical vacuolar form of *Blastocytis* sp. from the goat isolate based on TEM analysis. Central body (CB) is filled with electron-dense granules with prominent nucleus (Nu) at the end of the peripheral cytoplasm.

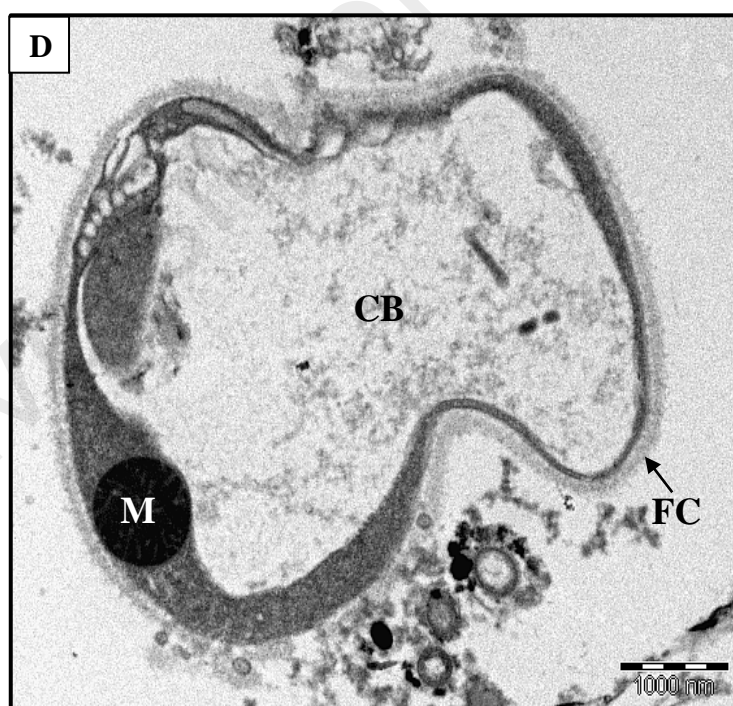


Figure 4.5D Irregular vacuolar form of *Blastocytis* sp. from the orang utan isolate based on TEM analysis. Central body (CB) contains less electron dense material with presence of MLO with cristae at one end of the peripheral cytoplasm. Thick fuzzy coat (FC) is seen surrounding the parasite.

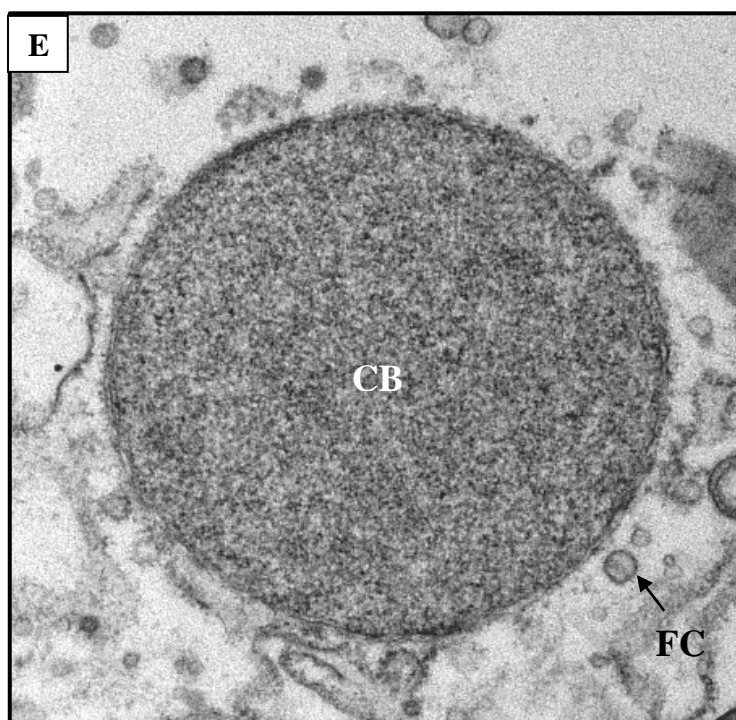


Figure 4.5E Spherical vacuolar form of *Blastocytis* sp. from the cow isolate based on TEM analysis. Central body (CB) contains high electron dense material. Thin fuzzy coat (FC) is seen surrounding the parasite.

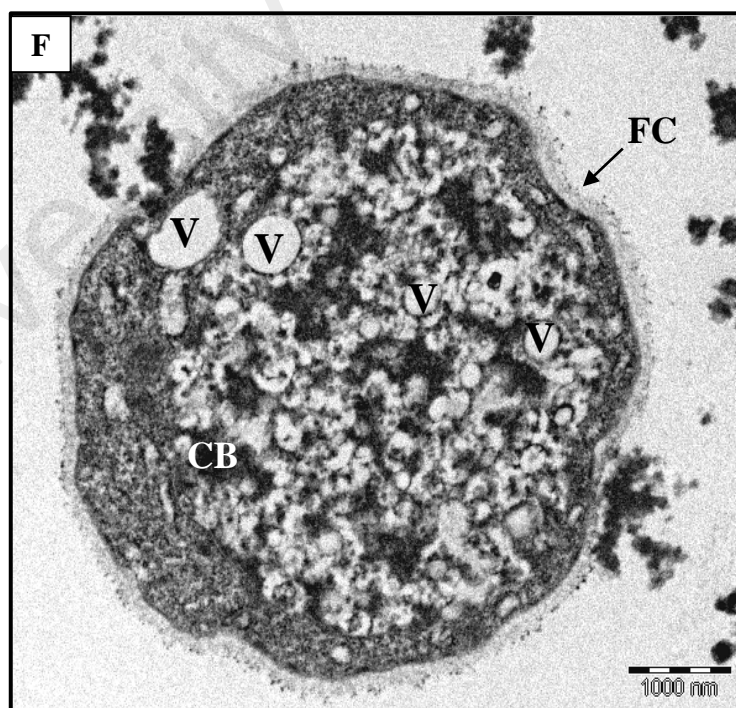


Figure 4.5F Irregular vacuolar form of *Blastocytis* sp. from the peacock isolate based on TEM analysis. Central body (CB) contains numerous granules and small vacuoles (V). Thick and intact fuzzy coat (FC) is surrounding the parasite.

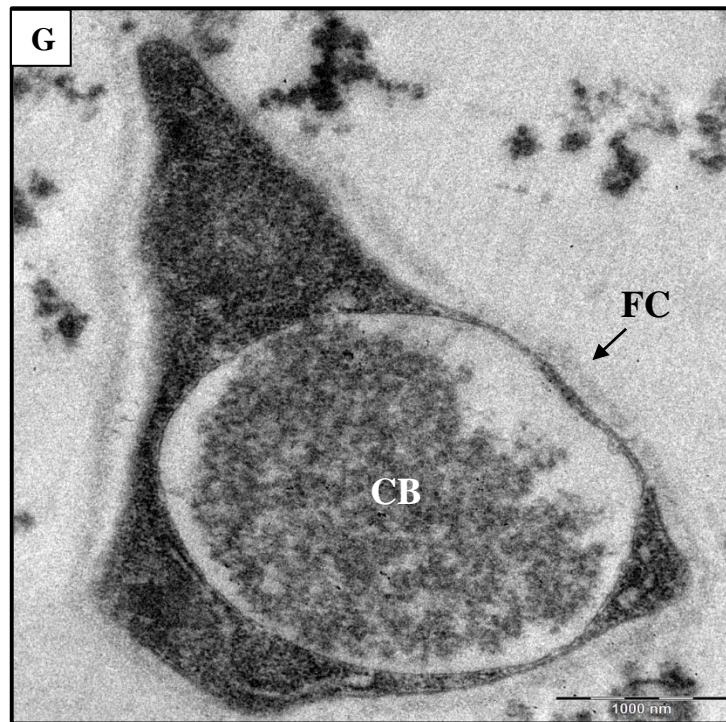


Figure 4.5G Irregular vacuolar form of *Blastocytis* sp. from the sheep isolate based on TEM analysis. High electron dense area is seen surrounding the central body (CB) containing electron dense granules. Thick fuzzy coat (FC) is observed surrounding the parasite.

Table 4.3: Detailed description of vacuolar forms of animal isolates (Transmission electron microscopy analysis).

Animals	Peacock	Pig	Cow	Sheep	Goat	Ostrich	Orang utan
Membrane thickness mean (nm) (range)	139.49 (118.72-167.99)	236.45 (227.99-258.22)	96.42 (77.18-119.33)	134.70 (104.84-157.47)	218 (214.89-232.71)	302.47 (235.48-345.22)	131.37 (110.45-142.50)
Shape	Irregular	Spherical	Spherical	Irregular	Spherical	Irregular	Irregular
Presence of nucleus	Absent	Present	Absent	Absent	Present	Present	Absent
Presence of MLO	Absent	Absent	Absent	Absent	Absent	Present	Present
Electron dense material	High	High	High	High	Less	High	Less

4.3.2.2 Typical characteristics of cystic forms of *Blastocystis* sp. from animal isolates based on transmission electron microscopic analysis

Cystic forms from ostrich isolates were spherical in shape with high electron dense observed in the central body and numerous prominent small vacuoles were seen within the parasite (Figure 4.6A). Cystic forms of pig isolates were spherical in shape with a central body which is less dense. Prominent nucleus was observed at the peripheral cytoplasm with a thick fuzzy coat surrounding the cyst (Figure 4.6B). Cystic forms of goat isolates were spherical in shape with the central body containing less electron dense material with prominent small vacuoles (Figure 4.6C). Spherical cystic forms of orang utan isolates have been shown to be surrounded by a thick fuzzy coat surrounding the central body which contained high electron dense materials. Irregular and elongated form of MLO with prominent cristae was also seen in the cytoplasm (Figure 4.6D). Cysts from the fresh faeces of the cow were spherical in shape with high electron dense area and numerous granules observed in the central body (Figure 4.6E). Cystic forms from peacock isolates were spherical in shape with thick fuzzy coat surrounding the cyst. Less electron dense material were observed in the central body with more than two prominent nuclei seen in the cytoplasm (Figure 4.6F). Cystic forms from sheep isolates were spherical in shape with thick fuzzy coat surrounding the cyst. Prominent nucleus was observed at the end of the peripheral cytoplasm with less electron dense material in the central body. (Figure 4.6G). The cyst wall layer of the cystic forms isolated from ostrich, pig, goat, orang utan, cow, peacock and sheep ranged from 298.15 to 365.45 nm, 274.19 to 292.58 nm, 136.23 to 183.97 nm, 125.56 to 152.92 nm, 97.89 to 141.15 nm, 150.22 to 180.72 nm and 130.44 to 178.47 nm respectively. The thickest cyst wall was observed in the ostrich isolate and the thinnest in the cow isolate. Detailed description of cystic forms of animal isolates is shown in Table 4.4.

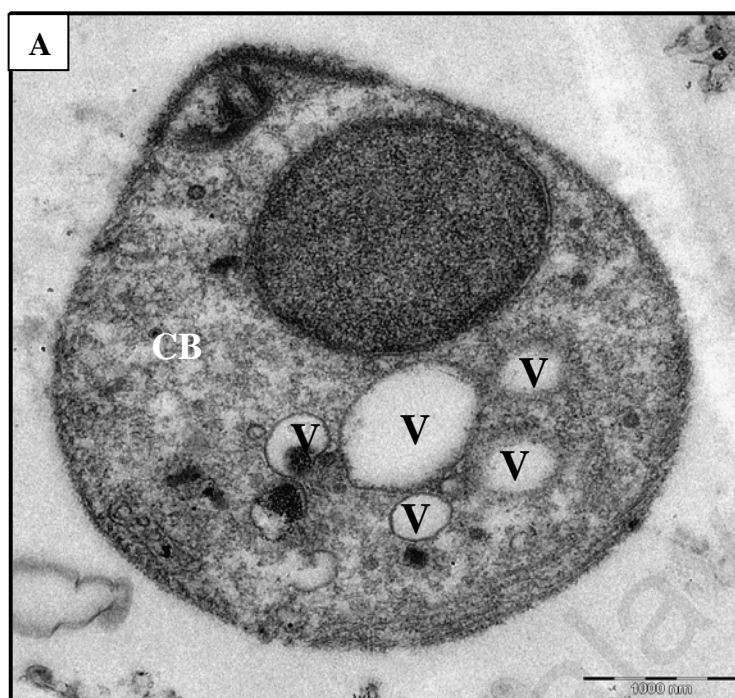


Figure 4.6A Cyst form from the ostrich isolate based on TEM analysis. Small vacuoles (V) were seen within the parasite. High electron dense area was observed in the central body (CB).

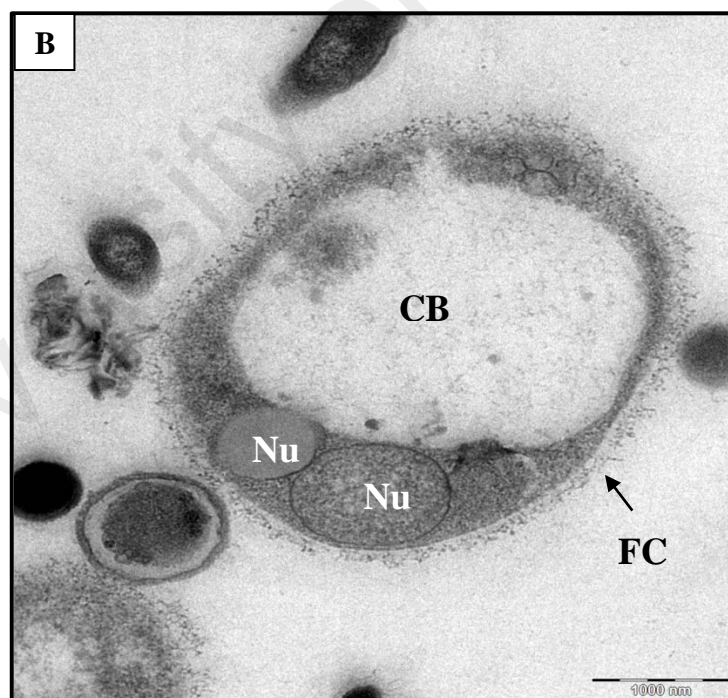


Figure 4.6B Cyst form from the pig isolate based on TEM analysis. Presence of thick and prominent fuzzy coat (FC) surrounding the parasite. Central body (CB) contains less electron dense material with presence of nucleus (Nu) at one end of the peripheral cytoplasm.

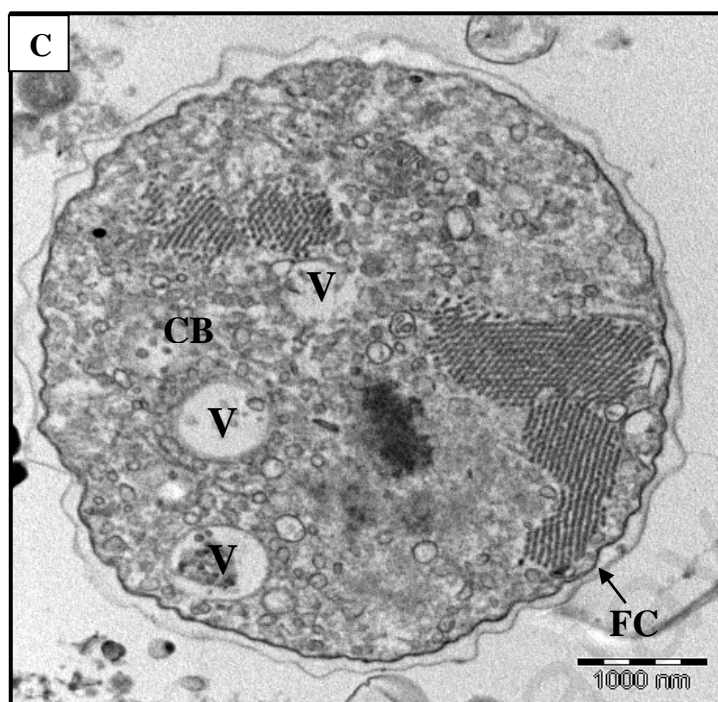


Figure 4.6C Cyst form from the goat isolate based on TEM analysis. Presence of thin fuzzy coat (FC) surrounding the parasite. Central body (CB) contains less electron dense material with presence of small vacuoles (V).

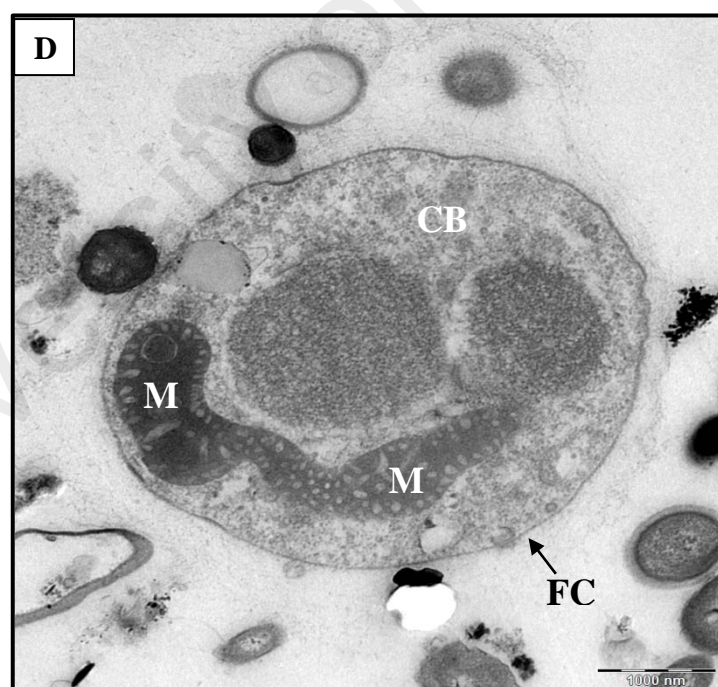


Figure 4.6D Spherical cystic forms of orang utan isolates are surrounded by a thick fuzzy coat (FC) and central body (CB) contains high electron dense materials based on TEM analysis. Irregular and elongated form of MLO (M) with prominent cristae was also seen in the cytoplasm

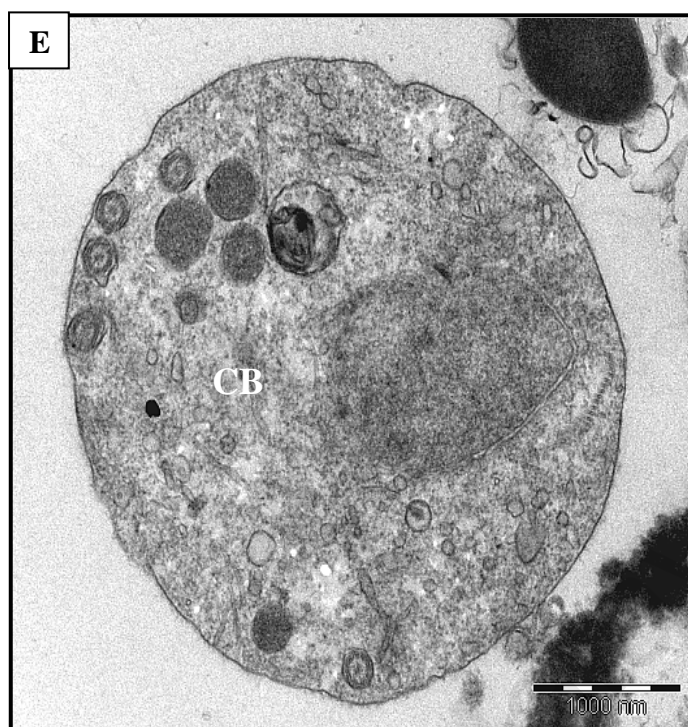


Figure 4.6E Cyst form from the cow isolate based on TEM analysis. Central body (CB) contains high electron dense granules.

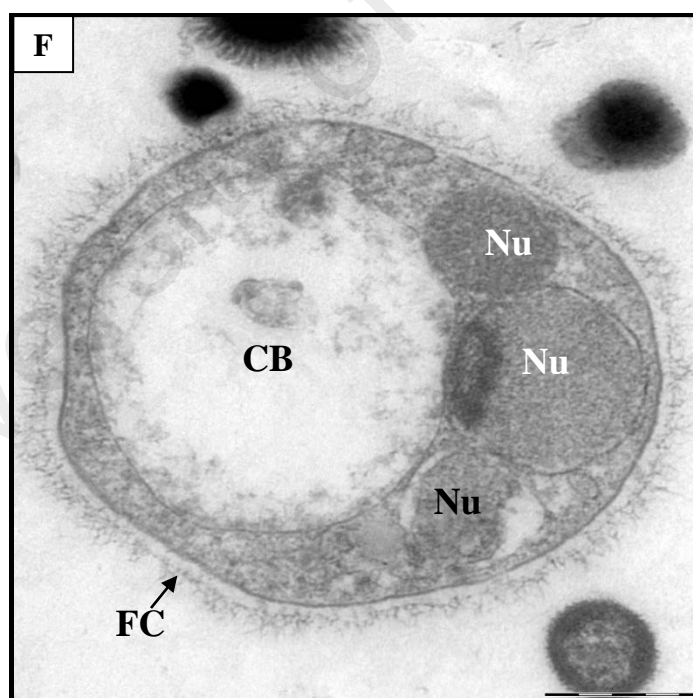


Figure 4.6F Cyst form from the peacock isolate based on TEM analysis. Central body (CB) contains less electron dense material with more than two prominent nuclei (Nu) at the end of the peripheral cytoplasm. Thick fuzzy coat (FC) is seen surrounding the parasite.

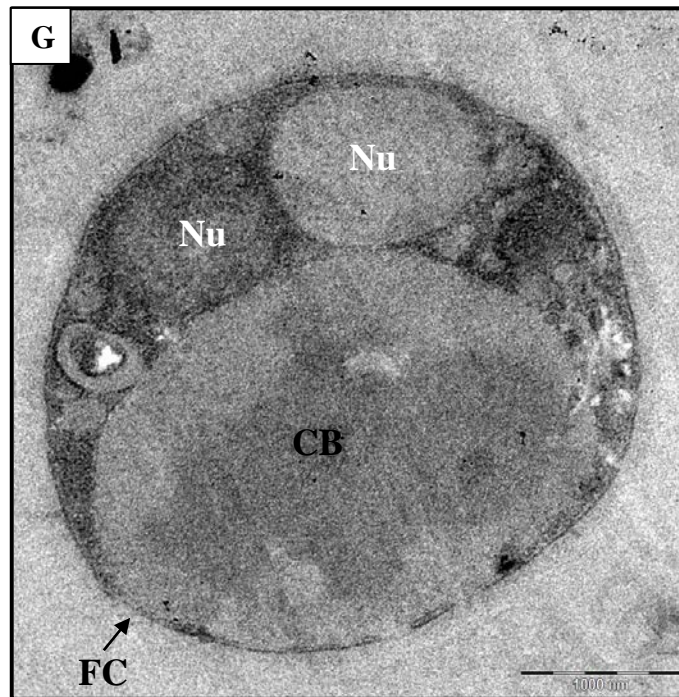


Figure 4.6G Cyst form from the sheep isolate based on TEM analysis. Central body (CB) contains less electron dense material with prominent nucleus (Nu) at the end of the peripheral cytoplasm. Thick fuzzy coat (FC) is seen surrounding the cyst.

Table 4.4: Detailed description of cystic forms of animal isolates (Transmission electron microscopy analysis).

Animals	Peacock	Pig	Cow	Sheep	Goat	Ostrich	Orang utan
Cyst wall thickness mean (nm) (range)	165.82 (150.22 -180.72)	283.28 (274.19-292.58)	115.22 (97.89-141.15)	148.93 (130.44-178.47)	161.59 (136.23-183.97)	328.98 (298.15-365.45)	136.98 (125.56-152.92)
Shape	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical
Presence of nucleus	Present	Present	Absent	Present	Absent	Absent	Absent
Presence of MLO	Absent	Absent	Absent	Absent	Absent	Absent	Present
Electron dense material	Less	Less	High	High	Less	Less	High

4.4 Discussion and conclusion

Based on the light microscopy, the diameter range of vacuolar forms from the animal isolates were comparatively smaller than human isolates which usually range from 2 to 200 μm (Stenzel & Boreham, 1996; Zierdt, 1991). The cystic forms of the animal isolates were comparatively larger than the human isolate which range from 3 to 6 μm (Zaman et al., 1995b). In the present study, ultrastructural findings on *Blastocystis* sp. of animal isolates concurred with the previous studies (Tan, 2004; Yoshikawa et al., 2007). Most of the *Blastocystis* sp. appeared to be rounded or slightly irregular in shape with a thick and compact surface coat seen to be surrounding the cell. One or more nuclei were seen in most of the cystic and vacuolar forms of the animal hosts.

Studies previously have shown that vacuolar forms of *Blastocystis* sp. isolated from the ostriches are consistently small with a diameter of 6-10 μm (Stenzel et al., 1994). However, in the present study, the size range of the vacuolar forms ranged from 9 - 42.9 μm which is remarkably larger, the finding of which contradicts with the previous finding. This could be due to the culture conditions where it has been reported previously that culture medium is capable of altering the general appearance of the organism (Stenzel et al., 1991). In the present study, the cyst wall of ostrich cyst was seen to be the thickest. Similar findings were also reported by Stenzel et al., (1994) where they showed that the ostrich isolate had the thickest surface coat relative to the cell diameter when compared to the other avian hosts. These findings imply that greater resistances of the cystic forms isolated from non-human hosts do have the ability to cause infection in other animal species and human. Besides that, the cell organelles such as MLO (mitochondria-like organism) and nucleus were very prominent with high

electron dense material often seen occupying the central vacuole of the ostrich isolate in the present study.

Cystic forms of *Blastocystis* sp. from the fresh faeces of pig have been studied previously (Cassidy et al., 1994) however there was very little information on the morphology reported. They described only the surface thickness of the cyst based on transmission microscopy which ranged from 250 to 500nm. In the present study, the surface thickness of the cystic stage from pigs showed a thickness of 274.19 to 292.58nm. Both cystic and vacuolar forms isolated from the pig were mostly spherical in shape and the central body of most vacuolar forms showed high electron dense material occupying the central body of the vacuolar forms.

The present study is the first to describe the ultrastructure of *Blastocystis* sp. cyst isolated from orang utan which was shown to be mostly spherical and surrounded by a thick fuzzy coat with a high electron dense material occupying in the central body. A previous study highlighted that most cystic forms from monkeys were round, except some which appeared elongated and irregular in shape (Stenzel et al., 1996). In comparison, cysts from orang utan in the present study were smaller based on light microscopic observation than the cystic forms of monkeys which ranged from 12 to 15 μm in diameter (Stenzel et al., 1996). In contrast, the vacuolar forms from orang utan in the present study were comparatively larger than *Blastocystis* sp. from the monkey isolate which is usually small (approximately $6\pm 10 \mu\text{m}$ in diameter). One very unique characteristic shown in the present study was the large, irregular and elongated form of MLO with prominent cristae in the cytoplasm than those seen in the vacuolar forms.

This finding concurred with the previous study seen in the monkey samples (Stenzel et al., 1996).

Despite previous studies that have reported on the prevalence of *Blastocystis* sp. in birds (Stenzel et al., 1994) such as peacock (Abe et al., 2002; Yoshikawa et al., 2004), there has been no study highlighting the morphological and ultrastructural features of *Blastocystis* sp. from the peacock isolate. In the present study, the smallest size range of *Blastocystis* sp. was observed in the peacock isolate for both vacuolar and cystic forms. The vacuolar forms ranged from 3 to 15 μm compared to those isolated from other avian hosts which was previously reported to be from 3 to 120 μm (Lee & Stenzel., 1999; Stenzel et al., 1994). However, the cystic forms were similarly small with a range from 3 to 4 μm (Lee & Stenzel., 1999). In the present study, the central body of parasite isolated from the peacock contains numerous granules and small vacuoles in the vacuolar forms, a similar description seen in *Blastocystis* sp. isolated from the duck (Stenzel et al., 1994). Most cystic forms from the peacock showed multiple nuclei. A similar finding was reported in the domestic chicken (Lee & Stenzel 1999).

Several studies have reported the prevalence of *Blastocystis* sp. among the livestock animals such as cattle, goat and sheep (Quilez et al., 1995; Abe et al., 2002; Lee et al., 2012), however none have described the morphological and ultrastructural features of *Blastocystis* sp. isolated from these animals. In the present study, both vacuolar and cystic forms of *Blastocystis* sp. isolated from the cow showed the thinnest membrane when compared to the other animals in the study group. Meanwhile, cystic forms from sheep and goat isolates mostly showed less electron dense materials, however the cystic forms from the sheep isolate contain prominent nucleus with a thick fuzzy coat often surrounding the cells, while nucleus was not seen in most of the cysts from the goat and

were surrounded by a thin membrane. However, there has been no previous study carried out to describe the ultrastructural features of *Blastocystis* sp. isolated from the livestock animals such as cow, sheep and goat.

In conclusion, the present study is the first to show distinct ultrastructural characteristics of cystic and vacuolar forms from a range of animals. Most studies confine the same attempt within one or two animal groups but this will not give an opportunity to compare and appreciate the morphological diversity that exists between various animal groups. Based on the description and the size range of cystic and vacuolar forms from a range of animals, it was possible to develop a schematic diagram to demonstrate major differences in the morphology of the vacuolar (Figure 4.7) and cystic (Figure 4.8) forms especially from these seven animals described in the schematic representation below in order to trace the source of the cysts when seen in aquatic sources. A simple guideline is included below to assist researches in using the diagram below for source tracking.

1.) Vacuolar forms:-

- a) Samples should be subjected for both light and electron microscopy studies.
- b) The sizes should be noted and based on the size eg: if more than 30µm, then it is possible that the parasite could belong to pig, cow and ostrich.
- c) Based on TEM analysis, if the membrane thickness of the parasite is lesser than 200nm and the shape of the parasite is spherical, the parasite could belong to cow.
- d) If the parasite does not have a prominent nucleus, then it could belong to cow.

- e) If the parasite had no prominent mitochondria-like organism (MLO), then it could belong to cow.
- f) If the central vacuole contain high electron dense material, the parasite can be confirmed as cow isolate.

2.) Cystic forms:-

- g) Samples should be subjected for both light and electron microscopy studies.
- h) The sizes should be noted and based on the size eg: if lesser than 6 μ m, then it is possible that the cyst could belong to peacock, sheep and goat.
- i) Based on TEM analysis, if cyst wall of the cyst is lesser than 200nm and the shape of the cyst is spherical, the cyst could belong to peacock, sheep or goat.
- j) If the cyst does not have a prominent nucleus, the cyst could belong to goat.
- k) If the cyst had no prominent mitochondria-like organism (MLO), the cyst could belong to goat.
- l) If the central vacuole contain less electron dense material, the cyst can be confirmed as goat isolate.

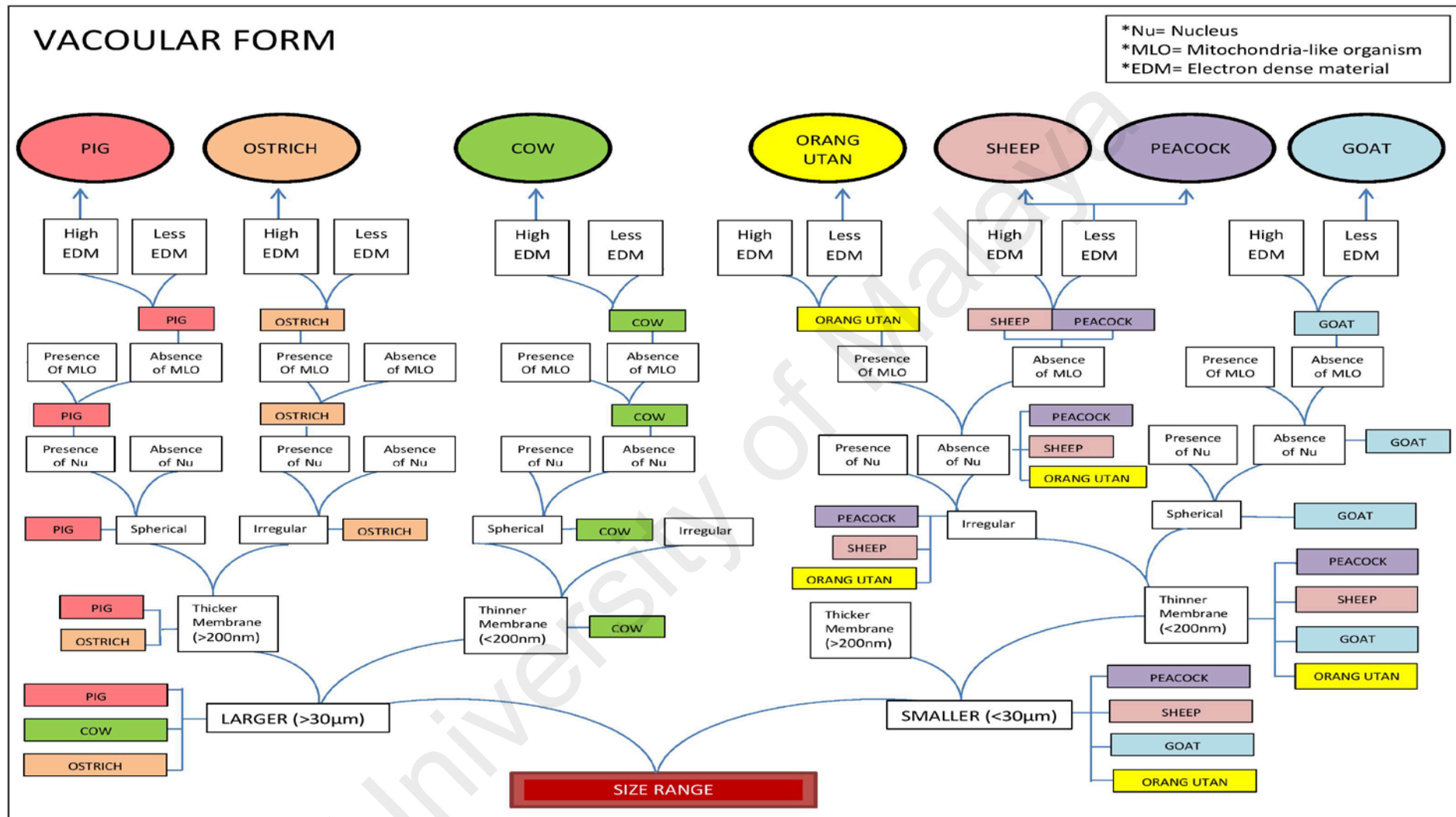


Figure 4.7 Schematic representation of the major differences in the morphology of the vacuolar forms of *Blastocystis* sp.

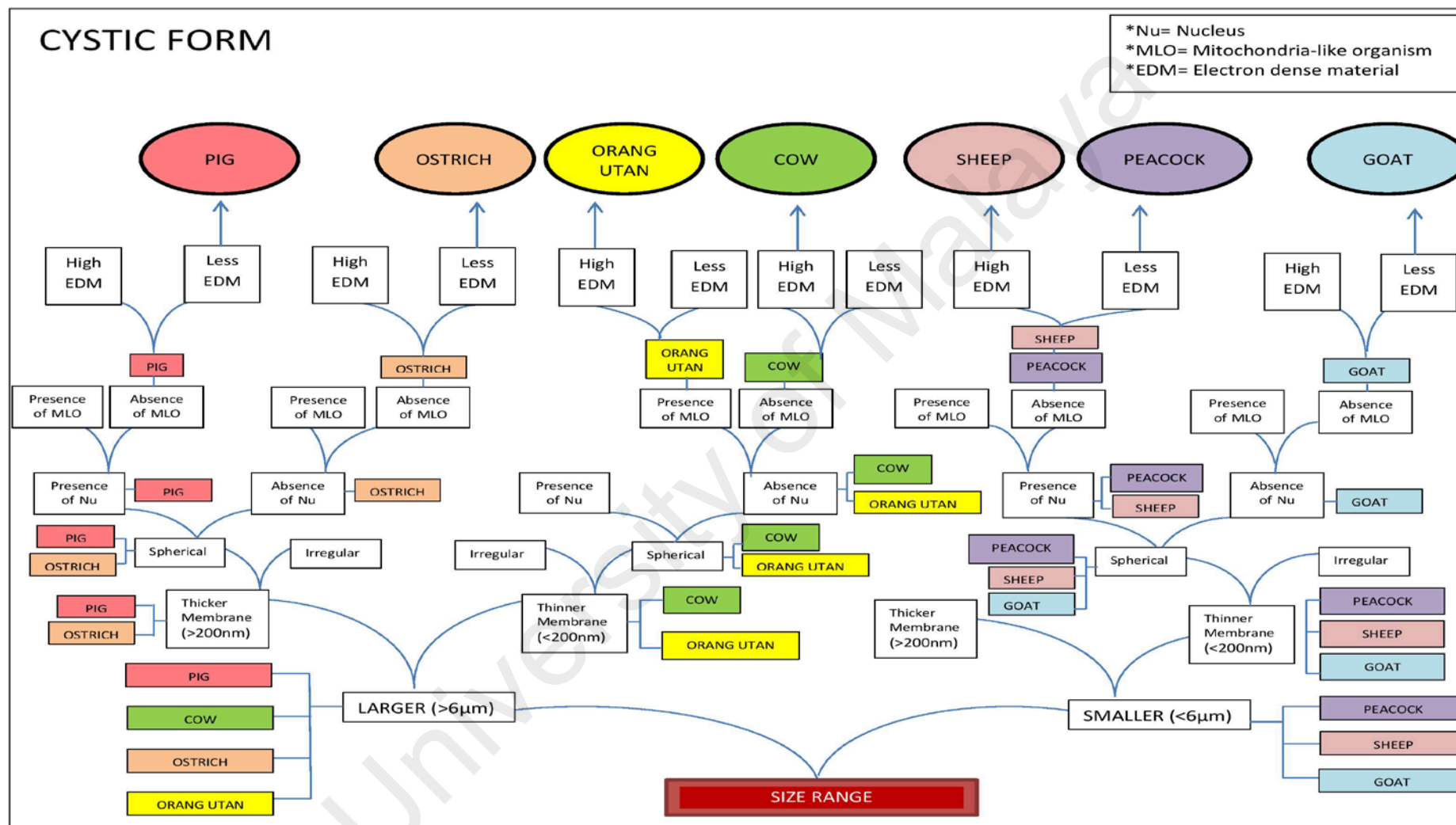


Figure 4.8 Schematic representation of the major differences in the morphology of the cystic forms of *Blastocystis* sp.

CHAPTER 5

EXPERIMENTAL INFECTION STUDIES IN SPRAGUE DAWLEY RATS USING *BLASTOCYSTIS* SP. FROM ANIMAL HOSTS

5.1 Introduction

Several molecular studies on *Blastocystis* sp. have shown that both human (Yoshikawa et al., 1996, Arisue et al., 2003; Yoshikawa et al., 2004b) and animal isolates (Abe et al., 2003a; Noel et al., 2003) display genetically polymorphic organisms (Arisue et al. 2003), however most of the isolates from humans (Mehlhorn, 1988; Stenzel & Boreham, 1991) and animals (Zaman et al., 1995; Cassidy et al., 1994) are identical or very close to each other on the basis of the morphological or ultrastructural observations.

To date, several new species have been proposed from various animal hosts based on the host origins, morphology, and molecular characterization which includes mammalian, amphibian (Belova & Krylov, 1997); reptilian (Teow et al., 1991; Singh et al., 1996), avian (Tanizaki et al., 2005), rodents (Chen et al., 1997) and insects (Zaman et al., 1993). Most of *Blastocystis* sp. from humans and animals has been suggested to exhibit low host specificity (Pakandl, 1992; Tanizaki et al., 2005). If *Blastocystis* sp. do have low host specificity or if the organisms from animal hosts are capable of transmitting the infection to humans, the number and range of animals found to be infected with *Blastocystis* sp. based on the recent prevalence studies (Alfellani et al., 2013; Roberts et al., 2013; Tan et al., 2013) indicate a vast potential reservoir for human infections to take place (Stenzel & Boreham, 1996).

In recent years, the number of livestock farms housing cattle, pigs goats and sheep have exponentially increased 23% between 1980 and 2010, based on research conducted by the Worldwatch Institute, (2013). This growth is driven by an increasing demand for livestock products due to population growth, urbanization and the increasing income

especially in developing countries (Delgado, 2005). Correspondingly there has been an increase in the rodent population which has been rising steadily during the past 30 years in rural dwellings and especially farms where food and nesting sites have been shown to be plentiful (Endepols & Klemann, 2004). These animals consume and in the process contaminate food meant for livestock, other animals, and human which eventually lead to disease transmission facilitated from rats to both humans and livestock. In the present study we aimed to obtain cysts of *Blastocystis* sp. from peacock, orang utan, ostriches, pig, cow, goat and sheep which previously (Chapter 3) were shown to be positive for *Blastocystis* sp. and cause experimental infection in rats to assess how host specific *Blastocystis* sp. really is.

5.2 Materials and methods

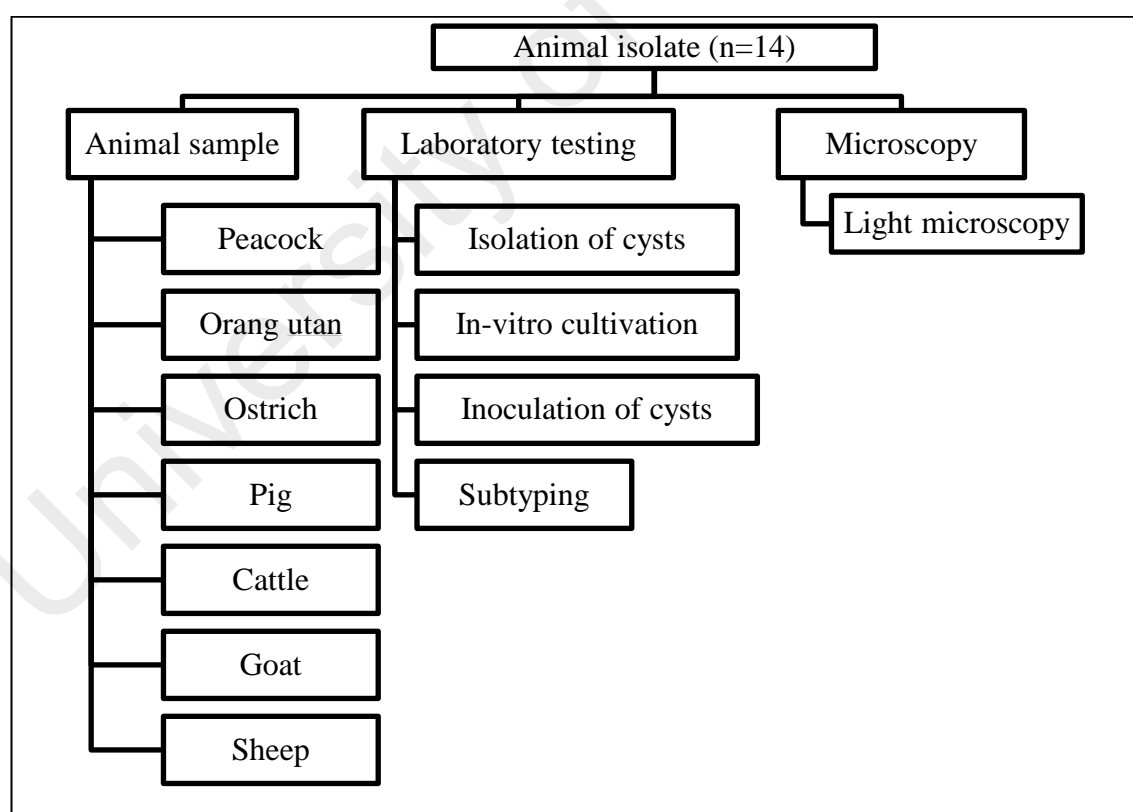


Figure 5.1: Schematic representation of the overall methodology emphasizing on the inoculation of *Blastocystis* sp. isolated from various animals into Sprague Dawley rats.

5.2.1 Source of *Blastocystis* sp. cyst

Fresh faecal samples of seven animal species which included peacock, ostrich, cow, goat and sheep were collected from Infoternak Sg.Siput farm located at Ipoh, while faecal samples of orang utan were collected from Bukit Merah Conservation centre. Fecal samples from pigs were collected from private farms in Ipoh. For the peacock, orang utan, ostrich, pigs and cows, sampling were carried out from faecal droppings from the ground housing the respective animals whereas rectal swabs were made directly on the smaller ruminant animals such as goats and sheeps. The fresh faecal samples from animals were stored in stool containers and processed soon after collection.

5.2.2 Laboratory testing

5.2.2.1 In vitro cultivation of *Blastocystis* sp. isolates

Blastocystis sp. was isolated from the fecal samples of various animals by *in-vitro* cultivation using Jones' medium supplemented with 10% heat-inactivated horse serum at 37°C. Subsequently after isolation, the cells were maintained in Jones' medium by sub-culturing once every 2 to 3 days (Suresh et al., 1994).

5.2.2.2 Isolation of *Blastocystis* sp. cysts

Fresh faecal samples were dissolved in normal saline and sieved into 15ml Falcon culture tubes. The culture tubes were centrifuged at 3000rpm for 10min. The sediment were layered on a 5ml of Ficoll-Paque solution in a culture tube and centrifuged at 3500rpm for 20 minutes using Beckman Coulter 365303 Spinchron DLX

centrifuge machine. The cystic stages, banded at 3 to 4cm are the third and fourth layer in the culture tube. These were pipetted into clean 14ml culture tubes. The cysts were washed twice by adding up to 14ml with phosphate buffer solution (PBS). The culture tubes were capped tight and inverted a few times and centrifuge at 3000rpm for 10 minutes for each washing. The supernatant was discarded completely and 1ml of PBS solution was added into the tube which contains the sediment. The cysts were enumerated and diluted to a concentration of 10^6 cysts/ml.

5.2.3 Inoculation of *Blastocytis* sp. cysts in Sprague Dawley rats

The cysts from each of the animal isolate were diluted to a concentration of 10^6 cysts/ml and inoculated orally, using 20G feeding needle of 1.5inch length into the seven batches of five rats each respectively. All the rats were anesthetized before inoculation

5.2.4 Microscopy

5.2.4.1 Light microscopy

Stool samples from all rats were checked for the parasite daily prior to post-infection. Faecal samples were collected and cultured in Jones' medium supplemented with 10% horse serum. The samples were microscopically examined 24, 48 and 72 hours post-inoculation (Suresh et al., 1994).

5.2.5 Molecular detection

5.2.5.1 Genomic DNA preparation

DNA was extracted from culture samples of all the inoculum and rats were infected with the animal isolates using QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol (Yoshikawa et al., 2004b).

5.2.5.2 Subtyping of *Blastocystis* sp. isolates

All parasite isolates were subjected to sequenced-tagged site (STS) primer-polymerase chain reaction using the following ten sets of primers (Yoshikawa et al., 2004b) (Table 3.2). Two to five μ l of DNA preparations were used to amplify the genomic sequences in a 20 μ l reaction containing 0.5mM of the dNTPs, 0.5mM of each primer, 1 x PCR buffer (75mM Tris-HCL, pH 8.8, 20mM $(\text{NH}_4)_2\text{SO}_4$ and 0.01% Tween 20), 2.5mM MgCl_2 and 1 U Taq DNA Polymerase (recombinant) (FERMENTAS, USA). PCR conditions consisted 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 10min chain elongation at 72°C (thermocycler Eppendorf, Germany). The amplification products were electrophoresed in 1.5% agarose gels (PROMEGA USA) and Tris-Borate-EDTA buffer. Gels were stained with ethidium bromide and photographed using ultra-violet gel documentation system (Uvitec, United Kingdom). The PCR amplification for each primer pair was repeated thrice for each isolate (Yoshikawa et al., 2004b). The classification of the subtypes for each *Blastocystis* sp. isolate was based on the standard terminology (Stensvold et al., 2007).

5.3 Results

Blastocystis sp. cysts from four animal species namely ostrich, pig, goat and peacock could cause experimental infection in rats. Table 5.1 shows that the cysts from the ostrich caused experimental infection within 2 days in all 5 rats, followed by cysts from the pig and goat which caused infection in 4 out of 5 rats within 2 days of infection. Cysts from the peacock infected 3 out of 5 rats within 4 days of infection. However, cysts from sheep, orang utan and cow were not able to cross-infect into the rats after 8 days of post-infection. Using sequenced-tagged site primer-PCR, only the ostrich isolates were confirmed to be subtype 6 (ST5). Meanwhile, the subtype of remaining animal isolates revealed no bands when amplified with PCR using sequence-tagged site (STS) when assessed using primers for subtypes 1 to 7 (Table 5.2). The cysts seen in the faeces from rats infected with cysts isolate from the ostrich were identified to be subtype 6 which was similar to subtype of the inoculum when amplified with PCR using sequence-tagged site (STS) primers (Figure 5.2).

Table 5.1: Days and numbers of rats infected with *Blastocystis* sp.

Hosts	Day of rats infected after oral inoculation	Number of rats infected (n=5)
Ostrich	2	5
Pig	2	4
Goat	2	4
Peacock	4	3
Sheep	No cross-infectivity	0
Orang utan	No cross-infectivity	0
Cow	No cross-infectivity	0

Table 5.2: Subtype distribution of *Blastocystis* sp. in infected animals and rats

Hosts	Subtype in animals	Subtype in infected rats
Ostrich	subtype 6	subtype 6
Pig	Not detected	Not detected
Goat	Not detected	Not detected
Peacock	Not detected	Not detected

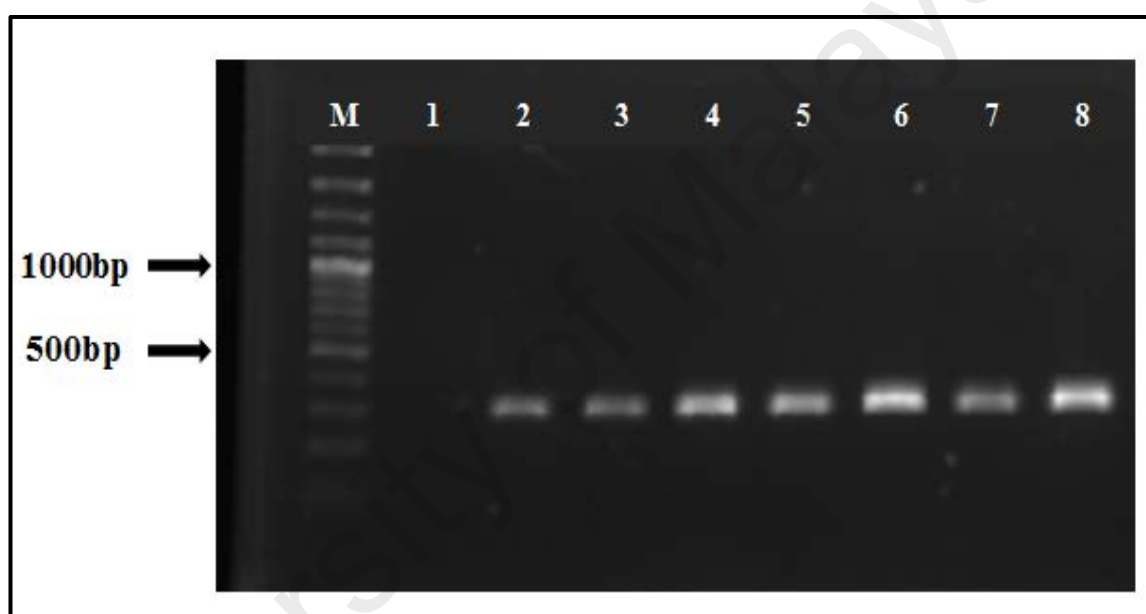


Figure 5.2: Agarose gel image of *Blastocystis* sp. from cross-infection *in-vivo* study amplified by sequenced-tagged site (STS) primers. Lane M = DNA markers of 100bp plus DNA ladder (Fermentas, USA); lane 1 = negative control; lane 2 = positive control; lane 3 = *Blastocystis* sp. cyst of ostrich isolate (inoculum); Lane 4-8 = *Blastocystis* sp. isolates of infected rats. The amplicon size of subtype 6 = 317bp.

5.4 Discussion and conclusion

In the present study, cysts of *Blastocystis* sp. from ostrich, pig, goat and peacock were able to cause infections in rats providing evidence—that the parasite exhibit low

host specificity which concurred with the findings of Pakandl (1992) and Tanizaki et al., (2005). *Blastocystis* sp. isolated from all the non-human hosts in the present study was confirmed to be not any subtype between 1 and 7 except for the ostrich isolate which was identified to be subtype 6. Most importantly, none of the infected rats were subtype 7 which naturally was found in the rodent population. Cysts from *Blastocystis* sp. isolated from ostrich could cause experimental infection in Sprague Dawley rats as evidenced by cysts in stools after infection were also subtype 6 possibly imply that *Blastocystis* sp. is subtype-specific. Subtype 6 was shown to be prevalent in pigs and cows (Stensvold et al., 2009b) have also been shown to be seen in humans (Stensvold et al., 2009) providing evidence that *Blastocystis* sp. exhibits low host specificity.

Till this date, numerous publications report of cross-infection of *Blastocystis* sp. from human isolate (Moe et al., 1998; Yoshikawa et al., 2004; Chandramathi et al., 2012). Meanwhile very limited cross-infectivity studies have been achieved using animal isolates where in a study by Pakandl (1992), mice and gerbils were successfully infected with porcine strains of *Blastocystis* sp. and chickens were infected experimentally with chickens, quails and geese isolates (Tanizaki et al., 2005). Therefore, this is the first study to provide evidence that *Blastocystis* sp. cysts isolated from various animals can cause experimental infection in rats. Since rodents are increasing menace to humans and the fact that rats can carry this infection justifies further that the animal farms with wild rats can be a reservoir for human infections since *Blastocystis* sp. exhibits low host specificity. Hence, the possibility of zoonotic transmission to humans cannot be ruled out.

CHAPTER 6

***IN-VIVO* EXPERIMENTAL INFECTION STUDIES USING SYMPTOMATIC AND ASYMPTOMATIC HUMAN SUBTYPE 3 *BLASTOCYSTIS* SP. ISOLATES**

6.0 *In-vivo* experimental infection studies using symptomatic and asymptomatic human subtype 3 *Blastocystis* sp. isolates

6.1 Introduction

The pathogenicity of *Blastocystis* sp. remains disputable as studies have shown that infected persons continue to be asymptomatic carriers (Tan et al., 2008; Mirza & Tan, 2012; Scanlan & Pauline, 2012). To date, *Blastocystis* sp. can be characterized into 17 distinct subtypes (ST1-ST17), of which at least nine have been found in humans (Scanlan & Pauline, 2012; Alfellani et al., 2013). Recent studies have incriminated *Blastocystis* sp. ST3 to be pathogenic and responsible for causing gastrointestinal symptoms (Katsarou-Katsari et al., 2008; Tan et al., 2008; Vassalos et al., 2010). This was further evidenced by the high occurrence of *Blastocystis* sp. ST 3 in symptomatic than asymptomatic patients (Babb & Wagener, 1989; Scanlan & Stensvold, 2013; Souppart et al., 2009).

Several studies have studied on the effects of parasitic infection influencing biochemical and haematological parameters in humans. Recently, Janarthanan et al., (2011) have reported that a large colonic ulcer patient suffered from persistent diarrhea had *Blastocystis* sp. as the sole pathogen showed normal hematological and biochemical profiles. In another study, HIV patients with multiple intestinal protozoans infection including *Blastocystis* sp. showed no significant changes at the levels of alkaline phosphatase and serum amylase (Cotte et al., 1993). Respondents in Nigeria infected with filariasis and intestinal protozoan infections have shown significant differences in liver parameters such as bilirubin, alanine and aspartate aminotransferase which indicate direct influence on the functions of the liver, however the haematological parameters

such as packed cells volume (PCV) and haemoglobin level showed no significant differences (Ojiako & Onyeze, 2008). In contrast, no significant changes were observed in the albumin and glucose levels in patients infected with *Blastocystis* sp. as compared to healthy individuals (Chen et al., 2003).

In earlier colonoscopic studies of humans, some authors reported no pathological lesions, while others showed non-specific inflammation without evidence of invasion with *Blastocystis hominis* (Chen et al., 2003; Garavelli et al., 1991; Horiki et al., 1977). Thus far, there is only one *in-vivo* study which showed significant rise in the haematological parameter comprising of mean corpuscular volume and platelet counts in rats infected with *Blastocystis* sp. (Li et al., 2012). On the other hand, histopathological studies carried out previously in *Blastocystis* infected mice suggested that this organism can invade (Moe et al., 1997). Both an essential number of parasites was shown to be necessary for experimental infection studies as well as the ability of *Blastocystis* sp. to invade lamina propria, submucosa and muscle layers of the large intestine were shown (Elwakil & Hewedi, 2010). In contrast, no obvious histopathological changes were observed in the cross-sectional tissue biopsies taken from the large intestine of a *Blastocystis* sp. infected pig (Wang et al., 2014). Moderate to severe degrees of pathological changes were seen in rats infected with symptomatic subtypes while mild degree in asymptomatic subtypes infected rats (Hussein et al., 2008),

There has been, thus far no studies carried out to correlate biochemical, haematological and histopathological changes in *in-vivo* experimental animal infections with *Blastocystis* sp. isolated from humans. Besides this, there has been no study to compare the transformational changes of *Blastocystis* sp. from the cyst to vacuolar

forms between subtype 3 of *Blastocystis* sp. from symptomatic and asymptomatic isolates. Encystation studies have been previously reported (Zhou et al., 2010; Suresh et al., 2009; Suresh et al., 1994). Subtype 3 has also been reported from asymptomatic patients (Tan et al., 2008; Scanlan & Pauline, 2012). The present study attempts to assess whether *Blastocystis* sp. subtype 3 isolated from symptomatic and asymptomatic persons can influence changes at the biochemical and haematological levels and further cause histological changes in *Blastocystis* sp. infected rats.

6.2 Materials and methods

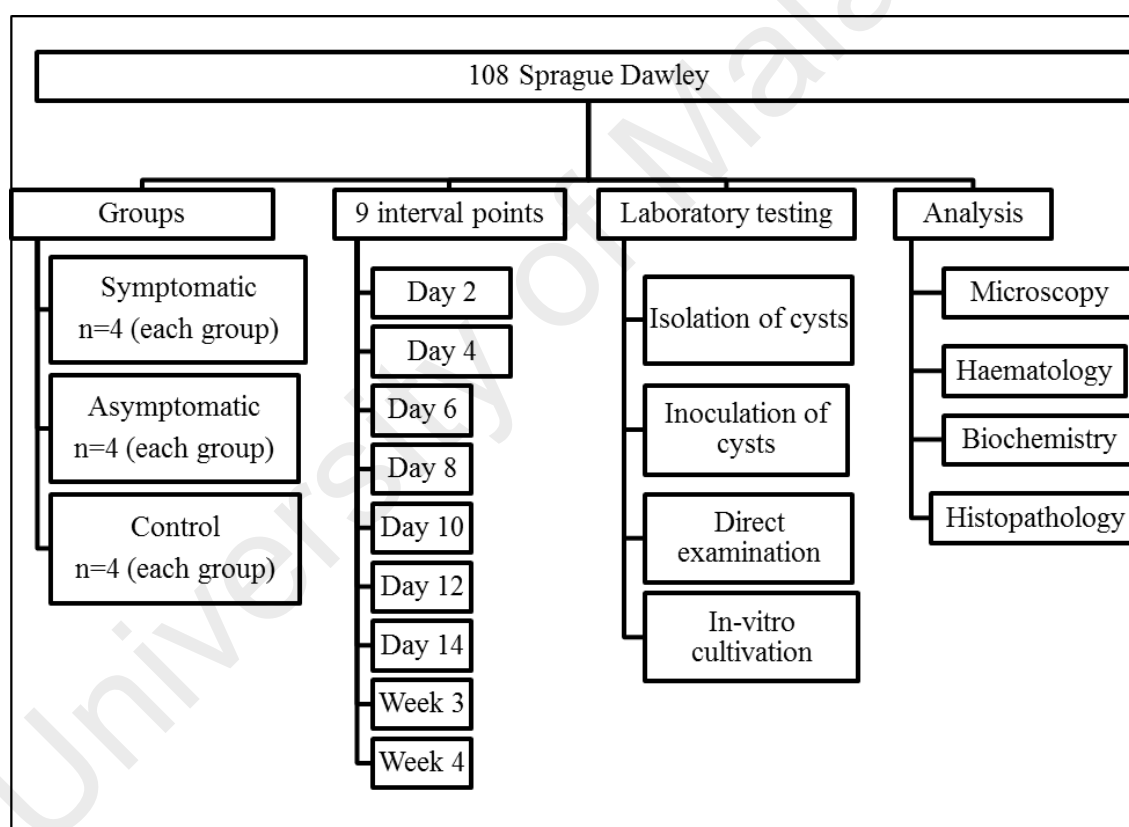


Figure 6.1: Schematic representation of the overall methodology emphasizing on the inoculation of *Blastocystis* sp. isolated from ST3 symptomatic and asymptomatic isolates into Sprague Dawley rats.

6.2.1 Experimental animals

108 healthy laboratory 2-3 weeks old inbred Sprague Dawley rats of both sexes and weight between 30 and 37 g, previously confirmed negative for *Blastocystis* sp. were used in this experiment. The rats were divided into three groups of four rats each namely group 1: control group without *Blastocystis* sp. infection, group 2: asymptomatic (rats infected with *Blastocystis* sp. ST3 isolated from asymptomatic persons who showed no symptoms and had no other pathogens) and group 3: symptomatic (rats infected with *Blastocystis* sp. isolated from symptomatic patients having only *Blastocystis* sp. as the only pathogen). The animals were kept in individual cages maintained in an animal experimental room at room temperature and were fed a normal diet of commercial pellets and potable water ad libitum.

6.2.2 Source and isolation of *Blastocystis* sp.

Fresh stool samples were collected during a survey from a person infected with subtype 3 *Blastocystis* sp. that showed no symptoms and had no other pathogens. As for the symptomatic groups, fresh stool samples were collected from a symptomatic patient infected with subtype 3 *Blastocystis* sp. attending to a private hospital complaining of persistent diarrhea and had no other pathogens. This study has been approved by the Institutional Animal Care and Use Committee (IACUC), University Malaya. The reference numbers are PAR/29/06/2012/LIL(R) and PAR/23/05/2013/HC(R). A pea size of the stool sample was cultured in Jones' medium supplemented with 10% horse serum. A direct smear of the fresh stool sample from both asymptomatic and symptomatic patients was made to examine for *Blastocystis* sp. as well as other parasites. The cultured stool samples were screened for *Blastocystis* sp. after 24 and 48 hours of incubation. The positive samples were subsequently maintained by sub-culturing in Jones' medium once every 3 to 4 days.

6.2.3 Experimental infection with Sprague dawley rats using cysts of *Blastocystis* sp.

Fresh stool samples were dissolved in normal saline and sieved into 15ml Falcon tubes. The culture tube was centrifuged at 3000rpm for 10min using Beckman Coulter 365303 Spinchron DLX centrifuge machine. The sediment were layered on a 5ml of Ficoll-Paque solution in a culture tube and centrifuged at 3500rpm for 20 minutes. The cystic stages were banded at 3 to 4 cm below the meniscus between the third and fourth layer. This was subsequently pipetted out into clean 14 ml culture tubes. The suspension containing the cysts was subsequently washed twice by adding 14ml of phosphate buffer solution (PBS). The culture tubes were then capped tight, inverted a few times and centrifuged at 3000rpm for 10 minutes (Beckman Coulter 365303 Spinchron DLX centrifuge) for each washing. The supernatant was discarded completely and 1ml of PBS solution was added into the culture tube containing the sediment. The cysts were then enumerated and diluted to a concentration of 10^6 cysts/ml. Three batches of 5 rats each were inoculated orally, using 20G feeding needle of 1.5inch length. The control groups were inoculated with PBS solution. All rats were anesthetized before causing the experimental infection using a syringe and a feeding tube orally. All three batches represent the symptomatic, asymptomatic and control groups respectively. This experiment was carried out for 4 weeks at 9 interval points namely at day 2, 4, 6, 8, 10, 12, 14, week 3 and week 4.

6.2.4 Detection of *Blastocystis* sp in stool samples

Stool samples from all rats in the three groups were cultured daily in Jones' medium with 10% horse serum to detect for the parasite daily for a period of 4 weeks post-infection. The weight gain of rats was measured daily from day 0 of pre-

inoculation, post-inoculation and before blood collection from the rats every alternate days. The culture samples were microscopically examined 24, 48 and 72 hours post-inoculation to detect for the presence of the parasites.

6.2.5 Direct fecal examination

The positive faecal smears were observed under light microscopy at 40x magnification, stained subsequently with 10% Giemsa stain and observed under microscopy at 100x magnification.

6.2.6 Haematological and Biochemical assays

Blood samples of rats from all three groups were collected by cardiac puncture on every alternate day till 2, 3 and 4 weeks post-infection. Blood samples were collected in culture tubes with and without EDTA for haematology and biochemistry evaluation respectively. The blood in EDTA tubes were immediately used for haematological evaluation including Red Blood Cell (RBC) count, Hemoglobin (Hb) concentration, hematocrit (Hct) value, Mean Cell Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), White Blood Cell (WBC) count and Differential WBC. All the parameters were measured using veterinary haematology analyzer (Scil Vet ABC Plus+, United States, Colorado). The Differential WBC count was calculated from Giemsa stained slide using the manual differential counter. For serum biochemical measurements, blood in tubes without anticoagulant was left for clotting for separating the serum. These samples were centrifuged at 1500rpm for 10min (Eppendorf Centrifuge Model 5702) and blood sera were collected and immediately used for biochemical measurements including glucose, calcium, total protein, phosphate, urea, ammonia, amylase, albumin and Alkaline phosphatase (ALP).

All the biochemical parameters were analysed using an automatic analyzer (Idexx VetTest 8008 chemistry analyzer, United States, California).

6.2.7 Histological examination

12 rats were sacrificed immediately after cardiac puncture at every interval points on alternate days namely on days 2, 4, 6, 8, 10, 12 and 14 and during week 3 and 4. Sections of caecum, large intestine and rectum were cut from every rat and fixed with 10% buffered formalin. Fixed tissues were dehydrated through ascending grades of ethanol to absolute ethanol. They were cleaned in xylene, embedded in paraffin wax (melting point 56°C). Sections were cut at 4µm using a microtome, flattened in warm water and mounted on to albumenized slides and dried overnight. The sections were dewaxed in xylene and hydrated through descending grades of ethanol to water. They were initially stained in Harris haematoxylin and differentiated in acid alcohol and thereafter stained with methylene blue. They were then dehydrated in 95% alcohol, stained in 10% alcohol eosin, dehydrated in absolute alcohol, cleaned in xylene and mounted in Canada Balsam. The resulting slides were then viewed under light microscopy for histological evaluation.

6.2.8 Statistical analysis

Biochemical and haematological results were expressed as means of 12 determinations of standard deviations (SD). Statistical analysis was performed as symptomatic infected group and asymptomatic infected group vs control group using the Bonferroni-One-way Anova Test. The chosen level of significance is $p < 0.05$.

6.3 Results

6.3.1 Profile on weight gain

The mean weight gained for every rat at every alternate day for all three groups was compared with the weight measured on day 0. All groups showed a significant increase in weight gain especially at week 2, 3 and 4 ($P < 0.05$) (Figure 1). Least weight gain were observed in the rats from the symptomatic group from day 6 till week 4 of infection which were significantly lower when compared to both asymptomatic and control groups ($P < 0.05$). None of the rats showed weight loss or lethargy during the entire period of observation.

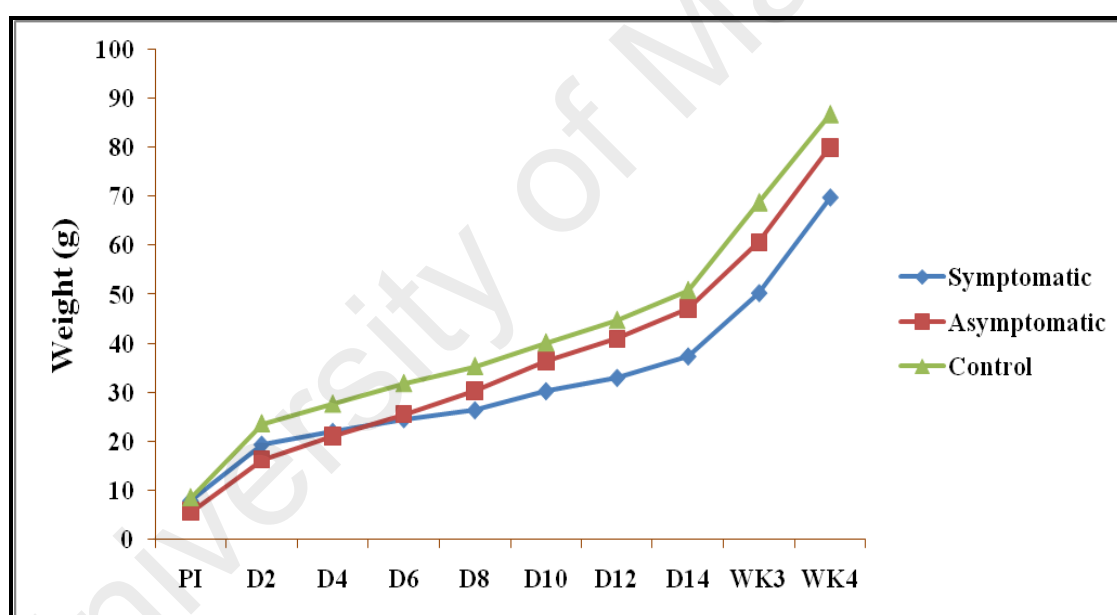
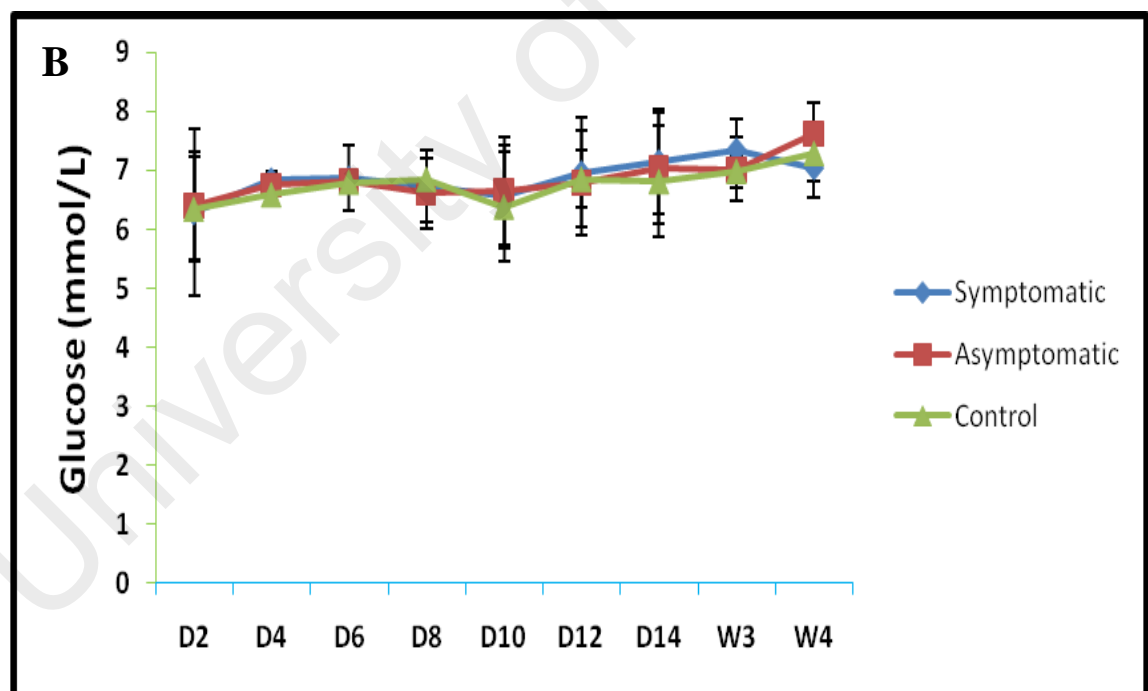
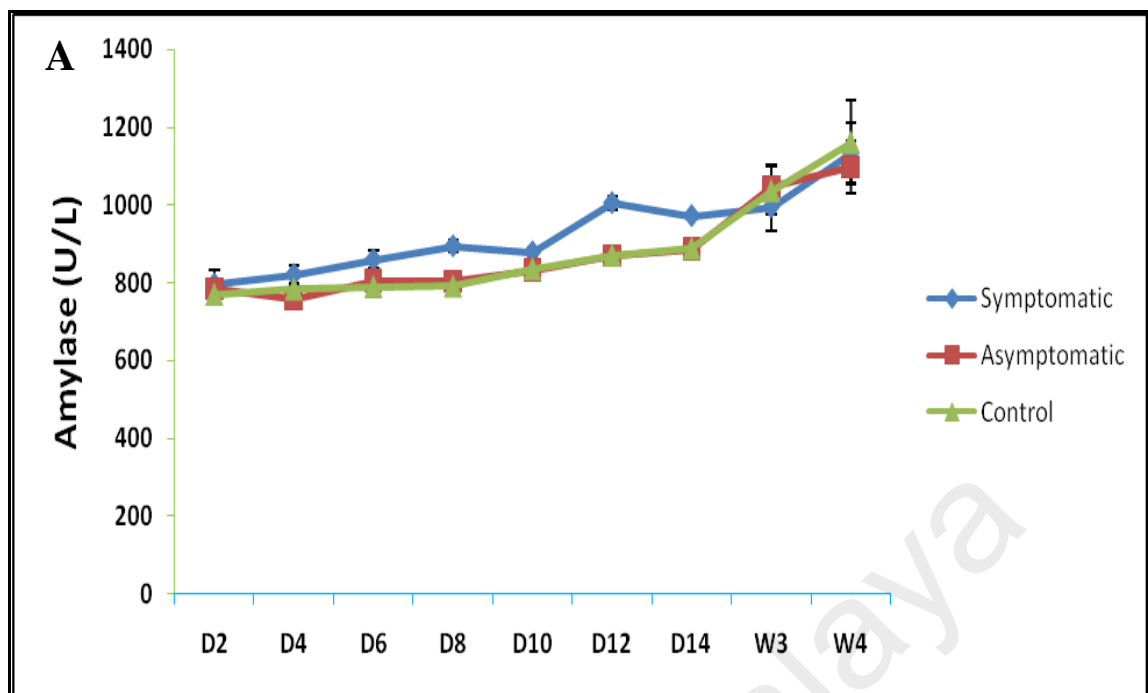
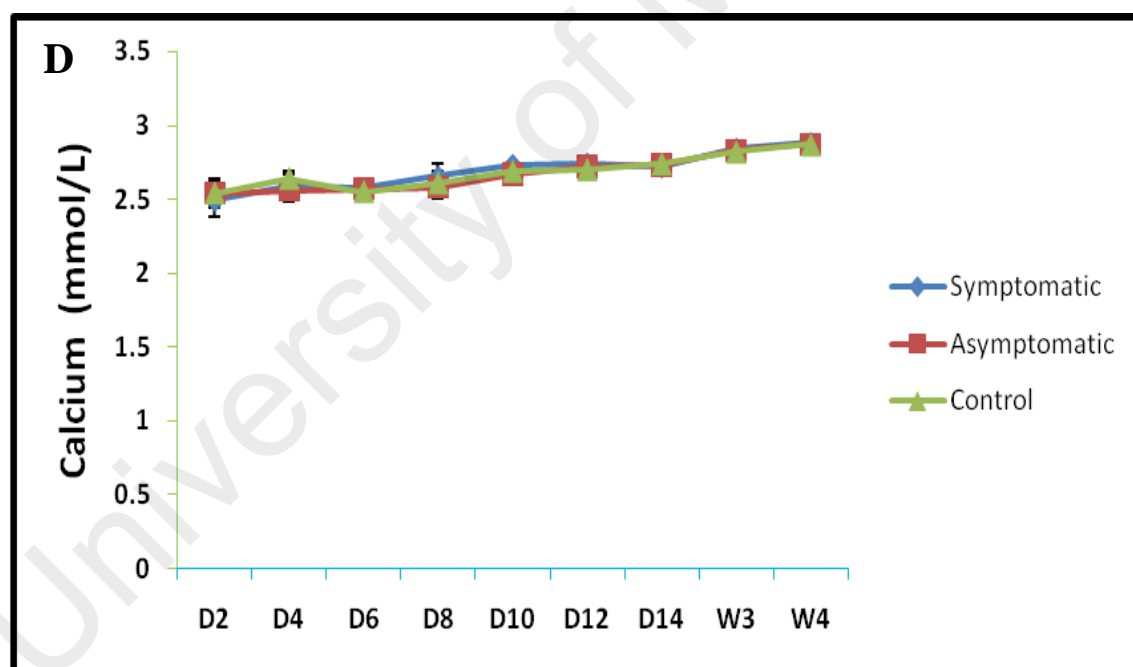
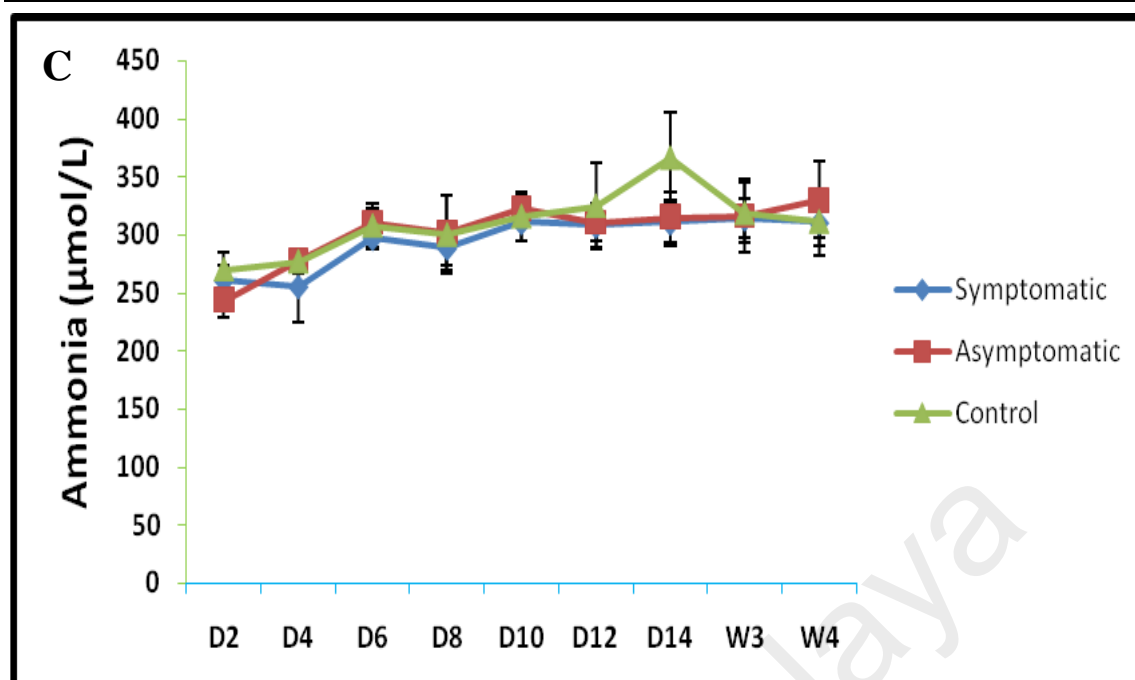


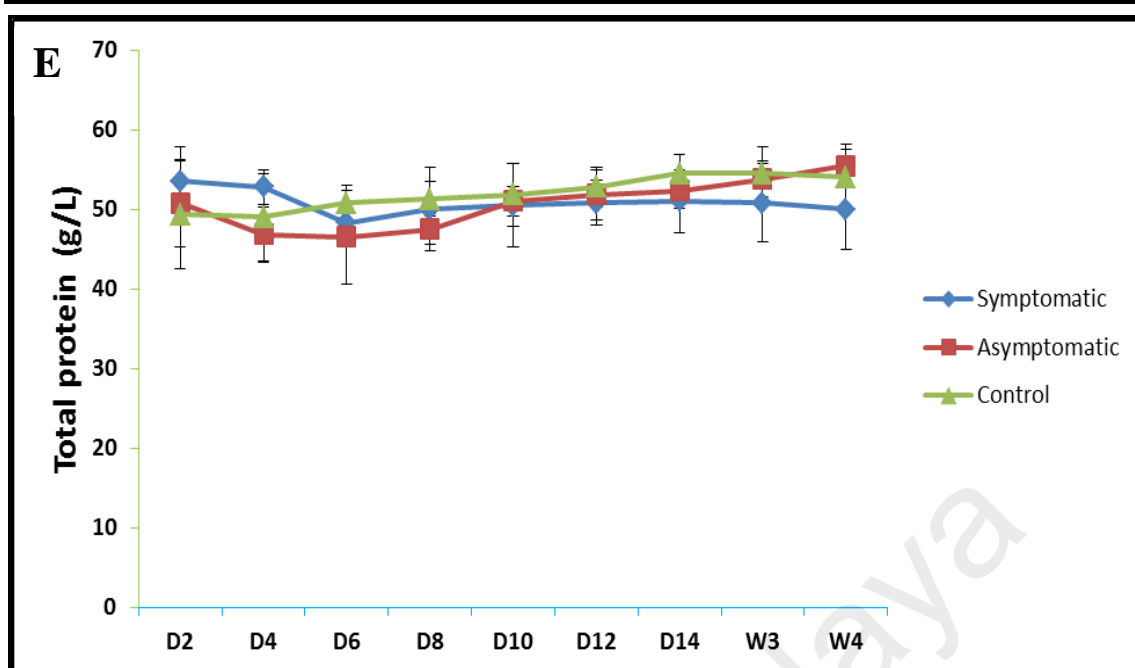
Figure 6.2: Effects of *Blastocystis* sp. infection on weight gain in the respective group of rats according to study duration. Mean weight gain is compared with their initial weight on Day 0. Data shown is in mean \pm SD. D = day; PI = Pre-inoculation; WK = week.

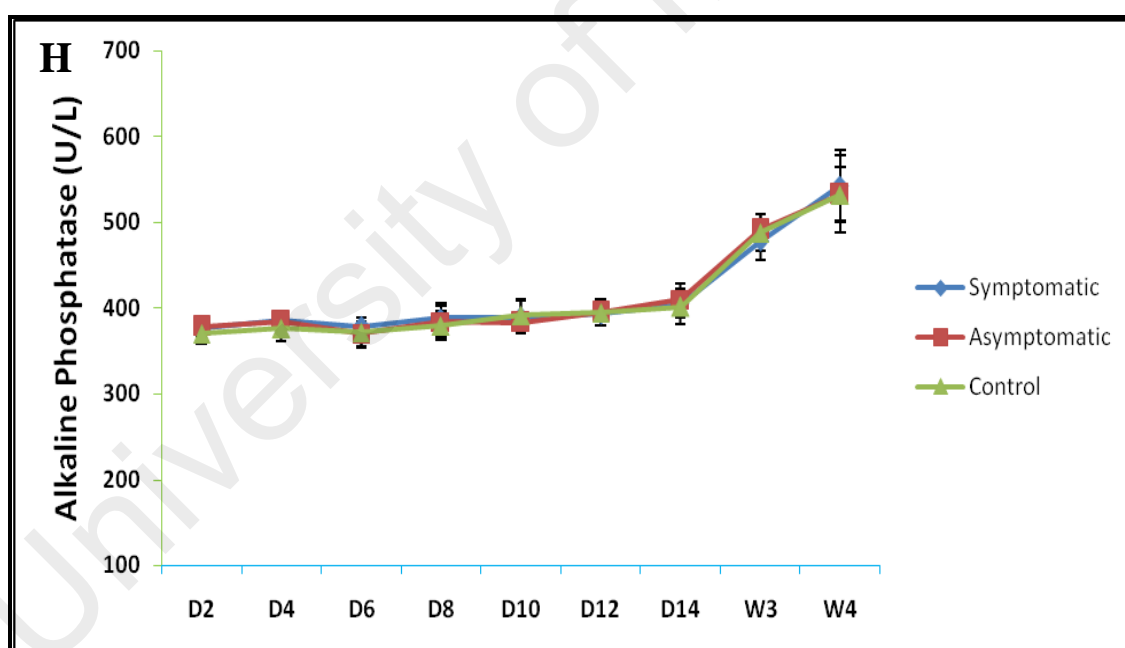
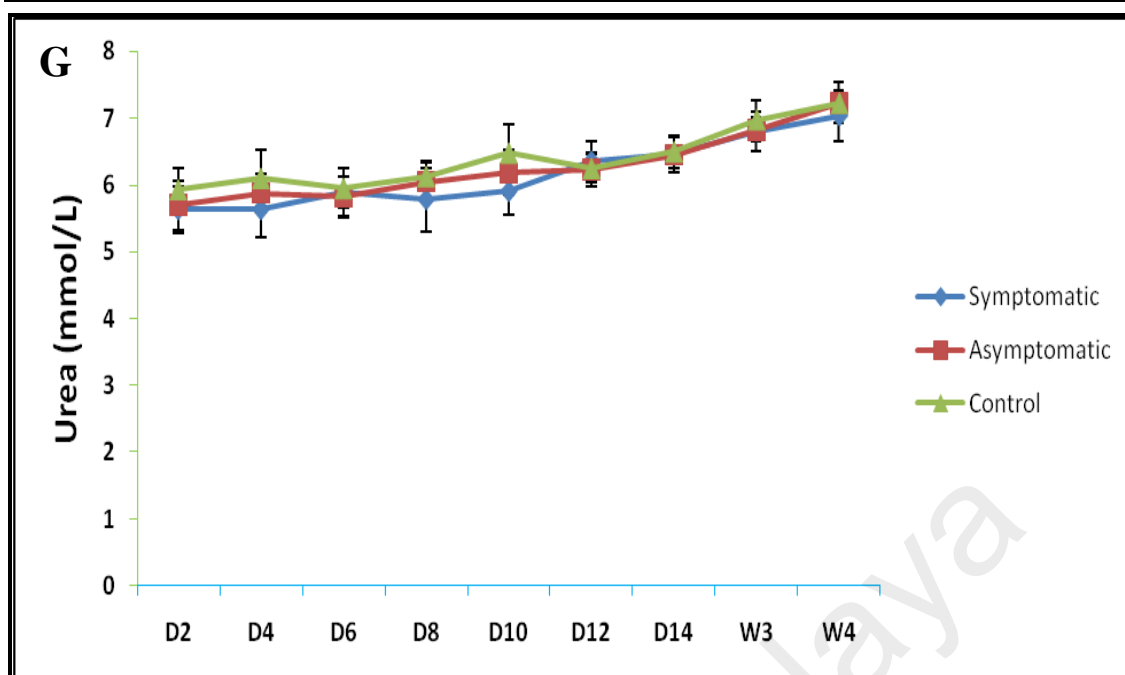
6.3.2 Biochemistry profile

A total of nine parameters which included serum amylase, glucose, ammonia, calcium, total protein, phosphate, urea, alkaline phosphatase and albumin were used in this study but significant changes were only observed in levels of amylase. The level of amylase in the symptomatic group were significantly higher followed by the asymptomatic and control groups with the levels increasing from day 10 to Week 4 ($P < 0.05$) (Figure 6.3A). The level of glucose and ammonia increased in all three groups without any significant changes between the three groups (Figure 6.3B and Fig 6.3C). The level of calcium in all three groups was fluctuating until day 12 followed by an increase until Week 4 (Figure 6.3D). The level of total protein in symptomatic group dropped on day 6 and was lower throughout the experiment till week 4 when compared to control and asymptomatic groups however was not significant (Figure 6.3E). In contrast, phosphate levels increased in all three groups, however from day 10 till week 4, the level of phosphate for all the groups was observed to be fluctuating (Figure 6.3F). Level of urea in the symptomatic group was lower in the first week of infection, however, a gradual increase was seen from day 12 to week 4 (Figure 6.3G). On the other hand, levels of alkaline phosphatase in all three groups increased throughout the experiment (Figure 6.3H). Albumin levels fluctuated in the symptomatic and asymptomatic group since the first week of infection and then subsequently dropped on day 6 and were seen to be lower when compared to the control group throughout the study period (Figure 6.3I).









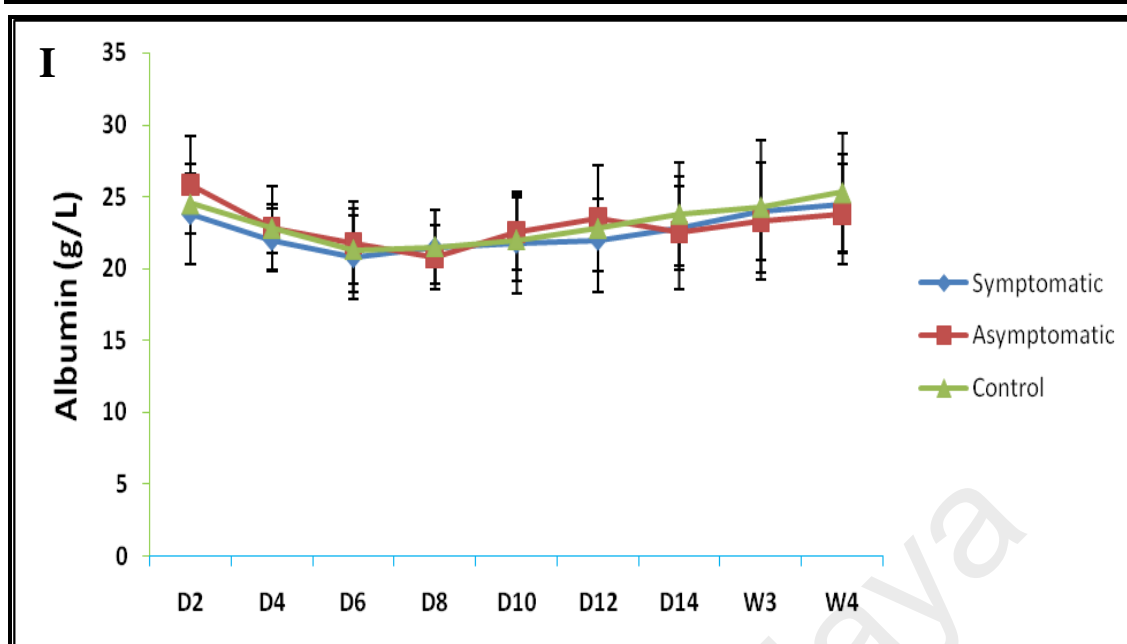
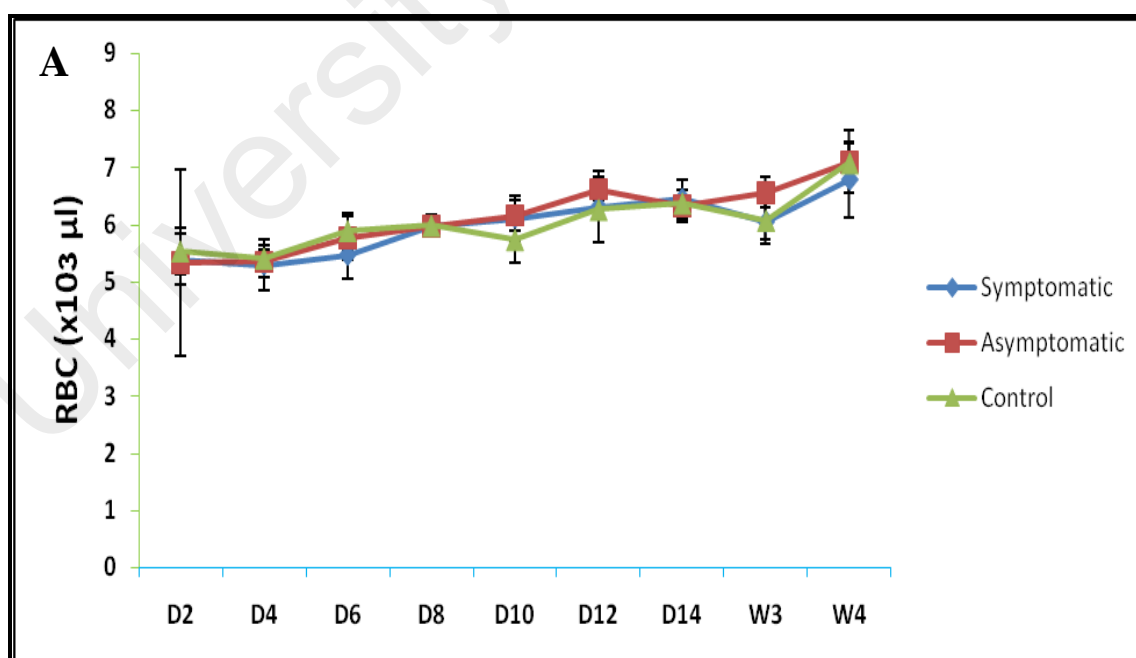


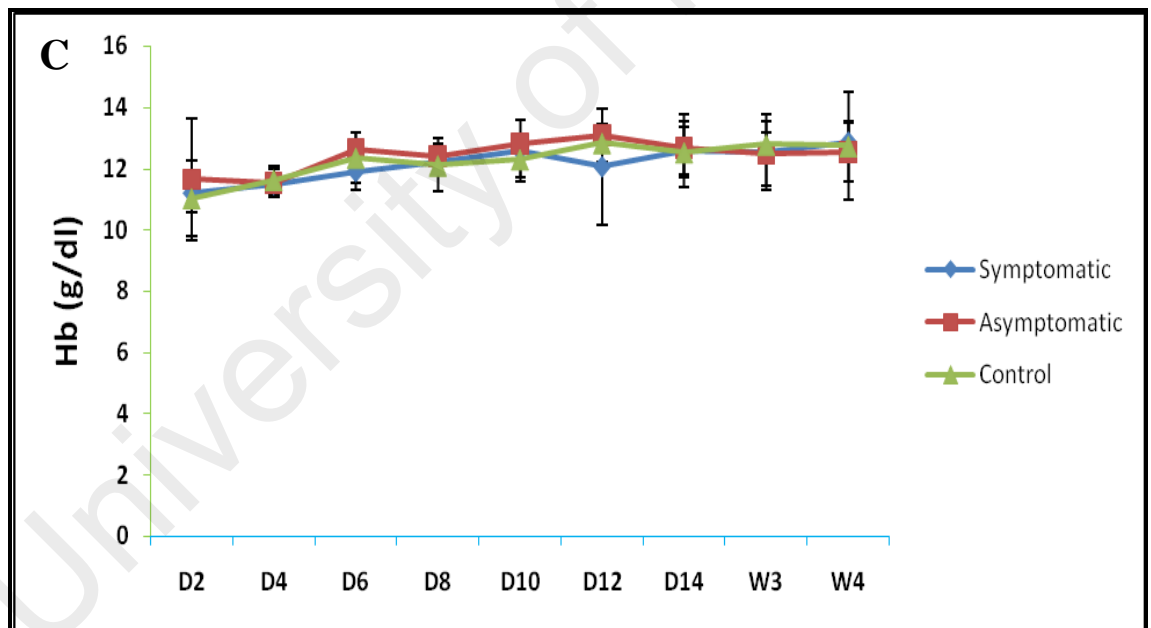
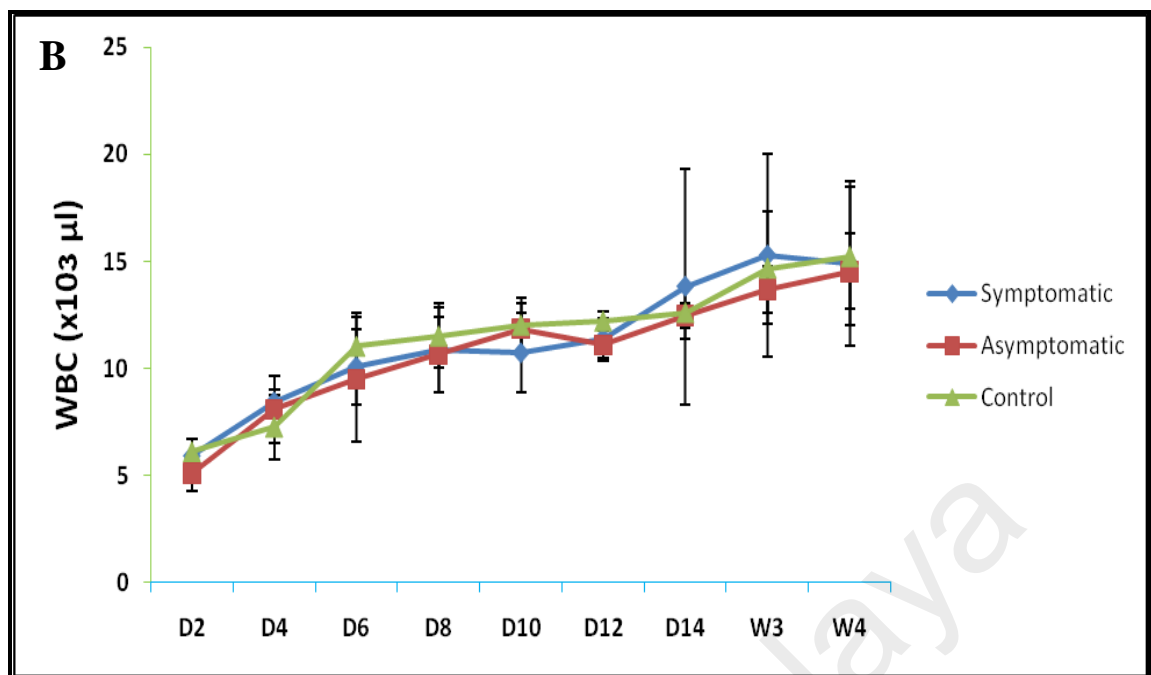
Figure 6.3: (A-I) The values of serum: A) amylase, B) glucose, C) ammonia, D) calcium, E) total protein, F) phosphate, G) urea, H) alkaline phosphatase and I) albumin in the three groups. Data is given in mean \pm SD. $P < 0.05$ is the comparison done against control group using Mauchly's Test. D = day; W = week.

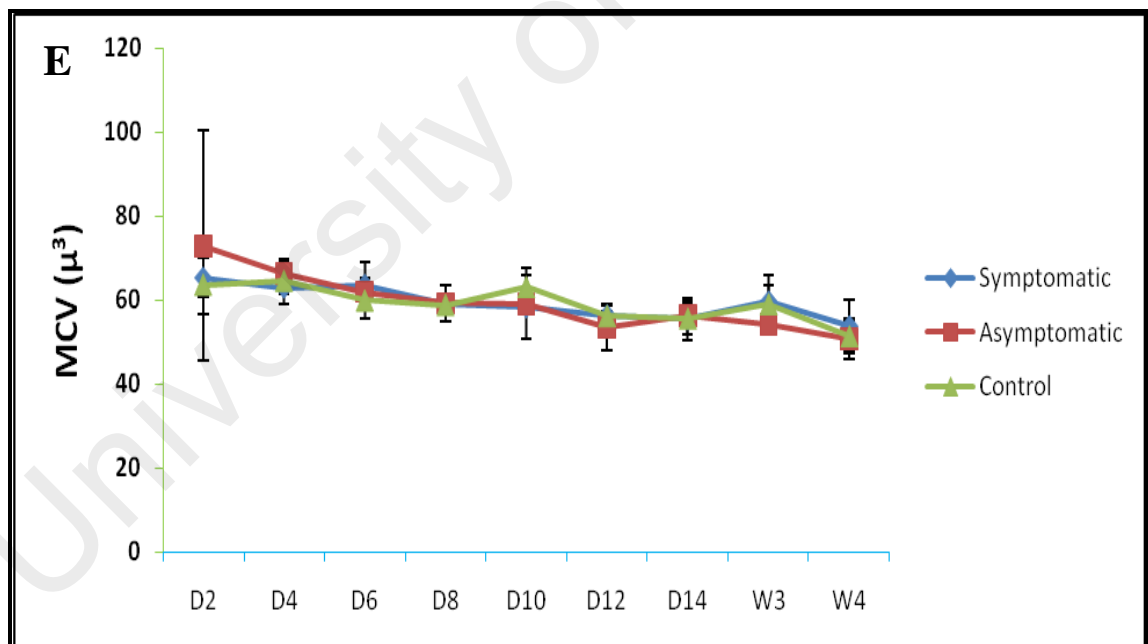
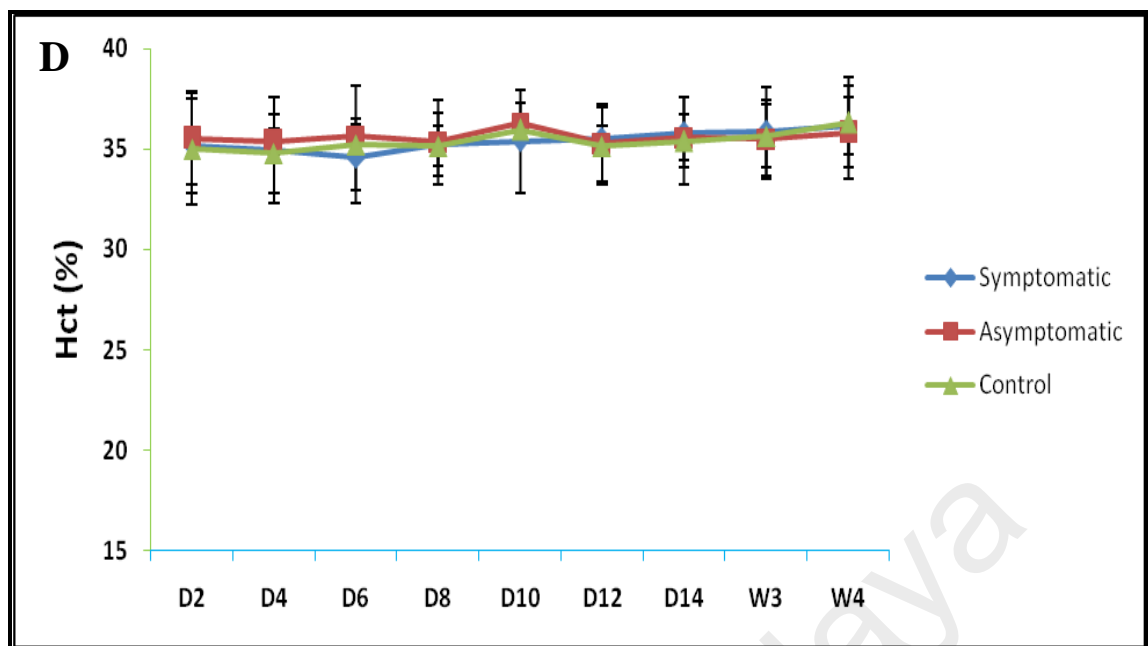
6.3.3 Haematological profile

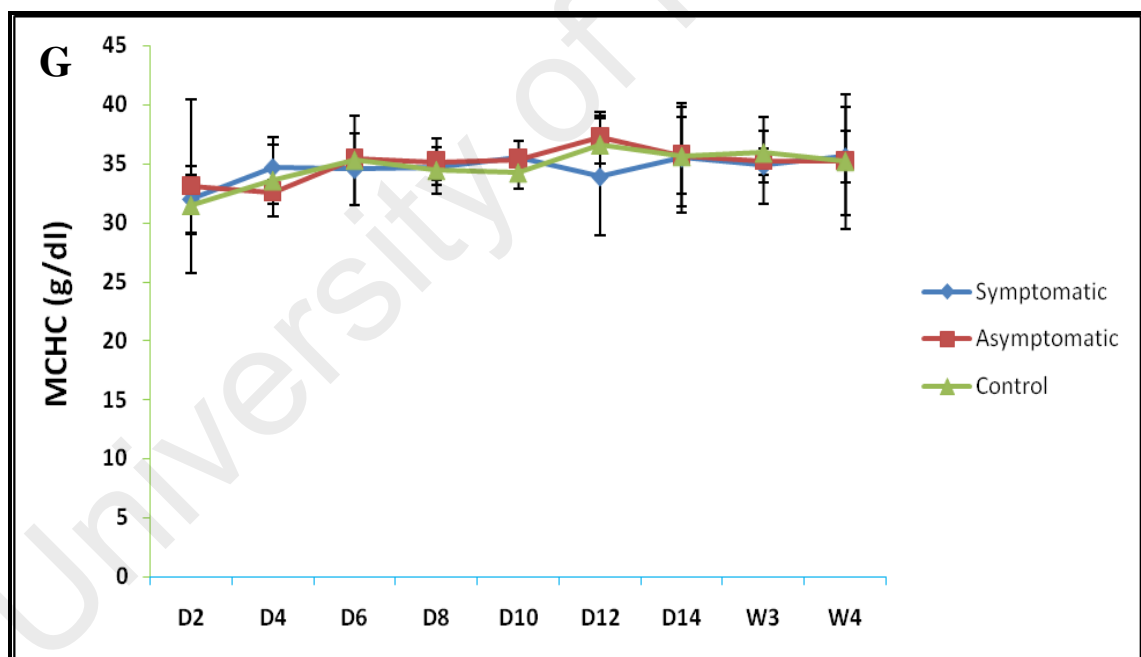
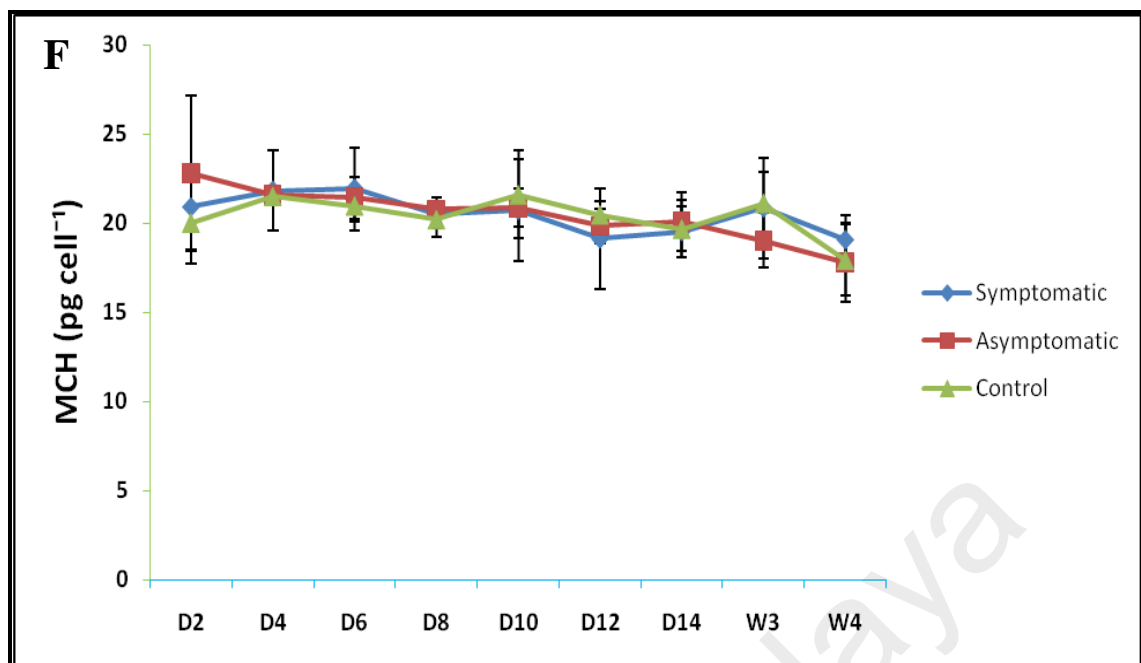
A total of 11 parameters which included red blood cells, white blood cells, haemoglobin, packed cell volume, mean cell volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, lymphocytes, neutrophils, monocytes and eosinophils level were assessed but there was no significant changes when observed in any of these parameters (Figure 6.4A-K). The level of red blood cell count was fluctuating in all the three groups (Figure 6.4A). In contrast, the level of white blood cell count was higher in the symptomatic group especially at week 2 and week 3 when compared to the other groups (Figure 6.4B). However, an increasing trend was observed in all the three groups from week 1 to week 4. Besides this, the level of haemoglobin in all three groups was observed to be fluctuating from the beginning of the experiment until week 4 (Figure 6.4C). On the other hand, the level of hematocrit increased in all

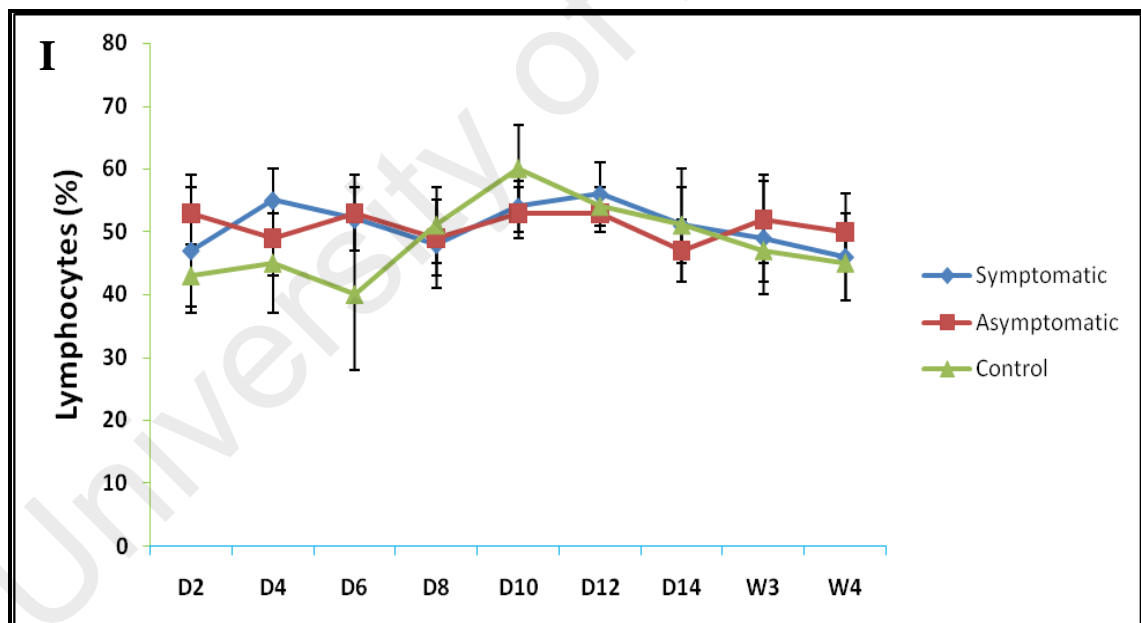
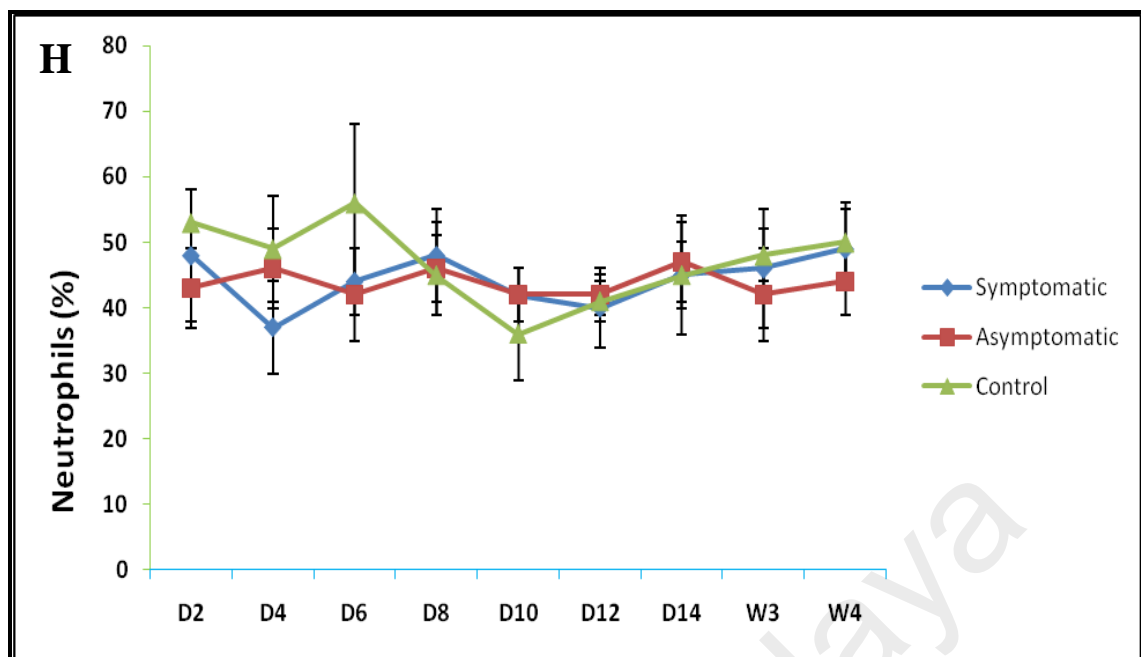
three groups without any significant changes between the three groups (Figure 6.4D). In contrast, the mean cell volume level dropped in all three groups from the day of infection (Figure 6.4E). The level of mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration in all three groups were constantly decreasing throughout the duration of study (Figure 6.4F and Figure 6.4G). The blood differential count showed that the level of neutrophils was fluctuating from day 2 to levels low and high as 40% and 55% respectively (Figure 6.4H). The level of lymphocytes was fluctuating in all the three groups (Figure 6.4I). Besides this, the level of monocytes in symptomatic group was higher than the other groups particularly at the 1st week of infection, however it did not show any significant changes between the groups till the final week (Figure 6.4J). Moreover, the level of eosinophils in the asymptomatic group was generally higher followed by the control and symptomatic group especially from day 2 of post-infection till week 4 (Figure 6.4K).











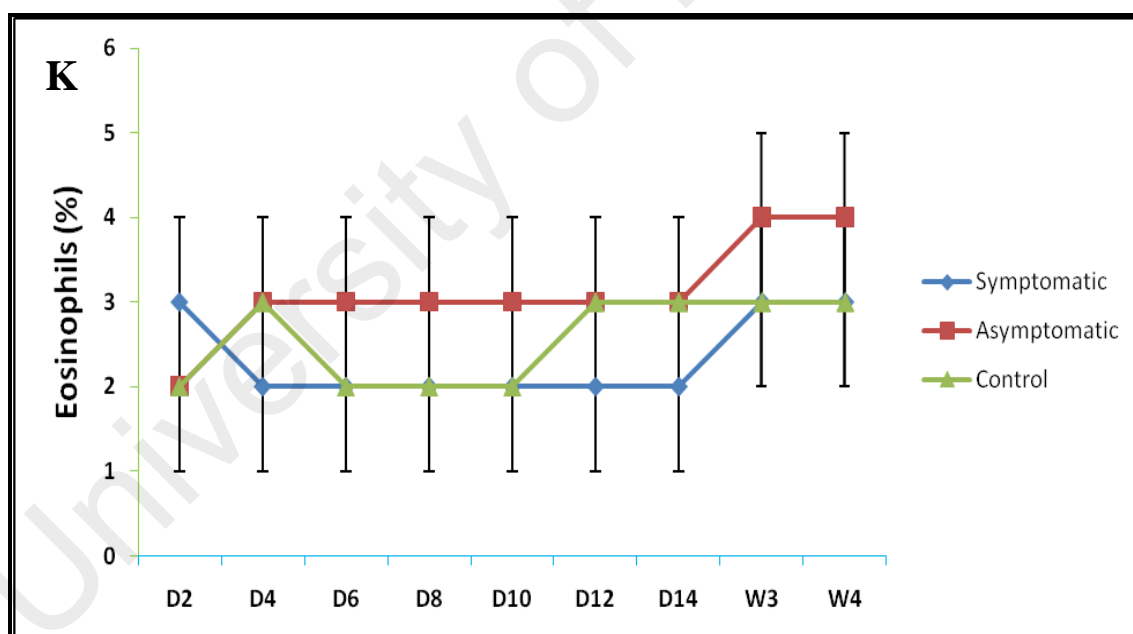
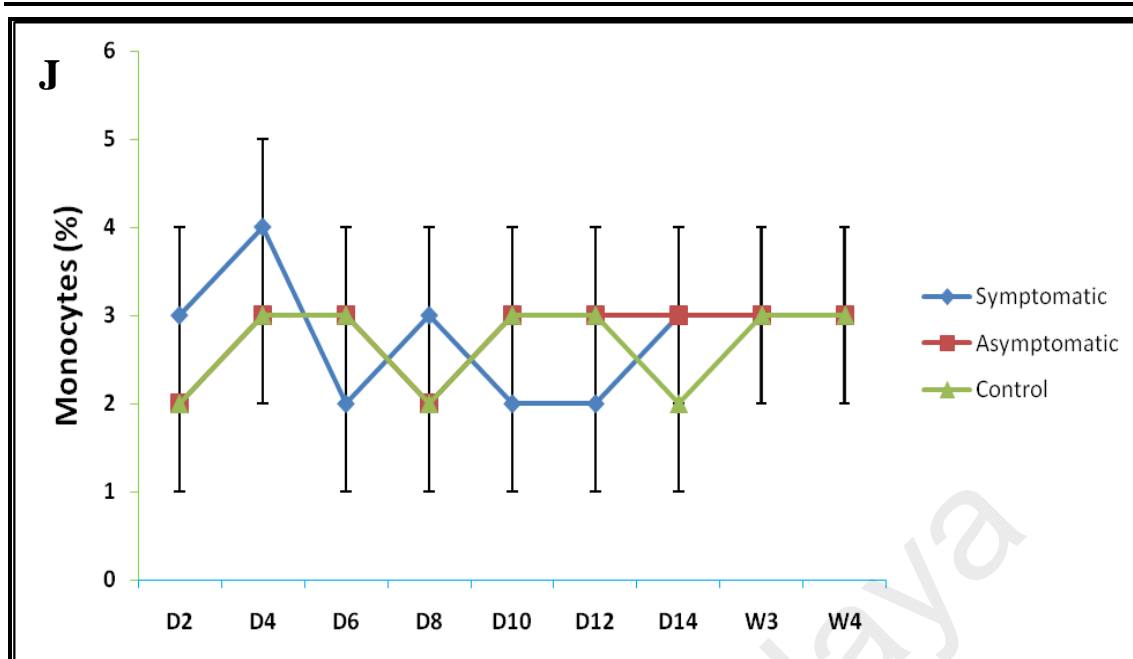


Figure 6.4: (A-K) The values of haematological parameters: A) RBC, B) WBC, C) Hb, D) Hct, E) MCV, F) MCH, G) MCHC, H) neutrophils, I) lymphocytes, J) monocytes and K) eosinophil in the groups. Data is given in mean \pm SD. $P < 0.05$ is the comparison done against control group using Mauchly's Test. D = day; W = week.

6.3.4 Direct examination of intestinal smears

Vacuolar forms of *Blastocystis* sp. with distinct cytoplasm were commonly seen in direct smears of caecum samples from both symptomatic and asymptomatic groups. Cystic forms were more commonly seen in the direct smears of colon and rectum samples from both groups when stained with Giemsa stain. Irregular vacuolar forms were frequently observed in the caecum and colon culture samples isolated from symptomatic rats. Typical vacuolar forms were usually seen in the caecum and colon culture samples isolated from the rats infected with asymptomatic isolates. Several multi-budding organisms with daughter cell-like structures within the central vacuole were observed in the parasites isolated from the cultures with the rectum section of rats infected with symptomatic isolates. The average mean diameter of vacuolar forms found in the stools and intestines cultures were 15 to 40 μm . In the direct examination of intestines, the mean diameter of cystic and vacuolar forms was 5 to 7 μm and 10 to 25 μm .

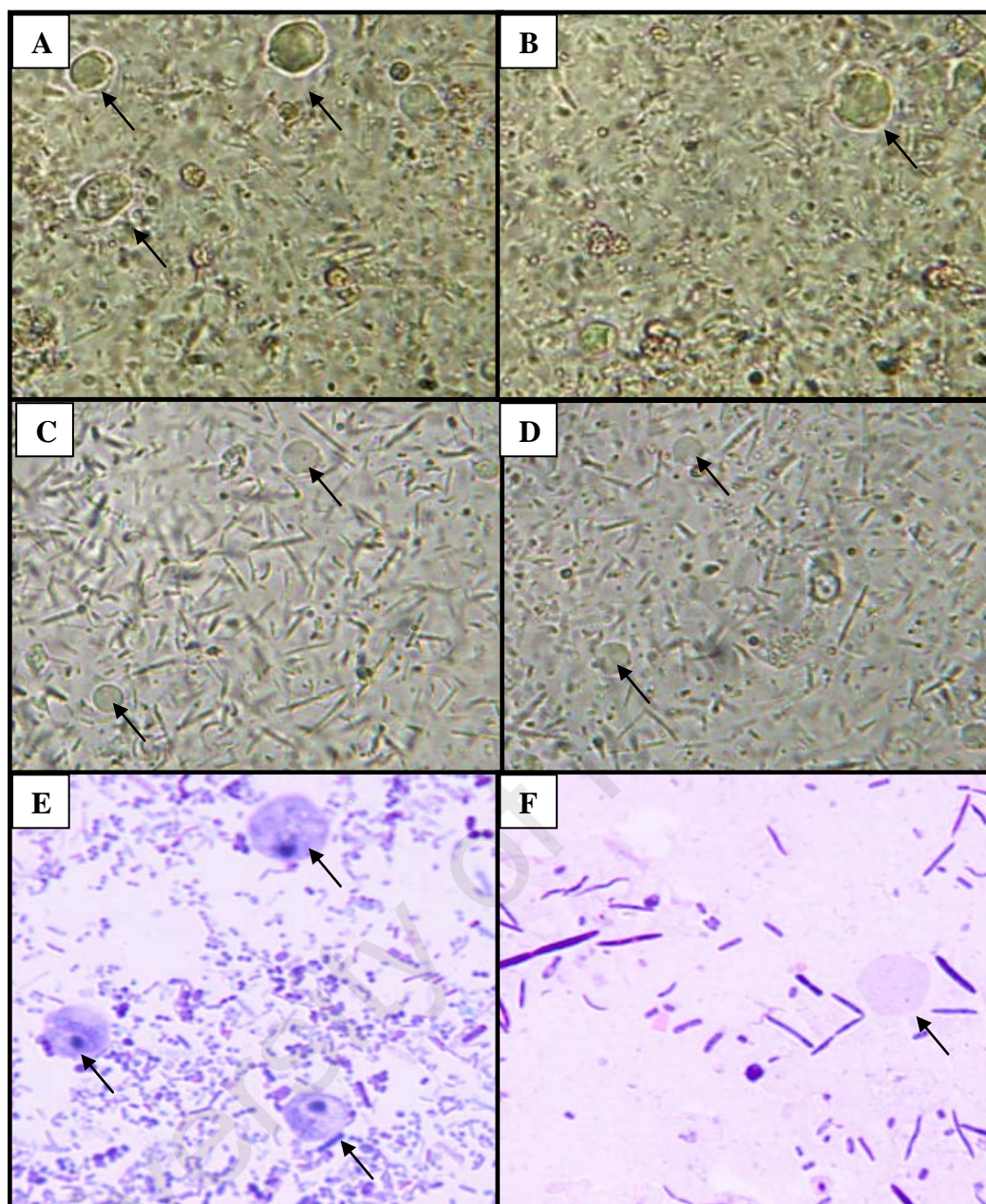


Figure 6.5: Comparison of direct examination of large intestine samples from both symptomatic and asymptomatic groups under light microscope at 40x magnification. **A,B:** Vacuolar forms of *Blastocystis* sp. with distinct cytoplasm seen in the caecum samples from both symptomatic and asymptomatic rats respectively; **C,D:** Cystic forms seen in the colon samples from both symptomatic and asymptomatic rats respectively. **E,F:** Rounded cystic forms observed in the rectum samples from both symptomatic and asymptomatic rats respectively stained with Giemsa stain.

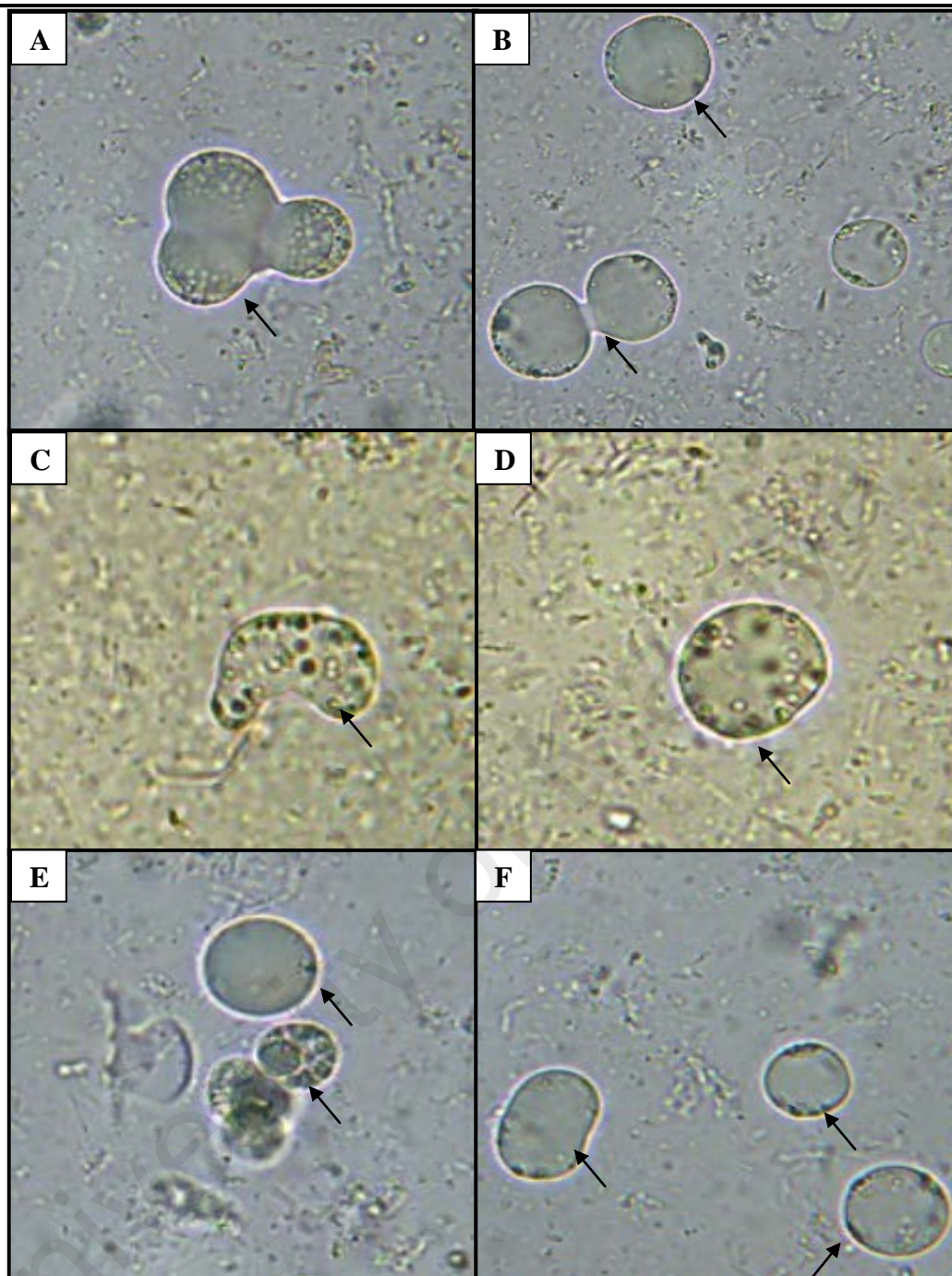


Figure 6.6: Comparison of examination of cultures from the large intestine samples from both symptomatic and asymptomatic groups under light microscope at 40x magnification. **A,C:** Irregular vacuolar forms observed in the caecum and colon culture samples of symptomatic rats respectively; **B,D:** typical vacuolar forms seen in the caecum and colon culture samples of asymptomatic rats respectively; **C,D:** Cystic forms seen rectum sample of both in the colon samples from both symptomatic and asymptomatic rats respectively. **E:** Multi-budding organisms with daughter cell-like

structures in central vacuole observed in the rectum culture of symptomatic rats; F: Typical vacuolar forms seen in the rectum culture samples of asymptomatic rats.

6.3.5 Comparison of gross changes in the intestines

Rats remained negative in the control group for *Blastocystis* sp. throughout the experiment. Infected symptomatic rats appeared healthy but excreted softer stools compared to infected asymptomatic rats. Meanwhile, the intestines collected on the 4th week from symptomatic rats showed intense reddishness and bloating as compared to the intestines from the asymptomatic rats which showed moderate reddishness. Control rats showed no gross changes.



Figure 6.7: Comparison of intestine from Sprague Dawley rats for control, asymptomatic and symptomatic groups. A: normal intestine of an uninfected rat; **B:** intestine of an infected asymptomatic rat; **C:** intestine of an infected symptomatic rat. Note the bloating of the caecum and colon in the infected caecum (arrows).

6.3.6 Histopathology

6.3.6.1 Comparison of histological sections of the large intestines among the study groups

Histology of the tissue from the colon (Figure 6.8A, 6.11A, 6.14A), caecum (Figure 6.9A, 6.12A, 6.15A), and rectum (Figure 6.10A, 6.13A, 6.16A) of control rats from both asymptomatic and symptomatic groups appeared normal without any sloughing or inflammation throughout the experiment from day 2 to week 4. On day 2 of infection, moderate mucosal sloughing was seen in the colon of two rats from the asymptomatic group (Figure 6.8B) and mild mucosal sloughing in the colon of two rats from the symptomatic group (Figure 6.8C). Meanwhile, extensive mucosal sloughing was seen in the caecum of one rat from the asymptomatic group (Figure 6.9B) and moderate mucosal sloughing in the caecum of two rats from the symptomatic group (Figure 6.9C). In the rectum, mild mucosal sloughing was seen in two rats from the asymptomatic group (Figure 6.10B) and only one rat from the symptomatic group showed moderate mucosal sloughing in the rectum (Figure 6.10C).

Subsequently, on day 4 of infection, extensive mucosal sloughing was observed in the colon of two rats from the asymptomatic group (Figure 6.11B) while, moderate mucosal sloughing was seen in the colon of two rats from the asymptomatic group (Figure 6.11C). In the caecum, extensive mucosal sloughing was seen in one rat from the asymptomatic group (Figure 6.12B), while mild mucosal sloughing was observed in two rats from the symptomatic group (Figure 6.12C). Extensive mucosal sloughing was seen in the rectum of two rats from the asymptomatic group (Figure 6.13B) while moderate mucosal sloughing was seen in the rectum of two rats from symptomatic group (Figure 6.13C).

Meanwhile, on the 4th week of infection, mild mucosal sloughing was seen in the colon of two rats from the asymptomatic group (Figure 6.14B), however, extensive mucosal sloughing was seen in the colon of two rats from the symptomatic group

(Figure 6.14C). Moderate mucosal sloughing was seen in the caecum of one rat from the asymptomatic group (Figure 6.15B), while extensive mucosal sloughing was seen in the caecum of two rats from the symptomatic group (Figure 6.15C). In rectum, however moderate mucosal sloughing was seen in the rectum of one rat from the asymptomatic group (Figure 6.16B) and extensive mucosal sloughing was seen in the rectum of two rats from symptomatic group (Figure 6.16). Only one rat from the symptomatic group on the 4th week of infection had excessive mucus in the lamina propria of caecum section (Figure 6.17A) and acute inflammation in the lamina propria of rectum section (Figure 6.17B).

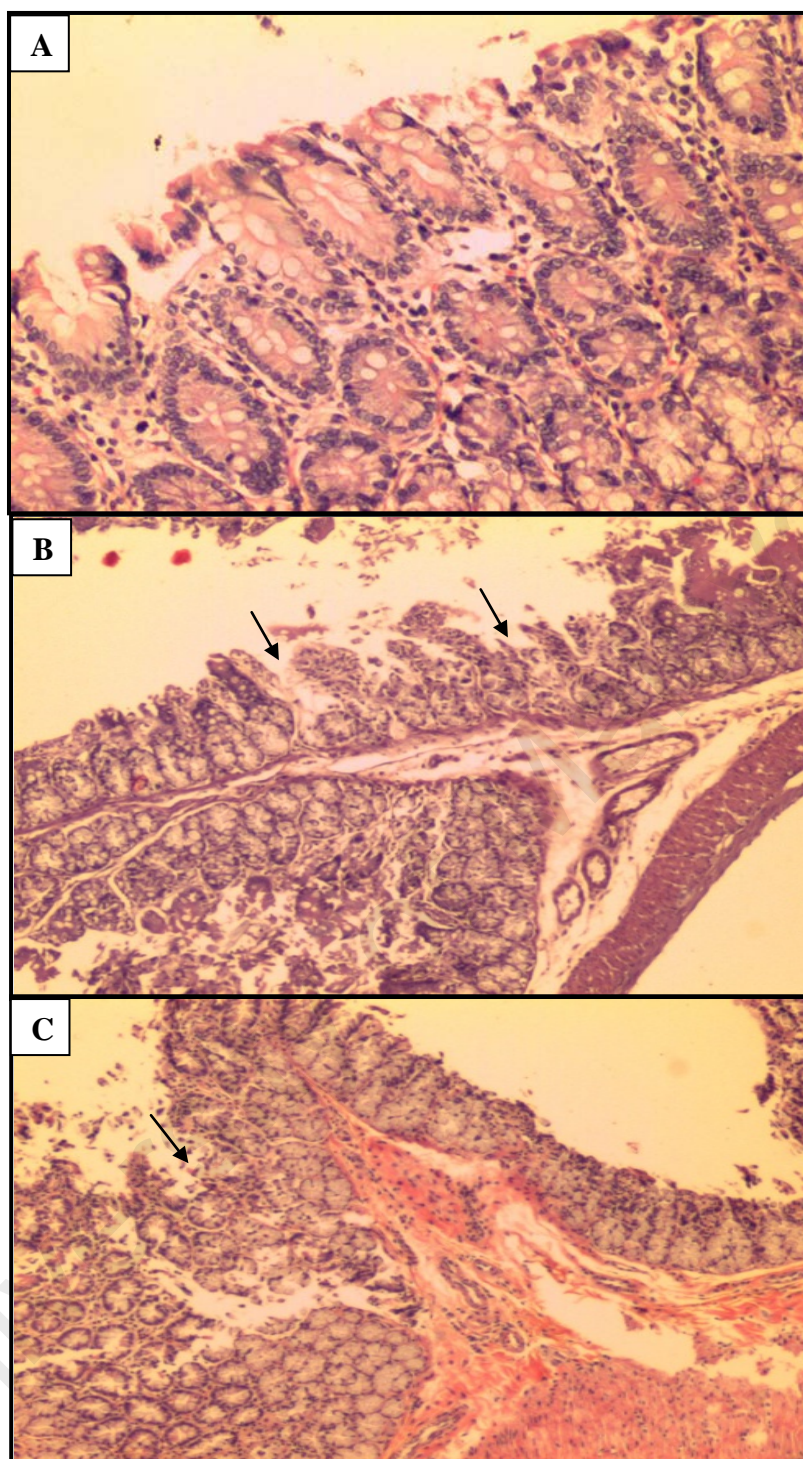


Figure 6.8: Cross section of colon from Sprague Dawley rats of all three groups on day 2 of infection. **A:** normal colon of an uninfected rat, H&E (x100 magnification); **B:** Moderate mucosal sloughing (*arrow*) was seen in the colon from asymptomatic rat, H&E (x100 magnification); **C:** Mild mucosal sloughing (*arrow*) was seen in the colon from symptomatic rat, H&E (x100 magnification).

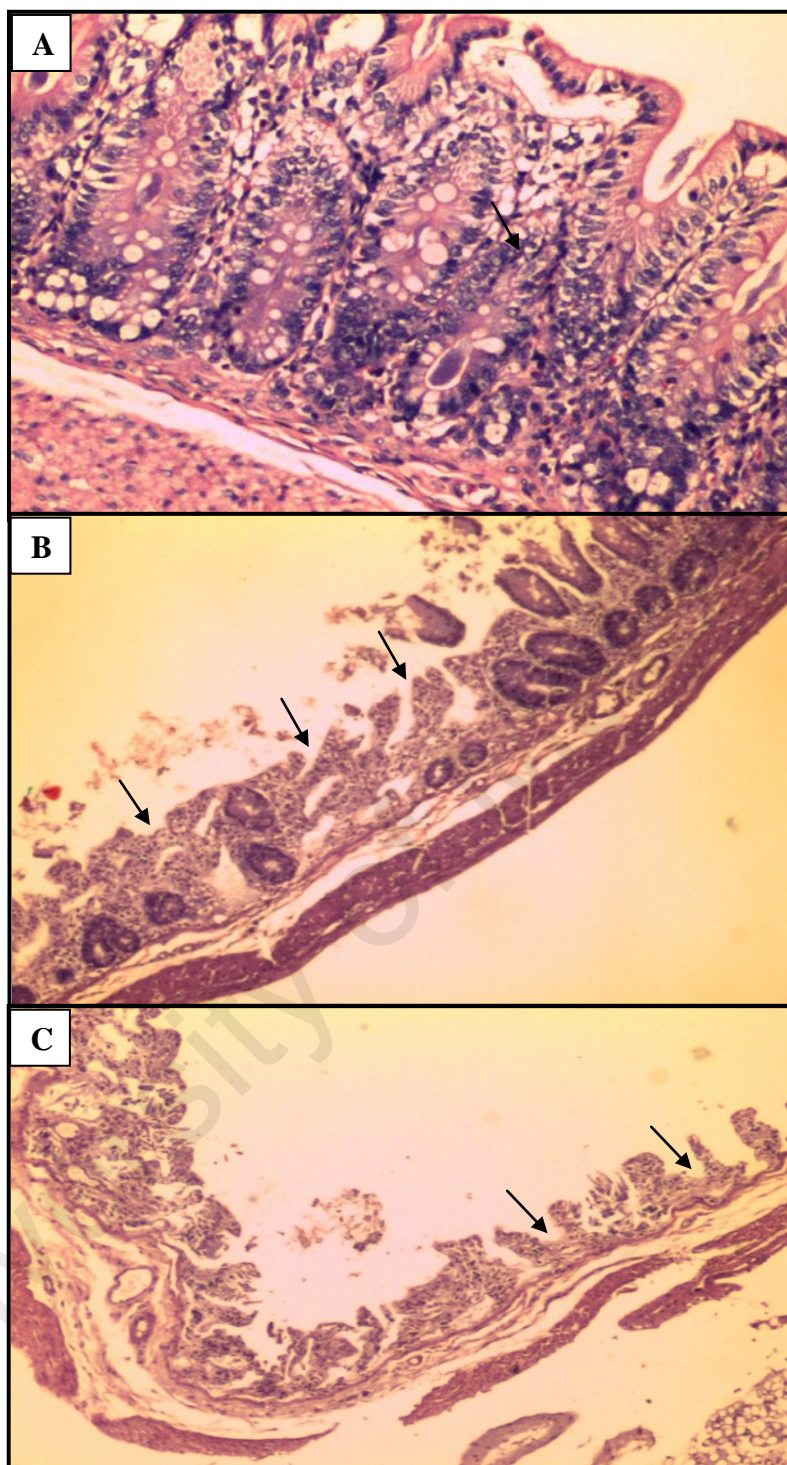


Figure 6.9: Cross section of caecum from Sprague Dawley rats of all three groups on day 2 of infection. A: normal caecum of an uninfected rat, H&E (x100 magnification); **B:** Extensive mucosal sloughing (*arrow*) was seen in the caecum from asymptomatic rat, H&E (x100 magnification); **C:** Moderate mucosal sloughing (*arrow*) was seen in the caecum from symptomatic rat, H&E (x100 magnification).

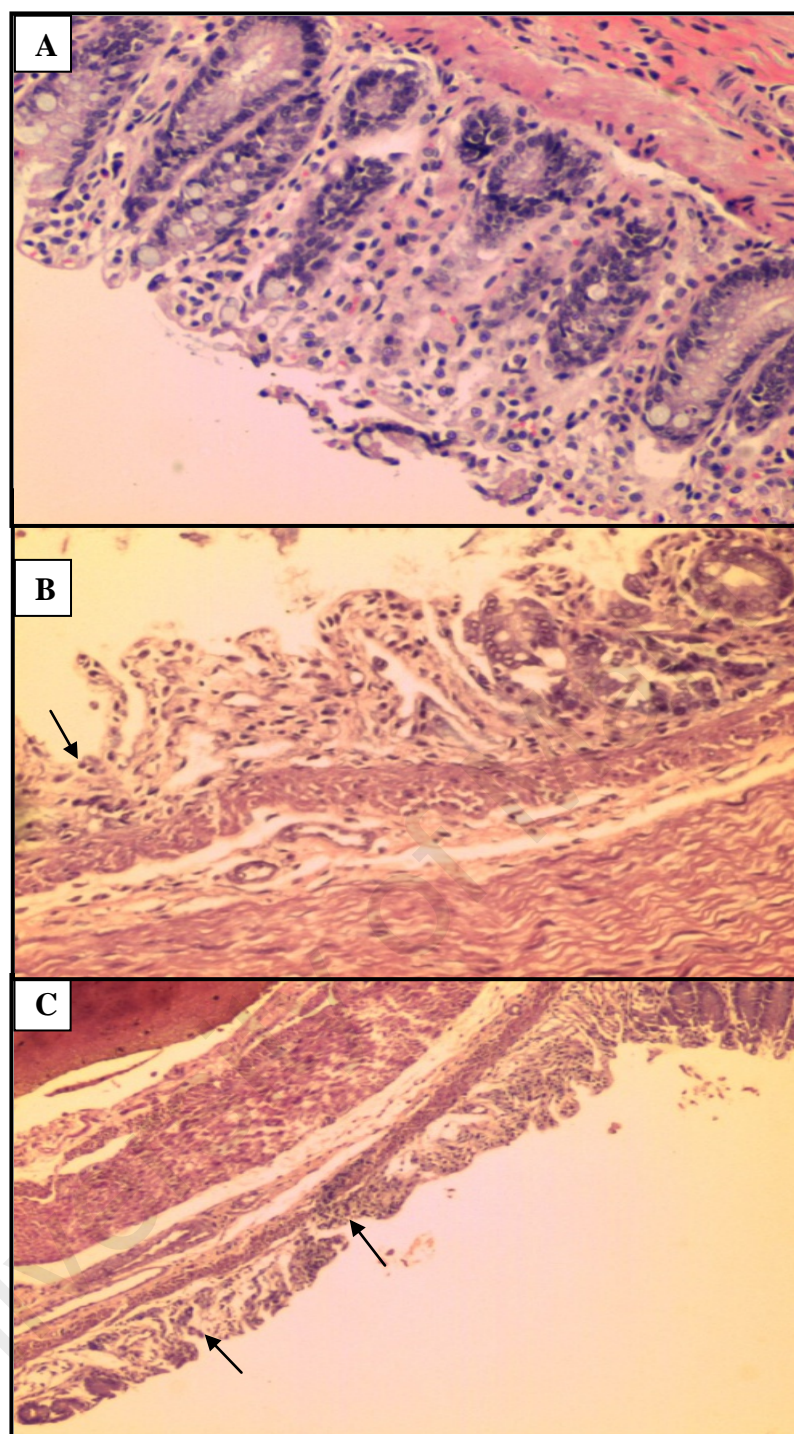


Figure 6.10: Cross section of rectum from Sprague Dawley rats of all three groups on day 2 of infection. A: normal rectum of an uninfected rat, H&E (x100 magnification); **B:** Mild mucosal sloughing (*arrow*) was seen in the rectum from asymptomatic rat, H&E (x100 magnification); **C:** Moderate mucosal sloughing (*arrow*) was seen in the rectum from symptomatic rat, H&E (x100 magnification).

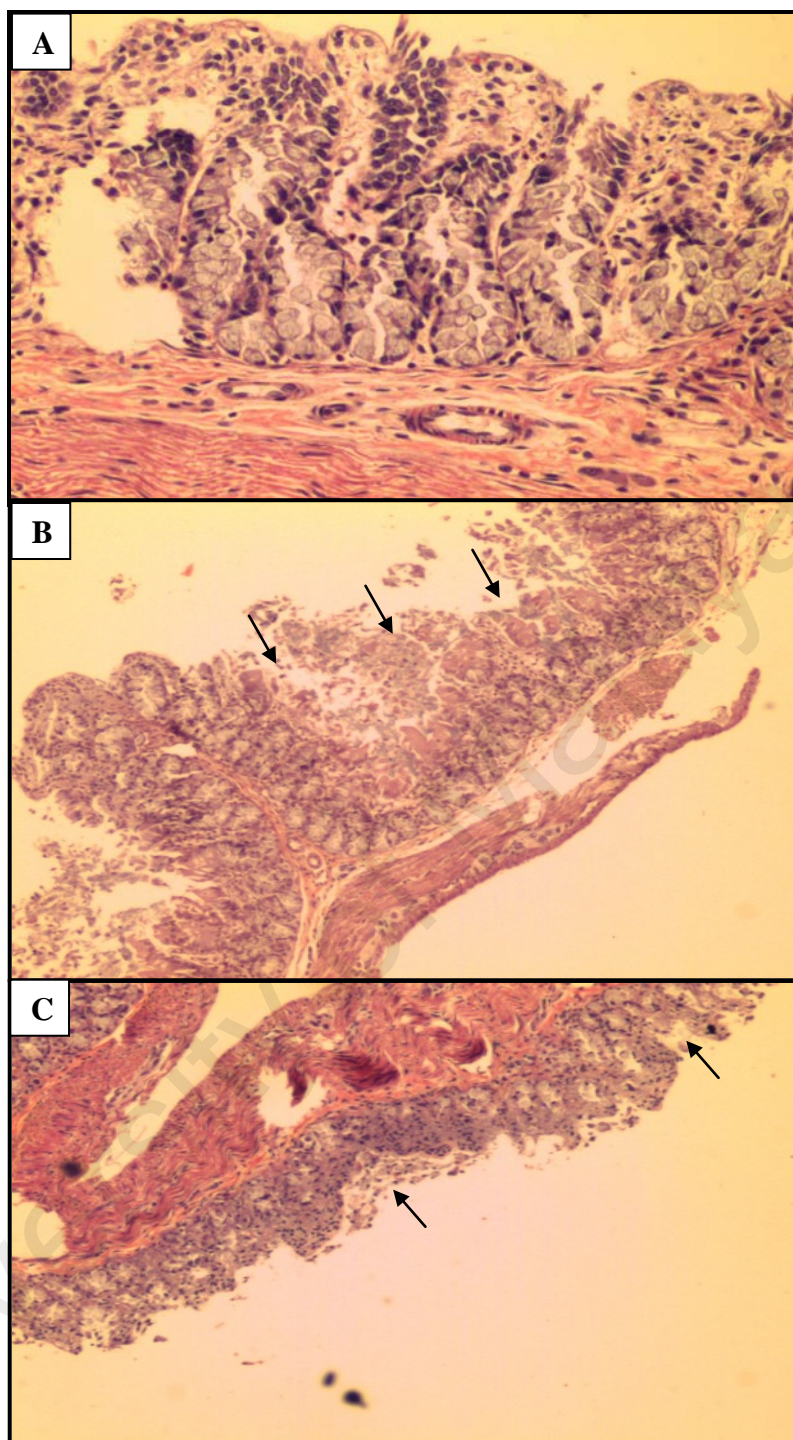


Figure 6.11: Cross section of colon from Sprague Dawley rats of all three groups on day 4 of infection. A: normal large intestine of an uninfected rat, H&E (x100 magnification); **B:** Extensive mucosal sloughing (*arrow*) was seen in the colon from asymptomatic rat, H&E (x100 magnification); **C:** Moderate mucosal sloughing (*arrow*) was seen in the colon from symptomatic rat, H&E (x100 magnification).

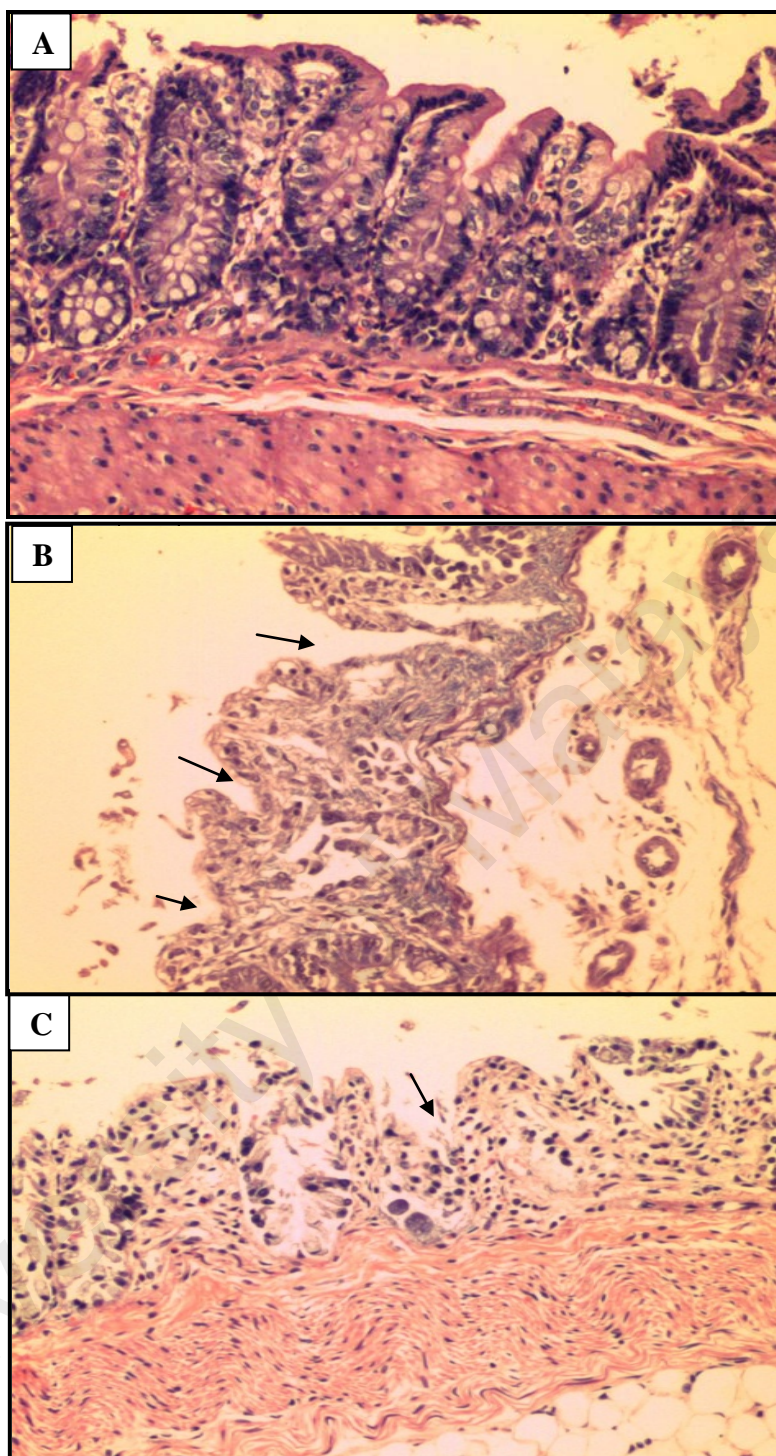


Figure 6.12: Cross section of caecum from Sprague Dawley rats of all three groups on day 4 of infection. A: normal caecum of an uninfected rat, H&E (x100 magnification); **B:** Extensive mucosal sloughing (*arrow*) was seen in the caecum from asymptomatic rat, H&E (x100 magnification); **C:** Mild mucosal sloughing (*arrow*) was seen in the caecum from symptomatic rat, H&E (x100 magnification).

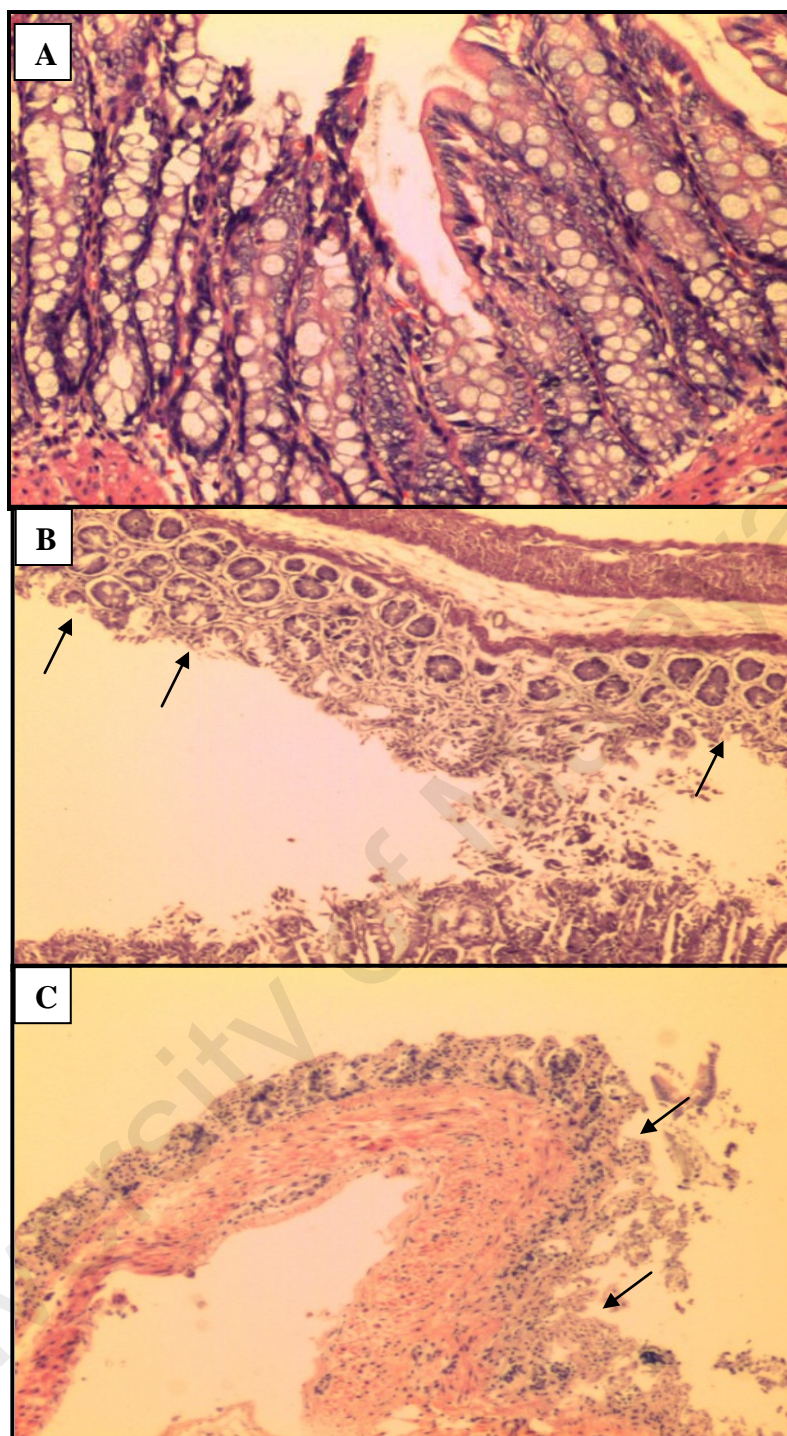


Figure 6.13: Cross section of rectum from Sprague Dawley rats of all three groups on day 4 of infection. A: normal rectum of an uninfected rat, H&E (x100 magnification); **B:** Extensive mucosal sloughing (*arrow*) was seen in the rectum from asymptomatic rat, H&E (x100 magnification); **C:** Moderate mucosal sloughing (*arrow*) was seen in the rectum from symptomatic rat, H&E (x100 magnification).

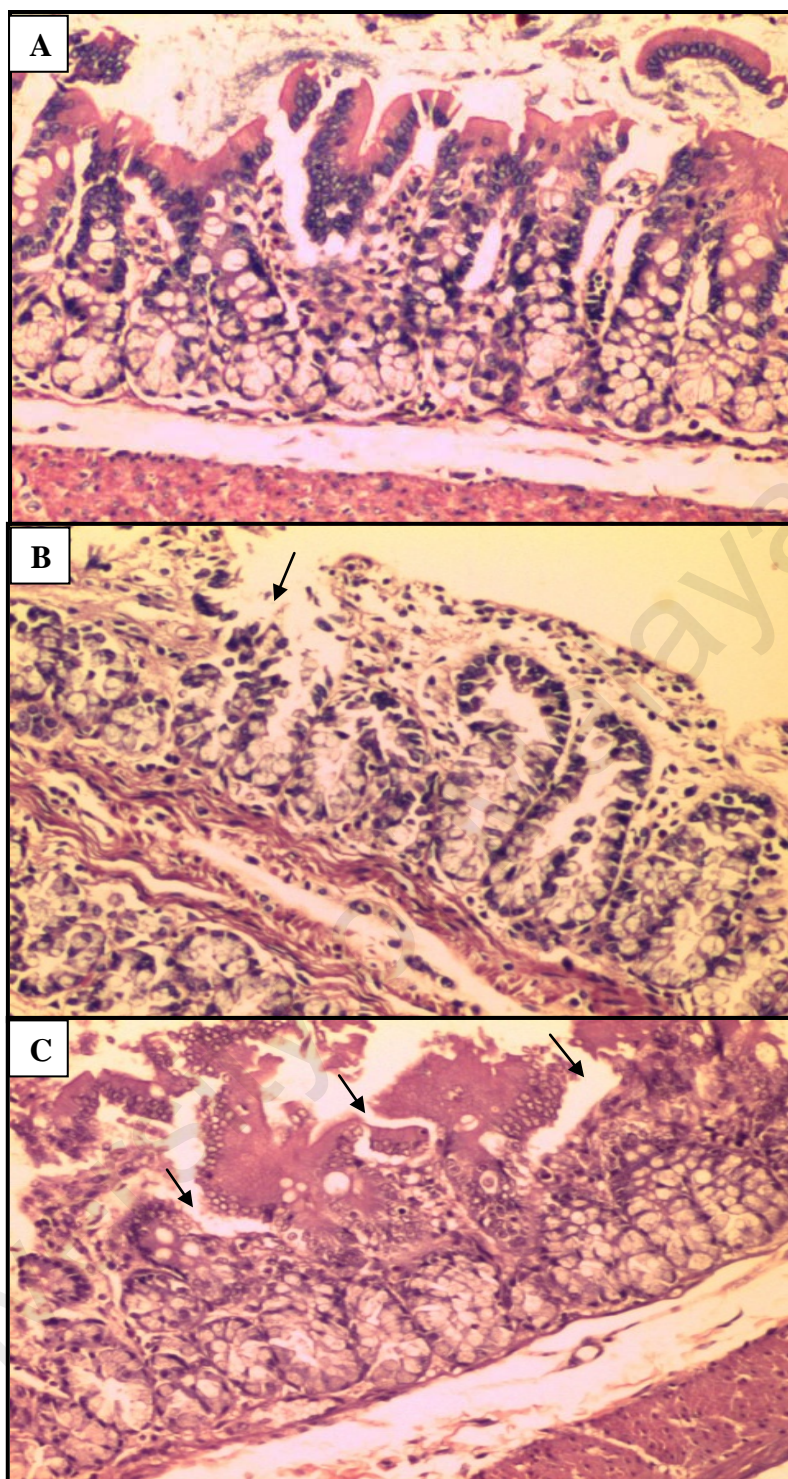


Figure 6.14: Cross section of colon from Sprague Dawley rats of all three groups on the 4th week of infection. A: normal large intestine of an uninfected rat, H&E (x100 magnification); **B:** Mild mucosal sloughing (*arrow*) was seen in the colon from asymptomatic rat, H&E (x100 magnification); **C:** Extensive mucosal sloughing (*arrow*) was seen in the colon from symptomatic rat, H&E (x100 magnification).

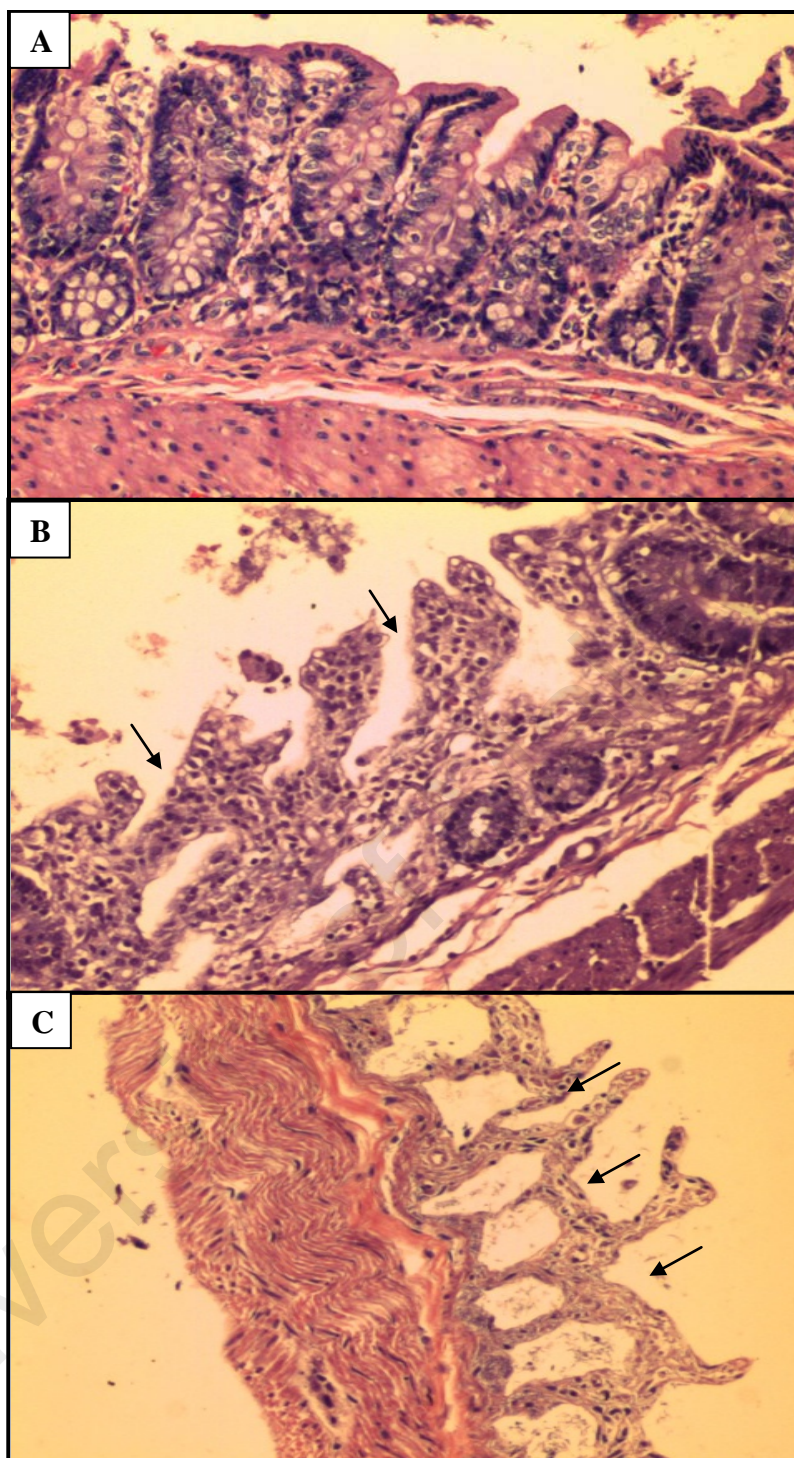


Figure 6.15: Cross section of caecum from Sprague Dawley rats of all three groups on the 4th week of infection. **A:** normal caecum of an uninfected rat, H&E (x100 magnification); **B:** Moderate mucosal sloughing (*arrow*) was seen in the caecum from asymptomatic rat, H&E (x100 magnification); **C:** Extensive mucosal sloughing (*arrow*) was seen in the caecum from symptomatic rat, H&E (x100 magnification).

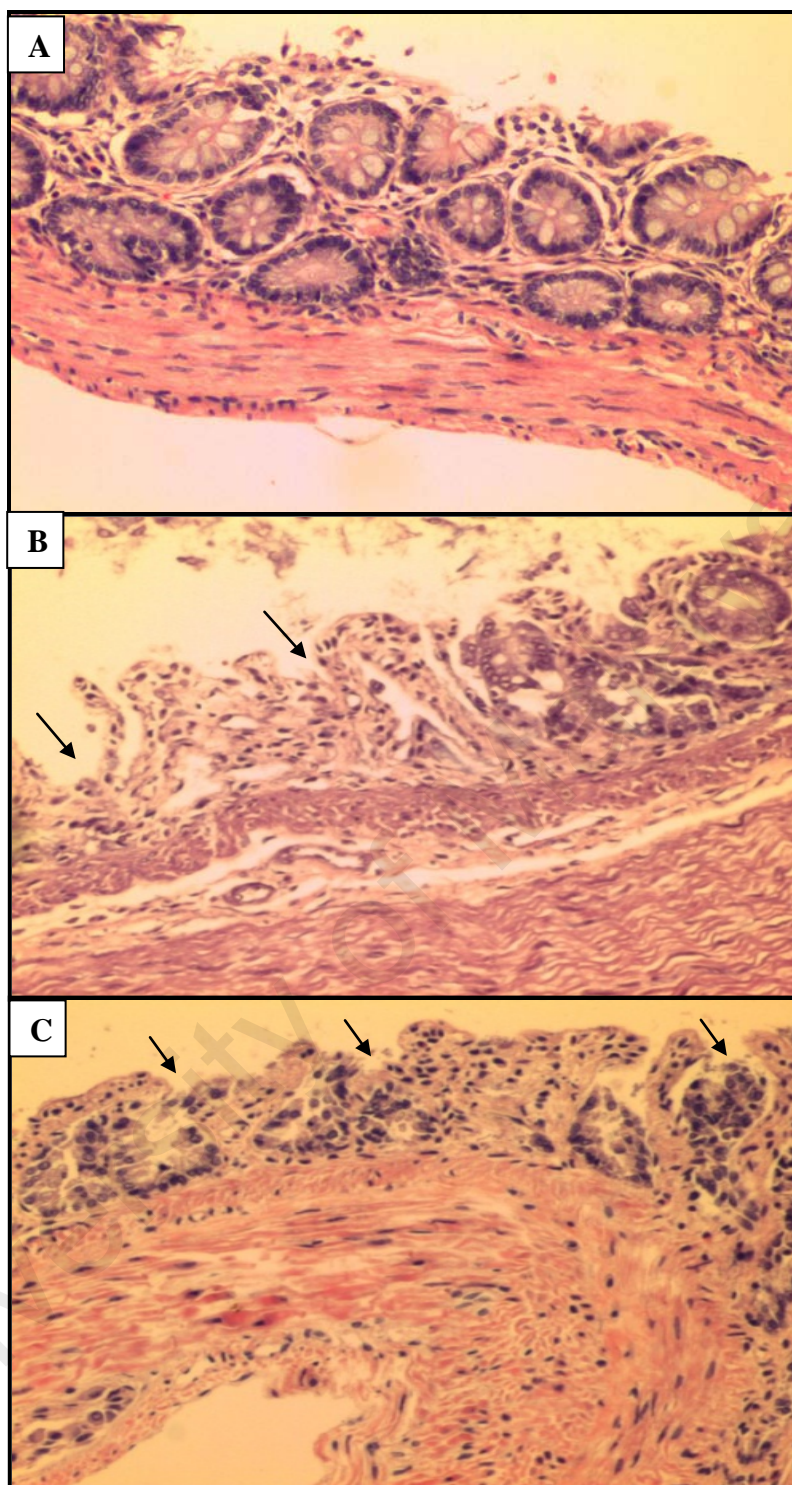


Figure 6.16: Cross section of rectum from Sprague Dawley rats of all three groups on the 4th week of infection. A: normal rectum of an uninfected rat, H&E (x100 magnification); **B:** Moderate mucosal sloughing (*arrow*) was seen in the rectum from asymptomatic rat, H&E (x100 magnification); **C:** Extensive mucosal sloughing (*arrow*) was seen in the rectum from symptomatic rat, H&E (x100 magnification).

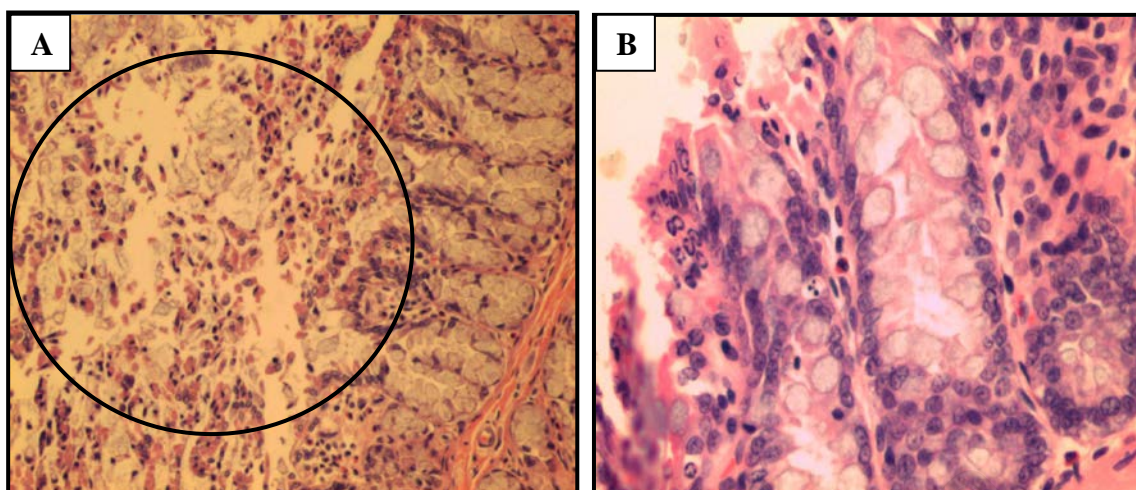


Figure 6.17: Specific histopathological changes of rat caecum section and rectum section from symptomatic group on the 4th week of infection. A: Excessive mucus (*circle*) was seen in the lamina propria of the rat caecum, H&E, (x200 magnification). **B:** Acute inflammation was observed in the lamina propria of the rat rectum H&E, (x200 magnification).

6.4 Discussion and conclusion

Budding was frequently seen in the fresh cultures from the intestines (Zhang et al., 2007; Zhang et al., 2012) of rats inoculated with symptomatic isolates of *Blastocystis* sp. implying that these reproductive processes have been previously reported but rarely seen, and this may be more in *in-vivo* infections. Another interesting point to note is that only the cystic stages were observed in the colon of the rats from both symptomatic and asymptomatic groups. These cysts must have developed from parasitic stages in the caecum probably from the vacuolar or granular form or through some intermediary stages (Moe et al., 1997). The average size of the vacuolar forms in the cultures with cut sections of the large intestines appeared similar to *Blastocystis hominis* with a size range from 5 to 50µm (Yamada & Yoshikawa, 2012). Meanwhile, the mean diameter of cystic forms in the colon and rectum of the rats from the symptomatic group ranged from 5 to

7µm in the present study which are slightly larger than the human isolate which usually range from 3 to 5µm (Zhang et al., 2007; Yoshikawa et al., 2003).

The level of amylase in the symptomatic group was significantly higher when compared to the other groups in the present study. Amylase plays an important role in the hydrolysis of carbohydrate and this enzyme has been shown to be released into blood if the pancreas is diseased or inflamed (Mande & Breslin, 2012). Serum amylase level is used as a diagnostic marker for acute pancreatitis and inflammatory bowel diseases (Triantafillidis & Merikas, 2010). Previous publications have reported that the elevation of serum amylase are commonly seen in patients with intestinal obstruction (Stephen et al., 2014), inflammatory bowel disease (Heikius et al., 1999; Bokemeyer, 2002) and acute pancreatitis disease (Triantafillidis & Merikas, 2010). Heikius et al., 1999 have further described that extensive involvement of the colon accompanied by significant histological activity indirectly induces the increase of serum amylase level. However, none of these studies showed that there is a correlation of serum amylase level with the parasitic diseases. Meanwhile, a study has shown that a child diagnosed of chronic pancreatitis had weight loss and elevated level of serum amylase in the blood examination (Davis & Kelsey, 1951). This finding, however, is not conclusive enough to correlate if an elevated level of serum amylase can be correlated with the least weight gain seen in the rats infected with the symptomatic isolate. The rest of the biochemical parameters showed were not significantly different which concurred with the findings of Chen et al., 2003 and Valsecchi et al., 2004.

Previous studies have reported that there were no significant changes observed in the five critical haematological parameters comprising of white blood cells, eosinophils, haemoglobin, platelets and erythrocyte sedimentation rate in patients infected with

Blastocystis sp., when compared to healthy individuals (Chen et al., 2003). There was also a report of an immunocompetent patient with large ulcers in the caecum due to *Blastocystis* sp. infection who showed normal haematological profiles (Janarthanan et al., 2011). These results concurred with our study that there was no significant changes observed in the haematological profiles of rats infected with symptomatic isolates when compared to the other groups.

Blastocystis sp. is also known to cause mucosal inflammation which can contribute to the gastrointestinal symptoms (Ustun & Turgay, 2006). Previous studies have shown that *Blastocystis* sp. can cause severe effect to the host especially when coupled with other intestinal disorder. This is the first study to elucidate histopathological aspects of the rats inoculated with *Blastocystis* sp. ST3 cysts from two different groups i.e. asymptomatic and symptomatic. In the present study, *Blastocystis* sp. from symptomatic isolates caused least weight gain with excretion of softer stools when compared with stool from other rats in the two other groups. The appearance of the histopathological features seen in the intestines of rats inoculated with cysts from symptomatic patients was different compared to rats inoculated with cysts from asymptomatic individual. The intestines of rats inoculated with cysts isolated from symptomatic patient appeared to show significant reddishness and bloating which concurred with the previous findings (Moe et al., 1997 and Chandramathi et al., 2014).

In the present study, extensive mucosal sloughing was observed on day 4 in asymptomatic isolates which indicate excystation of cysts and higher rate of parasite proliferation in the intestine as soon as infection took place in the asymptomatic group compared to when infected with symptomatic isolates. The findings correlated with a previous study which showed that asymptomatic isolates had the highest growth rate in

cultures when compared to symptomatic (Tan et al., 2008) and highest cell proliferation seen in the asymptomatic isolates was between days 3 and 6 in a growth profile study of human isolates (Nanthiney et al., 2014). On the other hand, the extent of mucosal sloughing was seen to be more pronounced in caecum, colon and rectum in rats infected with cysts from symptomatic patients compared to cysts from the asymptomatic patients. Furthermore, excessive mucus and acute inflammation observed in the intestines of one rat in the symptomatic group at the 4th week suggests that symptomatic patients harbouring *Blastocystis* sp. are severely affected and show greater symptoms than the asymptomatic persons.

In the present study, different degrees of mucosal sloughing were observed among all infected rats from both the asymptomatic and symptomatic groups. The ST3 symptomatic isolate induced a severe degree of pathological changes in the rats from the symptomatic group. These results concurred with the findings of Moe et al., 1997, who showed that mucosal sloughing in caecum, colon and rectum sections from rats infected with the symptomatic isolates ranged from moderate to extensive. It has been suggested that mucosal sloughing will cause destruction to the surface area resulting in poor absorption which in turn will cause digestive problems. Meanwhile, other pathological changes such as severe edema, hyperemia, and congestion were not observed in the present study, which have been previously reported in the cecum and colon of mice infected with symptomatic isolate (Abou & Negm, 2001; Yao et al., 2005).

The present study represents the first study to correlate the infection which induces changes at the biochemical and haematological levels with histopathological

changes in the large intestines of infected rats from the symptomatic and asymptomatic groups. The least weight gain and the gross changes in the intestines of rats from symptomatic group showed that ST3 *Blastocystis* from the symptomatic isolate had pathogenic potential. Previous studies have shown ST3 *Blastocystis* sp. in asymptomatic persons (Tan et al., 2008; Scanlan & Pauline, 2012) and (Eman et al., 2008) have further justified the presence of pathogenic and non-pathogenic strains in ST3 *Blastocystis* sp. Hence, the most important finding in the present study is that ST3 *Blastocystis* sp. isolated from both groups show a marked difference in the histopathological consequence despite being infected with the similar subtype. Therefore, it appears to be that pathogenicity may not be subtype related and there could be other host factors that could play a role in determining the pathogenicity of this organism.

CHAPTER 7

TRANSFORMATIONAL CHANGES DURING EXCYSTATION OF CYSTS OF *BLASTOCYSTIS* SP.

7.0 Transformational changes during excystation of cysts of *Blastocystis* sp.

7.1 Introduction

One of the earliest studies to document encystation in *Blastocystis* sp. was carried out in 1994 (Suresh et al., 1994). The detailed ultrastructural changes from vacuolar to cystic forms showed that the amebic forms were the intermediary forms between these two life cycle stages. The histopathological changes seen in *Blastocystis* sp. subtype 3 infected rats from symptomatic and asymptomatic isolates in the previous chapter reveal marked changes in the former group. The significant sloughing of cells seen in the lumen of rats infected with asymptomatic isolates compared to those infected with symptomatic ones in the first week of infection does imply that there could be transformational changes during excystation in parasites from these two isolates.

Very few studies have highlighted the details during excystation of *Blastocystis* sp. cysts (Moe et al., 1999; Chen et al., 1998). *Blastocystis* sp. from symptomatic and asymptomatic isolates have shown previously to have different growth profiles (Tan et al., 2008) and it is possible that excystation rates and transformational changes could be different between parasites from symptomatic and asymptomatic isolates. Till this date, there has been no comparison on the development of cystic forms into vacuolar forms isolated from symptomatic and asymptomatic patients. The present study attempts to compare the transformational changes seen in the cells from cysts to vacuolar forms isolated from both ST3 symptomatic and asymptomatic isolates using transmission electron microscopy which could substantiate the histopathological consequence seen in the infected rats shown in chapter 6.

7.2 Materials and methods

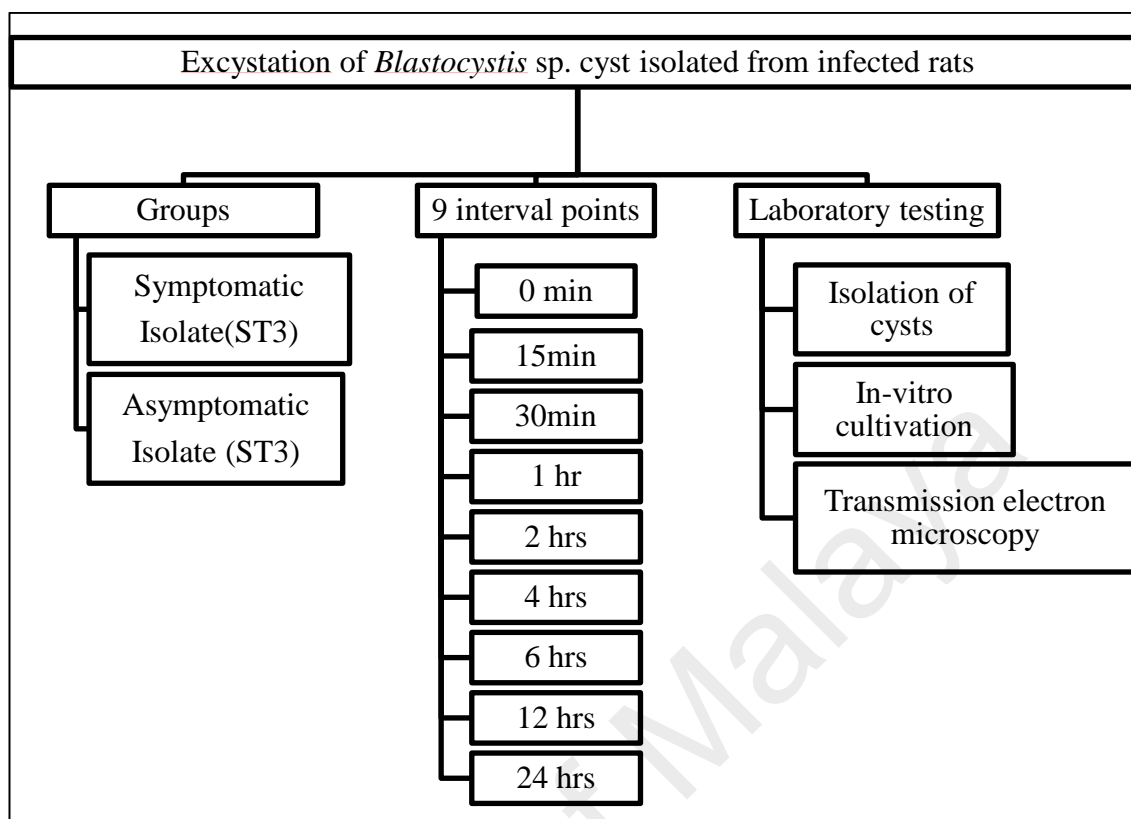


Figure 7.1: Schematic representation of the overall methodology emphasizing on the excystation of *Blastocystis* sp. isolated from ST3 symptomatic and asymptomatic isolates.

7.2.1 Source of *Blastocystis* sp. cyst

Fresh stool samples were collected from only one person infected with subtype 3 *Blastocystis* sp. out of 20 individuals who work closely with animals in an animal research institute during a small scale survey conducted in Ipoh. This individual showed no symptoms and had no other pathogens except *Blastocystis* sp. in the stool examination. As for the symptomatic groups, fresh stool samples were collected from a symptomatic patient infected with subtype 3 *Blastocystis* sp. at a private hospital complaining of persistent diarrhoea and had no other pathogens. The faecal samples were collected and dissolved in normal saline and sieved into 15ml Falcon tube. The

culture tubes were centrifuged at 3000rpm for 10min (Beckman Coulter 365303 Spinchron DLX centrifuge machine). The sediment was layered on a 5ml of Ficoll-Paque solution in a culture tube and centrifuged at 3500rpm for 20 minutes. The cystic stages banded at 3 to 4cm below the top meniscus, between the third and fourth layers. This then was pipetted out into clean 14ml culture tube. The cysts were washed twice by adding up to 14ml of phosphate buffer solution (PBS). The culture tube was capped tight, inverted a few times and centrifuged at 3000rpm for 10 minutes for each washing. The supernatant was discarded completely and 1ml of PBS solution was added into the culture tube which contained the sediment. The cysts were then enumerated and diluted to a concentration of 10^6 cysts/ml.

7.2.2 Experimental infection with *Blastocystis* sp. cysts in Sprague Dawley rats

The cysts from the symptomatic and asymptomatic faeces were diluted and made up to a concentration of 10^6 cysts/ml respectively and subsequently inoculated orally, using a 20G feeding needle of 1.5inch length into two groups of two rats each. All rats were anesthetized before inoculation.

7.2.3 Isolation of cysts from the rats

Fresh stool samples were collected from rats infected with *Blastocystis* sp. subtype 3 from symptomatic and asymptomatic isolates. The faecal samples were dissolved in normal saline and sieved into 15ml Falcon tube. The tube was centrifuged at 3000rpm for 10min. The sediment was layered on a 5ml Ficoll-Paque solution in a culture tube and centrifuged at 3500rpm for 20 minutes. The cystic stages banded between 3 and 4cm below meniscus which is the third and fourth layer. This was then pipetted out into

a clean 14ml culture tube. The cysts were then washed twice by adding up to 14ml of phosphate buffer solution (PBS). The culture tube was then capped tight and inverted a few times and subsequently centrifuged at 3000rpm for 10 minutes for each washing. The supernatant was then discarded completely and 1ml of PBS solution was then added into the culture tube which contained the sediment.

7.2.4 *In-vitro* cultivation

The cysts were then aliquoted as 1 ml suspension in each culture tube. Then, 2 ml of Jones' medium (Jones 1946) containing 10% horse serum was added to each culture tube and incubated at 37 °C. Excystation of cysts was monitored by taking a drop of the sediment at intervals of 0 min, 15min, 30min, 1hr, 2hrs, 4hrs, 6hrs, 12 hrs and 24 hrs respectively. Each set was prepared in triplicates and all the cultures were kept in airtight tubes.

7.2.5 Observations by transmission electron microscopy

At each time point, the parasites were processed for transmission electron microscopy (TEM) studies. The sediment containing the parasites was washed three times using phosphate buffered saline (PBS) pH 7.4. The sample was centrifuged at 3000rpm for 5 minutes. The pelleted cells were re-suspended 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 then dehydrated for 5 minutes in ascending series of ethanol (30%, 50%, 70%, 80%, 90% and 100%) and embedded in epoxy resin. Semi-thin sections were stained with toluidine blue. Ultrathin sections were cut, contrasted with

uranyl acetate and lead citrate. This was viewed using a transmission electron microscope (LEO Libra120) (Tan et al., 2006).

7.3 Results

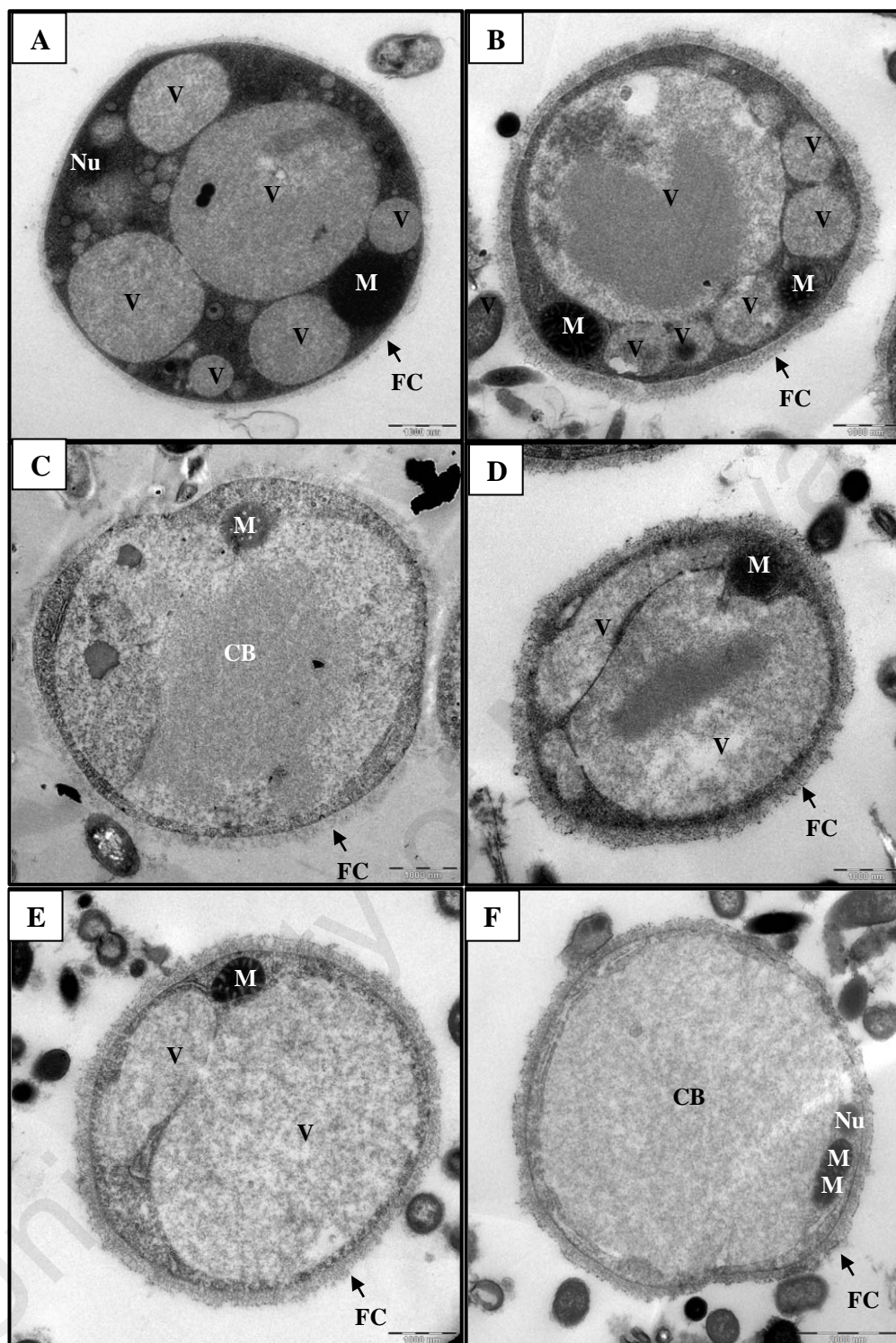
Based on light microscopy, most of the cysts appeared rounded or ovoid with a size range of 3 to 5 μm . The cyst wall was mostly thick comprising of an outer fuzzy coat with a clear inner wall. Generally, only one nucleus with a distinct nuclear membrane was seen in each cyst. Each cyst has at least one to three mitochondria with prominent cristae. Endoplasmic reticulum was rarely seen in the cytoplasm. Varying numbers and sizes of empty vacuoles with some inclusions were often observed.

For asymptomatic isolates, at 0 min, a thick fuzzy coat with prominent mitochondria and distinct nucleus were seen within the cysts. Numerous spherical vacuoles with inclusions were seen in the cytoplasm. Most of the cells at this interval time showed high electron dense material in the central vacuole (Figure 7.2A). At 15 min of culture, the fuzzy coat remained thick with a clear inner wall becoming more obvious in the cell. Two mitochondria with prominent cristae and numerous spherical vacuoles were observed in the cytoplasm of most of the cells. The central vacuole contained high electron dense material (Figure 7.2B). At 30 min, the rounded cysts showed the same fuzzy coat with some of the organisms showing only one mitochondrion in the cytoplasm. The small vacuoles were seen to coalesce forming one large vacuole within the central vacuole (Figure 7.2C). After 1 hour, thicker fuzzy coat with prominent mitochondria were seen in the cells. Some of the cells showed the clustering of vacuoles in the central body (Figure 7.2D). At 2 hours, thick fuzzy coat with a clear inner wall was observed. Only one mitochondrion with prominent cristae per cell was observed

(Figure 7.2E). Larger vacuoles with less electron dense material within were also observed. At 4 hours, typical round shaped parasites with an oval-shaped mitochondria and a large vacuole were observed. Electron dense material was observed to be scanty in some cells. (Figure 7.2F). After 6 hours, the fuzzy coat appeared thinner with a clear inner wall. Most of the cells had one prominent mitochondrion and showed more than one nucleus in the cytoplasm. Vacuoles clustered together forming a larger vacuole which was frequently seen within the parasites. The central vacuole contained less electron dense material (Figure 7.2G). At 12 hours of culture, the thinning of fuzzy coat was clearly seen and the small vacuoles were seen to be coming together to form a large vacuole. Some cells had more mitochondria with prominent cristae. Less electron dense material was observed in the central vacuole (Figure 7.2H). After 24 hours of culture, the thin fuzzy coat became even thinner. There were more mitochondria with prominent cristae observed. Several forms of *Blastocystis* sp. with large vacuole with a central body showing less electron dense material were often seen (Figure 7.2I).

For the symptomatic isolate, at 0 min, a thick fuzzy coat was seen with numerous prominent mitochondria with a clear inner wall. Numerous spherical vacuoles with inclusions were seen in the cytoplasm. The cells showed high electron dense material within the central vacuole (Figure 7.3A). At 15 mins, the size of the fuzzy coat appeared similar with a clear inner wall remaining obvious. The cytoplasm was filled with mitochondria, with distinct nucleus and numerous spherical vacuoles. High electron dense material was seen in most parts of the parasites (Figure 7.3B). At 30 min, the fuzzy coat remained as thick as before with the rounded cysts showing only one mitochondrion in the cytoplasm. Various sizes of small vacuoles were seen in the parasite with high electron dense material in the central vacuole (Figure 7.3C). After 1 hour, the fuzzy coat remained the same with mitochondria showing prominent cristae.

Numerous spherical vacuoles were seen with parasites showing only one nucleus in the cytoplasm. The central body of most of the cells contained high electron dense material (Figure 7.3D). At 2 hours, the fuzzy coat remained the same size with a clear inner wall observed in some of the parasites. Two nuclei were observed in some of the parasites. Some cells had larger spherical vacuoles with high electron dense material observed in the central body (Figure 7.3E). At 4 hours, a typical round shaped parasite with a clear inner wall was seen. Large spherical vacuoles were observed in the central body of some cells. Very few mitochondria with prominent cristae were seen in the cytoplasm of most of the cells (Figure 7.3F). At 6 hours, numerous lipid inclusions were seen with high electron dense material observed within the cell body of the parasite (Figure 7.3G). At 12 hours, more prominent mitochondria were seen in the cytoplasm. Spherical small vacuoles with high electron dense material was seen in the central body (Figure 7.3H). At 24 hours, the thick fuzzy coat of the parasite had become very thin (Figure 7.3I). The clusters of small vacuoles coalesced to form a large vacuole in the parasite with electron dense material becoming scanty within the central body. Most of the cells showed few mitochondria in the cytoplasm.



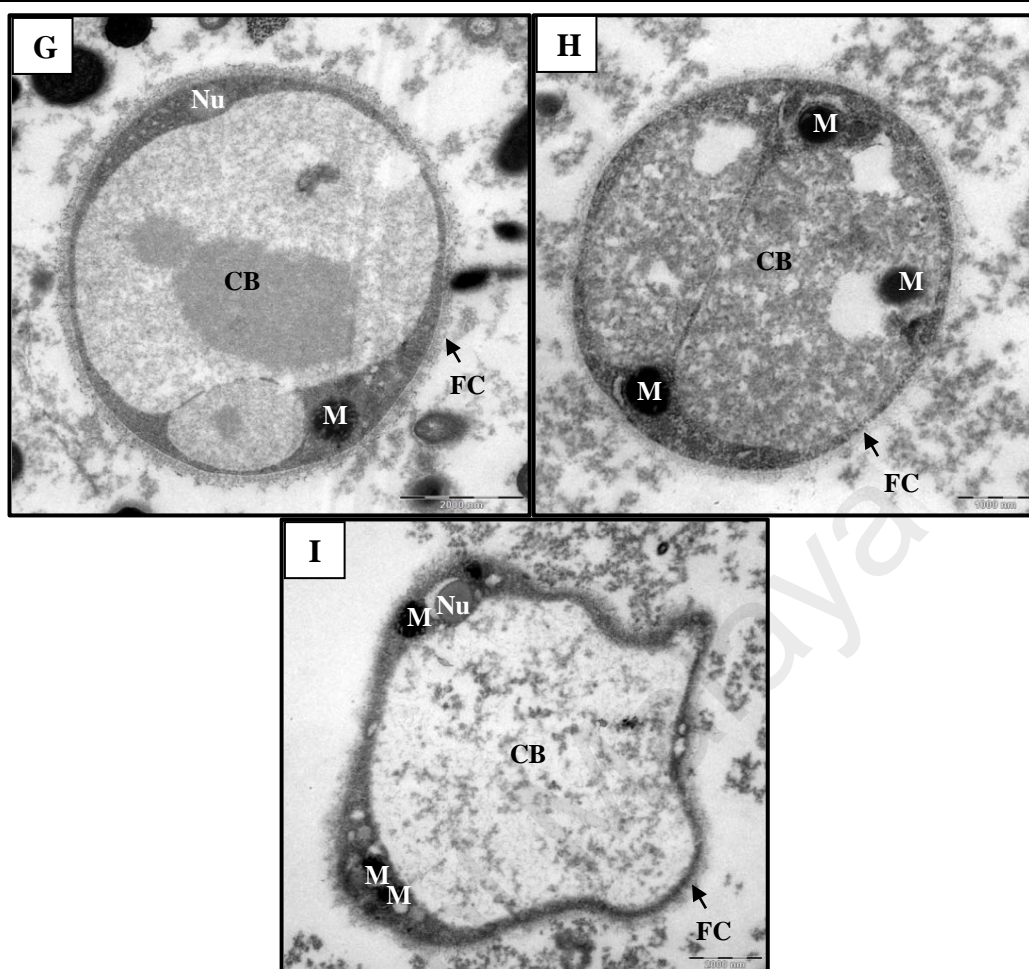
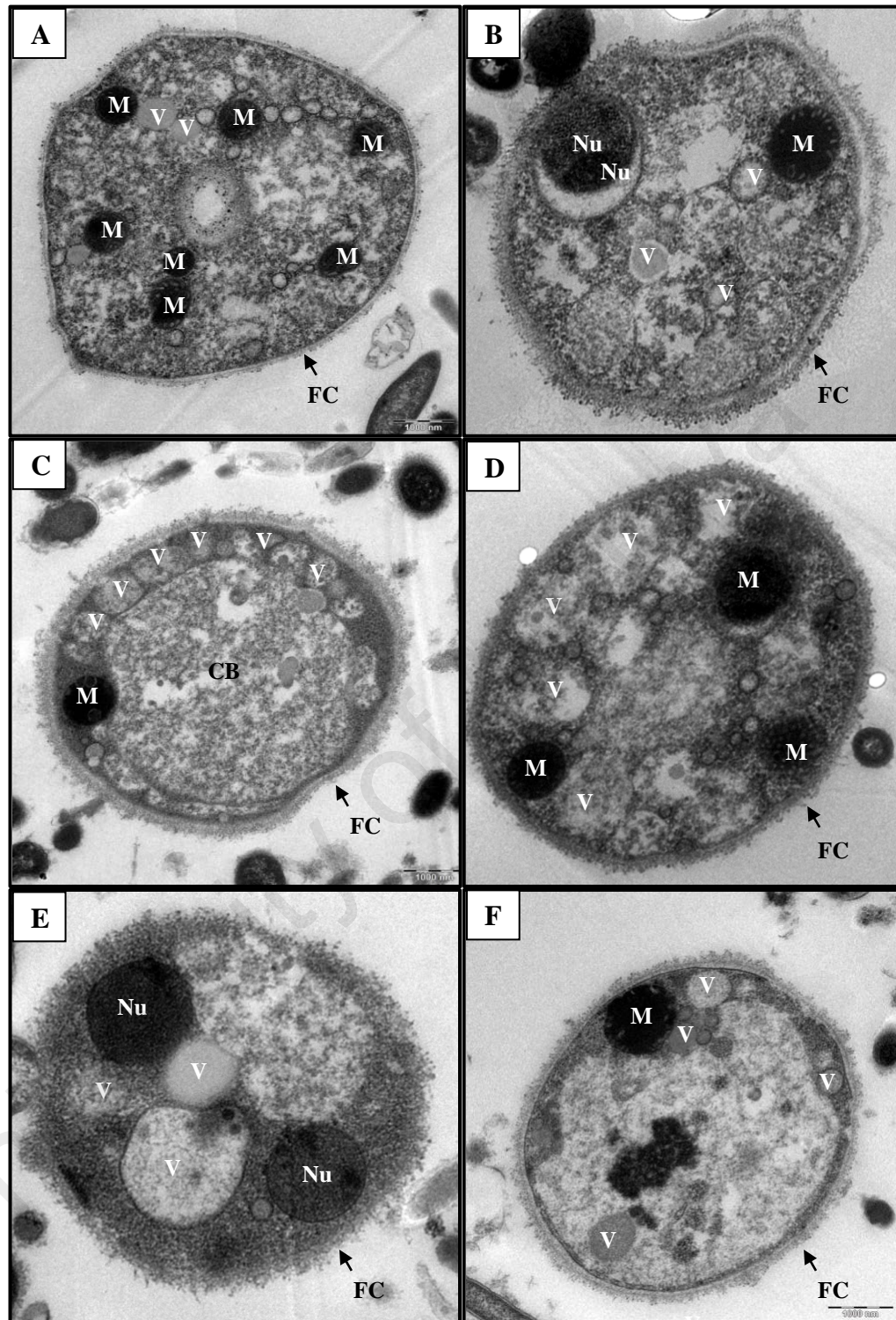


Figure 7.2: Transmission electron micrographs of *Blastocystis* sp. of ST3 asymptomatic isolate at different interval points. **A:** Cells at 0 min of culture, showing numerous spherical vacuoles (V) with inclusions in the central body with prominent mitochondria (M) and distinct nucleus (Nu) in the cytoplasm. Note the thick fuzzy coat (FC) (arrow) surrounding the cell; **B:** Cells at 15 min of culture, showing thick fuzzy coat (FC) surrounding the cell with prominent mitochondria (M) and numerous spherical vacuoles (V) in the cytoplasm; **C:** Cells at 30 min of culture, show thick fuzzy coat (FC) with one prominent mitochondria (M) in the cytoplasm. Small vacuoles slowly coalesced to form a larger vacuole in the central body; **D:** Cells at 1 hr of culture, showing a thick fuzzy coat (FC) with prominent mitochondria (M) in the cytoplasm. Note the cluster of vacuoles (V) in the central body; **E:** Cells at 2 hrs` of culture, showing thick fuzzy coat (FC) with a large vacuole in the central body. Less electron dense material is observed in the central vacuole with prominent mitochondria (M); **F:** Cells at 4 hrs of culture, showing cysts with a large vacuole and prominent mitochondria (M) is observed in the cytoplasm. Less electron dense material was observed in some of the cells; **G:** Cells at 6 hrs of culture, showing a thinner fuzzy coat (FC) with a clear inner wall and one prominent mitochondria and nucleus in the cytoplasm. Note the clustering of vacuoles forming a larger vacuole in the cell; **H:** Cells at 12 hrs of culture showing thinner fuzzy coat surrounding the cell with numerous prominent mitochondria in the cytoplasm. Note the small vacuoles coalescing to form a larger vacuole in the cell; **I:** parasites at 24 hrs of culture, showing thin fuzzy coat almost completely dissolved. More mitochondria and a large vacuole with less electron dense material were observed.



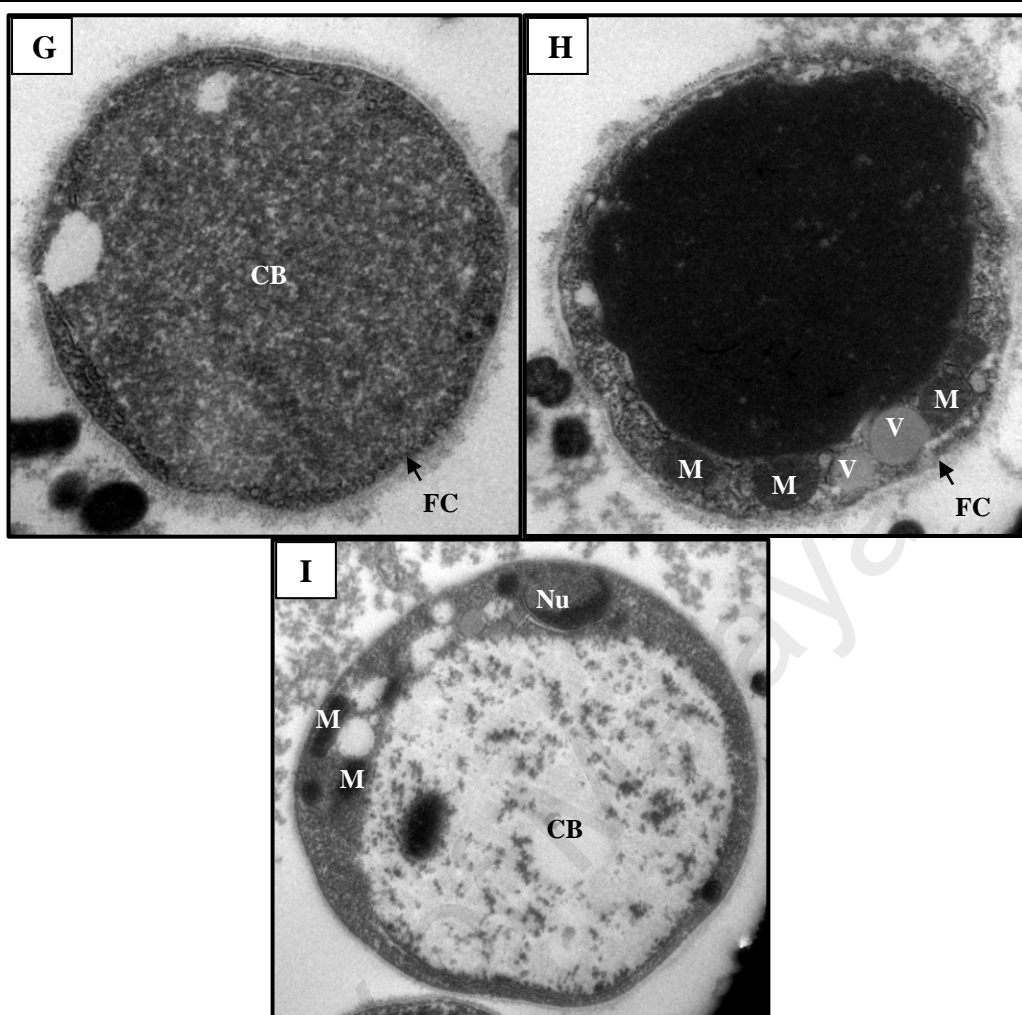


Figure 7.3: Transmission electron micrographs of *Blastocystis* sp. of ST3 symptomatic isolate at different interval points. **A:** Cells at 0 min of culture, thick fuzzy coat (FC) is observed with numerous prominent mitochondria (M) and spherical vacuoles (V) in the cytoplasm. Central vacuole contains high electron dense material; **B:** Cells at 15 min of culture, showing thick fuzzy coat (FC) with the cytoplasm filled with mitochondria (M), distinct nucleus (Nu) and numerous spherical vacuoles (V). High electron dense material was observed in the central vacuole; **C:** Cells at 30 min of culture show thick fuzzy coat (FC) with prominent mitochondria (M) seen in the cytoplasm. Small vacuoles (V) were seen to coalesce to form a larger vacuole within the central body; **D:** Cells at 1 hr of culture show a thicker fuzzy coat (FC) surrounding the parasite and more mitochondria (M) with prominent cristae were seen in the cells. The central body (CB) contains numerous spherical vacuoles (V) and high electron dense material; **E:** Cells at 2 hrs of culture, showing thick fuzzy coat (FC) with a clear inner wall. High electron dense material and larger spherical vacuoles (V) were observed in the central body with the presence of two nuclei (Nu) in the cytoplasm; **F:** Cells at 4 hrs of culture, show rounded parasite with a clear inner wall observed. The central body contains large spherical vacuoles (V) with very few mitochondria (M) seen in the cytoplasm; **G:** Cells at 6 hrs of culture, show thick fuzzy coat (FC) surrounding the parasite with numerous lipid inclusions and high electron dense material within the central body (CB); **H:** Cells at 12 hrs of culture show thick fuzzy coat (FC) observed with numerous prominent mitochondria (M) in the cytoplasm. Note the small vacuoles (V) forming a large

vacuole in the cell; **I:** Cells at 24 hrs of culture, show a thinner fuzzy coat (FC). Very few mitochondria (M) and a large vacuole with less electron dense material were observed.

7.4 Discussion and conclusion

Several authors have shown the presence of cystic forms (Mehlhorn, 1988; Suresh et al., 1994; Zaman et al., 1995). However the transformational changes during excystation were never compared between the cysts isolated from symptomatic and asymptomatic isolate. Hence, the present study provides the ultrastructural evidence of the development of cysts into vacuolar forms in *in-vitro* culture. There are very few publications which have highlighted on the excystation of *Blastocystis* cysts and when reported these parasites were isolated from only symptomatic patients at different interval point (Moe et al., 1996; Moe et al., 1999). In the present study, the transformational changes of the cystic forms into vacuolar forms were compared in parasites isolated from both symptomatic and asymptomatic isolates. Within 1hr, a thick fuzzy coat was shown which was similar to those cysts isolated from the fresh faeces at 0min. Similar finding was also reported by Moe et al., 1999. The prominent thicker fuzzy coat observed in the symptomatic isolate which slowly became thinner only after 24 hrs in the present study. However, Moe et al., 1999 have shown that the symptomatic isolate excyst at 12hrs which is 6hrs later than the asymptomatic isolate shown in the present study.

Numerous spherical vacuoles were seen with parasites showing one to two nuclei in the cytoplasm of the symptomatic isolates from 0 min onwards which were also observed in the earlier study especially at 6-9hrs of culture (Moe et al., 1999). Meanwhile, numerous lipid inclusions observed within the cell body of the parasite appeared as granules. An earlier study described three types of granules namely

metabolic, lipid, and reproductive granules (Tan & Zierdt., 1973). Metabolic granules were found in the cytoplasm, lipid vacuoles in cytoplasm and central vacuole, while reproductive granules were shown to be present in the central vacuole (Zierdt, 1973). In the present study, lipid-like inclusions were seen more at 6 hrs of culture while the same were reported to be seen at 48hrs culture in the symptomatic isolate (Moe et al., 1999). These lipid granules have been suggested to represent a form of energy storage (Dunn et al., 1989).

Meanwhile, the prominent mitochondria frequently seen in the cytoplasm of the symptomatic isolate at every interval point in the present study was only seen at 3hrs culture of the symptomatic isolate studied previously (Moe et al., 1999). The more prominent mitochondria observed in the symptomatic isolate suggest that mitochondria become more activated during the process of excystation to provide energy that is required for the structural changes in the parasite. In the present study, high electron dense material was observed continuously to be present in the central vacuole of the parasites between 0min till 12 hrs of culture. However, this was not highlighted or described in detail in the previous study by Moe et al., (1999). Meanwhile, endoplasmic reticulum previously shown in the parasites (Moe et al., 1999) was not seen in cells from both the asymptomatic and symptomatic isolates in the present study.

To date, none have described the excystation process from the cyst to vacuolar forms isolated from asymptomatic patient. This study provides for the first time ultrastructural evidence of the excystation process which had almost similar processes but differed in time when compared to the symptomatic and asymptomatic isolates, the most obvious appears to be the length of time taken for the thick fuzzy coat to become thinner. *Blastocystis* sp. from asymptomatic isolate took 6 hours compared to the

symptomatic isolate which took 24 hours. This probably explains why asymptomatic isolates grow faster than symptomatic isolates in *in-vitro* cultures (Tan et al., 2008) (Appendix 1). The authors further suggested that these isolates may undergo different modes of reproduction during *in-vitro* cultivation. The faster proliferation of the asymptomatic isolate accounts for the larger mucosal sloughing seen in rats infected with the asymptomatic isolate than symptomatic isolate (see chapter 6). Hence, it can be concluded that cells from asymptomatic isolate can cause greater consequence soon after infection to the host because the cell proliferates faster into vacuolar forms than symptomatic isolates.

The limitation of this study is that the observation was made based on only one isolate from symptomatic and asymptomatic patients. Due to the cumbersome nature of the experiment and the meticulous observation needed as well the limited time usage possible at the TEM room, the study had to limit the numbers. However this study forms the basis for more studies to be carried out to assess if other subtypes follow this same pattern of excystation. The present study provides clarity to substantiate the histopathological consequence seen in the infected rats as shown in the previous study. It is obvious that this study was necessary to provide the basis as to why extensive sloughing was seen in rats infected with asymptomatic isolates compared to rats infected with symptomatic isolates.

CHAPTER 8

GENERAL DISCUSSION AND

CONCLUSION

8.1 General Discussion and Conclusion

The present study showed that the overall prevalence of *Blastocystis* sp. among pets, domestic and zoo animals is 35.6% which implies that these animals could be a potential source for transmission to humans especially when they come in close contact with animals. Results from Chapter 3 showed that prevalence in livestock group comprising of cow, deer, gaur, goat, sheep and pig was 51.2%. Most studies confine their reports to one or two groups of animals. In such cases it would be difficult to compare the life cycle stages of *Blastocystis* sp. isolated from animals from different regions or countries as environment and farm practices could cause changes in the different parasite. In the present study however, comparisons were made from 7 different groups of animals which is probably the largest compared in recent years.

The study clearly provides evidence that the thickness of the cyst wall of *Blastocystis* sp. does exert an influence in the prevalence of the parasite within the animal group as well as the capability of infecting rats. The cyst wall of cysts of *Blastocystis* sp. isolated from the goat showed a mean thickness of 161.59 nm and was observed to be the second thickest after the pig isolate when compared to the other livestock animals such as the cow and the sheep highlighted in Chapter 4. *Blastocystis* sp. goat isolate could cause experimental infection in rats within 2 days of infection (described in Chapter 5).

Despite few studies showing previously a high prevalence of *Blastocystis* sp. in the pig population with a percentage of 68 to 93% (Pakandl., 1991) and 95% (Abe et al., 2002) respectively, there are no other studies providing the morphological description of the cystic forms of *Blastocystis* sp. isolated from the pigs. Hence, the present study

showed that the cyst wall of *Blastocystis* sp. isolated from the pigs show a mean thickness of 283.28 nm which was reported to be the second thickest cyst wall among seven groups of animals studied. This probably accounts for the 100% prevalence seen in pigs as the robust wall described in Chapter 3 could have helped facilitate the transmission. This parasite isolates from pigs also caused experimental infection in 80% of the rats within 2 days of infection.

The cysts from the peacock showed a surrounding thick cell wall with a mean thickness of 165.82 nm which could cause infection in rats within 4 days of the infection. 100% prevalence seen in ostriches, could be due to the thick wall seen in the *Blastocystis* sp. cysts which measure a mean thickness of 328.98 nm, the thickest measured among the seven groups of animals studied. The cysts from the ostrich isolate were able to cause experimental infection in all the 5 rats inoculated, within 2 days of infection. On the contrary, cyst wall of *Blastocystis* sp. isolated from the cow, sheep and orang utan were comparatively thinner with a mean thickness of 115.22 nm, 148.93 nm and 136.98 nm respectively. These isolates were not able to cross-infect the rats.

The study of classical parasitology involving the appreciation of the diversity of morphologies as well as understanding the biological features do have an important role in understanding the transmission patterns of *Blastocystis* sp. in animals. It was possible to correlate the prevalence of *Blastocystis* sp. and the ability to cross infect rats to the thickness of the cyst wall of the parasite. Many of the recent publications highlight findings that are more skewed to elucidating molecular mechanisms and biochemical pathways. Although important but unless accompanied by a proper insight into the classical and more fundamental aspects such as the understanding of the biology and the

morphology there will never be a complete and holistic understanding of disease transmission.

In chapter 4, the results showed that the vacuolar forms of the animal isolates ranged from 3 to 55 μm which had comparatively smaller size range than the human isolates (2 to 200 μm) based on light microscopy reported previously by (Stenzel & Boreham, 1996; Zierdt, 1991). The vacuolar forms isolated from humans have been shown to be almost as large and if not almost double in size of the vacuolar forms isolated from animals. Meanwhile, the cystic forms from animal isolates range from 2 to 7 μm which were relatively similar in size when compared to the human isolate which usually range from 3 to 6 μm (Zaman et al., 1995b). On the other hand, the thickness of the cyst walls of all seven animal groups studied in the present study were found to be comparatively smaller than the human ones (<1000 nm) (Zaman et al., 1995b; Moe et al., 1996; Khalifa., 1999) and this concurred with studies carried out on animal isolates (250-500 nm) Cassidy et al., 1994; Stenzel et al., 1997).

Carrying out studies of this nature from a larger range of animal groups confers the ability to appreciate morphologies and even compare organelles these parasites have. For instance the present study showed an unusual form of mitochondria seen in the orang utan isolate which were irregular in shape than those mitochondria classically recognized in both human (Zaman et al., 1995b; Khalifa., 1999) and animal isolates (Stenzel et al., 1997; Cassidy et al., 1994). Mitochondria have been previously shown in monkey samples (Stenzel et al., 1996) as well as in humans (Tan et al., 2006; Suresh et al., 2009) but never of a kind that is unique in terms of unusual prominent cristae with a shape that is uniquely irregular. Whether this is a result of cellular activity due to encystment process as suggested by Suresh et al., (2009) or this is a regular feature of

Blastocystis sp. isolated from orang utan cannot be determined. Further studies need to be carried out to determine the exact role of the surface coat of *Blastocystis* sp in causing infection.

This is also the first study to establish a schematic diagram highlighting the differences in the morphology of the vacuolar (Figure 4.6) and cystic (Figure 4.7) forms so that we can use these distinct morphological differences for source tracking especially in settings where molecular analysis becomes a challenge. By no means this is comprehensive but provides a basis for a comparison to be made and in future when more *Blastocystis* sp. are isolated from a wider range of animals a similar approach may be used to further refine the schematic diagram to help further classify the parasites.

The thick cyst wall has the ability to cause infection in rats within shorter days as cysts from ostriches only took 2 days to cause the infection in rats whereas *Blastocystis* sp. isolated from the peacock with a less thicker cyst wall almost took double the time. The finding has important implication in that rats which have increased numbers in farms (Endepols & Klemann, 2004) could transmit the infection to farmers. This implies that rats can be naturally infected if they eat the feces of the animals in the farms containing the robust *Blastocystis* cyst which then facilitates these rodents to be natural carriers in human dwellings (Chapter 5). This study provides a basis to show such a transmission is possible, as there is evidence that *Blastocystis* sp. does have low host specificity. Therefore, the possibility of zoonotic transmission from infected rats should be taken into consideration especially when animals are also known to harbour similar subtypes found in humans.

Recent studies have implicated subtype 3 to be the pathogenic subtype because it has been predominantly reported in patients with gastrointestinal symptoms. The present finding with regard to infecting the same subtype 3 from symptomatic and asymptomatic isolates has important landmark implications when assessing the biochemical and histopathological findings. This comes in view of several publications which have reported the presence of ST3 *Blastocystis* sp. in asymptomatic persons and have further confirmed the presence of pathogenic and non-pathogenic strains in ST3 *Blastocystis* sp. (Tan et al., 2008; Scanlan & Pauline, 2012; Eman et al., 2008). Previous histopathological studies have revealed the invasive ability of the organism and to further cause different degrees of pathological changes among rats infected with different subtypes (Moe et al., 1997; Hussein et al., 2008; Elwakil & Hewedi., 2010).

Subtype 3 *Blastocystis* sp. isolated from symptomatic patient showed an elevated amylase level in infected rats which also showed histopathological changes such as excessive mucus, mucosal sloughing, inflammation and necrosis. Elevated levels of amylase suggest an inflammation in the pancreas infected with subtype 3 from the symptomatic group and this showed a significant elevated level implying that pancreas may have been affected. This is an important finding and future studies should study the histopathological changes if any in the pancreas.

The extensive mucosal sloughing seen in the intestines of asymptomatic rats concurs with the findings on the ultrastructural changes during excystation which showed that subtype 3 from asymptomatic isolate has a shorter duration i.e. 6 hours when compared to 24 hours in symptomatic isolate and proliferate soon after infection compared to symptomatic group. This concurred with the proliferation seen to be the highest in *in-vitro* cultures of the asymptomatic isolates between days 3 and 6 (described in Chapter

7). This is the first study to compare the excystation of *Blastocystis* sp. ST3 isolated from symptomatic and asymptomatic persons. The rapid multiplication of parasites must have exerted an influence on the more intense sloughing seen in the intestines of infected rats when inoculated with cysts from asymptomatic patients. Thus, the finding from this study provides evidence that rapid excystation and proliferation in asymptomatic isolates could cause intense host reaction with heightened symptoms shown when initially acquiring the infection when infected with asymptomatic parasites. However, the long term effect by the symptomatic isolate with extensive intestinal mucosal sloughing might cause serious consequence in the host especially if immunocompromised. Thus, the present study postulates that pathogenicity may not be subtype related and there could be other host factors that could play a role in determining the pathogenicity of this organism.

8.2 Research achievements

1. This is the first study to assess the prevalence of *Blastocystis* sp. among pets, domestic and zoo animals as these animals could be a potential source for transmission to humans especially when they come in close contact with animals association.
2. This study has shown a high prevalence of *Blastocystis* sp in the ostrich and pig population. This is the first study to demonstrate subtype 6 seen in ostrich isolates.
3. This is the first study to provide a schematic drawing which provides a key-like guidance to differentiate the morphology of the vacuolar and cystic forms of *Blastocystis* sp. isolated from a group of animals. This can provide information for source tracking. The schematic diagram provides a basis for the differentiation and is by no means comprehensive. Parasites must be isolated from animal species to refine further this schematic diagram.
4. The present study establishes a role for the cyst wall and based on its thickness, transmission patterns appears to be influenced. The high prevalence seen in ostriches and pigs is mainly attributed to the robustness of the cysts.
5. This study has also provided evidence that the parasite's thick cyst wall is primarily responsible for causing the infection in rats and the thickness of the cyst wall could be corresponded to the number of days taken to cause the infection.

6. The present study also suggests that *Blastocystis* sp. does have low host specificity as cysts from animal isolates could cause infection in rats.
7. This study also provides evidence that histopathological and biochemical changes do change even in infections with the same subtype. *Blastocystis* sp. isolated from symptomatic patient does cause a change as indicated by the amylase level in rats. These trigger inflammation and necrosis as evidenced by the histopathological changes in *Blastocystis* infected rats compared to ST3 isolated asymptomatic persons showing pathogenicity may not be subtype related.
8. This present study demonstrates marked differences in the excystation process between subtype 3 *Blastocystis* sp. isolated from asymptomatic and symptomatic persons demonstrating that there could be intra-specific variation within the same subtype.

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**LIST OF PUBLICATIONS AND
PAPER PRESENTED**

PUBLICATIONS:

1. **Hemalatha C**, Suresh KG, Chandrawathani P, Premaalatha B, Kalyani R, Gaythri T. (2014). **Subtype 6 in *Blastocystis* sp. From Ostrich - Lipid storage in vacuolar forms and cross-infectivity of cysts.** (Parasites and Vectors)
2. **Hemalatha C**, Suresh KG, Chandrawathani P, Premaalatha B, Geethamalar S, Lily Rozita MH, Farah Haziqah MT, Sabapathy D, Ramlan M. (2014). **The diagnosis of *Blastocystis* sp. from animals – an emerging zoonosis.** (Malaysian Journal of Veterinary Research).
3. Farah Haziqah MT, Chandrawathani P, Mohd Zain SN, Suresh KG, **Hemalatha C**, Premaalatha B. (2014). **A Preliminary Study of *Blastocystis* sp. from Chicken in Perak and Selangor, Malaysia.** (Malaysian Journal of Veterinary Research).
4. **Hemalatha C**, Suresh KG, Chandrawathani P, Jayalakshmi P. (2016). **Elevated levels of amylase and histopathological changes in rats infected with subtype 3 *Blastocystis* sp.** (Manuscript in preparation).

PAPER PRESENTED:

1. **Prevalence of *Blastocystis* sp. Isolated from Different Animal Hosts**
Hemalatha C., Suresh KG, Chandrawathani P, Premaalatha B, Geethamalar S & Lily RM. Poster presentation at the 48th Annual Scientific Conference of the Malaysian Society of Parasitology and Tropical Medicine (MSPTM 2012).

2. **The diagnosis of *Blastocystis hominis* from Animals - An Emerging Zoonosis**

Chandrawathani P, **Hemalatha C.**, Suresh KG, Premaalatha B, Geethamalar S & Lily RM. Oral presentation at the International Conference On One Health and 24th Veterinary Association Malaysia Congress (VAM 2012).

3. ***In- Vivo* experimental infection with *Blastocystis hominis***

Hemalatha C, Suresh KG, Chandrawathani P, Premaalatha B, & Nanthiney DR. Poster presentation at the 49th Annual Scientific Conference of the Malaysian Society of Parasitology and Tropical Medicine (MSPTM 2013).

4. ***Blastocystis* sp. in ostrich**

Hemalatha C, Suresh KG, Chandrawathani P, Premaalatha B, & Kalyani R. Poster presentation at the 49th Annual Scientific Conference of the Malaysian Society of Parasitology and Tropical Medicine (MSPTM 2013).

5. **A Preliminary Study of *Blastocystis* sp. Isolated from Chicken in Perak and Selangor, Malaysia.**

Farah Haziqah MT, Chandrawathani P, Mohd Zain SN, Suresh KG, **Hemalatha C**, & Premaalatha B. Oral presentation at the 25th Veterinary Association Malaysia Congress (VAM 2013).

6. **Infectivity of *Blastocystis* isolates from different animals hosts in Sprague Dawley rats.**

Hemalatha C, Suresh KG, Chandrawathani P, Premaalatha B, & Farah Haziqah MT. Poster presentation at the 50th Annual Scientific Seminar of Malaysian Society of Parasitology and tropical Medicine (ACTMP 2014).

7. Subtype 6 *Blastocystis* sp. in ostrich population in Malaysia.

Hemalatha C, Suresh KG, Chandrawathani P, Premaalatha B, & Farah Haziqah MT. Oral presentation at the 50th Annual Scientific Seminar of Malaysian Society of Parasitology and tropical Medicine (ACTMP 2014).

8. A Survey of *Blastocystis* sp. in Various Animals from Wildlife Park.

Farah Haziqah MT, Chandrawathani P, Mohd Zain SN, Suresh KG, Sabapathy D, **Hemalatha C**, & Premaalatha B. Poster presentation at the 50th Annual Scientific Seminar of Malaysian Society of Parasitology and tropical Medicine (ACTMP 2014).

9. Detection of *Blastocystis* sp. in Companion Animals, Wild Rat and Chicken Population.

Farah Haziqah MT, Mohd Zain SN, Suresh KG, Chandrawathani P, **Hemalatha C**, & Premaalatha B. Oral presentation at the 50th Annual Scientific Seminar of Malaysian Society of Parasitology and tropical Medicine (ACTMP 2014).

10. Infectivity of *Blastocystis* isolates from different animals hosts in Sprague Dawley rats.

Hemalatha C, Suresh KG, Chandrawathani P, Premaalatha B, & Farah Haziqah MT. Oral presentation at the 26th Annual Conference of Veterinary Association Malaysia (VAM 2014).

11. **Natural Occurrence of *Blastocystis* sp. from the Wild Rats.** Farah Haziqah MT, Mohd Zain SN, Suresh KG, Chandrawathani P, **Hemalatha C**, & Premaalatha B. Oral presentation at the 26th Annual Conference of Veterinary Association Malaysia (VAM 2014).

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