COMPARISON BETWEEN CONVENTIONAL AND MOLECULAR METHODS TO DETECT PARASITIC INFECTIONS IN PATIENTS WITH NEUROLOGICAL SYMPTOMS FROM CEREBROSPINAL FLUID

VANITAH SUPRAMANIAM

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2016

COMPARISON BETWEEN CONVENTIONAL AND MOLECULAR METHODS TO DETECT PARASITIC INFECTIONS IN PATIENTS WITH NEUROLOGICAL SYMPTOMS FROM CEREBROSPINAL FLUID

VANITAH A/P SUPRAMANIAM

DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF BIOTECHNOLOGY

INSTITUTE OF BIOLOGICAL SCIENCES
UNIVERSITY OF MALAYA
KUALA LUMPUR

2016

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Vanitah a/p Supramaniam

Registration/Matric No: SGF110010

Name of Degree: Master of Biotechnology

Title of Project Paper/ Research Report/ Dissertation/ Thesis ("This Work"):

COMPARISON BETWEEN CONVENTIONAL AND MOLECULAR METHODS TO DETECT PARASITIC INFECTIONS IN PATIENTS WITH NEUROLOGICAL SYMPTOMS FROM CEREBROSPINAL FLUID

Field of Study: Medical Biotechnology

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of copyrighted works has been done in a fair and appropriate manner and for a purpose allowed for any extracts or quotations. References or reproduction from or to any produced work containing copyright has been clearly and completely identified and acknowledgement of the title of said Work and its author/writer has been stated in this Work;
(4) I do not have any actual knowledge that this produced Work violates any copyright of any other work;
(5) With this I relinquish each and every right in the produced Work to the University of Malaya ("UM"). Beginning from this day UM owns the copyright to this produced Work and any reproduction or use in any form or any manner whatsoever is prohibited unless written permission is obtained from UM;
(6) I am fully aware that if, in the production of this Work, I have violated any copyright of another Work with intention or otherwise, I may be subjected to legal action or any other action as decided by UM.

Candidate's signature Date:

Subscribed and solemnly declared before,

Witness's signature Date:

Name:

Designation:

ABSTRACT

Neurological problems in patients require rapid diagnosis for accurate detection and timely treatment for better management and cure. Rarely, clinicians correlate the problem to presence of parasitic infections. Active metabolite, cerebrospinal fluids (CSF) was exploited to assess if parasitic infections in patients with neurological symptoms can be detected. Total of 238 cerebrospinal fluids specimens was investigated using conventional staining and polymerase chain reactions (PCR) using primers targeted for *Acanthamoeba* spp, *Entamoeba* spp, *Blastocystis* spp and *Toxoplasma gondii* spp infections. Eight out of 238 specimens, show some parasite-like microorganism from conventional staining methods. However, polymerase chain reaction subjected to identified parasite-like microorganism shows negative results. Conversely, eleven samples were identified to have *Toxoplasma gondii* infections from nested polymerase chain amplification. Data collected from medical record office, University of Malaya Medical Center (UMMC) indicated, all these 11 specimens show elevated leucocytes and protein level in cerebrospinal fluid analysis and decrease in glucose in few cases. Although with various medical history, these patients can be grouped under immuno-compromised category and shows some common neurological symptoms, such as seizure, fever, vomiting, generalized body weakness and slurred speech. Cerebrospinal fluids can be used for the detection of parasite *Toxoplasma* using nested PCR. Patients with neurological symptoms especially, immuno-compromised patients negative for microbiological and other routine preliminary diagnosis could be positive for opportunistic parasite infections such as *Toxoplasma gondii*. Thus, detection of *Toxoplasma gondii* infection by molecular method should be considered and implemented at preliminary stage to specimens with unknown etiologic agent with prolonged symptoms.

ABSTRAK

Masalah saraf pada pesakit memerlukan diagnosis segera untuk pengesanan dan rawatan tepat pada masanya. Sering kali, doktor jarang mengaitkan masalah ini dengan jangkitan parasit yang mungkin puncanya. Cecair serebrospina (CSF) telah dieksploitasi untuk mengenalpasti jika jangkitan parasit pada pesakit dengan gejala neurologi boleh dikesan dengan menggunakannya. Sejumlah 238 spesimen cecair serebrospina dikaji dengan menggunakan pemeriksaan tradisional dan tindak balas rantai polymerase (PCR) untuk mengenalpasti jangkitan disebabkan oleh *Acanthamoeba* spp, *Entamoeba* spp, *Blastocystis* spp dan *Toxoplasma gondii*. Lapan daripada 238 spesimen, menunjukkan beberapa mikroorganisma mirip parasit daripada teknik pewarnaan. Tetapi, tindak balas rantai polymerase terhadap mikroorganisma mirip parasit gagal menunjukkan keputusan positif. Sebaliknya, sebanyak 11 sampel telah dikenal pasti mempunyai jangkitan *Toxoplasma gondii* daripada tindak balas rantai polymerase bersarang. Data diambil dari pejabat rekod perubatan, Pusat Perubatan Universiti Malaya (PPUM) menunjukkan, semua 11 spesimen menunjukkan tahap leukosit dan protein yang tinggi dalam analisis cecair serebrospina. Tahap glukosa yang rendah juga direkod. Walaupun latar belakang perubatan pesakit berlainan, mereka boleh dikumpulkan di bawah kategori imunisasi dikompromi dan menunjukkan beberapa gejala neurologi, seperti sawan, demam, muntah, lemah badan umum dan pertuturan tidak jelas. Cecair serebrospina boleh digunakan untuk mengesan jangkitan parasit *Toxoplasma* dengan menggunakan tindak balas rantai polymerase bersarang terutamanya untuk pesakit imunisasi dikompromi yang negatif untuk ujian mikrobiologi dan diagnosis awal. Oleh itu, pengesanan jangkitan *Toxoplasma gondii* dengan kaedah molekul perlu dipertimbangkan dan dilaksanakan pada peringkat awal untuk spesimen dengan agen etiologi yang tidak diketahui dengan gejala yang berpanjangan.

ACKNOWLEDGEMENTS

This manuscript would not have been possible without the help of many kind hearts in various ways. I am pleased to acknowledge their contributions.

I would like to express my sincere appreciation to my project supervisor, Prof. Dr. Suresh Kumar A/L Govind. I am indebted to him beyond measure for his remarkable guidance; incredible patience and constructive feedback which never failed to encourage me during hard times make me realize the truth of life. Likewise, I also would like to thank Associate Prof. Dr. Subha A/P Bhassu, the co-supervisor of the project, for the guidance and rendering the facility in her lab. Not forgetting, Associate Prof. Dr. Rishya A/L Manikam from Faculty of Medicine for his guidance and help in getting specimen and patient's data for analysis from University of Malaya Medical Center (UMMC). Thousand thanks to Dr. Saharuddin of Department of Bioinformatics on his guidance on sequencing and phylogeny analysis. During my study, I had wonderful friends who went through thick and thin with me and therefore deserve mention, namely, Neelaveni, Kasthuri, Kavimalar and Revati. Together their influences and support over my study and research period are inestimable. My loving family, especially my husband, Ravin and my mother, Sarasah through their presence and patience, their tender care and comforting and encouraging words, has made this sometimes trying effort much more palatable. Lastly, my heartfelt sincere thanks to all out there who involved directly or indirectly to fulfillment of my research and this manuscript.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
°C	degree celcius
AIDS	acquired immune deficiency syndrome
AK	Amoebic keratitis
ALA	amoebic liver abscess
BBB	blood-brain barrier
BLASTn	Basic Local Alignment Search Tool (nucleotide)
BSCB	blood-spinal cord barrier
bp	base pair(s)
CIE	counter-immunoelectrophoresis
CNS	central nervous system
CSF	cerebrospinal fluid
DNA	deoxyribonucleic acid
DT	Sabin-Feldman dye test
ELISA	enzyme-linked immunosorbent assay
et al.	and others
FLA	free living amoeba
g	gram
GAE	Granulomatous amebic encephalitis
HIV	human immunodeficiency virus
IAAT	immunosorbent agglutination assay test
IFA	indirect immunofluorescence assay
IHA	indirect haemagglutination

kg	kilogram
LAT	latex agglutination test
LP	lumbar puncture
M	molar
MAT	modified agglutination test
ml	millilitre
mM	millimolar
MTS	modified trichrome stains
ng	nanogram
PAS	periodic acid Schiff
PCR	polymerase chain reaction
pH	potential hydrogen
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
STS	sequence tagged site
TBE	Tris Borate EDTA
TEM	Transmission electron microscopy
U	Unit
µg	microgram
µl	microliter
µm	micro molar
UMMC	University Malaya Medical Centre
UV	ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Neurological disorders are generally referred to any disorder which involves or affects the brain system, spinal cords, central and the peripheral nervous system in the body (Scheld et al., 2004). There are a number of factors that contribute to the development of this disorder including heredity, tumors, congenital abnormalities, lifestyles, malnutrition, brain injury, spinal cord and other nerve injury (Scheld et al., 2004).

Among others, infections of central nervous system (CNS) remains a major cause for neurological disorders which presents in clinical symptoms in both immunocompetent and compromised hosts. However due to its diverse manifestation, the possibility of easily being confused or misdiagnosed with other disorders or diseases is high (Scheld et al., 2004).

Infections of central nervous system are largely known to cause by bacteria, viruses, and in rare cases, fungi and parasites. Acute bacterial and viral meningitis including meningococcal, pneumococcal, *Haemophilius influenza* and *Listeria monocytogenes* meningitis and encephalopathy have attracted attention from clinicians (Chong & Tan, 2005).

Besides, a broad range of parasitic diseases including, strongyloidiasis, neurocysticercosis, schistosomiasis, toxoplasmosis and trypanosomiasis have been involved in neurological disorders but often diagnosed generally after fatal death. Toxoplasmosis is a good example of this which has been diagnosed after autopsy in transplant patients (Medeiros et al., 2001).

The recurrence of neurological symptoms and exacerbated neurological complication in both healthy and impaired immune individuals may be due to neglected and undiagnosed parasitic infection (Jones et al., 2014; Montoya et al., 2002). Thus the present study aims to elucidate parasitic infections in patients with prolonged neurological symptoms which could have been missed being diagnosed, using cerebrospinal fluids (CSF) (Hotez, 2008; Townsend et al., 1975).

1.2 Research Questions

This present study was carried out with the following research questions;

1. Is the unknown cause for neurological disorder a result of the failure to diagnose parasitic infections?
2. Can CSF be used as a sample source to detect these parasites either through direct stained smears or through PCR?

1.3 Objectives of Study

1. To identify the parasites from cerebrospinal fluid (CSF) through staining and molecular methods in patients with neurological symptoms
2. To correlate the occurrence and symptoms of parasite infections among patients showing neurological symptoms

CHAPTER 2

LITERATURE REVIEW

2.1 Central Nervous System and Neurological Disorders

The central nervous system consists of the brain and spinal cord which acts as the main 'processing center' and activity controller of the body. Lately, disease affecting the brain and components of central nervous system are among the most complex conditions affecting mankind worldwide (Walker & Zunt, 2005). There are various disease of central nervous system including brain tumor, epilepsy, Parkinson's disease, stroke, migraine and acute headache. What causes these conditions? Diverse factors contribute to the development of these diseases, including a climate change that favors transmission of insect-borne pathogen, increase number of immunosuppressive individuals due to human immunodeficiency virus (HIV), organ transplant, alcoholic, diabetes and others.

2.2 Central Nervous System Barriers and Parasitic Infections

The central nervous system (CNS) consists of highly specialized physical barrier that separate it from blood circulatory system. There are three main barriers in central nervous system which includes the blood - brain barrier (BBB) which separates brain and bloodstream, it's sister barrier, the blood-spinal cord barrier (BSCB) that separates spinal cord and bloodstream and an epithelial cell barrier separating the bloodstream and the cerebrospinal fluid (CSF) (Abbott, 2005). These barriers within central nervous system give few dynamic protective functions to brain. The blood-brain barrier (BBB)

protects brain from potential damage while regulating transport of essential nutrients and metabolites in and out to maintaining a stable microenvironment (Banks & Erickson, 2010; Masocha & Kristensson, 2012). Besides, this barrier also shields the CNS from neurotoxic substances circulating in the blood, which may be endogenous metabolites or protein, xenobiotic ingested from diet or otherwise acquired from the environment. The barrier also serves as protective agent against unwanted pathogens and controls the immunologic status of the brain. It was reported, pathogens must break this barrier to enter the central nervous system regardless of the host immune condition (Banks & Erickson, 2010).

2.3 Cerebrospinal Fluids (CSF) Analysis

The cerebrospinal fluid (CSF) is a dynamic and active metabolic substance which has an important role in investigating various neurological diseases. The clinical use of routine CSF analysis includes total protein, albumin, glucose, lactate, cytological staining and microscopic examination and this CSF is obtained by lumbar puncture (LP). In addition to that, cerebrospinal fluid polymerase chain reaction (PCR) can be performed for more reliable diagnosis method in medical practice. The cerebrospinal fluid need to be analyzed immediately after 6-8 hours of collection for better result. The CSF should be stored at 4-8°C for short term or at -20°C for long term analysis. It is recommended to store approximately 3-4ml at 4°C for general investigation, cultivation, microscopic examination and PCR assay. Bigger volumes (10-15ml) are needed for identification of certain pathogens such as some bacteria, fungi and parasite (Deisenhammer et al., 2006).

2.4 Parasitic Infections of Central Nervous System

Common infections of nervous system caused by cestodes, trematodes and protozoans such as *Echinococcus* spp., *Spirometra* spp., *Schistosoma* spp., *Trypanosoma* spp., *Naegleria fowlerii*, *Acanthamoeba* spp and *Balammuthia mandrillaris*. Following are parasitic infections associated with Central Nervous System (CNS) discussed in this study;

a) *Acantamoeba* Infections
b) Microsporidia Infections
c) *Entamoeba* Infections
d) *Toxoplasma gondii* Infections
e) *Trypanosoma* Infections

2.4.1 *Acantamoeba* spp Infections

Acantamoeba is common free living amoebae (FLA) with worldwide distribution. *Acantamoeba* was detected as a contaminant in *Cryptococcus pararoseus*, a yeast culture in 1930 by Castellani and grouped in genus *Acanthamoeba*, a year later by Volkonsky (Volkonsky, 1931). This opportunist FLA amoeba have been found in a variety of habitats including soil, fresh and brackish water, swimming pools, dust in air, and as contaminant in bacteria, fungal and mammalian cell cultures. Besides, *Acantamoeba* also has been isolated from cornea scraping swabs, brain, skin, of infected individual (Marciano-Cabral & Cabral, 2003). Visvesvara et al. (2007) reported more than 24 species of *Acanthamoeba* have been categorized and named based on their morphological features. Generally, *Acanthamoeba* spp classified as three different, as Group I, II and III according to their morphology and cyst size (Visvesvara et al., 2007). *Acanthamoeba* spp are able to survive and invade wide range of habitats as they tolerant of broad range of osmolarity (Marciano-Cabral & Cabral, 2003).

2.4.1.1 Life-cycle and Morphology of *Acantamoeba* spp

Acanthamoebae consist of two forms of life-cycle, vegetative trophozoite stage and dormant cyst stage. The vegetative stage is the stage where the trophozoite actively divides and cyst form is protected from harsh conditions in the environment (Khan, 2007). Both life cycle stages were reported to be found in tissue of infected individuals and environment. The trophozoites vary in size ranging from 25 to 40 µm and they feed on bacteria, algae and can be axenically grown in culture media. The distinguishing features of trophozoite are the presence of spiny surface projections called acanthopodia, contractile vacuole and nucleus with central nucleolus. Besides, the cysts have double walled wrinkled cysts which consist of an ectocyst and an endocyst. The size ranges from 13 to 20 µm and which varies from species to species (Marciano-Cabral & Cabral, 2003). Advance molecular technology enables more precise classification of genus *Acanthamoeba* based on rRNA gene sequences and is grouped under 17 different genotypes (T1-T17). Among other genotypes, T4 genotype is reported to cause most the infections in human (Siddiqui & Khan, 2012). For example, life threatening Granulomatous amebic encephalitis (GAE), a fatal disease of central nervous system and *Acanthamoeba* keratitis was reported to be associated with T4 genotype.

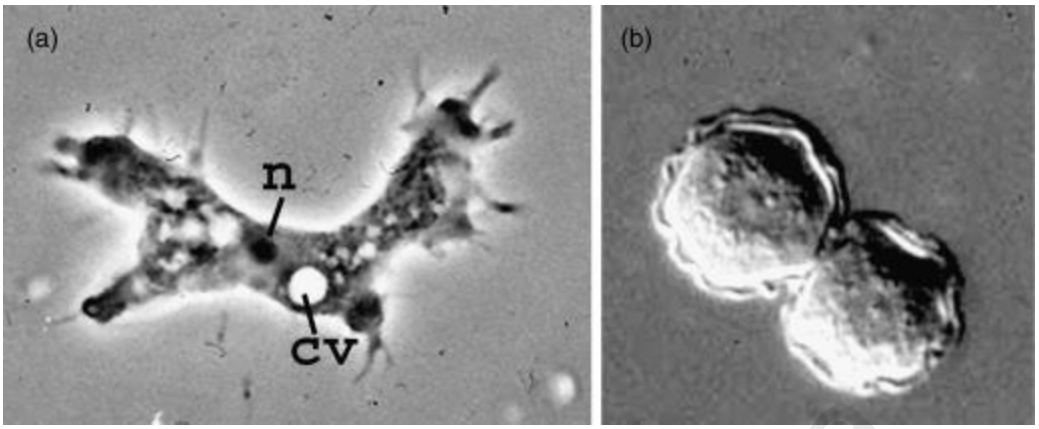


Figure 2.1: *Acanthamoeba castellanii*, trophozoite (a) and cysts (b): n, nucleus; cv, contractile vacuole. Both images captured at x1000 magnification. Adapted from "Pathogenic and opportunistic free-living amoebae*: Acanthamoeba* spp.*, Balamuthia mandrillaris, Naegleria fowleri,* and *Sappinia diploidea*", by Visvesvara et al., 2007, *FEMS Immunology & Medical Microbiology, 50*(1), p. 5.

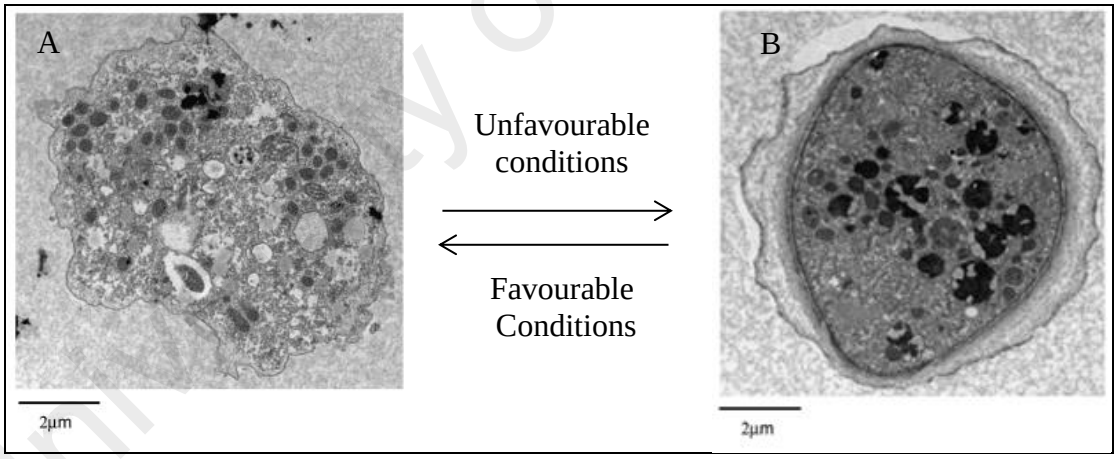


Figure 2.2: The life cycle of *Acanthamoeba* spp under transmission electron micrograph. Trophozoite stage (A) which actively multiply and causes infection under favorable conditions while under unfavorable conditions trophozoite differentiate into dormant double-walled cyst form (B). Adapted from "Biology and pathogenesis of *Acanthamoeba*", by Siddiqui and Khan, 2012, *Parasite and Vector, 5*(6), p. 4.

2.4.1.2 Clinical Manifestation of *Acanthamoeba* spp Infections

Granulomatous amebic encephalitis (GAE) is a rare opportunistic central nervous infection which leads to mostly fatal conditions. Largely, GAE infection is associated with human immunodeficiency virus (HIV) infection and patients under immunosuppressive therapy. Adding to that, patients with diabetes mellitus, autoimmune disease, cirrhosis, renal complication, organ transplantation, malignancies chemo and radiotherapy are also the potential groups at risk for the infection (Siddiqui & Khan, 2012). However, Amoebic keratitis (AK) a vision-threatening infection of cornea has been generally observed in immuno-competent individual, especially in contact lens users. Clinical manifestation of GAE infection can mimic other meningitis caused by viral, bacteria and microbes. The symptoms can be different within infected individuals which include headache, fever, seizures, neck stiffness, nausea, vomiting, behavioral changes, confusion, increased intracranial pressure and coma (Siddiqui & Khan, 2012).

2.4.1.3 Diagnosis of *Acanthamoeba* spp Infections

The diagnosis for GAE can be complicated since; the symptoms are close to other central nervous infection. However, as laboratory routine, diagnosis test for *Acanthamoeba* can be done through cerebrospinal fluid (CSF) investigation by wet mount microscopy and staining method. But, in certain cases, trophozoite present in wet mount, might be undetectable or unrecognized as they are similar to features of macrophages. Due to some limitation in routine conventional microscopy and staining method, molecular methods are widely used to diagnosis *Acanthamoeba* infection. Polymerase chain reaction (PCR) analysis has been widely used in detection and identification of *Acanthamoeba* infection and was reported to be 100% sensitive and specific in comparison to culture methods (Gatti et al., 2010).

2.4.1.4 Treatment and Prevention of *Acanthamoeba* spp Infections

As for treatment in human infections, combination of few drugs are required and reported to be more efficient than single drug in treating *Acanthamoeba* spp infections. Besides that, no single drug has been documented for successful treatment of both dormant cyst and trophozoite stages infections in human (Marciano-Cabral & Cabral, 2003). Generally, combination of ketoconazole, fluconazole, sulfadiazine, pentamidine isethionate, amphotericin B, azithromycin, itraconazole or rifampin have been reported to be effective against central nervous system (CNS) infections due to amebic infections, although with some severe side effect (Khan, 2006). A brief report by Helton et al. (1993) reported a successful treatment for an AIDS patient with cutaneous and sinus lesions using 40 mg of 5-fluorocytosine per kg for 2 weeks. However, disseminated *Acanthamoeba* infection in HIV negative patients that underwent renal transplants were successfully treated with 1 month course of IV pentamidine isethionate, topical chlorhexidine gluconate, and 2% ketoconazole cream. Khan (2006) reported hexadecylphosphocholine, an alkylphosphocholine compound which shows anti-*Acanthamoeb*a properties and can be used as a potential drug in treating GAE infection as it posses the ability to cross the blood–brain barrier. Although there are many combinations of the drug available for the treatment of *Acanthamoeba* spp infection, only early treatment have been shown to be successful before the parasite gets to be disseminated to the entire central nervous system (Marciano-Cabral & Cabral, 2003).

Commonly, central nervous system infections due to *Acanthamoeba* spp, including GAE and *Acanthamoeba* granulomatous encephalitis (AGE) in human occur among immuno-suppressive individuals. Therefore, there is no specific defined prevention strategy to combat infections of these protozoan parasites among weakened immune

functions in patients. However infections due to *Acanthamoeba* keratitis mostly among contact lens users can be prevented by few practices. Self education for proper care and usage of contact lenses among contact lens users can prevent the infections. In addition to that, they also should be aware of the risk of getting the infection through any of water activities including swimming, watersport games, spa activities which can prevent the infection. Moreover, avoiding wearing contact lens during these water based activities also can save these contact lens users from infections.

2.4.2 Microsporidia Infections

Microsporidia refer to a group of obligate, intracellular protozoan parasite that belongs to phylum *Microspora*. *Nosema bombycis* was reported as the first recognized microsporidia in 1857 by Nageli and described as a pathogen that causes pebrine diseases severely affecting silkworm. Microsporidium infections have been also associated with agriculture industry particularly to fish and honeybee as well as laboratory rodents, rabbits, fur-bearing animals and primates (Didier et al., 2004). Although it was identified initially in mid-17th century, incidents of human infection of microsporidia was only reported a century later in 1959. In 1985, human infection of microsporidia was widely investigated after the new discovery of *Enterocytozoon bieneusi* in HIV infected patients associated with chronic diarrhea and weight loss (Desportes et al., 1985). To date, there are approximately 144 genera and over 1200 of species in phylum Microsporidia that infect a wide range animal groups, but only a few genera causes infection to human (Didier, 2005; Garcia, 2002; Weiss, 2001). These include, *Enterocytozoon*, *Encephalitozoon*, *Pleistophora*, *Trachipleistophora*, *Vittaforma*, *Brachiola* and *Nosema* as well as unclassified microsporidia. Microsporidia posses prokaryotic-like 70S ribosomes, lack of peroxisomes, simple Golgi body and

mitochondria (Didier, 2005; Dunn, & Smith, 2001). On the other hand, the microsporidial genome is relatively small and less complex compared to eukaryotes. However they are considered as true eukaryotes as they posses enclosed nucleus, cytoplasmic membrane and true nuclear division through mitotic spindles. Phylogeny and sequence analysis proposed that their potential relation is to fungi as they consist of chitinous spore wall, showed the presence of some important genes including a mitochondrial HSP70 gene and genes encoding beta tubulin (Didier, 2005; Garcia, 2002; Weiss, 2001).

2.4.2.1 Microsporidia Spore

The important feature of phylum microsporidia is the highly specialized and organized spore. The spores are the only viable stage of microsporidia that exists outside the host cell and their active stages of life cycle relatively occurs in human or animal host cells. The spores are unique, species specific and generally usable to species differentiation. The size of the spores is wide in range and dependent to species. Commonly, the microsporidia spores infecting human range from 1-4 µm, although there is the large spore which measures 12µm as reported by Weiss (2001). The shape of spores is mostly ovoid with varied shapes such as spherical, rod shaped or crescent shaped. However, in some species the morphology of spores shows certain variation in different stages of their life cycle, although fairly regular. The nucleus of spore exist as single nuclei in certain species including in *Enterocytozoon*, *Pleistophora*, *Trachipleistophora*, *Encephalitozoon* or as of two closely adjoined nuclei functioning as a single unit as in *Nosema*, *Vittaforma* and *Thelohania* (Didier et al., 2004). The spore contains outer electron-dense exospore made of glycoprotein and inner electron-lucent endospore made of chitin (Weiss, 2001). Distinct feature of the mature spore is a specific posterior

vacuole with anterior regions consisting of extrusion apparatus which comprises of polar tube that is attached to the inside of the anterior region by an anchoring disc. The polar tube coiled around the sporoplasm, forming 6 to 10 coils in genus *Enterocytozoon* and *Brachiola* and 5 to 11 coils in genus of *Encephalitozoon*, *Trachypleistophora* and *Vittaforma.* The polar tube coils are species dependent and up to 30 coils has been described in certain species (Bigliardi & Sacchi, 2001; Didier et al., 2004; Didier & Weiss, 2006). The spores are environmentally resistant due to the present of chitinous wall (Didier et al., 2004; Didier & Weiss, 2006). Spores are able to survive upon freezing and in various range of pH, whereby Didier et al. (2004) reported *E. cuniculi* spores survived for minimal of 24 hours after incubation at extreme pH 4 and pH 9. Li et al. (2003) reported that *E. intestinalis* and *E. hellem* remain infectious after incubation in water at temperatures ranging from 10 ^{o} to 30 °C for weeks to months as observed in *E. cuniculi.* Similarly, Weber et al. (1994b) reported that *N. bombycis* was able to survive up to 10 years in distilled water.

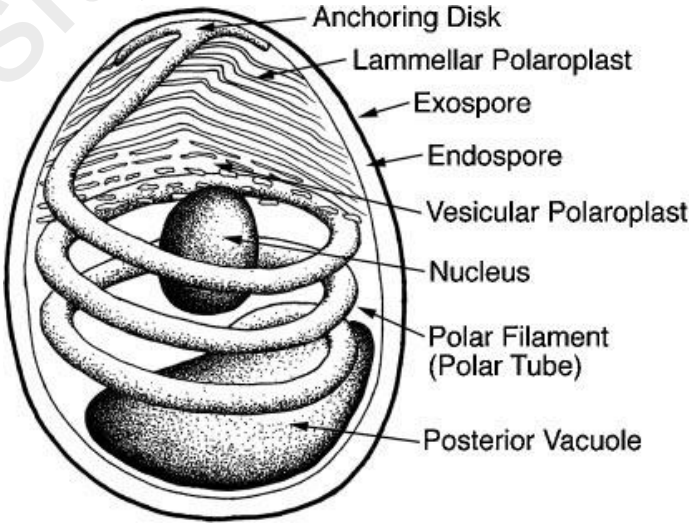


Figure 2.3: Diagram of Microsporidian spore. Adapted from "Microsporidia: biology and evolution of highly reduced intracellular parasites", by Keeling and Fast, 2002, *Annual Reviews in Microbiology, 56*(1), p. 95.

2.4.2.2 Life Cycle and Invasion of Microsporidia

Infection of Microsporidia acquired by ingestion or inhalation of the microsporidium spores, which was the only stage in it's life cycle outside the host cell. The life cycle comprises of three definite stages; infective spore stage; merogony, a proliferative stage; and sporogony stage which will develop into spores. Firstly, the germination of spore starts when they are triggered by environmental stimuli or condition that generally vary among species. These stimuli include changes in pH, effect of ions, rehydration or UV radiation (Weiss, 2001). These stimuli cause an increase in spore's internal osmotic pressure and results in water flow into spore which leads to swelling of polaroplast and posterior vacuole. The swelling forces the spores to discharge and the polar tube extruded from anterior region infect host cell by injecting the infective sporoplasm through polar tube in an explosive reaction as fast as 2 seconds (Figure 2.4) (Bigliardi & Sacchi, 2001; Weiss, 2001).

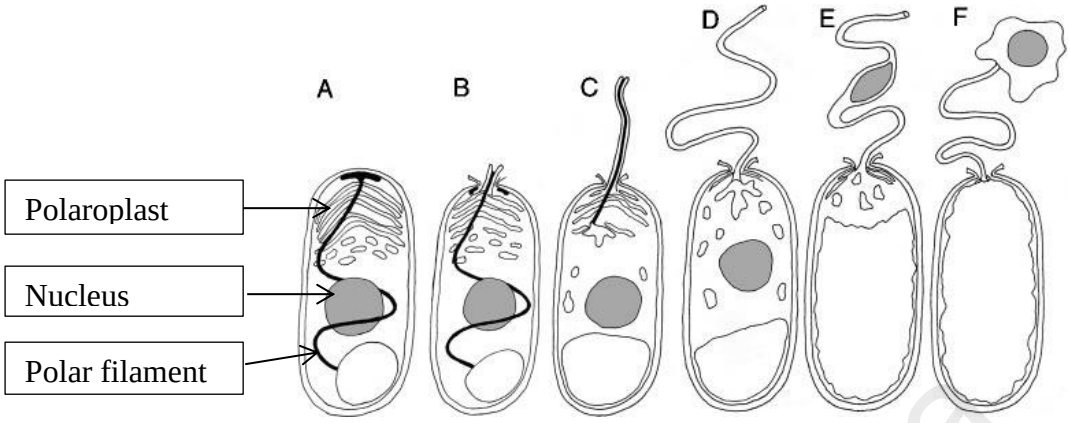


Figure 2.4: Diagram shows polar tube extrusions of microsporidia spore during spore germination. (*A*) Dormant spore, showing polar filament (*black*), nucleus (*gray*), polaroplast and. (*B*) Polaroplast and posterior vacuole swelling, anchoring disk ruptures, and polar tube begins to extrude out and (*C*) continues to extrude out. (*D*) As polar tube is fully everted, the sporoplasm is forced into and (*E*) through the polar tube. (*F*) Sporoplasm emerges from the polar tube and infects other host cell. Adapted from "Microsporidia: biology and evolution of highly reduced intracellular parasites", by Keeling and Fast, 2002, *Annual Reviews in Microbiology, 56*(1), p. 97.

Next, inside the host cell, injected sporoplasm start their extensive proliferation and develop into meronts that bound by atypical unit membrane and this stage known as merogony. At this stage, they multiply by binary fission as in *Encephalitozoon*, *Nosema*, *Vittaforma* or as multiple fission depending on the species. Also, nuclear division may occur before or without cytokinesis, resulting in formation of multinucleated cells termed merogonial plasmodia as observed in *Enterocytozoon*, *Pleistophora* and *Trachipleistophora* (Bigliardi & Sacchi, 2001; Weber et al., 1994b). This development can occur either in direct contact with the host cell cytoplasm as in *Nosema*, *Enterocytozoon bieneusi* or inside a vacuole termed parasitophorous vacuole as in *Encephalitozoon intestinalis* (Bigliardi & Sacchi, 2001; Franzen, 2004; Keeling and Fast, 2002).

Following merogony, the next stage will be sporogony whereby microsporidia develop into a sporont from a meront either freely in the cytoplasm or inside a parasitophorous vacuole. The sporont divides to produce sporoblast which will eventually develop into mature spores. During sporogony, a thick wall is formed around the spore which provides resistance to adverse environmental conditions. Eventually, cytoplasmic organelle formation and differentiation also took place at this stage. When the spores increase in number and completely fill the host cell cytoplasm, the cell membrane burst to release the spores to the surroundings. These free mature spores can then infect new cells thus continuing the cycle (Bigliardi & Sacchi, 2001; Franzen, 2004; Keeling and Fast, 2002).

Invasion of microsporidia to host cell is thought to be initiated by the injection of protoplasm through polar tube into host cell but the mechanism was unclear. However, recent studies proposed that, the polar tube penetrate into host cell by a phagocyctic process upon contact of protoplasm with host cell membrane (Bigliardi & Sacchi, 2001; Franzen, 2004). At the last stage of the invasion process, the spore was placed within a large vacuole.

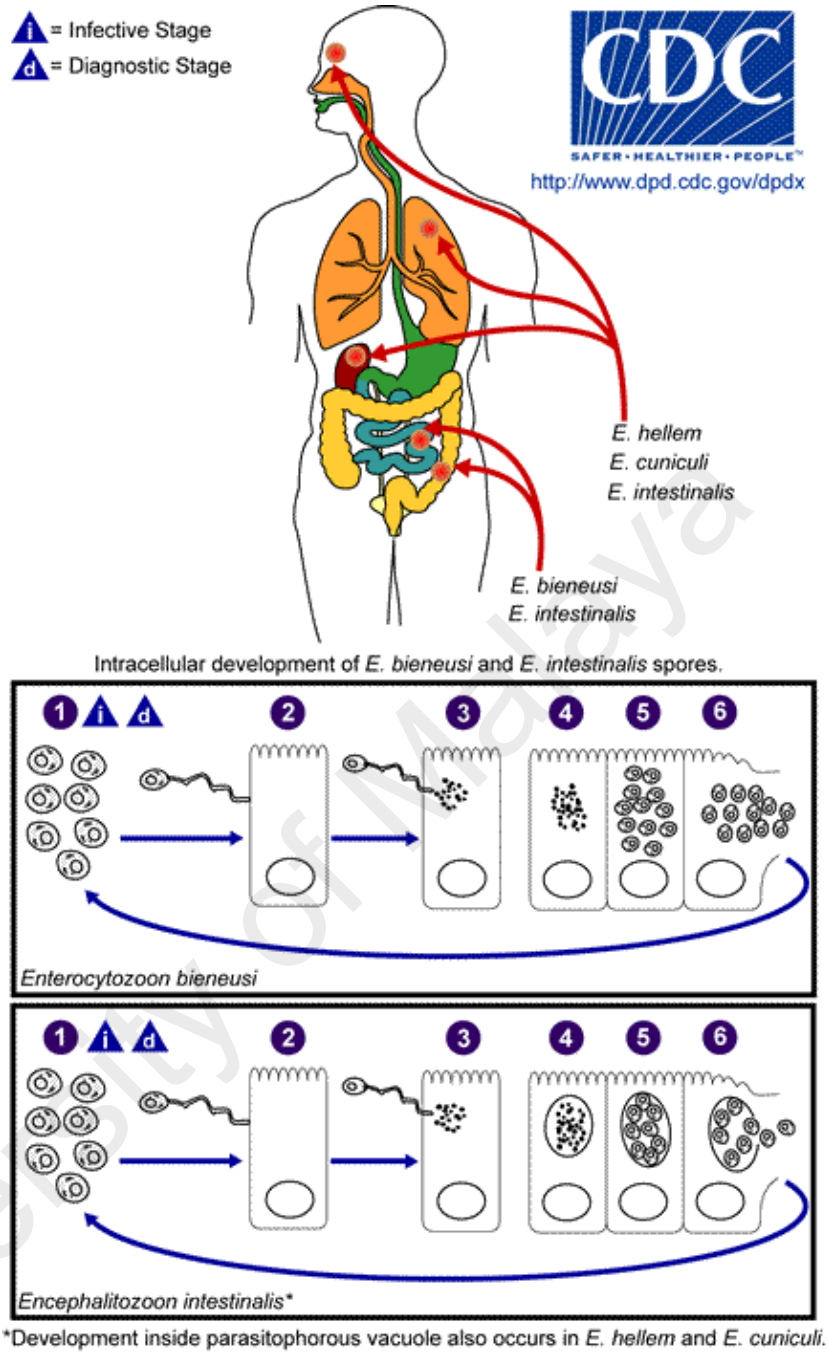


Figure 2.5: Life cycle of *Enterocytozoon* and *Encephalitozoon* species of microsporidia in humans. Adapted from *Microsporidiosis*,Retrivied Dec 30, 2014 from http://www.cdc.gov/dpdx/microsporidiosis/

2.4.2.3 Transmission Microsporodia Infections

Transmission of microsporodial infection from human is possible through horizontal transmission which includes fecal-oral route, oral-oral route, ingestion of contaminated food and water (Didier, 2005). Homosexual practice, intravenous drug use, and exposure to swimming pool water also increases the risk factor for horizontal transmission among humans but vertical transmission from mother to fetus in humans has not been reported. In addition to that, zoonotic transmission is also possible for microsporodiosis, as a wide range of microsporodian species infecting humans also infect animals (Didier, 2005) which, implicates a possible zoonotic transmission. Furthermore, many species of microsporodia infecting humans has been identified in various water sources and this probably causes microsporodiosis spread through water. Moreover, National Institutes of Health and the Centers for Disease Control and Prevention listed microsporidia as Category B priority pathogens of concern for waterborne transmission (Didier et al., 2004). Hutin et al. (1998) reported that eating of undercooked beef at least once a month was associated with microsporidiosis in HIV-infected individuals. In other hand, *E. intestinalis* organisms have been detected in irrigation water used for crop production and this evidence supports a possibility for foodborne transmission of microsporidiosis (Thurston-Enriquez et al., 2002). Dascomb et al. (2000) reported the possibility for vector-borne transmission of microsporidiosis in HIV-infected individuals associated with risk factor being stung by a bee, wasp, or hornet.

2.4.2.4 Clinical Manifestation of Microsporidiosis in Human

A wide range of clinical manifestations and diseases caused by microsporidiosis in humans have been documented as it is dependent to the types of microsporidian species and immune status of the infected individual. Of note, *Encephalitozoon cuniculi* has been associated with sinusitis, hepatitis, encephalitis and disseminated disease. It was reported that a 2 year old infant admitted with generalized convulsive seizures and light facial trauma was associated with *Encephalitozoon cuniculi* infection (Bergquist et al., 1984; Franzen & Muller, 2001). In other case, a 9 year old Japanese boy was reported to struggle from headache, vomiting and convulsive seizures and was associated with cerebral infection due to *Encephalitozoon* species (Franzen & Muller, 2001). In addition to that, *Encephalitozoon hellem was* reported to cause superficial keratoconjunctivitis, sinusitis, respiratory disease, prostatic abscesses and disseminated infection. Similarly, *Encephalitozoon intestinalis* also was implicated to cause diarrhea, superficial keratoconjunctivitis and disseminated infection. Other species of microsporodia such as *Nosema*, *Vittaforma* and *Brachiola* have been documented to cause keratitis in immunocompetent individual as well. Furthermore, rare clinical manifestations such as urethritis, tongue ulcer, skeletal involvement and cutaneous microsporodiosis also have been reported to be associated with microsporidian infection (Franzen & Muller, 2001). In immunocompetent individuals such as travelers and children, infection by *E. bieneusi* was reported to cause self- limited diarrhea. Moreover, similar symptoms were observed in patients that underwent organ transplant such as liver and bone marrow transplantation (Weber and Bryan, 1994a).

2.4.2.5 Diagnosis of Microsporidiosis in Human

There are numerous methods or techniques established for identification and detection of microsporidiosis in human and animal. Primarily, spores are generally demonstrated through light microscopy using various staining methods. Among the other, modified trichrome stains and Giemsa stain are the two staining techniques effectively used to detect microspodiosis. Modified trichrome stain appear to be more specific in detecting the organism in a shorter time from fluid and stool specimens whereas Giemsa stain was suitable to detect organism from body fluids cytology and intestinal cytology specimens (Didier, 2005; Franzen & Muller, 2001; Garcia, 2002). However, microsporidian spores can be overlooked as it quite small and very dependent on the expertise of microscopists. Microsporidium detection through immunofluorescent reagents has been used particularly useful for detecting *Encephalitozoon* species spores, but this is not commercially available. Unfortunately, the background staining and cross-reactions with yeast species and bacteria makes the immunofluorescent technique to be not applicable for routine diagnostic use (Didier & Weiss, 2006; Gracia, 2002). Serological assays also have been used to identify *E. cuniculi* infections in humans, but these assays have become complicated by the emergence of new species of microsporidia and the increasing number microspodiosis incidents in immune-deficient individuals who may not express significant or specific antibody responses (Didier, 2005; Gracia, 2002). Application of electron microscopy is considered gold standard in the detection of microsporidia, but limited facilities and variation in sensitivity in different types of specimen for detection offers a challenge (Gracia, 2002). Transmission electron microscopy (TEM) has been employed to characterize the ultrastructural features of developing mature spore of microsporidia and also for taxonomic classification of new species. TEM is not routinely used as it costly, time-consuming, and relatively

insensitive and requires expert for optimal operation (Didier et al., 2004). Molecular based method, such as polymerase chain reaction (PCR) and nested PCR, have been used as important tool in the detection of microsporidia to species level and for taxonomic classification (Didier, 2005; Franzen & Muller, 2001; Garcia, 2002). Previously, it was reported PCR was used to identify infection of *E. bieneusi*, *E. cuniculi*, *E. intestinalis* and *E. hellem* (Franzen & Muller, 2001).

2.4.2.6 Therapy and Prevention of Microsporodiosis in Human

Albendazole that has anti-helmintic and anti-fungal activities, as well as fumagillin, an antibiotic produce by fungus *Aspergillus fumigatus*, are the two common drugs that has been widely used in treating microsporodiosis in both human and animals (Didier et al., 2004; Weber et al., 2004). Albendazole was effective in treating *Encephalitozoon* species infection in human but shows variation in effectiveness against *E. bieneusi*. Administration of fumagillin to patient with keratoconjunctivitis due to *Encephalitozoon* species is highly effective when administered systemically to humans at a dose of 20 mg three times per day. However, it causes some side effects such as neutropenia and thrombocytopenia in some patients although it highly effective against *E. bieneusi* (Didier, 2005; Molina et al., 2002). Besides these two drugs, there are other drugs which have been reported to treat microsporodiosis including furazolidone, sinefungin, atovaquone, azithromycin, itraconazole, octreotide, and sulfa drugs (Didier, 2005; Conteas et al., 2000). Due to some side effects and variable effectiveness of the drugs, there is a search for new drugs for treating microsporodiosis.

Preventional strategies for microsporodiosis are not specified since the modes of transmission and source of infection is not clear. However, common strategy is likely to reduce the possibilities to exposure of the spores to avoid its ingestion, particularly among patients at high risk. This high risk group comprises mainly immunocompromised patients including HIV-infected patients; patient undergoes organ transplant as well as chemotherapy (Didier et al., 2004; Weber et al., 1994b). They advised to drink boiled or bottled drinks, consume well cooked meat, fish and washed fruits and vegetables. Apart form that, various strategies implemented to reduce survival and infectivity of microsporidian spores in environment. Boiling water at least for 5 minutes and application of disinfectants able to kill and completely destroyed *E. cuniculi* organisms (Didier, 2005; Didier et al., 2004).

2.4.3 *Entamoeba* spp Infections

2.4.3.1 Morphology and Life Cycle of *Entamoeba* spp

Life cycle of *Entamoeba histolytica, E. dispar* and *E. moshkovskii* consists of an infective cyst and trophozoite stage. The cysts ingested into human by fecal contaminated food, water, or hands where it travels through gut lumen to small intestine. Excystation take place in the small intestine releasing four daughter trophozoites. Trophozoites will convert into pre-cyst and then mature into tetra-nucleated cyst whereby it migrates down at the large intestine (Tanyuksel & Petri, 2003). The cysts when passed through the faeces can survive in the external environment for few weeks before being transmitted back to humans. However, trophozoites will be destroyed once out of the body. Normally, trophozoites will remain in the intestinal lumen of humans while in certain cases it will invade intestinal mucosa

where it is able to migrate to other organs resulting in extraintestinal infection. Apart from infecting the organs, it also can migrate to the brain and infect the central nervous system resulting in brain abscesses. A case has been reported on a patient without liver or brain abscesses but yet there was the presence of trophozoites in the cerebrospinal fluid caused by *E. histolytica meningoencephalitis* (Goh & Marrone, 2013).

The diameter of trophozoites of *E. histolytica, E. dispar* and *E. moshkovskii* ranges between 15 µm to 20µm and then cysts between 12 µm to 15 µm. Trophozoites and cysts of *E. hartmanni* are the smallest of *Entamoeba* species which range between 8 µm to 10µm and 6 µm to 8µm respectively. In contrast, *E. coli* has the largest diameter with the size of trophozoites and cysts ranging between 20 µm to 25µm and 15 µm to 25µm respectively. The quantity of nucleus varies according to the cyst of the *Entamoeba* species. However, all trophozoites of *Entamoeba* have a nucleus. The mature cyst of *E. histolytica, E. dispar, E. moshkovskii* and *E. hartmanni* has four nuclei while the immature cyst has one or two nuclei. However, *E. coli* cyst has eight nuclei (Fotedar et al., 2007).

2.4.3.2 Clinical Manifestation of *Entamoeba* spp Infections

Entamoeba genus consists of six species including *E. histolytica, E. dispar, E. moshkovskii, E. coli, E. hartmanni and E. polecki* that infect humans and reside in human intestinal lumen. *E. histolytica* is only species pathogenic to human among all the species. The untreated asymptomatic colonization with *E. histolytica* can lead to amoebic dysentery. The asymptomatic individuals ranging from 4% to 10% which are colonized with *E. histolytica* will be developed into colitis or extra intestinal disease according to Gathiram & Jackson (1987) and Haque et al. (2001). Common symptoms

of acute amoebic colitis are abdominal pain or tenderness with watery, bloody, or mucous diarrhea. Commonly, 80% of patients will complain of localized abdominal pain while in certain cases they may have only intermittent diarrhea alternating with constipation (Fotedar et al., 2007). Other symptoms are weight loss and anorexia. The additional symptoms also include inflammatory bowel disease, ischemic colitis and diverticulitis. Feces have been show positive for occult blood as it invades colonic mucosa.

Apart from that, extensive fulminant necrotizing colitis, toxic mega colon and perianal ulceration also have resulted from acute intestinal amoebiasis. Normally, patients develop fulminant amoebic colitis with profuse bloody diarrhea, pronounced leucocytosis, fever, and widespread abdominal pain, (Takahashi et al., 1997) which can lead to mortality. Those who are at greater risks of developing fulminant amoebic disease is malnutrition, compromised innate immunity and treatment with high-dose corticosteroids.

Moreover, dysenteric stool, diffuse abdominal pain with high fever and severe dehydration indicate severe cases of amoebic colitis in patients. They would look very ill at this stage. Another intestinal amoebiasis condition is the formation of annular colonic granulation tissues in the caecum and ascending colon which is known as ameboma (Adams & MacLeod, 1977). Clinical syndromes for extra intestinal amoebasis are amoebic liver abscess (ALA), perforation and peritonitis, pleuropulmonary amoebiasis, amoebic pericarditis and cutaneous amoebiasis. ALA patients usually having fever, right upper quadrant pain and hepatic tenderness. There are possibilities of cough, jaundice, dullness and rales in the right lung base.

According to Allason et al. (1986), roughly 20% to 30% of homosexual males are colonized with *E. dispar* in Western countries due to oral-anal sex practices while in certain cases amebiasis in homosexual men from Taiwan and Korea (Hung et al. 1999; Oh et al. 2000) and Australia (Fotedar et al., 2007; Stark et al., 2006) have been reported.

2.4.3.3 Transmission of *Entamoeba* spp Infections

Infection of *Entamoeba* species can be transmitted to human through various ways from the immigrants, travellers to areas of endemicity and institutionalized population. Moreover, other transmissions route is through ingestion of contaminated food and water by polluted water supply with feces of *E. histolytica* cyst from human feces or feces of infected wild or domestic animals. Dirty handling by infected individuals and infected food handlers may contribute to high prevalence of *Entamoeba* infection (Mahmud et al., 2013). According to Ngui et al. (2012), low sanitation and hygiene also plays a vital role in transmitting the infection. Hung et al. (2012) reported that these infection increases among male homosexuals who engage in oral-anal sex. This infection is common among male homosexuals in Japan based on Takeuchi et al. (1990) and Ohnishi et al. (2004) analysis. 80% of ameabiasis cases occurred in male homosexuals in Japan (Nozaki et al., 1989). On the other hand, the human immunodeficiency virus (HIV)-positive male homosexuals are at a greater risk of acquiring an *E. histolytica* infection than the other HIV-positive individuals (Hung et al., 2008). Among 34 000 HIV infected patients in United State, roughly 111 (0.3%) patients were diagnosed having of *E. histolytica, E. dispar* infection (Lowther et al., 2000).

2.4.3.4 Diagnosis of *Entamoeba* spp Infections

There are numerous techniques to identify and detect infection of *Entamoeba* species in humans such as microscopy examination, culture methods, isoenzyme analysis, antibody detection tests, antigen detection tests and polymerase chain reaction. Initially, the stool samples were examined under light microscopy via direct saline mount or permanent stain smears which included trichrome or iron hematoxylin (Tengku & Norhayati, 2011) but the challenge was to differentiate between *E. histolytica, E. dispar* and *E. moshkovskii* as this was easily confused with macrophages and other *Entamoeba* species (Pillai et al., 1999). Gonzalez-Ruiz et al. (1994) reported that culture methods are more sensitive than microscopy examination. *E. histolytica, E. dispar* and *E. moshkovskii* could be differentiated by isoenzyme analysis on cultured samples of amoeba (Sargeaunt et al., 1980). Unfortunately, both analyses require few weeks to complete and delay in sample processing which results in false negative result in numerous microscopy positive samples (Strachan et al., 1988).

Antibody detection tests, including indirect haemagglutination (IHA), latex agglutination, immuno-electrophoresis, counter-immunoelectrophoresis (CIE), amoebic gel diffusion test, immuno-diffusion, complement fixation, indirect immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA) can be used to detect infection of *Entamoeba* species in humans. These assays however are costly to perform, less sensitive, nonspecific or time consuming (Fotedar et al., 2007) except enzyme-linked immunosorbent assay (ELISA) which is easy to perform and appears to be a rapid method for *E. histolytica* identification. There are several advantages of using antigen based ELISA as it can easily distinguish *E. histolytica* from *E. dispar*, possess excellent sensitivity and specificity and can be handled by non-

experienced laboratory personnel. Polymerase chain reaction (PCR) exhibited a high sensitivity and specificity to detect infection of *Entamoeba* species (Tanyuksel & Petri, 2003).

2.4.3.5 Treatment and Prevention of *Entamoeba* spp Infections

Nitroimidazole derivatives such as metronidazole, tinidazole, ornidazole have been used to treat amoebiasis. Treatment is usually with metronidazole which is eventually followed by a luminal agent such as paromomycin, iodoquinol or diloxanide furoate in order to eradicate colonization (Mahmud et al., 2013). Asymptomatic patients should also be treated to avoid transmission. Rapid clinical improvement of amoebic liver abscess results from oral or intravenous metronidazole (Irusen et al., 1992). Open surgical drainage will only be implemented after the cavity ruptures into adjacent viscera or peritoneum due to high surgical mortality (Sharma & Ahuja, 2003).

Communities should be given health education inculcating healthy personal habits, sanitary disposal of feces and hand washing to help control infections to humans (Ngui et al., 2012). This is because most of the infection is transmitted to humans via contaminated food and water due to dirty handling habits. Apart from that sexual practices which involve fecal-oral contact should be avoided in order to reduce infection in homosexuals. *Entamoeba* cysts can be killed by iodine, boiling, desiccation and freezing below -5°C even though it is resistant to standard chlorine treatment (Mahmud et al., 2013). Besides this, *Entamoeba* cysts can be effectively removed by sedimentation and filtration processes.

2.4.4 *Toxoplasma gondii* Infections

2.4.4.1 History and Life Cycle *Toxoplasma gondii*

Toxoplasma gondii is an obligate intracellular protozoan parasite that is grouped under Phylum Apicomplexa. In 1908, Nicolle and Manceaux described *Toxoplasma* for the first time, when they discovered it while experimenting on *Ctenodactylus gundii* for Leishmaniasis research and suggested genus *Toxoplasma* for it (Nicolle & Manceaux, 1908). *Toxoplasma gondii* is only one species in this genus and named after it's isolation from a rodent, *Ctenodactylus gundi*. The organism is reported to have wide range of host and is world-wide distributed (Dubey, 2008). The life cycle of *Toxoplasma gondii* consist of two main stages which includes asexual described previously before 1970s and a sexual stage that was reported after 1970s only. The asexual stage demonstrates two distinct phases involving tachyzoites or trophozoites and bradyzoites or cystzoites while sexual stage involved oocyst which was environmentally resistant (Figure 2.6) (Tenter et al., 2000).

Tachyzoite form of *Toxoplasma* is commonly oval or crescent shape and is measured approximately 6 mm long and 2 mm wide with pointed anterior region and more rounded posterior region (Black & Boothroyd, 2000; Dubey, 2008; Smith, 1995). Tachyzoite consists of various organelles and inclusion bodies including, rhoptries, micronemes, mitochondrion, microtubules, Golgi complex, ribosomes, rough and smooth endoplasmic reticula, nucleus, amylopectin granules, and apicoplast and other as in (Figure 2.7). The nucleus is usually observed at central area of cell and contains clumps of chromatin and a centrally-located nucleolus. Generally they rapidly multiply by repeated endodyogeny and infect adjacent cells during acute phase of infection and in rare case by binary fission (Smith, 1995). They move by mean of gliding, flexing,

undulating, and rotating, without any specific locomotion features like cilia, pseudopodia or flagella (Black & Boothroyd, 2000; Dubey, 2008).

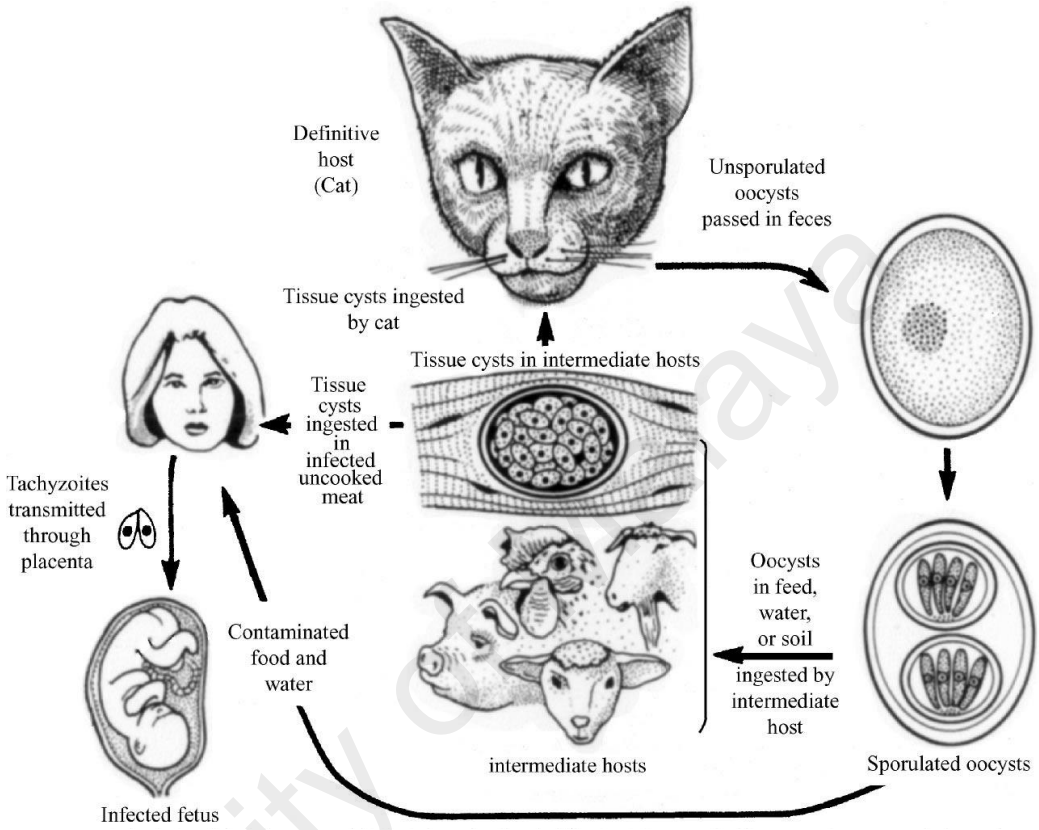


Figure 2.6: Life cycle of *Toxoplasma gondii.* Adapted from "*Toxoplasma gondii*: transmission, diagnosis and prevention", by Hill and Dubey, 2002, *Clinical Microbiology and Infectious Diseases, 8*, p. 635.

Bradyzoites is the differentiated form of tachyzoite and exist as dormant cysts in the central nervous system and muscle tissues (Black & Boothroyd, 2000; Smith, 1995). Of note, bradyzoites are only slightly different from tachyzoites structurally, whereby they show some variation in position of nucleus and rhoptries content (Figure 2.7). The nucleus of bradyzoites located more toward posterior region while the contents of rhoptries are more electron dense, although they vary with the age of the tissue cyst. Moreover, bradyzoites are more slender than tachyzoites (Dubey, 2008).

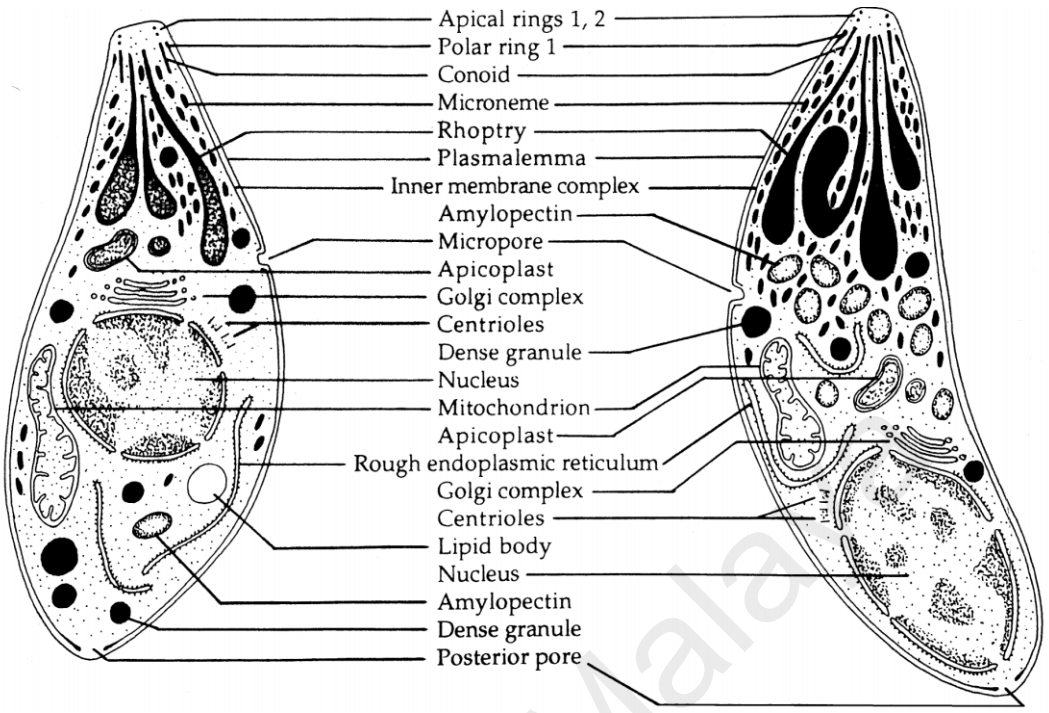


Figure 2.7: Ultrastructure drawings of a tachyzoite (left) and a bradyzoite (right) of *Toxoplasma gondii*. The drawings are based on electron microscope. Adapted from "Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts", Dubey et al., 1998, *Clinical Microbiology Reviews, 11*(2), p. 269

Oocysts, only develop in the feline host after ingestion of any three *Toxoplasma gondii* infectious stages, and there are commonly oval in shape and vary in size. Dubey et al. (1998) documented oocysts in the brain which are often spherical in shape and is relatively small, while oocysts in muscles are elongated and about 100mm long. Oocysts remain unsporulated (non-infective stage) outside the feline host, after it's exposure to environment after shedding but they remain sporulated (infectious stage) between 1 to 5 days depending upon aeration and temperature.

2.4.4.2 Transmission of *Toxoplasmsa gondii* Infections

Transmission of sexual stages of *Toxoplasmsa gondii* is limited to feline host, but the asexual form is able to invade a wide range of warm blooded nucleated cells. Generally, there are two considerable routes of transmission to humans, i.e. oral and congenital transmission. Oral transmission mainly takes place by ingestion of tissue cysts in raw or undercooked meat. Besides that, ingesting other foods and water contaminated with feline feces also contribute to the disease transmission. Congenital transmission of toxoplasma occurs from mother to fetus, if the mothers acquire infection during pregnancy and the severity of disease complication to fetus depends upon which trimester the mother acquired the infection (Hill & Dubey, 2002). Toxoplasmosis can also be acquired by contaminated blood transfusion and organ transplantation from infected donor.

2.4.4.3 Clinical symptoms and burden of *Toxoplasmsa gondii* Infections

The symptoms and signs of toxoplasmosis depends on several criteria, whether the disease is acquired or congenital, whether the infected patients an immunocompetent or an immunodeficient, or the infection is limited to eye or is systemic. Thus, we can divide the patients into three different groups including (*a*) immuno-competent patients with acquired infection, (*b*) immuno-deficient patients with acquired or reactivated infection and (*c*) patients with congenital infection.

In immuno-competent patients with acquired infection, most cases are asymptomatic (Hill & Dubey, 2002). The patients normally remain asymptomatic except if the parasite gets reactivated due to immunosuppression condition. Lymphadenopathy that refers to enlarged lymph node is the most significant clinical manifestation and may be associated with fever, fatigue, muscle pain, sore throat and headache (Hill & Dubey,

2002, Hill et al., 2005). Toxoplasmosis in immuno-deficient patients causes severe damage and reactivation of latent infection causing symptomatic disease, such as encephalitis. Infected patients may present with symptoms including headache, disorientation, drowsiness, reflex changes and convulsions, and many end in comatose. On the other hand, toxoplasmosis has been established as one of major opportunistic parasite in AIDS patients and causes toxoplasmic encephalitis (Dubey, 2009; Hill & Dubey, 2002; Hill et al., 2005). In congenital toxoplasmosis the most of infected pregnant women do not experience obvious symptoms, but some may experience malaise, low-grade fever, and lymphadenopathy. Infected mother can vertically transmit the parasite to developing fetus. A broad range of clinical manifestation can be observed in congenitally infected infants. As for mild diseases, infants may experience slightly diminished vision. However, in severe infection infants can develop retinochoroiditis, hydrocephalus, convulsions and intracerebral calcification and ocular disease. Hydrocephalus is rare disease, but most dramatic, lesion of toxoplasmosis.

2.4.4.4 Invasion of *Toxoplasmsa gondii*

Toxoplasma gondii is well known to invade a wide range of nucleated cells of warm-blooded host and their invasion to host is multi-step complex process. Two distinct specialized secretory organelles of *Toxoplasma gondii*, termed micronemes and rhoptries, which are characteristic of the Apicomplexa phylum, play a vital role in invasion of this parasite to its host. The multistep invasion involves, initial contact of parasite to host membrane, parasite attachment, parasite motility and then penetration to host cell. Firstly, low affinity interaction took place, whereby parasites loosely attach to surface of host cell which was regulated by parasite surface antigen. Then, an unknown signal was triggered and stimulates the release of micronemes which consist of protein

that involves in adhesion. Following micronemal adhesion to host cell, the tight junction is also known as moving junction forming the apical membrane antigen 1 (AMA1), secreted from micronemes and andrhoptry neck (RON) proteins, secreted from rhoptries which play a vital role in the parasite entry to the host cell. Later, parasites invade the host cell using the unique gliding motility where it is propelled to enter into host cell by means of an internal actomycin motor. This invasion further leads to the formation of parasitophorous vacuole which is surrounded by its membrane inside the host cell where they reside in the host cell throughout its time. (Smith, 1995; Blader & Saeij, 2009; Montoya, 2002)

2.4.4.5 Diagnosis of *Toxoplasma gondii* Infections

Diagnosis of toxoplasmosis generally presumptively is on the basis of clinical symptoms and signs which might mimic other central nervous system (CNS) infections leading to inaccurate diagnosis or simply being overlooked. Established diagnosis for toxoplasmosis using various serological procedures including the Sabin-Feldman dye test (DT), the modified agglutination test (MAT), the IHAT, the indirect fluorescent antibody assay (IFA), the direct agglutination test, the latex agglutination test (LAT), the ELISA, and the immunosorbent agglutination assay test (IAAT). In addition, diagnosis also conducted by detecting the parasite from the biopsy of infected host and microscopic examinations. Staining methods such as Giemsa, periodic acid Schiff (PAS), immuno-histochemical staining with fluorescence has been used in diagnosing toxoplasmosis. Lately, there are other various type of polymerase chain reaction (PCR), widely used to diagnosis toxoplasmosis from cerebrospinal and amniotic fluids (Hill & Dubey, 2002; Cinque, et al., 1997).

2.4.4.6 Treatment and Prevention of *Toxoplasma gondii* Infections

Sulfadiazine and pyrimethamine (Daraprim) are two popular drugs used for the treatment of *Toxoplasma gondii* infection (Goldstein et al., 2008). However these drugs are not successful to eradicate the infection, although they exhibit beneficial action in controlling proliferation of the parasite in acute stage. Besides, in complicated cases, few more drugs like diaminodiphenylsulfone, atovaquone, spiramycin, and clindamycin also widely used to combat toxoplasmosis (Dubey, 2009; Hill & Dubey, 2002; Hill et al., 2005). In the current management of toxoplasmosis in pregnant ladies, spiramycin doses is introduced if, infection occurred at trimester, prior to conduct amniocentesis to identify fetus infection. However, if infection is detected in the fetus, sulfadiazine and pyrimethamine are alternatively used to treat toxoplamosis, instead of spiramycin (Goldstein et al., 2008).

Primary prevention of toxoplasmosis in humans is by implementing a good hygiene practice as transmission occurs when the shedding of fecal matter through cats contaminate food and water with either tachyzoites, bradyzoitess or oocysts of *Toxoplasma gondii*. On the other hand, exposure of meat to extreme heat and cold can prevent diseases transmission. It was reported previously that, tissue cyst can be killed by heating up to 66-67°C and cooling to -13°C (Hill et al., 2005; Goldstein et al., 2008). Pregnant women should be aware of the complication of toxoplasmosis and be more careful in dealing with undercooked meat and wear protective gloves while gardening.

2.4.5 *Trypanosoma* spp Infections

Trypanosoma spp is a protozoan flagellate parasite that belongs to the family of Trypanosomatidae. The name of the parasite was derived from the Greek word, trypano (borer) and soma (body) due to their corkscrew-like locomotion. Previously, numerous species and sub-species of trypanosomes have been described to infect a variety of different vertebrates, including animals and humans. Insects are the primary host for transmission. *Trypanosoma* spp infection caused by the species of *Trypanosoma brucei* group and *Trypanosoma cruzi was* reported to infect millions of people worldwide including in Africa, Asia, parts of Europe, and Latin America. They reported to cause life threating diseases like sleeping sickness and Chagas' disease (Andrade & Andrews 2005; de Souza, et al., 2010).

2.4.5.1 Life Cycle and Morphology *Trypanosoma* spp

Generally, one specific feature of family of Trypanosomatidae, is their ability to transform their general shape during their life cycle. Among the other species, *Trypanosoma cruzi*, that causes Chagas's disease has the most complex life cycle with several development phase, which enable them to survive in vertebrate and invertebrate host (de Souza, et al., 2010). The life cycle of *Trypanosoma cruzi* in insect vector tse tse fly and human (Figure 2.8) shows that infected insect vector, inject metacyclic trypomastigote into host cell acquired through blood sucking of infected vertebrate. In the insect vector such as triatomines bug, trypomastigote of *Trypanosoma cruzi* transform into epimastigotes that rapidly multiply extracellularly in the insect's midgut, upon intake of infected blood meal with *Trypanosoma cruzi* trypomastigote. Then, the parasite continues to multiply and reaches the posterior intestine. In the intestine they attach to the wall of the rectum and differentiate into infective metacyclic

trypomastigotes, which will be released in the feces when the bug takes a blood meal. The metacyclic trypomastigotes then penetrate into mammalian host cells through a few routes including bite wound, open skin lesion or mucosal surfaces and the subsequently invade host cells. In the host, trypomastigotes infect cells from a variety of tissues and transform into intracellular replicative amastigotes stage in new infection sites. The amastigote stage undergoes many rounds of binary division transforming into elongated, motile trypomastigote stages. Eventually, this motile trypomastigote is released when the host cell ruptures. The trypomastigotes are then disseminated in the blood and lymph where they infect virtually any nucleated cell or be taken up by the insect vector to complete the life cycle (Andrade & Andrews 2005; Minning et al., 2009; de Souza, et al., 2010).

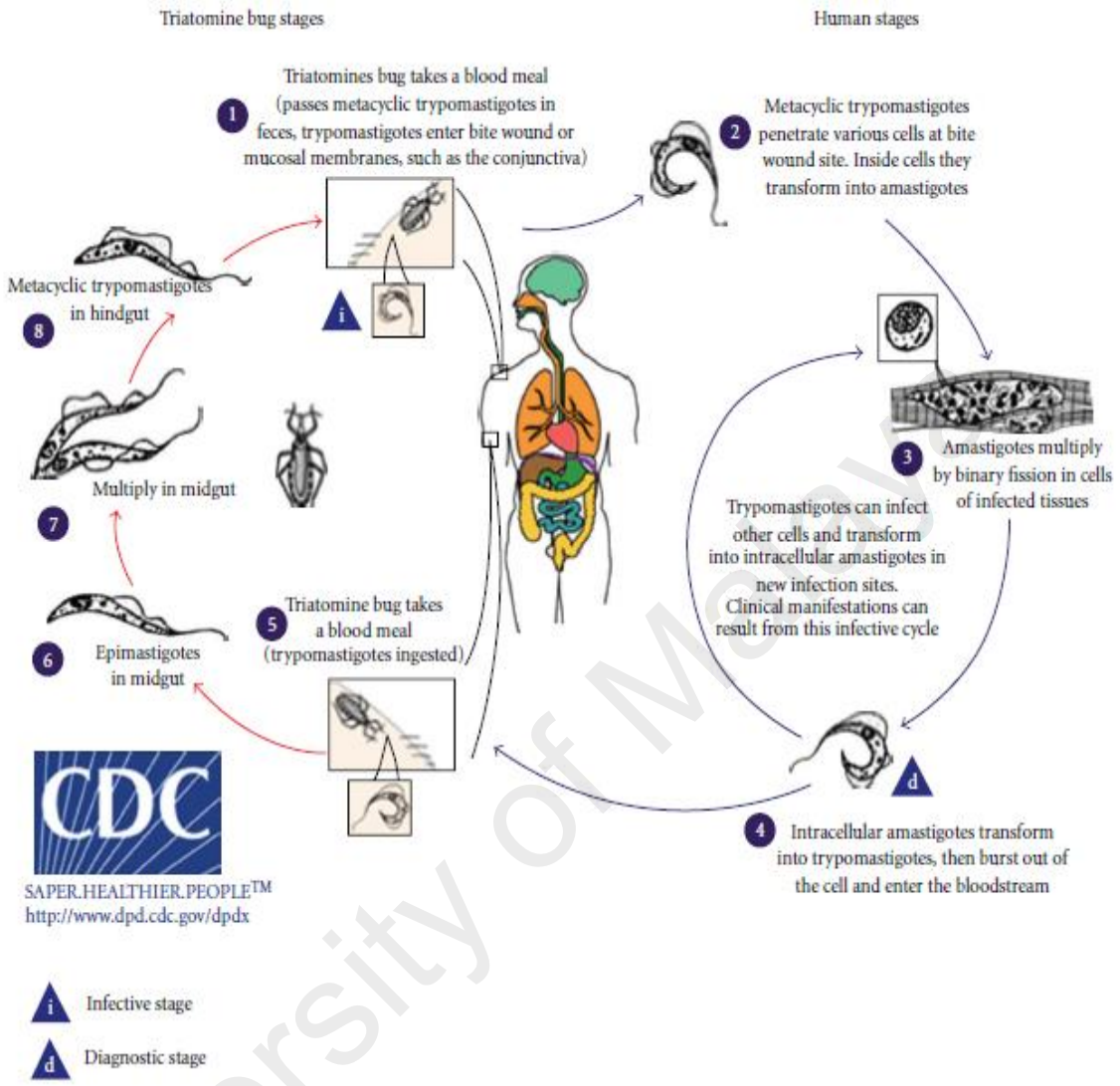


Figure 2.8: Life cycle of *Trypanosoma cruzi* and its developmental stages in insect vector, triatomine bug and in humans. Adapted from *American Trypanosomiasis*, Retrieved Jan 12, 2015 from http://www.cdc.gov/dpdx/trypanosomiasisAmerican/

2.4.5.2 Invasion of *Trypanosoma* spp into Host Cell

Host cell invasion, generally in vertebrates including human, involves a multistep process, which can be divided into three main phases of adhesion and recognition, signaling, and invasion. However, the invasion process is dependent on the strains of trypanosome developmental stage as well as on the host. Firstly, the adhesion of the *Trypanosome cruzi* occurs through the recognition of surface molecules in both parasite and host cells as well as some molecules secreted by parasite. De Souza et al. (2010) reported that, different strains of *Trypanosome cruzi* as well as different developmental phase of the parasite, express different surface molecules that interact with host components to invade mammalian cells. These molecules include various glycoprotein such as sgp90, gp82 and gp35/50 molecule; mucins, a surface glycoproteins that also act as ligands; trans-sialidases; Gp83, a ligand used to attach and enter phagocytic and nonphagocytic cells; and other protease like cruzipain, oligopeptidase B, and Tc80 are documented to play vital role for invasion of *Trypanosome cruzi*, especially in trypomastigote stage. As *Trypanosome cruzi* invasion is a multifactorial process, whereby molecules present in the membrane of the host cell, also act as potential partners for recognition.

2.4.5.3 Transmission of *Trypanosoma* spp Infections

Transmission of *Trypanasoma* spp can occur through various routes which include transmissions involving vector, congenital, blood-borne, and organ-derived as well as oral transmission. The vector-borne transmission remains one of major transmission routes that cause new infections in human. Transmission through this route generally take place when feces of infected insect vector comprises of infective metacyclic trypomastigotes enter or penetrate into host cell or human cell through bite wound or other mucous membrane (Bern et al., 2011). Congenital transmission can occur from infected pregnant women to their fetus. Congenital transmission rates vary according to geographic areas, from 1% to 10% (Hermann et al., 2004). *Trypanasoma* spp infections also can be acquired through infected blood transfusion. Among the other, platelet transfusion is believed to pose a higher risk than transfusion of other components of blood for blood-borne transmission. However, transmission through this route has been minimized via screening serological and blood donors prior to blood transfusions (Bern et al., 2011; Hermann et al., 2004). Transmission through organ transplant involves kidney, pancreas, liver, and heart. However, Bern et al. (2011) reported infection through heart transplant believed to express significant infection compared to other organ transplantation. Besides that, oral transmission of *Trypanasoma* spp, especially in *Trypanasoma cruzi* attain a great consideration as several outbreaks of *Trypanasoma cruzi* infection associated with contaminated fruit, sugar cane and others. Nobrega et al. (2009) through their retrospective cohort study reported outbreak of *Trypanasoma cruzi* infections through consumption acai palm fruit, in Brazilian Amazon region.

2.4.5.4 Diagnosis of *Trypanosoma* spp Infections

Trypanasoma spp infection can be diagnosed by microscopy, isolation of parasite from culture of direct specimens, various serological tests and through application of molecular techniques. Microscopy examination can utilize to detect parasite from fresh specimens or stained smear. However, this technique not efficient in detecting the parasite in an acute phases of *Trypanasoma cruzi* infections (Bern et al., 2011; Rosenblatt et al., 2009). In addition to that, various serological tests also employed to diagnosis chronic infection that include, detection IgG antibodies, most commonly the enzyme-linked immunosorbent assay (ELISA) and immunofluorescent-antibody assay. Polymerase chain reaction (PCR) technique allows the most sensitive tool to diagnose acute phase and early congenital infection as well as in organ transplant recipients. The technique enables the detection of infection as early days to a week before the infective circulating parasitic phase detectable in blood smears (Bern et al., 2011; Castro, et al., 2002).

2.4.5.5 Treatment and Prevention of *Trypanosoma* spp Infections

Nifurtimox and benznidazole are only drugs are efficiently used to treat *Trypanasoma* spp infection especially against *Trypanasoma cruzi*. However nifurtimox is commonly associated with gastrointestinal side effects accounting in 30% to 70% patients that comprises of weight loss, nausea, vomiting and abdominal discomfort. In addition, neurological toxicity has also been associated with nifurtimox. Benznidazole derivate of nitroimidazole studies to be more trypanocidal than nifurtimox and commonly express some dermatological side effects. In rare cases, the drug also exhibit exfoliative dermatitis or dermatitis associated with fever and lymphadenopathy. Side effects are dependent to the dosage and patient's age, and being more common in adults than in children, for both nifurtimox and benznidazole drug treatment (Bern et al., 2011; Estani & Segura, 1999).

2.5 Staining for Parasite Identification

2.5.1 Modified Field's Stain

Modified Field's Stain was developed with some modification of Field's Stain which was widely used in malarial staining in earlier years. The stain is made up from methylene blue and Azure 1 dissolved in phosphate buffer solution and Eosin Y in buffer solution. Whist, in Modified Field's Stain, methanolic Eosin Y was used for replacing buffered Eosin solution (Pirehma et al, 1999). Modified Field stain technique allowed rapid identification which gave results in 20 seconds and was comparable to other staining methods that was complex procedures and time consuming. The best part of this stain was the ability to confer a good color contrast by differentiating nucleus, cytoplasm, nucleolus and cell membrane and it was comparable to Giemsa and Gram staining (Pirehma et al., 1999; Afzan, et al., 2010; Ithoi et al., 2011). It stains the nucleus and nucleolus in pink and dark blue color respectively. Furthermore, the stain is

very effective and useful in determining false positive results, since it able to identify and differentiate contaminant and debris in target culture or specimen (Pirehma et al., 1999). The stain is also reported to be very useful in determining the life-cycle of parasites, since it stains nucleus division as well.

2.5.2 Modified Trichome Stain and Giemsa Stain

There are various methods developed in order to identify microsporidial spores and it's infections in man. However, examination of body tissues and fluids remain one of important diagnosis method for identification of microsporidiosis. Thus, microscopy examination using modified trichrome stains (MTS) was used for microsporidial spores detection. The spore's wall stain pinkish to red, while the interior part of spore will be clear showing diagonal stripe representing the polar tube. Giemsa Staining has been generally used in histopathological studies and the diagnosis of malaria and other parasites.

2.6 Polymerase Chain Reaction for Parasite Identification

Polymerase chain reaction assay can be performed for diagnosis purpose to detect various parasitic infections in clinical specimen. The reliable and rapid PCR assay widely used in diagnosis *Acanthamoeba* spp infections upon completion of 18S ribosomal RNA gene (18S rDNA) sequencing. Moreover, it is reported that, mitochondrial DNA polymerase chain reaction (PCR) has been used successfully to identify *Acanthamoeba* spp in cerebrospinal fluids (CSF) and brain tissues.

2.7 Rationale of the Study

The study was undertaken to assess for the presence of parasitic infections that usually associated with central nervous system (CNS) from cerebrospinal fluids (CSF). These samples were obtained from the laboratory submitted for routine diagnosis.

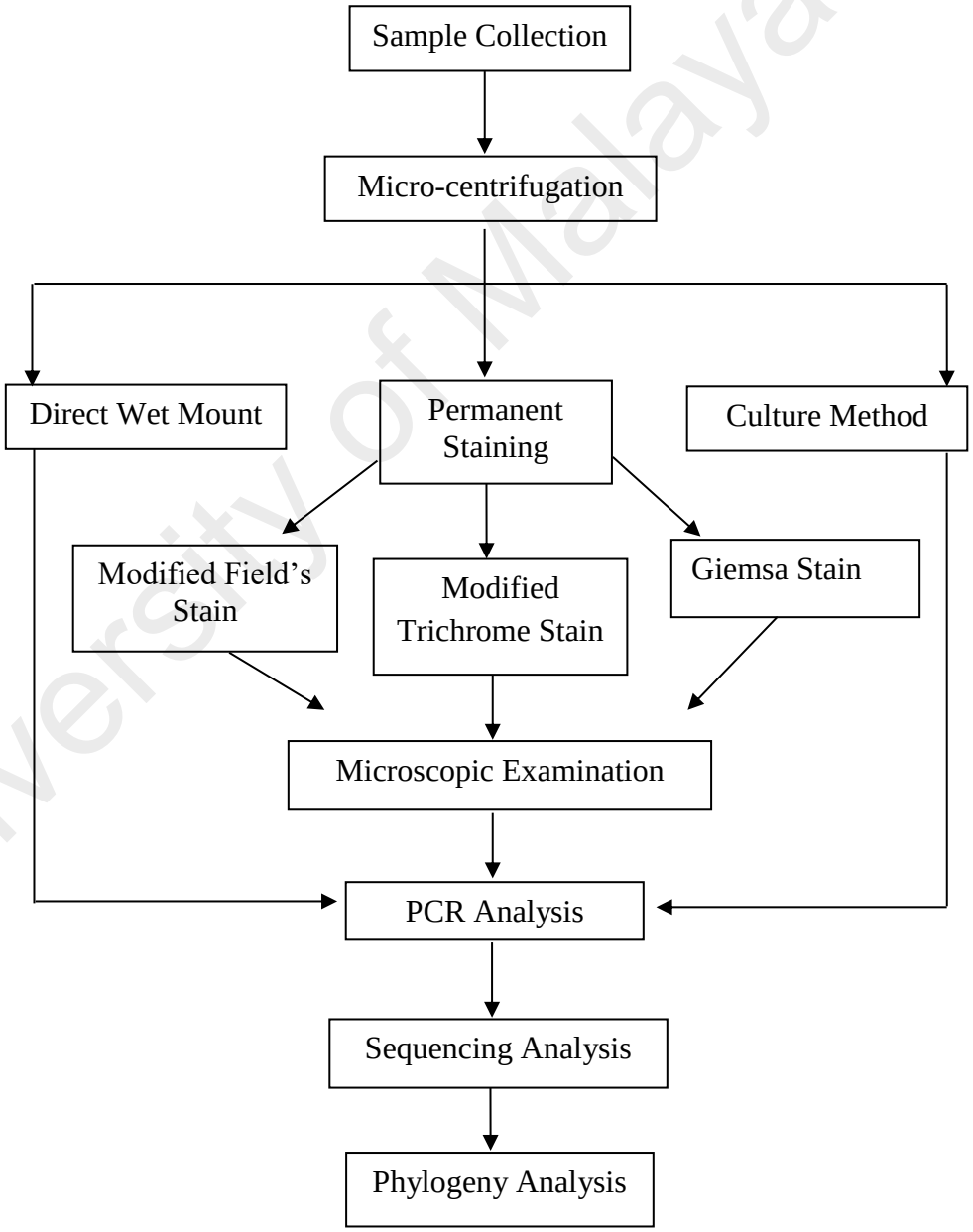
All the samples assessed for parasitic infections in the present study were reported negative for some of routine diagnosis test for viral, bacterial and fungal infections. Often, diagnosis for parasitic infections were limited and rarely conducted at preliminary stage. Despite, limited diagnosis for parasitic infections, they were generally diagnosed using stool or serum samples only.

Therefore, we relooked at these samples for the parasitic infections listed above through direct microscopy and polymerase chain reaction (PCR) for targeted pathogen. Then, validate the obtained results using sequencing and bioinformatics analysis.

CHAPTER 3

METHODOLOGY

3.1 Overview of Methodology



3.2 Source of Specimens

Cerebrospinal fluid (CSF) samples were received from respective clinics from University Malaya Medical Centre (UMMC). Patient identity, sampling date and time was recorded. In addition to that, the color and the sample condition was also described and recorded.

3.3 Detection of Parasite by Conventional Diagnostic Approaches

3.3.1 Direct Wet Mount Procedures

Prior to direct wet mount procedure; about 200 µL cerebrospinal fluid (CSF) samples were centrifuged at 2000 rpm for 10 minutes (Kubota Model 2010, Japan) at room temperature (Dunbar et al., 1998). About three quarter of the supernatant was then discarded and the remaining fluid mixed with a sterile serological pipette. Microscope slides were cleaned with 70% alcohol and a small drop of sediment or pellet was then placed on it. A cover slip was placed over the drop and examined by light microscopy under 40 X magnifications for the detection of possible parasites present in the cerebrospinal fluid (CSF) specimens.

3.3.2 Staining Procedures

3.3.2.1 Modified Trichrome Stains

Modified trichrome stain (MTS) procedure was initiated by heating the prepared stain at 56°C for one hour prior to commencing the staining procedure. Meanwhile, about 200 µL of specimens were centrifuged for 10 minutes at 2000 rpm (Kubota Model 2010, Japan) prior to smear preparation (Garcia, 2000). The smears were, fixed with methanol and then allowed to air dry. The dried smears were then stained with modified trichrome staining for 10 minutes, rinsed with acid alcohol for 3 seconds followed by rinsing again with 95% alcohol for 3 seconds. The slides were then, left in 95% alcohol for 5 minutes and continuously dried in ascending concentrations of ethanol till 100% alcohol. The

slides were then finally, cleared in xylene for 10 minutes and subsequently air dried for 2 hours before mounting with DPX and examining under oil immersion with x1000 magnification.

3.3.2.2 Modified Fields' Stain

The prepared smears were air dried and fixed with 3 to 5 drops of Field B stain (modified methanolic eosin). Following this, 6-10 drops of Field A stain was added on top of Field B stain. The slides were tilted for 10 to 15 seconds and washed under the slow flow of running water and air dried prior to examination by light microscopy under x 400 magnification (Afzan, 2010).

3.3.2.3 Giemsa Stain

Smears were made on slides and fixed with pure methanol for 30 seconds. The slides were then immersed in a freshly prepared Giemsa Stain solution for 10 to 20 minutes and subsequently flushed with tap water, left to air dry and examine under light microscopy under x 400 magnification (Afzan, 2010).

3.3.*3 In vitro* culture Methods

3.3.3.1 *In vitro* culture Media for *Acanthamoeba* spp

The ingredients (Appendix II and Appendix IIA) were dissolved in distilled water and sterilized by autoclaving at 121°C for 15 minutes to prepare ameba saline. Sterilized saline was cooled and kept at 4°C until further use. 100 ml of ameba saline prepared was mixed with 900 ml sterile distilled water to make 1x ameba saline. Later, 15.0 g of Bacto agar was added to this solution and heated until fully dissolved. Following that, the media was sterilized by autoclaving at 121°C for 15 minutes. After sterilization the media was cooled down approximately to 60°C and aseptically poured into sterile petri dishes. Solidified agar plates were stored at 4°C until further cultivation.

3.3.3.2 Cultivation *Acanthamoeba* spp

Prior to cultivation, the CSF specimen was centrifuged at 250 x g for 10 minutes. Then, 0.5 ml of supernatant was transferred to a sterile tube and stored at 4°C until further use. Next, the sediment was mixed well and 2 to 3 drops of mixed fluids was placed on the center of the non-nutrient agar which has been pre-coated with bacteria prepared earlier. The plates were then sealed and incubated in an upright position at 37 °C. The plates were then examined under microscopy for presents of cysts or trophozoites every day continuously for 10 days.

3.3.3.3 Culture Media for *Blastocystic spp*

All ingredients listed (Appendix I) for Jones medium preparation was periodically added with 960 ml sterile distilled water and mixed well to dissolve. Then 12.5ml of solution was pipetted out followed by addition of 100 ml of 1% of yeast solution. The pH of solution was adjusted to pH 7.4 and sterilized by autoclaving 121°C for 15 minutes. Sterilized media was then added with 10% horse serum and stored at 4°C until further use.

3.3.3.4 Cultivation of *Blastocystic spp*

About 0.25 ml of centrifuged CSF sediment was inoculated into culture tube containing 3ml of Jones' Medium supplemented with 10% horse serum (Gibco Laboratories, Life Technologies) and incubated at 37^{o}C (Tan et al., 2008). The cultures were then examined 24 hours later under light microscopy for the detection of *Blastocystic spp*. Cultures were then sub-cultured into fresh medium once every three days.

3.4 Identification of Parasite by Molecular Method

3.4.1 DNA Extraction from Cerebrospinal Fluid (CSF)

Cerebrospinal fluid specimens were centrifuged at 250 x g for 10 minutes to obtain the pellet material. The DNA extraction was done in accordance with manufacture's direction using QIA amp DNA Mini and Blood Mini Kit (Qiagen). Firstly, 200µL of CSF pellet was added with 200µL of proteinase K and mixed well. Next, 200µL of lysis buffer was added to sediment or pellet. Then, the suspension was vortex for 15 seconds. Following that, the suspension was incubated for 10 minutes at 56°C in the water bath and subsequently the samples were centrifuged. Later, the entire suspension was transferred to QIA amp Mini Spin Column and centrifuged at 8000rpm for 1 minute. Filtered suspension was then discarded and 500µL of AW1 buffer subsequently added and centrifuged at 8000rpm for 1 minute. The filtered suspension was then again discarded before adding another 500µL of AW2 buffer and centrifuged at 14000rpm for 3 minutes. 200µL of elution buffer was then added and incubated at room temperature for 5 minutes. The incubation time was increased from 7 to 10 minutes for a better yield. The spin column was centrifuged again at 8000 rpm for 1 minute with DNA extracted, collected and stored at -20°C until further use.

3.4.2 DNA Quantification

The yield and the concentration of extracted DNA was quantified using NanoDrop 2000 ultraviolet (UV) spectrophotometer (Thermo scientific, MA, USA). The concentration of purified DNA was recorded in ng/µl. The blank used for spectrophotometer reading was the elution buffer used in extraction procedures.

3.4.3 Storage of Genomic DNA

Quantified DNA samples were labeled and kept in freezer at 4°C for short term storage and at -20^{o}C for longer storage period.

3.4.4 PCR Amplification

3.4.4.1 PCR Amplification Procedure for *Acanthamoeba* spp

PCR amplification for *Acanthamoeba* spp was done using a primer set of Aca16Sf1010 and Aca16Sr1180 as reported by Yagi et al. (2007). The forward primer sequence was as follow, Aca16Sf1010 (5'-TTATATTGACTTGTACAGGTGCT-3'), whereas the reverse primer sequence was Aca16Sr1180 (5'-CATAATGATTTGACTTCTTCTCCT-3'). The primer set amplifies 161bp fragment of mitochondrial 16S rRNA gene of *Acanthamoeba* spp.

Prior to preparation of master mix solution, primer stocks (100µM/µL) were diluted with deionized water for a final concentration of 10µM/µL. PCR reaction mixture consists of 5 µL of template DNA and 20 µL of master mix solution (Table 3.1), which made up of a total of 25µL of reaction mixture for each reaction and this was prepared using Promega Reagent Kit. As for the negative control 5 µL of template DNA was replaced with deionized water to make up total of 25µL of reaction mixture. Table 3.1 shows the master mix solution for PCR amplification with their components and concentration used. PCR Amplification was conducted using thermal cycler (Eppendorf AG 22331, Hamburg, Germany). Amplification was carried out with minor modification of standard cycling protocol reported by Kao et al. (2012) as follows; an initial denaturation at 94°C for 5 minutes, followed by 40 cycles of: denaturation at 94 °C for 1 minute, annealing at 60 °C for 1 minute and extension at 72 °C for 1 minute. The amplification was ended with final extension at 72 °C for 10 minutes.

Table 3.1: PCR Master Mix Recipe for *Acanthamoeba* spp

Components	Stock Concentration	Working Concentration	Master Mix [Volume (µL)/reaction]
H2O (Deionized)			14.3
PCR Buffer	10X	1X	2.0
MgCl2	25mM	1mM	1.0
dNTPs	40mM	0.2 mM each	0.5
Taq Polymerase	5U	0.2 U	0.2
Forward Primer	10µM	0.4 µM	1.0
Reverse Primer	10µM	0.4 µM	1.0

3.4.4.2 PCR Amplification Procedure for *Entamoeba* spp

Amplification procedure for *Entamoeba* spp was carried for three different species including for *E. histolytica*, *E. dispar* and *E. moshkovskii* targeting fragment of small subunit ribosomal RNA (SSU rRNA). Conserved region in all three *Entamoeba* species, designed as sequence of forward primer, EntaF (5'-ATG CAC GAG AGC GAA AGC AT-3'). The reverse primer was designed specifically for each species, EhR (5'-GAT CTA GAA ACA ATG CTT CTC T-3'), EdR (5'-CAC CAC TTA CTA TCC CTA CC-3') and EmR (5'-TGA CCG GAG CCA GAG ACA T-3') for *E. histolytica*, *E. dispar* and *E. moshkovskii* respectively. (Anuar et al., 2012; Hamzah et al., 2006). Combination of forward primer and specific reverse primer give a amplicon size, 166 bp for *E. histolytica, 580* bp for *E. moshkovskii* and 752 bp for *E. dispar.*

PCR amplification was conducted in total volume of 50µL, made up from 5 µL template DNA and 45µL of reaction mixture for each reaction. As for negative control 5 µL of template DNA was replaced with deionized water to make up total 45µL of reaction mixture. Table 3.2 shows the master mix solution for PCR amplification with their components and concentration used. Amplification was performed as reported by (Anuar et al., 2012; Hamzah et al., 2006) as follow; an initial denaturation at 94°C for 3 minutes, followed by 30 cycles of: denaturation at 94 °C for 1 minute, annealing at 58 °C for 1 minute and extension at 72 °C for 1 minute. The amplification was ended with final extension at 72 °C for 7 minutes.

Table 3.2: PCR Master Mix Solution for *Entamoeba* spp

Components	Stock Concentration	Working Concentration	Volume (µL)/reaction
H2O (Deionized)			24.9
PCR Buffer	10X	1X	5.0
$MgCl_2$	25mM	6mM	12.0
dNTPs	40mM	0.8 mM	1.0
Taq Polymerase	5U	0.5U	0.1
Forward Primer	10µM	0.1µM	0.5
Reverse Primer (Eh)	10µM	0.1µM	0.5
Reverse Primer (Ed)	10µM	0.1µM	0.5
Reverse Primer (Em)	10µM	0.1µM	0.5

3.4.4.3 PCR Amplification Procedure for *Blastocyctis* spp

Amplification for *Blastocyctis* spp was conducted, targeting 7 subtypes previously reported by Yoshikawa et al., 2004. Table 3.3 shows sequence tagged site (STS) primer sets used for *Blastocyctis* spp amplification. Amplification for genomic sequences was performed by using 5µl of template DNA in total volume of 25µl reaction, which consist of 1X PCR buffer (Fermentas, USA). The cycling condition used was initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturing at 95 °C for 1 minute, annealing at 56.3°C for 1 minute 30 seconds, an extension at 72°C for 1minute, and a final extension at 72°C for 10 minute (Thermocycler Bio-Rad). The end products of amplification were then electrophoresed in 1.5% agarose gels (Promega, USA)

3.4.4.4 PCR Amplification Procedure for *Toxoplasma* spp

Nested PCR amplification was performed targeting B1 gene, which is conserved in *Toxoplasma gondii* for the detection of *Toxoplasma gondii* infections. Primer designed for the first amplification was outer primers B1F1 (5'-CCG TTG GTT CCG CCT CCT TC-3') and B1R1 (5'-GCA AAA CAG CGG CAG CGT CT-3'). Besides, primer designed for second amplification was internal primers B1F2 (5'-CCG CCT CCT TCG TCC GTC GT-3') and B1R2 (5'-GTG GGG GCG GAC CTC TCT TG-3') that corresponded to 213bp amplicon as end product (Puviarasi et al., 2010). Amplification was performed in a total volume of 25µl which consisted of 5µl of template DNA. Table 3.4 showed the master mix solution for PCR amplification with their components and concentration used. Five microliter of template DNA in amplification reaction mixture was replaced with deionized water and used as negative control to monitor cross contamination. The amplification was performed in thermal cycler (Eppendorf AG 22331, Hamburg, Germany). The cycling condition for amplification was 7 minutes at 94°C for initial denaturation, followed by 35 cycles whereby each cycle consist of

denaturation at 94°C for 60 seconds, annealing at 54°C for 30seconds, extension at 72°C for 60 seconds and final extension at 72°C for 7minutes. For second round of amplification, 4µl of end product of first amplification was used as template DNA in total volume of 25 µl under the same cycling conditions as in the first round, except for annealing at 60°C for 30 seconds, using internal primers. The amplified PCR product will be run on 2% agarose gel and compared with ladder DNA markers.

Table 3.4: PCR Master Mix Solution for *Toxoplasma gondii* spp

Components	Stock Concentration	Working Concentration	Volume (µL)/reaction
H2O (Deionized)			8.0
PCR Buffer	10X	1X	2.5
$MgCl_2$	25mM	6mM	6.0
dNTPs	40mM	200 µM each	0.5
Taq Polymerase	5U	0.5U	2.5
Forward Primer	10µM	0.1µM	0.25
Reverse Primer	10µM	0.1µM	0.25

3.4.5 Analysis of PCR Amplicons

3.4.5.1 Preparation of Agarose Gel

2% agarose gel was prepared by completely dissolving 2.1g agarose powder (Seakem, Germany) in 70 ml 1X Tris Borate EDTA (TBE) buffer and boiled in microwave oven until it melted and became colorless. Subsequently, 1.5 µl of 10 µg/ml ethidium bromide was added to the gel solution and swirled to mix well before pouring into a horizontal casting tray. Gel comb was used to form a row of 14 wells. After cooling and polymerization, the gel was loaded in a horizontal electrophoresis apparatus and submerged in buffer in a single chamber. Solution and reagent used for agarose gel electrophoresis are shown in Appendix II.

3.3.5.2 Gel Electrophoresis

Gel Electrophoresis system was used to resolve the PCR product or the amplicons. The agarose gel wells prepared earlier were loaded with samples to be analyzed. At each of the well, a mixture of 5µL of PCR product and 1µL of loading dye was loaded prior to the run in the horizontal electrophoresis system (Major Science, MP 300V). The electrophoresis system was run at constant voltage (100V and 400A) for 35 minutes at room temperature. The amplicons sizes were then compared with 100 bp DNA ladder (Fermentas International Inc, Ontario, CA) and the gel was then visualized using UV transilluminator (G-Biosciences, MO, USA) after the completion of gel electrophoresis system run.

3.4.6 DNA Sequencing

Purified PCR amplicons were subsequently sent to My TACG Bioscience Enterprise for sequencing service. About 20 µl of each purified PCR product was sent for sequencing with respective forward and reverse primers to aid bidirectional sequencing. The result was received in softcopy which was found to be compatible with a freeware known as *BioEdit* Sequence Alignment Editor to facilitate sequence analysis for the identification of respective target gene.

3.4.6.1 Analysis of DNA Sequencing

The sequences obtained from sequencing were aligned with sequence in GeneBank database using BLASTn program search analysis on the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/. Aligned Blastn sequences with highest percentage of identity, lowest E-value and highest maximum score were chosen.

3.4.6.2 Phylogeny Analysis

Phylogeny tree was aligned and constructed using the MEGA software program version 6.0.6 (Mega Software, Tempe, Arizona, USA). Phylogenetic relationships were analyzed by the neighbor-joining analysis from pairwise comparisons.

3.5 Analysis of Patient's Symptoms

Patients' demographic profile, relevant laboratory data, and clinical presentation of patient's data from the respective positive specimens for parasite were collected from medical record office, University of Malaya Medical Center (UMMC) to further correlate the findings to patients' symptoms with the identified parasite.

CHAPTER 4

RESULTS

4.1. Specimen Collections

A total of 238 cerebrospinal fluid specimens were received from the respective clinics at University Malaya Medical Centre (UMMC), collected from patients with some neurological symptoms. The condition of the specimen upon receipt was recorded and tabulated as shown below. The specimens were observed and recorded in four categories as; clear white, turbid white, slight yellowish fluids and blood stained. Present of mucus and bacterial contamination was also noted.

Table 4.1: Number and Conditions of the Specimens collected

The Conditions of Specimens	**Number of Specimens**
Blood stained	14
Clear white fluids	109
Turbid white fluids	52
Yellowish clear fluids	63
Total	**238**

4.2 Detection of Parasites by Conventional Diagnostic Approaches.

4.2.1 Identification by Wet Mount and Microscopy

Preliminary and conventional detection method on wet mounts under light microscopy revealed some parasite-like microorganisms. Three out of 238 of total specimens examined through wet mount examination for possible parasite detection, showed some microorganisms which resembled protozoan-like structures as tabulated (Table 4.2) below. In addition to that, there were also bacteria, debris, mucus and blood cells noted in some specimens. *Acanthamoeba*-like trophozoite (Fig 4.1) with acanthapodia –like projections was observed by direct microscopy. Besides, Figure 4.2 and Figure 4.3 show vacuolar (Fig 4.2) and granular (Fig 4.3) stage of *Blastocytis*-like organisms respectively.

Table 4.2: Type of Parasite-like Microorganisms Detected from direct microscopy

Types of Microorganism	**Number of Specimens**
Acanthamoeba spp	
Trophozoites form	1
Blastocytis hominis spp	
Vacuolar form	1
Granular from	1
Bacteria	21
Negative	214
Total	**238**

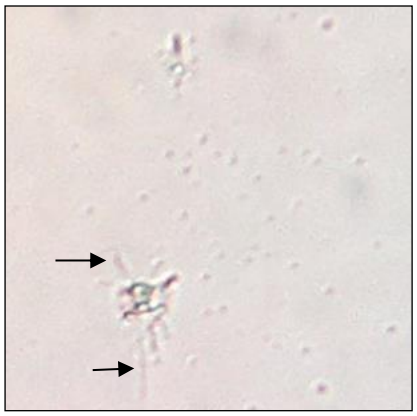


Figure 4.1: Wet mount examination under light microscopy (x400). Microorganism detected resembles trophozoite of *Acanthamoeba* spp. The arrow (→) shows acanthapodia-like projection observed in this microorganism.

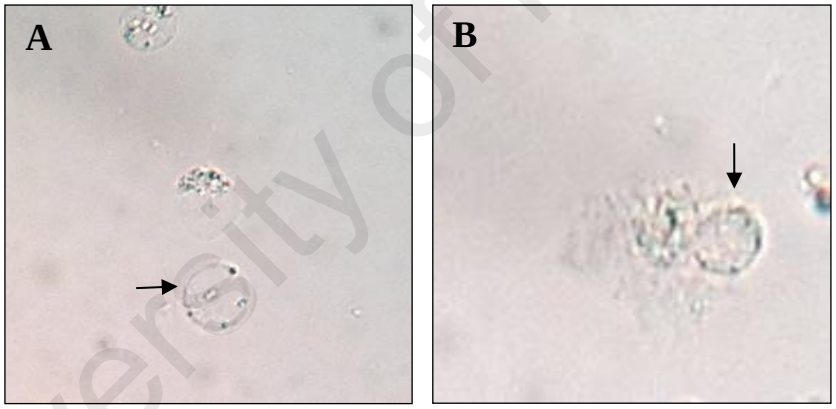


Figure 4.2: Wet mount examination under light microscopy (x400). Microorganism in A and B as shown by arrow (→) resembles vacuolar stage of *Blastocystis*-like microorganism.

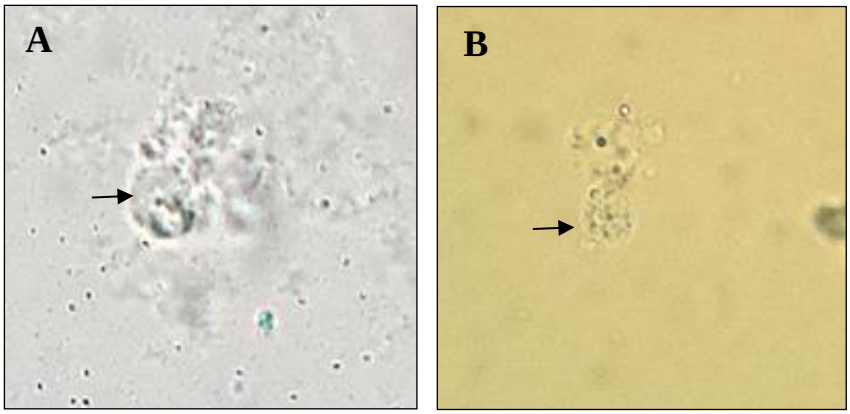


Figure 4.3: Wet mount examination under light microscopy (x400). Microorganism illustrated in A and B as shown by arrow (→) resembles granular stage of *Blastocystis*-like microorganism.

4.2.2 Identification by Staining Methods

Giemsa stain, Modified Field's stain and Modified Trichome stains were used in this study for identification of possible parasite present in specimens which could have remained undiagnosed. These stains are routinely used for identification of etiological agents in clinical samples. Staining procedures carried out in the study showed eight out of 238 specimens showing parasite-like microorganism (Table 4.3). Among that, two were observed to be *Acanthamoeba*-like microorganisms (Figure 4.4A) by Modified Field's stain. Another two were detected to be *Entamoeba*-like microorganisms (Figure 4.4B), two as spore-like microorganisms (Figure 4.4C) and the remaining two as *Blastocystis*-like microorganisms by Giemsa stain (Figure 4.4D). There was no parasite-like microorganism detected through Modified Trichome staining method.

Table 4.3: Types and Number of Parasite-like Microorganism Detected through Giemsa, Modified Field's and Modified Trichome stains

Type of Staining	**Types of microorganism**	**Number of specimens**
Modified Field's stain	*Acanthamoeba*-like microorganism	2
Giemsa stain	*Entamoeba*-like microorganism	2
	Blastocystis-like microorganism	2
	Spore-like microorganism	2
Modified Trichome stain	None	0
Negative		230
Total		**238**

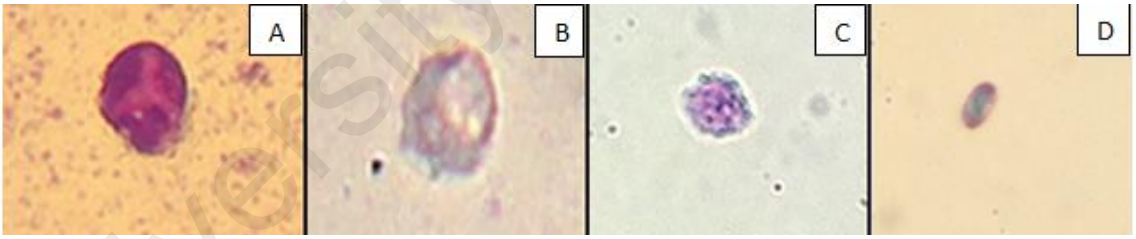


Figure 4.4: Parasite-like microorganisms detected through various conventional staining methods. Specimen stained with Modified Fields' stain shows *Acanthamoeba*-like microorganism (cyst) (Figure 4.4 A); Specimen stained with Giemsa stain showed *Entamoeba*-like microorganism (cyst) (Figure 4.4 B); Specimen stained with Giemsa stain showed *Blastocystis*-like microorganism (granular) (Figure 4.4 C); Specimen stain with Giemsa stain showed spore-like microorganism (Figure 4.4 D). All specimens examined under light microscopy (x400).

4.2.3 Identification by Culture Methods

In vitro culture with respective culture medium carried out to samples identified with some parasite-like microorganism through staining method shows no growth even after one week of culture.

4.3 Detection of Parasites by Molecular Diagnostic Approaches

Conventional and nested polymerase chain reactions (PCR) were carried out using species-specific primers that were conserved in targeting parasite for their identification by molecular diagnostic method.

4.3.1 Quantification of DNA Extracted from Cerebrospinal Fluids

DNA from cerebrospinal fluids was extracted using QIA amp DNA Mini and Blood Mini Kit (Qiagen). Extracted DNA was quantified using NanoDrop 2000 ultraviolet (UV) spectrophotometer and their concentration was as in table below (Table 4.4)

Table 4.4 Concentration of DNA extracted (ng/µL) from Cerebrospinal Fluids

Concentration of DNA extracted (ng/µL)	Number of Specimens
0.0-5.0	106
05.1- 10.0	71
10.1-15.0	26
15.1-20.0	16
20.1-25.0	11
25.1-30.0	8
Total	**238**

4.3.2 PCR and Gel Electrophoresis for Identification of *Acanthamoeba* spp Infections

Conventional PCR carried out targeting 161bp fragment of mitochondrial 16S rRNA gene of *Acanthamoeba* spp did not give rise to any band at expected base pair through gel electrophoresis analysis.

4.3.3 PCR and Gel Electrophoresis for Identification of *Entamoeba* spp Infections

Amplification was conducted for three different species of *Entamoeba* spp targeting fragment of small subunit ribosomal RNA (SSU rRNA) for the detection of *E. histolytica, E. dispar* and *E. moshkovskii.* However, the PCR amplicon did not show any band at the expected base pair size which was 166 bp for *E. histolytica, 580* bp for *E. moshkovskii* and 752 bp for *E. dispar* respectively through gel electrophoresis analysis.

4.3.4 PCR and Gel Electrophoresis for Identification of *Blastocystis* spp Infections

PCR Amplification were carried out for identification of *Blastocyctis* spp infections in CSF targeting 3 subtypes, including subtypes 2, 3 and subtypes 7 by sequence tagged site (STS) primer sets showed no significant band at the expected base pair through gel electrophoresis analysis of PCR amplicons.

4.3.5 PCR and Gel Electrophoresis for Identification of *Toxoplasma* spp Infections

Eleven out of 238 total specimens, showed band approximately at 213 base pair correspond to the ladder marker for the detection of *Toxoplasma gondii* infection targeting 35 fold repetitive B1 gene. Gel electrophoresis for genotyping *Toxoplasma gondii* infections was presented (Figure 4.5) below.

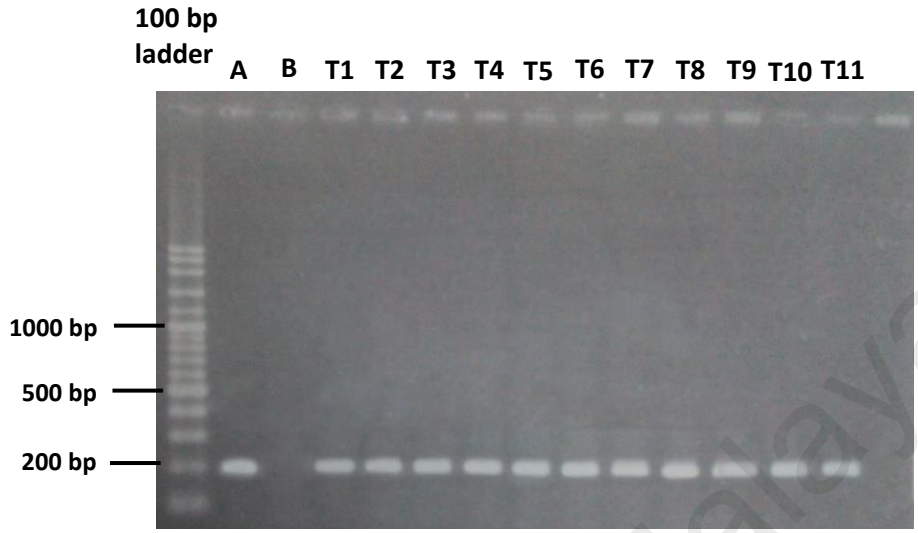


Figure 4.5: Gel electrophoresis for genotyping *Toxoplasma gondii* infections from CSF. Amplification of PCRs products for *Toxoplasma gondii* from specimen T1 (lanes 1), specimen T2 (lanes 2), specimen T3 (lanes 3), specimen T4 (lanes 4), specimen T5 (lanes 5), specimen T6 (lanes 6), specimen T7 (lanes 7), specimen T8 (lanes 8), specimen T9 (lanes 9), specimen T10 (lanes 10) and specimen T11 (lanes 11) and positive control (lanes A) show bands approximately at expected base pair 213bp. Lanes B shows negative control of reaction (master mix and distilled water replacing DNA template).

4.4 Sequencing Analysis

A total of eleven (T1-T11) specimens that showed the expected band for *Toxoplasma gondii* infection through gel electrophoresis were sent for sequencing. The sequences obtained from sequencing were aligned with sequence in GeneBank database using BLASTn program search analysis. Aligned BLASTn sequences with highest percentage of identity, lowest E-value and highest maximum score were tabulated (Table 4.5) below.

Table 4.5: Sequencing Analysis using BLASTn Software Analysis

Specimen	Parasite Aligned from BLASTn	Identity (%)	BLAST match Accession No
T1	*Toxoplasma gondii* B1 gene, partial sequence	98	AF179871.1
T2	*Toxoplasma gondii* B1 gene, partial sequence	99	EU340878.1
T3	*Toxoplasma gondii* B1 gene, partial sequence	100	EU340878.1
T4	*Toxoplasma gondii* B1 gene, partial sequence	98	AF179871.1
T5	*Toxoplasma gondii* B1 gene, partial sequence	99	EU340878.1
T6	*Toxoplasma gondii* B1 gene, partial sequence	100	EU340878.1
T7	*Toxoplasma gondii* B1 gene, partial sequence	100	EU340878.1
T8	*Toxoplasma gondii* B1 gene, partial sequence	100	EU340878.1
T9	*Toxoplasma gondii* B1 gene, partial sequence	100	EU340878.1
T10	*Toxoplasma gondii* B1 gene, partial sequence	99	AF179871.1
T11	*Toxoplasma gondii* B1 gene, partial sequence	100	EU340878.1

4.5 Phylogeny Analysis

The BLASTn analysis shows that all eleven specimens positive with *Toxoplasma gondii* showed high confident levels from 98% to 100%. However, certain variation in the identity from 98 to 100% was observed and phylogeny analysis was subsequently carried out to illustrate the pattern and the relationships that may present between the identified *Toxoplasma gondii* spp. Constructed phylogeny tree using the MEGA software program version 6.0.6 (Mega Software, Tempe, Arizona, USA) by neighbor-joining analysis was illustrated (Figure 4.6) as below.

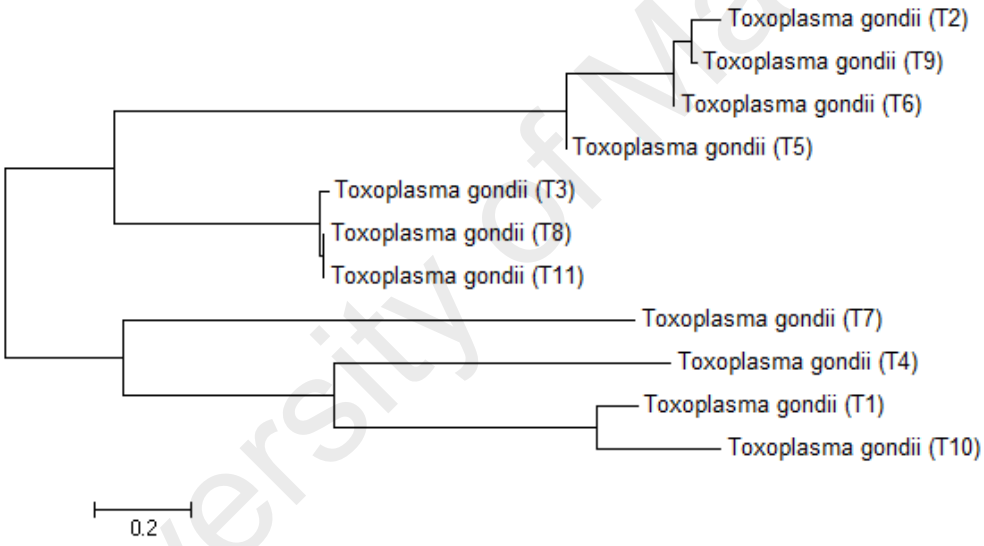


Figure 4.6: Phylogeny tree constructed by neighbor-joining analysis for *Toxoplasma gondii* positive specimens.

4.6 Analysis of Patient's Symptoms

Table 4.6: Analysis of Patient's Symptoms associated with *Toxoplasma gondii* infections.

Specimen	Age/ Gender	Past Medical History	CSF Analysis	CSF for Organism	Symptoms During Admission
T1	27/M	Nil	Elevated Leucocytes Elevated Protein	No Growth	Nil
T2	64/F	Diabetes Melitus Hypertension	Elevated Leucocytes Elevated Protein	No Growth	Found unconscious
T3	58/M	Nil	Elevated Leucocytes Elevated Protein Low Glucose	No Growth	Nil
T4	12/M	Primitive Neuroctodermal Tumour with Hydrocephalus	Elevated Leucocytes Elevated Protein		Fever, muscle weakness, seizure
T5	1+/M	Underlying premature hydrocephalus Chronic Lung Disease Retinopathy of Prematurity	Elevated Leucocytes Elevated Protein	No Growth	Fever and general weakness
T6	34/F	SLE with Lupus Nephritis on regular dialysis, chronic hepatitis C	Low Glucose	No Growth	High grade fever, right sided shoulder pain
T7	56/F	Diabetes Melitus Hypertension Surgical history of breast lumpectomy	Elevated Leucocytes Elevated Protein	No Growth	Slurred speech, Impaired memory, generalized body weakness
T8	3/M	Diagnosed for cerebello-pontine angle tumour	Nil	No Growth	Slurred speech, instability
T9	Nil	Nil	Nil	No Growth	Fever, right sided weakness, neck pain
T10	1+/F	Underlying Dandy Walker Syndrome, Congenital Hydrocephalus	Elevated Leucocytes Elevated Protein	No Growth	Vomiting, Fever, Seizure
T11	50/M	Underlying Hypertension, SLE with lupus nephritis		No Growth	Headache, fever, muscle weakness

CHAPTER 5

DISCUSSION

There is an increasing and significant rise in the numbers of immuno-compromised population globally (Ascioglu et al., 2002; Snydman et al., 2005). This population includes malnourished individuals, those with acquired or congenital immuno-deficiencies, wide range of group receiving immunosuppressive therapy and organ transplantation, individual with cardiovascular diseases and an ever growing list of groups with malignancy. In both immuno-competent and immuno-compromised individuals, infections of the central nervous system play an important role in exacerbating symptoms (Cunha, 2001). Diagnosing the etiologic cause among patients with certain neurological symptoms remains as one of challenging problem among physicians and healthcare professionals. Generally, most of the neurological symptoms mimic symptoms of meningitis and encephalitis caused by bacterial and viral infections. Therefore, the usual mode of diagnosis is from common specimens such as blood, urine and cerebrospinal fluids for the detection of possible etiological agents such as bacteria, virus, parasites and fungus. Often, screenings for parasitic infections from such samples are only conducted when requested by the doctor in certain cases. The present study was carried out to elucidate parasitic infections in patients with some neurological symptoms from cerebrospinal fluids (CSF). These samples were previously subjected to routine diagnosis test but proved negative for the usual etiologic pathogens.

A total of 238 cerebrospinal fluid (CSF) specimens were screened in this study and specimens were gross examined for their color and degree of turbidity upon arrival from respective clinic. Out of 238 specimens, 109 specimens showed clear CSF, 63 slightly yellowish, 52 were turbid and 14 specimens were blood stained. Normal cerebrospinal fluids (CSF) were clear and colorless. Specimens which are slightly yellowish, turbid and blood stained were considered abnormal and indicate the presence of bilirubin, hemoglobin, and red blood cells respectively. The turbid specimens indicate an increased number of cells in the specimen which signal a sign of infections (Majumdar et al., 2013; Wright et al., 2012). Thus about 54 % of the specimens examined in this study were abnormal due to some infections.

Microscopic and staining procedures are still employed as gold standard for diagnosis of infections involving central nervous, particularly with parasitic involvement (Mohapatra et al., 2014; Moody & Chiodini, 2000; Montoya, 2002). However, its sensitivity varies depending on observer's skill and expertise, which can give rise to false positive and false negative results (Montoya, 2002). In the present study, wet mount and microscopic examinations show some parasite-like microorganisms such as *Acanthamoeba*-like trophozoite and *Blastocytis*-like microorganism. However, cultivation of *Acanthamoeba*-like trophozoite and *Blastocytis*-like microorganism in non-nutrient agar coated with *E.coli* and Jones' medium respectively, did not show any growth. Thus, direct wet mount examination may show false positive results as evidenced by the negative molecular method identification. Besides that staining by Modified Fields' stain and Giemsa stain also showed some parasite-like microorganisms as illustrated (Figure 4.4), but failed to show positive results through cultivation and molecular method identification.

Detection by microscopy in some cases should correspond to verification through molecular methods. The application of polymerase chain reaction chain (PCR) assays to identify and detect parasites confer greater sensitivity in detecting parasites independent of the immuno-competence or previous clinical history of the patient, although it is technically more complex. This assay also can distinguish between organisms that are morphologically similar.

PCR based assays have also been reported previously to detect DNA of one parasite in clinical specimens (Weiss, 1995; Monis & Giglio, 2006). In this study, PCR was carried out to detected parasites that might causes symptoms related to CNS in patients including *Acanthamoeba* spp, *Entamoeba* spp, *Blastocystis* spp and *Toxoplasma gondii* infections. Amplicons obtained from PCR were subjected to gel electrophoresis. The gel electrophoresis show negative results for *Acanthamoeba* spp, *Entamoeba* spp, *Blastocystis* spp targeting gene. However the present study demonstrated 11 specimens (T1 to T11) at 213 base pair targeting B1 gene of *Toxoplasma gondii* through gel electrophoresis analysis which accounted for 4.6% of total specimens screened. Targeted B1 genes were 35 copies in the genome of *Toxoplasma gondii* with unknown function but these are mostly used for detection of *Toxoplasma gondii* with high specificity (Switaj, et al., 2005). B1 gene of *Toxoplasma gondii* genome was also used to detect *Toxoplasma gondii* infections in cerebrospinal fluids successfully.

To validate the PCR results of all 11 specimens (T1 to T11) that showed the expected band corresponding for *Toxoplasma gondii* infections, further studies were carried out using sequencing analysis. Sequences obtained from sequencing were aligned with sequences in GeneBank database using BLASTn program search analysis. Aligned BLASTn sequences showed the highest percentage of identity from 98% to 100% with lowest E-value and maximum score. All 11 specimens (T1 to T11) were aligned to *Toxoplasma gondii* B1 gene which was validated through gel electrophoresis analysis.

Phylogenetic analysis was carried out to illustrate the pattern or relationships that may be present between the identified *Toxoplasma gondii* spp. using the MEGA software program version 6.0.6 (Mega Software, Tempe, Arizona, USA) by neighbor-joining analysis. This analysis illustrated three distinct closely related groups (Figure 4.6). Firstly, *Toxoplasma gondii* from specimen T2, T9, T6 and T5 were closely related to each other. On the other hand *Toxoplasma gondii* from specimen T3, T8 and T11 were shown to be closely related and grouped together while *Toxoplasma gondii* from specimen T7, T4, T1 and T10 were shown to be far related from the first group.

The data collected from medical records at Medical Record Unit of University Malaya Medical Centre (UMMC), revealed most of the patients identified positive to *Toxoplasma gondii* infection can be grouped as immuno-compromised individual with wide range of age group from 1 to 64 years old. The patients were presented with history of underlying premature hydrocephalus showing chronic lung diseases, primitive neuroctodermal tumour and hydrocephalus, Systemic Lupus Erythematosus (SLE) with Lupus nephritis and chronic hepatitis C, cerebello-pontine angle tumour, underlying Dandy Walker Syndrome showing congenital hydrocephalus, diabetes mellitus and hypertension.

It has been reported that life-threatening condition of toxoplasmosis is more commonly found in immuno-compromised patients, affecting particularly the brain (Dupon et al., 1995). *Toxoplasma gondii* infection is recognized as one of more common opportunist protozoan parasite that manifests a wide range of clinical symptoms (Jones et al., 2014; Dubey, 2008). *Toxoplasma gondii* infection is a well known acquired disease sometimes from the hospital (Ibebuike et al., 2012). In addition to that, *Toxoplasma gondii* infection has also been described to be acquired by latent reactivation in immuno-compromised patients, congenitally and during pregnancy (Dubey, 2008). High prevalence of latent disseminated toxoplasma observed in immunosuppressive patient has been due to adaption manipulating the innate immune system, despite the arrival of leucocytes and other cells of the innate system (Pollard et al., 2009).

Data collected for cerebrospinal fluid analysis of patients positive for *Toxoplasma gondii* infection showed elevated leucocytes and protein levels in seven patients. Furthermore, some patients also showed low levels of glucose. Generally, individuals presenting neurological symptoms with raised leucocytes and plasma protein level, have been postulated to have bacterial meningitis, viral meningitis, subarachnoid hemorrhage or even as multiple sclerosis in clinical setting to diagnosing possible etiology agent (Scheld et al., 2004).

However, elevated plasma leucocytes and protein can be associated with parasitic infections that might be concurrent with other clinical complication seen in immuno-compromised and immuno-competent hosts. Previously, Maclean et al. (2001) reported plasma white blood cell (WBC) count and significantly increased levels of plasma protein in trypanosomiasis at early and late stage of prognosis of disease. Low glucose levels in patients with neurological signs may be associated with multiple infections including bacterial and viral meningitis (Scheld et al., 2004).

In this study, the symptoms of patients with *Toxoplasma gondii* infections includes fever, muscle weakness, seizures, high grade fever and right sided shoulder pain, slurred speech, impaired memory, generalized body weakness, neck pain and headache. Most of these symptoms are similar to common symptoms of neurological diseases including diseases causes by bacteria and viral. Thus, it is confusing to determine or conclude the diagnosis by symptoms only.

To date, data collected from patient's medical record showed negative for the diagnosis of toxoplasmosis despite being subjected to routine follow up, providing evidence that unless doctors make formal request technicians seldom or never screen as a routine diagnosis despite. Toxoplasmosis was being identified as one of major neglected parasitic infection in USA affecting significantly the countries' economic and healthcare management (Jones et al., 2014; Hotez, 2012).

Besides that, a good clinical management should implement for earlier diagnosis and better treatment on time for patients with neurological symptoms. Urgent hospital admission is vital for better treatment and cure. Moreover in certain severe cases, intensive care, including ventilation may necessary to reduce brain swelling. In addition to that, intravenous broad-spectrum antibiotics may be given to treat secondary bacterial infections among the patients. However, this intravenous fluids need to be given very carefully in order not to aggravate cerebral oedema. Treatments including anticonvulsants and sedatives may consider for patients to reduce agitation.

CHAPTER 6

CONCLUSION

Prolonged neurological symptoms present in immuno-compromised and immuno-competent patients can be due to parasitic infections which sometimes can not be diagnosed leading to the worsening of the complication. Often routine diagnosis may not capture the entire scope of diseases. Therefore, the present study was carried out to assess the occurrence of parasitic infection from cerebrospinal fluids in patients with some neurological symptoms which were already proven negative when diagnosed previously. The screening was conducted using conventional microscopy and polymerase chain reaction (PCR) with nested PCR.

Although conventional staining methods showed negative results, the sensitive polymerase chain reaction (PCR) enabled the detection of *Toxoplasma gondii* infections in patients with some neurological symptoms despite showing negative results for parasitic infections previously. Thus, detection of parasite infections by molecular methods should be considered and implemented at the preliminary stage when neurological symptoms are seen remain undiagnosed to avoid further complications.

BIBLIOGRAPHY

Abbott, N. J. (2005). Dynamics of CNS barriers: evolution, differentiation, and modulation. *Cellular and Molecular Neurobiology, 25*(1), 5-23.

Adams, E., & MacLeod, I. (1977). II. Amebic Liver Abscess and its Complications. *Medicine, 56*(4), 325-334.

Afzan, M. Y., Sivanandam, S., & Kumar, G. S. (2010). Modified Field's staining a rapid stain for *Trichomonas vaginalis*. *Diagnostic Microbiology and Infectious Disease, 68*(2), 159-162.

Allason-Jones, E., Mindel, A., Sargeaunt, P., & Williams, P. (1986). *Entamoeba histolytica* as a commensal intestinal parasite in homosexual men. *New England Journal of Medicine, 315*(6), 353-356.

Andrade, L. O., & Andrews, N. W. (2005). The *Trypanosoma cruzi*–host-cell interplay: location, invasion, retention. *Nature Reviews Microbiology, 3*(10), 819-823

Anuar, T. S., Al-Mekhlafi, H. M., Ghani, M. K. A., Osman, E., Yasin, A. M., Nordin, A., . . . Bernadus, M. (2012). Prevalence and risk factors associated with *Entamoeba histolytica*/*dispar*/*moshkovskii* infection among three Orang Asli ethnic groups in Malaysia. *PLoS One, 7*(10), e48165.

Ascioglu, S., Rex, J., De Pauw, B., Bennett, J., Bille, J., Crokaert, F., . . . Erjavec, Z. (2002). Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. *Clinical Infectious Diseases, 34*(1), 7-14.

Banks, W. A., & Erickson, M. A. (2010). The blood–brain barrier and immune function and dysfunction. *Neurobiology of Disease, 37*(1), 26-32.

Bergquist, N., Stintzing, G., Smedman, L., Waller, T., & Andersson, T. (1984). Diagnosis of encephalitozoonosis in man by serological tests. *British Medical Journal (Clinical research ed.), 288*(6421), 902.

Bern, C., Kjos, S., Yabsley, M. J., & Montgomery, S. P. (2011). *Trypanosoma cruzi* and Chagas' disease in the United States. *Clinical Microbiology Reviews, 24*(4), 655-681.

Bigliardi, E., & Sacchi, L. (2001). Cell biology and invasion of the microsporidia. *Microbes and Infection, 3*(5), 373-379. doi: http://dx.doi.org/10.1016/S1286-4579(01)01393-4

Black, M. W., & Boothroyd, J. C. (2000). Lytic cycle of *Toxoplasma gondii*. *Microbiology and Molecular Biology Reviews, 64*(3), 607-623.

Blader, I. J., & Saeij, J. P. (2009). Communication between *Toxoplasma gondii* and its host: impact on parasite growth, development, immune evasion, and virulence. *Apmis*, *117*(5-6), 458-476.

Castro, A., Luquetti, A., Rassi, A., Rassi, G., Chiari, E., & Galvão, L. (2002). Blood culture and polymerase chain reaction for the diagnosis of the chronic phase of human infection with *Trypanosoma cruzi*. *Parasitology research*, *88*(10), 894-900.

Chong, H.T., & Tan, C.T. (2005). Epidemiology of central nervous system infections in Asia, recent trends. *Neurology Asia*, *10*, 7 – 11.

Cinque, P., Scarpellini, P., Vago, L., Linde, A., & Lazzarin, A. (1997). Diagnosis of central nervous system complications in HIV-infected patients: cerebrospinal fluid analysis by the polymerase chain reaction. *Aids*, *11*(1), 1-17.

Conteas, C., Berlin, O., Ash, L., & Pruthi, J. (2000). Therapy for human gastrointestinal microsporidiosis. *The American Journal of Tropical Medicine and Hygiene*, *63*(3), 121-127.

Cunha, B. A. (2001). Central nervous system infections in the compromised host: a diagnostic approach. *Infectious Disease Clinics of North America*, *15*(2), 567-590.

Dascomb, K., Frazer, T., Clark, R. A., Kissinger, P., & Didier, E. (2000). Microsporidiosis and HIV. *JAIDS Journal of Acquired Immune Deficiency Syndromes*, *24*(3), 290-292.

Deisenhammer, F., Bartos, A., Egg, R., Gilhus, N., Giovannoni, G., Rauer, S., & Sellebjerg, F. (2006). Guidelines on routine cerebrospinal fluid analysis. Report from an EFNS task force. *European Journal of Neurology*, *13*(9), 913-922.

De Souza, W., de Carvalho, T. M. U., & Barrias, E. S. (2010). Review on *Trypanosoma cruzi*: host cell interaction. *International Journal of Cell Biology*, *2010*.

Desportes, I., Charpentier, Y. L., Galian, A., Bernard, F., Cochand-Priollet, B., Lavergne, A., . . . Modigliani, R. (1985). Occurrence of a New Microsporidan: *Enterocytozoon bieneusi* ng, n. sp., in the Enterocytes of a Human Patient with AIDS1. *The Journal of Protozoology*, *32*(2), 250-254.

Didier, E., Stovall, M., Green, L., Brindley, P., Sestak, K., & Didier, P. (2004). Epidemiology of microsporidiosis: sources and modes of transmission. *Veterinary Parasitology*, *126*(1), 145-166.

Didier, E. S. (2005). Microsporidiosis: an emerging and opportunistic infection in humans and animals. *Acta Tropica*, *94*(1), 61-76.

Didier, E. S., & Weiss, L. M. (2006). Microsporidiosis: current status. *Current Opinion in Infectious Diseases*, *19*(5), 485.

Dubey, J. (2009). History of the discovery of the life cycle of *Toxoplasma gondii*. *International Journal for Parasitology*, *39*(8), 877-882.

Dubey, J., & Jones, J. (2008). *Toxoplasma gondii* infection in humans and animals in the United States. *International Journal for Parasitology, 38*(11), 1257-1278.

Dubey, J. P. (2008). The history of *Toxoplasma gondii*—the first 100 years. *Journal of Eukaryotic Microbiology, 55*(6), 467-475.

Dubey, J., Lindsay, D., & Speer, C. (1998). Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. *Clinical Microbiology Reviews, 11*(2), 267-299.

Dunbar, S. A., Eason, R. A., Musher, D. M., & Clarridge, J. E. (1998). Microscopic examination and broth culture of cerebrospinal fluid in diagnosis of meningitis. *Journal of Clinical Microbiology, 36*(6), 1617-1620.

Dunn, A. M., & Smith, J. E. (2001). Microsporidian life cycles and diversity: the relationship between virulence and transmission. *Microbes and Infection, 3*(5), 381-388.

Dupon, M., Cazenave, J., Pellegrin, J.-L., Ragnaud, J.-M., Cheyrou, A., Fischer, I., . . . Lacut, J.-Y. (1995). Detection of *Toxoplasma gondii* by PCR and tissue culture in cerebrospinal fluid and blood of human immunodeficiency virus-seropositive patients. *Journal of Clinical Microbiology, 33*(9), 2421-2426.

Estani, S. S., & Segura, E. L. (1999). Treatment of *Trypanosoma cruzi* infection in the undetermined phase. Experience and current guidelines of treatment in Argentina. *Memórias do Instituto Oswaldo Cruz, 94*, 363-365.

Fotedar, R., Stark, D., Beebe, N., Marriott, D., Ellis, J., & Harkness, J. (2007). PCR detection of *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii* in stool samples from Sydney, Australia. *Journal of Clinical Microbiology, 45*(3), 1035-1037.

Franzen, C. (2004). Microsporidia: how can they invade other cells? *TRENDS in Parasitology, 20*(6), 275-279.

Franzen, C., & Müller, A. (2001). Microsporidiosis: human diseases and diagnosis. *Microbes and Infection, 3*(5), 389-400.

Garcia, L. S. (2002). Laboratory identification of the microsporidia. *Journal of Clinical Microbiology, 40*(6), 1892-1901.

Gathiram, V., & Jackson, T. (1987). A longitudinal study of asymptomatic carriers of pathogenic zymodemes of *Entamoeba histolytica*. *South African Medical Journal, 72*(10), 669-672.

Gatti, S., Rama, P., Matuska, S., Berrilli, F., Cavallero, A., Carletti, S., . . . Di Cave, D. (2010). Isolation and genotyping of *Acanthamoeba* strains from corneal infections in Italy. *Journal of Medical Microbiology, 59*(11), 1324-1330.

Goh, L., & Marrone, J. (2013). *Entamoeba histolytica* meningoencephalitis diagnosed by trophozoites in cerebrospinal fluid. *New Microbes and New Infections, 1*(1), 16-17.

Goldstein, E. J., Montoya, J. G., & Remington, J. S. (2008). Management of *Toxoplasma gondii* infection during pregnancy. *Clinical Infectious Diseases, 47*(4), 554-566.

Gonzalez-Ruiz, A., Haque, R., Aguirre, A., Castanon, G., Hall, A., Guhl, F., . . . Warhurst, D. (1994). Value of microscopy in the diagnosis of dysentery associated with invasive *Entamoeba histolytica*. *Journal of Clinical Pathology, 47*(3), 236-239.

Hamzah, Z., Petmitr, S., Mungthin, M., Leelayoova, S., & Chavalitshewinkoon-Petmitr, P. (2006). Differential detection of *Entamoeba histolytica, Entamoeba dispar*, and *Entamoeba moshkovskii* by a single-round PCR assay. *Journal of Clinical Microbiology, 44*(9), 3196-3200.

Haque, R., Ali, I. M., Sack, R. B., Farr, B. M., Ramakrishnan, G., & Petri, W. A. (2001). Amebiasis and mucosal IgA antibody against the *Entamoeba histolytica* adherence lectin in Bangladeshi children. *Journal of Infectious Diseases, 183*(12), 1787-1793.

Helton, J., Loveless, M., & White Jr, C. R. (1993). Cutaneous acanthamoeba infection associated with leukocytoclastic vasculitis in an AIDS patient. *The American Journal of Dermatopathology, 15*(2), 146-149.

Hermann, E., Truyens, C., Vega, C. A., Rodriguez, P., Berthe, A., Torrico, F., & Carlier, Y. (2004). Congenital transmission of *Trypanosoma cruzi* is associated with maternal enhanced parasitemia and decreased production of interferon-γ in response to parasite antigens. *Journal of Infectious Diseases, 189*(7), 1274-1281.

Hill, D., & Dubey, J. (2002). *Toxoplasma gondii*: transmission, diagnosis and prevention. *Clinical Microbiology and Infection, 8*(10), 634-640.

Hill, D. E., Chirukandoth, S., & Dubey, J. (2005). Biology and epidemiology of *Toxoplasma gondii* in man and animals. *Animal Health Research Reviews, 6*(01), 41-61.

Hotez, P. J. (2008). Neglected infections of poverty in the United States of America. *PLoS Neglected Tropical Diseases, 2*(6), e256.

Hotez, P. J. (2012). Fighting neglected tropical diseases in the southern United States. *BMJ-British Medical Journal, 345*(7875), 10.

Hung, C.-C., Chen, P.-J., Hsieh, S.-M., Wong, J.-M., Fang, C.-T., Chang, S.-C., & Chen, M.-Y. (1999). Invasive amoebiasis: an emerging parasitic disease in patients infected with HIV in an area endemic for amoebic infection. *Aids, 13*(17), 2421-2428.

Hung, C.-C., Ji, D.-D., Sun, H.-Y., Lee, Y.-T., Hsu, S.-Y., Chang, S.-Y., . . . Liu, W.-C. (2008). Increased risk for *Entamoeba histolytica* infection and invasive amebiasis in HIV seropositive men who have sex with men in Taiwan. *PLoS Neglected Tropical Diseases, 2*(2), e175.

Hung, C.-C., Chang, S.-Y., & Ji, D.-D. (2012). *Entamoeba histolytica* infection in men who have sex with men. *The Lancet Infectious Diseases, 12*(9), 729-736.

Hutin, Y. J., Sombardier, M.-N., Liguory, O., Sarfati, C., Derouin, F., Modaï, J., & Molina, J.-M. (1998). Risk factors for intestinal microsporidiosis in patients with human immunodeficiency virus infection: a case-control study. *Journal of Infectious Diseases, 178*(3), 904-907.

Ibebuike, K., Mantanga, L., Emereole, O., Ndolo, P., Kajee, A., Gopal, R., & Pather, S. (2012). Cerebellar toxoplasmosis in HIV/AIDS infant: case report and review of the literature. *Neurological Sciences, 33*(6), 1423-1428.

Irusen, E., Jackson, T., & Simjee, A. (1992). Asymptomatic intestinal colonization by pathogenic *Entamoeba histolytica* in amebic liver abscess: prevalence, response to therapy, and pathogenic potential. *Clinical Infectious Diseases, 14*(4), 889-893.

Ithoi, I. (2011). Morphological characteristics of developmental stages of *Acanthamoeba* and *Naegleria* species before and after staining by various techniques. *Southeast Asian Journal of Tropical Medicine and Public Health, 42*(6), 1327-1338

Jones JL, Parise ME, Fiore AE: Neglected Parasitic Infections in the United States: Toxoplasmosis. *The American Journal of Tropical Medicine and Hygiene* 2014, 90(5): 794-799.

Kao, P.-M., Hsu, B.-M., Chen, N.-H., Huang, K.-H., Huang, S.-W., King, K.-L., & Chiu, Y.-C. (2012). Isolation and identification of *Acanthamoeba* species from thermal spring environments in southern Taiwan. *Experimental Parasitology, 130*(4), 354-358.

Keeling, P. J., & Fast, N. M. (2002). Microsporidia: biology and evolution of highly reduced intracellular parasites. *Annual Reviews in Microbiology, 56*(1), 93-116.

Khan, N. A. (2006). *Acanthamoeba*: biology and increasing importance in human health. *FEMS Microbiology Reviews, 30*(4), 564-595.

Khan, N. A. (2007). *Acanthamoeba* invasion of the central nervous system. *International Journal for Parasitology, 37*(2), 131-138.

Li, X., Palmer, R., Trout, J., & Fayer, R. (2003). Infectivity of microsporidia spores stored in water at environmental temperatures. *Journal of Parasitology, 89*(1), 185-188.

Lowther, S. A., Dworkin, M. S., & Hanson, D. L. (2000). *Entamoeba histolytica*/*Entamoeba dispar* infections in human immunodeficiency virus-infected patients in the United States. *Clinical Infectious Diseases, 30*(6), 955-959.

MacLean, L., Odiit, M., & Sternberg, J. M. (2001). Nitric oxide and cytokine synthesis in human African trypanosomiasis. *Journal of Infectious Diseases, 184*(8), 1086-1090.

Mahmud, R., Ibrahim, J., Moktar, N., & Anuar, T.-S. (2013). *Entamoeba histolytica* in Southeast Asia *Parasites and their Vectors* (pp. 103-129): Springer.

Majumdar, A., Jana, A., Jana, A., Biswas, S., Bhatacharyya, S., & Bannerjee, S. (2013). Importance of normal values of CSF parameters in term versus preterm neonates. *Journal of Clinical Neonatology, 2*(4), 166.

Marciano-Cabral, F., & Cabral, G. (2003). *Acanthamoeba* spp. as agents of disease in humans. *Clinical Microbiology Reviews, 16*(2), 273-307.

Masocha, W., & Kristensson, K. (2012). Passage of parasites across the blood-brain barrier. *Virulence, 3*(2), 202-212.

Medeiros BCD, Werner B, Loddo G, Pasquini R, Bleggi-Torres LF: Disseminated toxoplasmosis after bone marrow transplantation: report of 9 cases. *Transplant Infectious Diseases* 2001, 3(1): 24-28.

Minning, T. A., Weatherly, D. B., Atwood, J., Orlando, R., & Tarleton, R. L. (2009). The steady-state transcriptome of the four major life-cycle stages of *Trypanosoma cruzi*. *BMC Genomics, 10*(1), 370.

Mohapatra, S., Sharma, D., Gupta, K., Deb, M., & Gaind, R. (2014). A simple and rapid staining method for detection of hemozoin pigment by methylene blue stain. *Tropical Parasitology, 4*(1), 59.

Molina, J.-M., Tourneur, M., Sarfati, C., Chevret, S., de Gouvello, A., Gobert, J.-G., . . . Derouin, F. (2002). Fumagillin treatment of intestinal microsporidiosis. *New England Journal of Medicine, 346*(25), 1963-1969.

Monis, P. T., & Giglio, S. (2006). Nucleic acid amplification-based techniques for pathogen detection and identification. *Infection, Genetics and Evolution, 6*(1), 2-12.

Montoya, JG: Laboratory Diagnosis of *Toxoplasma gondii* Infection and Toxoplasmosis. *The Journal of Infectious Diseases* 2002, 185(1):S73–82.

Moody, A., & Chiodini, P. (2000). Methods for the detection of blood parasites. *Clinical & Laboratory Haematology, 22*(4), 189-201.

Ngui, R., Angal, L., Fakhrurrazi, S. A., Lian, Y., Ling, L. Y., Ibrahim, J., & Mahmud, R. (2012). Differentiating *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii* using nested polymerase chain reaction (PCR) in rural communities in Malaysia. *Parasit Vectors, 5*, 187.

Nicolle, C., & Manceaux, L. (1908). Sur une infection à corps de Leishman (ou organismes voisins) du gondi. *CR Acad Sci, 147*(763).

Nobrega, A. A., Garcia, M. H., Tatto, E., Obara, M. T., Costa, E., Sobel, J., & Araujo, W. N. (2009). Oral transmission of Chagas disease by consumption of açaí palm fruit, Brazil. *Emerging Infectious Diseases, 15*(4), 653.

Nozaki, T., Motta, S. R., Takeuchi, T., Kobayashi, S., & Sargeaunt, P. G. (1989). Pathogenic zymodemes of *Entamoeba histolytica* in Japanese male homosexual population. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 83*(4), 525.

Oh, M.-d., Lee, K., Kim, E., Lee, S., Kim, N., Choi, H., . . . Choe, K. (2000). Amoebic liver abscess in HIV-infected patients. *Aids, 14*(12), 1872-1873.

Ohnishi, K., Kato, Y., Imamura, A., Fukayama, M., Tsunoda, T., Sakaue, Y., . . . Sagara, H. (2004). Present characteristics of symptomatic *Entamoeba histolytica* infection in the big cities of Japan. *Epidemiology and Infection, 132*(01), 57-60.

Pillai, D. R., Keystone, J. S., Sheppard, D. C., MacLean, J. D., MacPherson, D. W., & Kain, K. C. (1999*). Entamoeba histolytica* and *Entamoeba dispar*: epidemiology and comparison of diagnostic methods in a setting of nonendemicity. *Clinical Infectious Diseases, 29*(5), 1315-1318.

Pirehma, M., Suresh, K., Sivanandam, S., Anuar, A. K., Ramakrishnan, K., & Kumar, G. S. (1999). Field's stain–a rapid staining method for *Acanthamoeba* spp. *Parasitology Research, 85*(10), 791-793.

Pollard, A. M., Knoll, L. J., & Mordue, D. G. (2009). The role of specific *Toxoplasma gondii* molecules in manipulation of innate immunity. *Trends in Parasitology, 25*(11), 491-494.

Puviarasi ,M., P., Singh, S., Ling, L. Y., Singh, J., Subrayan, V., & Nissapatorn, V. (2010). Detection of *Toxoplasma gondii* DNA by PCR following microwave treatment of serum and whole blood. *Southeast Asian Journal of Tropical Medicine and Public Health, 41*(2), 265-273.

Rosenblatt, J. E., Reller, L. B., & Weinstein, M. P. (2009). Laboratory diagnosis of infections due to blood and tissue parasites. *Clinical Infectious Diseases, 49*(7), 1103-1108.

Sargeaunt, P., Williams, J., Kumate, J., & Jimenez, E. (1980). The epidemiology of *Entamoeba histolytica* in Mexico City. A pilot survey I. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 74*(5), 653-656.

Scheld WM, Whitley RJ, Marra CM: Infections of the Central Nervous System. 3rd Edition. Lippincott Williams & Wilkins, 2004: Available from :http://books.google.com.my/

Sharma, M., & Ahuja, V. (2003). Amebiasis. *The New England Journal of Medicine, 349*(3), 307.

Siddiqui, R., & Khan, N. A. (2012). Biology and pathogenesis of *Acanthamoeba*. *Parasit Vectors, 5*(6), 262.

Smith, J. E. (1995). A ubiquitous intracellular parasite: The cellular biology of *Toxoplasma gondii*. *International Journal for Parasitology, 25*(11), 1301-1309.

Snydman, D. R., Walker, M., & Zunt, J. R. (2005). Parasitic central nervous system infections in immunocompromised hosts. *Clinical Infectious Diseases, 40*(7), 1005-1015.

Stark, D. J., Fotedar, R., Ellis, J. T., & Harkness, J. L. (2006). Locally acquired infection with *Entamoeba histolytica* in men who have sex with men in Australia. *The Medical Journal of Australia, 185*(8), 417.

Strachan, W., Spice, W., Chiodini, P., Moody, A., & Ackers, J. (1988). Immunological differentiation of pathogenic and non-pathogenic isolates of *Entamoeba histolytica*. *The Lancet, 331*(8585), 561-563.

Switaj, K., Master, A., Skrzypczak, M., & Zaborowski, P. (2005). Recent trends in molecular diagnostics for *Toxoplasma gondii* infections. *Clinical Microbiology and Infection, 11*(3), 170-176.

Takahashi, T., Gamboa-Dominguez, A., Gomez-Mendez, T. J., Remes, J. M., Rembis, V., Martinez-Gonzalez, D., . . . Sierra-Madero, J. (1997). Fulminant amebic colitis. *Diseases of the Colon & Rectum, 40*(11), 1362-1367.

Takeuchi, T., Miyahira, Y., Kobayashi, S., Nozaki, T., Motta, S. R., & Matsuda, J. (1990). High seropositivity for *Entamoeba histolytica* infection in Japanese homosexual men: further evidence for the occurrence of pathogenic strains. *Transactions of the Royal Society of Tropical Medicine and Hygiene, 84*(2), 250-251.

Tan, T., Suresh, K., & Smith, H. (2008). Phenotypic and genotypic characterisation of *Blastocystis hominis* isolates implicates subtype 3 as a subtype with pathogenic potential. *Parasitology Research, 104*(1), 85-93.

Tanyuksel, M., & Petri, W. A. (2003). Laboratory diagnosis of amebiasis. *Clinical Microbiology Reviews, 16*(4), 713-729.

Tengku, S., & Norhayati, M. (2011). Review Paper Public health and clinical importance of amoebiasis in Malaysia: A review. *Tropical Biomedicine, 28*(2), 194-222.

Tenter, A. M., Heckeroth, A. R., & Weiss, L. M. (2000). *Toxoplasma gondii*: from animals to humans. *International Journal for Parasitology, 30*(12), 1217-1258.

Thurston-Enriquez, J. A., Watt, P., Dowd, S. E., Enriquez, R., Pepper, I. L., & Gerba, C. P. (2002). Detection of protozoan parasites and microsporidia in irrigation waters used for crop production. *Journal of Food Protection, 65*(2), 378-382.

Townsend, J. J., Wolinsky, J. S., Baringer, J. R., & Johnson, P. C. (1975). Acquired toxoplasmosis: a neglected cause of treatable nervous system disease. *Archives of Neurology, 32*(5), 335-343.

Visvesvara, G. S., Moura, H., & Schuster, F. L. (2007). Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. *FEMS Immunology & Medical Microbiology, 50*(1), 1-26.

Volkonsky, M. (1931). *" Hartmannella Castellanii" Douglas et classification des Hartmannelles (" Hartmanellinae" nov. subfam.," Acanthamoeba" nov. gen.," Glaeseria" nov. gen.), par M. Volkonsky*: H. Le Soudier.

Yagi, S., Schuster, F. L., & Bloch, K. (2007). Demonstration of presence of Acanthamoeba mitochondrial DNA in brain tissue and cerebrospinal fluid by PCR in samples from a patient who died of granulomatous amebic encephalitis. *Journal of Clinical Microbiology, 45*(6), 2090-2091.

Yoshikawa, H., Wu, Z., Kimata, I., Iseki, M., Ali, I. K. M., Hossain, M. B., . . . Takahashi, Y. (2004). Polymerase chain reaction-based genotype classification among human *Blastocystis hominis* populations isolated from different countries. *Parasitology Research, 92*(1), 22-29.

Walker, M., & Zunt, J. (2005). *Neuroparasitic infections: cestodes, trematodes, and protozoans.* Paper presented at the Seminars in neurology.

Weber, R., & Bryan, R. T. (1994a). Microsporidial infections in immunodeficient and immunocompetent patients. *Clinical Infectious Diseases, 19*(3), 517-521.

Weber, R., Bryan, R. T., Schwartz, D. A., & Owen, R. L. (1994b). Human microsporidial infections. *Clinical Microbiology Reviews, 7*(4), 426.

Weber, R., Deplazes, P., & Schwartz, D. (2004). Diagnosis and clinical aspects of human microsporidiosis.

Weiss, L. M. (2001). Microsporidia: emerging pathogenic protists. *Acta Tropica, 78*(2), 89-102.

Weiss, J. B. (1995). DNA probes and PCR for diagnosis of parasitic infections. *Clinical Microbiology Reviews, 8*(1), 113-130.

Wright, B. L., Lai, J. T., & Sinclair, A. J. (2012). Cerebrospinal fluid and lumbar puncture: a practical review. *Journal of Neurology, 259*(8), 1530-1545.