PHYLOGENETIC STUDY OF *ACANTHAMOEBA* ISOLATED FROM STRAY ANIMAL CORNEAS (CATS & DOGS) AND ENVIRONMENTAL SAMPLES

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ABSTRACT

Acanthamoeba is a ubiquitous free-living protozoa, inhabitants in all environment worldwide. *Acanthamoeba* infection caused serious diseases of granulomatous amoebic encephalitis (GAE) and acanthamoeba keratitis (AK). This study was to determine the occurrence of *Acanthamoeba* genotypes in naturally infected corneas of stray cats and dogs and environmental samples. Infected stray cats and dogs from Animal Welfare Society (PAWS) and alleyways (cats only) were identified of having eye infection by the signs of watery eyes, grey discharge and redness. While from the environment, samples were collected from animal bedding, food containers and drinking containers of animals in PAWS, recreational rivers (water, wet debris, soil), children's playground (soil) and University of Malaya Medical Faculty (indoor and outdoor dust). All samples were subjected to cultivation on non-nutrient agar lawned with *Escherichia coli* followed by PCR, cloning, sequencing and phylogenetic analysis.

Positive culture showed *Acanthamoeba* trophozoites with spike-like acanthopodia and cysts with wrinkled double thick walled which were detected as early as 2nd and 5th day, respectively. The occurrence of cultivable *Acanthamoeba* from corneal samples of both stray cats and dogs was 24.8% (56/225), in which 24% (48/200) in cats and 32% (8/25) in dogs. While from environmental samples, *Acanthamoeba* was detected at a higher prevalence of 85% (136/160).

Phylogenetic analysis revealed that *Acanthamoeba* isolates in corneas of stray cats and dogs were assemblage under *A. castellanii* (U07401) genotype T4 with two homologous groups with different bases. For environmental samples, three genotypes were detected (T4, T5 and T15) in PAWS environment, four genotypes (T3, T4, T5 and T15) in recreational rivers, one genotype in dry soil of playgrounds (T4) and two in dust at University of Malaya Medical Faculty (T4 and T15).

Of the four genotypes (T3, T4, T5 and T15) detected in this study, T4 was found to be the most predominant in infected corneal swabs, soil and dust samples. While genotype T5 was predominant in water samples. Furthermore, genotype T4 was well known to be the main genotype, followed by T3, T5 and T15 in causing human diseases of AK and GAE. The presence of these four genotypes in the environment (water, soil, wet debris and dust) may contribute to *Acanthamoeba* infection, especially in contact lens wearers and immunocompromised subjects. Rubbing eyes after entering of contaminated soil/dust particles and water during daily activities could lead to eye abrasion, contributing to a high risk of acquiring AK. Furthermore, *Acanthamoeba* genotype T4 found in infected corneas of stray cats/dogs in this study was possibly due to the entering of soil particles which were highly contaminated with genotype T4.

The existence of potential pathogenic genotypes T3, T4, T5 and T15 in the domestic environment can be considered a public health issue. Awareness of *Acanthamoeba* infections among clinicians is important in order to diagnose AK and GAE in infected patients. Finally, awareness of the health risk on the distribution of *Acanthamoeba* species in the environment should be made known to the public especially those with high risk of acquiring it.

Other than *Acanthamoeba*, 17.5% (28/160) are other free-living amoebas (FLA) such as *Naegleria* and *Hartmannella* species which were detected and were found growing together with *Acanthamoeba* in the main and sub-culture plates. All of these FLA isolates were subjected to PCR amplification and eight (8) types amplicon size were obtained which were *Naegleria* species [Type 1 (363 bp), Type 2 (315 bp), Type 3 (315 bp), Type 4 (310 bp), Type 5 (370 bp) and Type 6 (310 bp)] and *Hartmannella* species [Type 7 (667 bp) and Type 8 (553 bp)]. *Naegleria* species were found in recreational water samples

and dry soil in playground while *Hartmannella* species were found in playground dry soil and indoor dust samples at University of Malaya Medical Faculty.

All *Naegleria* species found in this study were not pathogenic to human and to date, pathogenic *N. fowleri* was not reported from Malaysia. However, there should be awareness on the risk of *N. fowleri* infection when dealing with water activities such as swimming in recreational natural rivers and Muslims who perform ablution may also be exposed to this FLA that enter via the nostrils. As for *Hartmannella* more attention should be given in order to know the current status of their existence in the local environment and their ability to associate with other pathogens and cause diseases to humans and animals. Public health education about preventive and control measures on FLA should be given to the community especially when dealing with water activities. Awareness among the medical practitioners and adequate treatment of public water supplies must be emphasized.

ABSTRAK

Acanthamoeba adalah sejenis protozoa hidup bebas yang terdapat persekitaran di seluruh dunia. Jangkitan *Acanthamoeba* menyebabkan penyakit serius iaitu ensefalitis granuloma amebik (GAE) dan keratitis acanthamoeba (AK). Kajian ini bertujuan untuk mengenalpasti kehadiran genotip *Acanthamoeba* di dalam sampel kornea terjangkit semulajadi pada kucing dan anjing terbiar dan juga dari sampel alam sekitar. Kucing dan anjing terbiar yang terjangkit dari rumah perlindungan haiwan PAWS dan dari kawasan lorong-lorong (kucing sahaja) telah dikenalpasti mengalami jangkitan mata dengan terdapatnya tanda-tanda seperti mata berair, discaj keabuan dan kemerahan. Manakala bagi alam sekitar, sampel dikutip dari tempat tidur haiwan, bekas makanan dan bekas minuman haiwan di kawasan PAWS, dari sungai rekreasi (air, debu lembap, tanah), taman permainan kanak-kanak (tanah) dan Fakulti Perubatan Universiti Malaya (debu dalaman dan luaran). Semua sampel dikultur pada agar tanpa nutrien dengan meletakkan *E. coli* dan diikuti dengan PCR, pengklonan, penjujukan dan analisis filogenetik.

Kultur positif menunjukkan trofozoit *Acanthamoeba* mempunyai akantopodia seperti duri dan sista dengan dinding berkedut setebal dua lapis yang dikesan masing-masing seawal hari ke-2 dan hari ke-5. *Acanthamoeba* yang dikultur daripada sampel kornea kedua-dua kucing dan anjing terbiar adalah 24.8% (56/225), di mana 24% (48/200) dari kucing dan 32% (8/25) dari anjing. Manakala dari sampel alam sekitar, *Acanthamoeba* dikesan pada prevalens yang lebih tinggi iaitu 85% (136/160).

Analisis filogenetik menunjukkan bahawa pencilan *Acanthamoeba* dari kornea kucing dan anjing terbiar dihimpunkan dibawah *A. castellanii* (U07401) genotip T4 dengan dua kumpulan homolog dan perbezaannya terdapat pada bes. Untuk sampel alam sekitar, tiga genotip dikesan (T4, T5 dan T15) pada persekitaran PAWS, empat genotip (T3, T4, T5

dan T15) di sungai rekreasi, satu genotip pada tanah taman permainan kanak-kanak (T4) dan dua pada debu di Fakulti Perubatan Universiti Malaya (T4 dan T15).

Daripada empat genotip (T3, T4, T5 dan T15) yang telah dikesan dalam kajian ini, T4 didapati paling predominan dalam swab sampel kornea yang terjangkit dan dalam sampel tanah dan debu. Manakala genotip T5 adalah predominan dalam sampel air. Tambahan pula, genotip T4 juga dikenali sebagai genotip utama, diikuti oleh T3, T5 dan T15 yang menyebabkan penyakit pada manusia iaitu AK dan GAE. Kehadiran empat genotip ini dalam persekitaran (air, tanah, debu lembap dan debu) boleh menyumbang kepada jangkitan *Acanthamoeba*, terutama dalam pemakai kanta lekap dan pesakit berimunokompromi. Menggosok mata selepas dimasuki zarah tanah/debu dan air yang tercemar semasa aktiviti harian boleh menyebabkan lelasan mata dan menyumbang kepada risiko yang tinggi bagi memperoleh AK. Tambahan pula, genotip T4 *Acanthamoeba* yang terdapat dalam kornea kucing/anjing terbiar yang terjangkit dalam kajian ini mungkin kerana dimasuki zarah tanah yang tinggi pencemarannya dengan genotip T4.

Kehadiran genotip T3, T4, T5 dan T15 yang berpotensi berpatogen dalam persekitaran domestik boleh dianggap sebagai isu kesihatan awam. Kesedaran jangkitan *Acanthamoeba* di kalangan doktor adalah penting untuk mendiagnosis AK dan GAE pada pesakit yang dijangkiti. Akhir sekali kesedaran mengenai risiko kesihatan pada taburan spesies *Acanthamoeba* dalam persekitaran perlu diberitahu kepada orang ramai terutama mereka yang mempunyai risiko tinggi untuk dijangkiti.

Selain dari *Acanthamoeba*, 17.5% (28/160) adalah amoeba hidup bebas (FLA) lain seperti spesies *Naegleria* dan *Hartmannella* yang dikesan dan dijumpai hidup bersama *Acanthamoeba* didalam plat kultur utama dan plat pengkulturan semula. Semua isolat FLA ini tertakluk kepada amplifikasi PCR dan lapan (8) jenis saiz amplicon didapati iaitu

spesies *Naegleria* [Jenis 1 (363 bp), Jenis 2 (315 bp), Jenis 3 (315 bp), Jenis 4 (310 bp), Jenis 5 (370 bp) dan Jenis 6 (310 bp)] dan spesies *Hartmannella* [Jenis 7 (667 bp) dan Jenis 8 (553 bp)]. Spesies *Naegleria* dijumpai pada sampel air di kawasan rekreasi dan tanah kering di taman permainan manakala spesies *Hartmannella* dijumpai pada tanah kering di taman permainan dan pada sampel pada debu dalaman di Fakulti Perubatan, Universiti Malaya.

Kesemua spesies *Naegleria* yang dijumpai didalam kajian ini adalah bukan berpatogen kepada manusia dan buat masa ini *N. fowleri* yang berpatogen belum pernah dilaporkan di Malaysia. Walaubagaimanapun, mesti ada kesedaran terhadap risiko jangkitan *N. fowleri* apabila melakukan aktiviti air seperti berenang di kawasan rekreasi sungai semulajadi dan bagi orang Muslim yang mengambil wuduk mungkin terdedah kepada FLA yang masuk melalui lubang hidung. Bagi *Hartmannella* lebih perhatian harus diberikan untuk mengetahui status terkini kehadirannya didalam alam sekitar tempatan dan keupayaan untuk berasosiasi dengan patogen lain dan menyebabkan penyakit kepada manusia dan haiwan. Pendidikan kesihatan awam mengenai langkah-langkah pencegahan dan kawalan FLA mesti diberikan kepada masyarakat terutama berkaitan dengan aktiviti-aktiviti air. Kesedaran di kalangan pengamal perubatan dan rawatan bekalan air yang mencukupi untuk kegunaan awam perlu diberi penekanan.

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LIST OF ABBREVIATIONS AND SYMBOLS

X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
AIDS	Acquired immunodeficiency syndrome
AK	*Acanthamoeba* keratitis
bp	Base pair
BLAST	Basic local alignment search tool
BBB	Blood-brain barrier
BAL	Bronchoalveolar lavage
$CaCl_2$	Calcium chloride
CNS	Central nervous system
CSF	Cerebrospinal fluid
CHX	Chlorhexidine digluconate
CT	Computerized tomography
°C	Degree Celsius
DNA	Deoxyribonucleic acid
DF3	Diagnosis fragment 3
Na_2HPO_4	Dibasic sodium phosphate
dH_2O	distilled water
E. coli	*Escherichia coli*
FLA	Free-living amoeba
FRIM	Forest Research Institute Malaysia
GC	Genome composite
g	Gram
GAE	Granulomatous amoebic encephalitis
HAART	HIV/AIDS Alliance for Region Two
HIV	Human Immunodeficiency virus

Ig	Immunoglobulin
IIF	Indirect immunofluorescence
IPTG	Isopropyl-β-D-thiogalactopyranoside
ITS	Internal transcribe spacers
K	Pottasium
Kb	Kilo base pair
L	Litre
LB	Luria-Bertani
LSU	Large subunit
$MgSO_4$	Magnesium sulfate
MRI	Magnetic resonance imaging
MBP	Mannose binding protein
ML	Maximum likelihood
Mb	Mega base pair
µm	Micrometer
mL	Millilitre
mm	Millimeter
mM	Millimole
NJ	Neighbour-joining
BLASTn	Nucleotide Basic local alignment search tool
NA	Nutrient agar
NNA	Non-nutrient agar
ORF	Open reading frames
PAS	Page's amoeba saline / phosphate-amoeba saline solution
pg	Picogram
%	Percent

PBS	Phosphate buffered saline
PHMB	Polyhexamethylene biguanide
PCR	Polymerase chain reaction
KH_2PO_4	Mono Potassium phosphate
PAM	Primary amoebic meningoencephalitis
RNA	Ribonucleic acid
rDNA	Ribosomal DNA
rpm	Rotation per minute
SJKC	Sekolah Jenis Kebangsaan Cina
SK	Sekolah Kebangsaan
SMK	Sekolah Menengah Kebangsaan
SRA	Sekolah Rendah Agama
SSU	Small subunit
SSU rRNA	Small subunit Ribosomal RNA
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Na_2HPO_4	Dibasic Sodium phosphate
sp.	Species
TAE	Tris-Acetate EDTA
tRNA	Transfer RNAs
Rnl	The large subunit rRNA gene
Rns	The gene encoding for 18S
UK	United Kingdom
US	United States of America
UV	Ultraviolet
WHO	World Health Organization

CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND OF THE RESEARCH

Acanthamoeba is one of the ubiquitous free living amoeba, inhabitants in all environments worldwide include soil, water, dust, air-conditioning units, domestic tap water, dental treatment units, dialysis units, contact lenses and lens cases, and as contaminants in tissue culture (Barbeau & Buhler, 2001; De Jonckheere, 1991; Mergeryan, 1991; Rodriquez-Zaragoza 1994; Szenasi *et al*., 1998; Visvesvara & Stehr-Green, 1990). Despite in many years of argument, the genus of *Acanthamoeba* was established in 1931 (Booton *et al*., 2002) and (Nagington *et al.*, 1974) was reported in patient with eye keratitis. Although the classification of *Acanthamoeba* showed substantial progress by the description of morphology, but however, their species were still under review (Marciano-Cabral & Cabral, 2003).

The life cycle of *Acanthamoeba* species consists of two stages which were the (active feeding and dividing) and a dormant cyst. Under favourable conditions, the cyst stage would transform into feeding trophozoite (diameter range 25 to 40 μm) and proliferates continuously. The trophozoite has a prominent large nucleolus at the centre of the nucleus and protruding specific spiny pseudopodia at the cell surface called acanthopodia. Under

harsh environments such as nutrients depletion, high temperatures and high osmolarity, the trophozoites transform into resistant cysts (diameter 10 to 25 µm). The cysts have a wrinkle double walled with variable shapes such as triangular, square, star, etc depending on their species. The cysts are resistant to the environmental changes, chemicals and therapeutic drugs and survive for years under adverse conditions, such as extreme pH, desiccation and chemical exposure (Byers *et al*., 1991; Ma *et al.*, 1990).

Despite special characteristic of acanthopodia (a spike-like pseudopodia), *Acanthamoeba* trophozoites also have pseudopodia (false feet) several secondary branches (usually absent) that are extended from the cell surface to achieve the distinctive amoeboid locomotion. Acanthopodia are usually used for both locomotion and feeding purposes (Khan, 2006). It obtains food through phagocytosis and this makes *Acanthamoeba* heterotroph. It consumed variety of food sources such as bacteria other protists, some detritivore and dead organic materials. Phagocytosis occurs when *Acanthamoeba* extends the pair of pseudopodia around the food which then fuses and add digestive chemical for digestion.

Pathogenic *Acanthamoeba* species are known to be causative agents of granulomatous amoebic encephalitis (GAE), a fatal disease of the central nervous system (CNS) in immunosuppressed patients (da Rocha-Azevedo *et al*., 2009; Marciano-Cabral & Cabral, 2003; Schuster and Visvesvara, 2004; Walochnik *et al*., 2008). Dissemination diseases such as skin lesions and sinusitis can also occurred in fatal GAE, and these usually found in patients with AIDS (Dunand *et al*. 1997; Gullet *et al*., 1979; Martinez & Janitschke, 1985). In healthy young people, *Acanthamoeba* can infect the eyes and produced disease called acanthamoebic keratitis (AK) (Jones *et al*., 1975; Martinez & Visvesvera, 1997; Nagington *et al*., 1974).

Acanthamoeba keratitis is a painful sight-threatening disease which had been increasingly diagnosed along with the spread of contact lens use. Many pathogenic species of *Acanthamoeba* were known to cause acanthamoebic keratitis, among which were morphologically recognized as *A. castellanii*, *A. polyphaga*, *A. culbertsoni*, *A. hatchetti*, *A. rhysodes*, *A. lugdunensis*, *A. quina* and *A.griffini* (Shuster and Visvesvara, 2004). Most of these pathogenic species were detected under genotype T4 after molecular analysis of diagnosis fragment 3 (DF3) of 18S rRNA gene, among which successfully detected 17 genotypes (T1-T17) (Schroeder *et al*., 2001) up to date.

In Malaysia, *Acanthamoeba* keratitis was first reported in 1995 involving a female contact lens user (Mohamed Kamel & Norazah, 1995). Following this, more cases were seen, though not reported and by the end of 2001, 10 cases have been diagnosed (Kamel *et al*., 2003). Despite, there was no report of GAE and dissemination case from Malaysia up to date, which may due to undiagnosed, misdiagnosed or overlooked by the clinicians or truly never occur.

Other than human, *Acanthamoeba* was detected in animal autopsy samples from wild squirrels (Lorenzo-Morales *et al*., 2007), dogs, monkeys, a bull, a kangaroo and an Indian buffalo (Schuster & Visvesvera, 2004). Most of the reported cases from dogs cases were chronic infections of GAE (Ayers *et al*, 1972; Bauer *et al*., 1993; Brofman *et al*., 2003; Pearce *et al*., 1985) and multisystemic (Dubey *et al*., 2005; Kent *et al*., 2011). Amoebiasis keratitis case is rarely reported in animal, although cornea abrasion is at high risk, due to accidental scratching or environmental particles entering the eyes. Common signs of eye infection occur in pet eyes including redness, watery eyes, squinting, discharges, difficulty in open the eyelids, cloudiness and attempts to rub and scratch the eye. In felines, there are case reports where eye infections are habitually experienced in veterinary clinics and can be precipitated by a variety of pathogens such as virus, bacteria

and fungi (Doyle, 2009). However, eye keratitis caused by *Acanthamoeba* is rarely reported or has not been naturally isolated from animal eyes except a patent note (Ledbetter, 2011). Only a single study showed that *Acanthamoeba* was detected in feline eye with keratitis symptoms (Ithoi *et al*., 2013). Free-living *Acanthamoeba* infection in domestic animals, especially cats and dogs could be consider as public health importance and human risk of acquiring infection with amoebic keratitis. Infected animals may harbour the pathogenic amoebae in the domestic environment which may finally infect human eye, especially those who keep pets and live in close contact in their homes. Stray cats and dogs were commonly seen in residential areas and back alleys in the city and this could be exposed humans to higher chance of acquiring free-living *Acanthamoeba* infection. Thus, the detection of free-living *Acanthamoeba* in animals with keratitis symptom was our interest. Furthermore, the environmental samples from the living areas of these domestic animals and humans were also included in this study.

1.2 JUSTIFICATION OF THE STUDY

Acanthamoeba infects the eye causing acanthamoeba keratitis is increasingly recognized especially in contact lens wearers. It can also infect the brain causing Granulomatous amoebic encephalitis (GAE), found commonly in immune compromised or severely debilitated individuals and this infection is often fatal. Outbreaks of *Acanthamoeba* keratitis (AK) was associated with contaminated water and contact lens wear (Ledee *et al*., 2009). *Acanthamoeba* keratitis has been reported worldwide including from Japan, Taiwan, India, Australia, Africa, North and South America, Europe and Israel (Govinda & Jeanette, 1990). It is not a notifiable disease and its true incidence is not known.

In the recent years, there are increasing cases of AK, especially among the contact lens users of which contributed high cases of 85% to 90% (Patel & Hammersmith, 2008). This high prevalence rate was possibly due to the used of contaminated contact lenses in

which being washed with pipe water instead of recommended sterile solution. In Malaysia, AK cases were reported from the contact lens users (Mohamed Kamel *et al*., 2000) as well as eye trauma by sand/dust particles with small abrasions in the corneas (Kamel *et al.*, 2005). Thus, the virulent *Acanthamoeba* could have been acquired from various natural environmental sources including water, soil and dust as a risk factors to cantaminate the contact lences. To date, very little information is available on the epidemiology of free living amoeba including *Acanthamoeba* in Malaysia.

The discrimination between pathogenic and non-pathogenic species of *Acanthamoeba* is critical for evaluating the risk of infection (Stehr-Green *et al*., 1989). To date, molecular study has identified 19 genotypes of *Acanthamoeba* based on DNA sequences of diagnosis fragment 3 (DF3) of the 18S ribosomal DNA (rDNA) gene region and genotype T4 was found to be the most associated with ocular keratitis, GAE and dissemination diseases in humans (Stothard *et al*., 1998). Besides, genotypes T2, T6, T10 and T11 were also occasionally detected in human keratitis (Booton *et al*., 2005; Nupraset *et al*., 2010). Investigating *Acanthamoeba* genotypes from infected human and environmental samples have been widely reported worldwide but however, lacking information from Malaysia.

Concerning keratitis in animals, most veterinarians were focus on viruses, bacteria and fungi but less research work on protozoa (*Acanthamoeba*). In majority of the cases, keratitis was often miss-diagnosed as herpes simplex virus or adenovirus infection (Martinez & Visvesvera, 1997), bacterial *Pseudomonas aeruginosa* (Clarke & Niederkorn, 2006) and *Staphylococcus* spp. (Giese & Weissman, 2002) and fungal including *Aspergillus flavus*, *Aspergillus fumigatus*, *Fusarium* spp., *Alternaria* spp. and yeast *Candida* (Halde, 1986). Indeed, *Acanthamoeba* infection in animal with keratitis symptoms are rarely reported in Malaysia and to the best of our knowledge there was only

one report on *Acanthamoeba* detected in naturally-infected feline corneas (Init *et al*., 2013). Therefore, this present study was conducted on animals (cats and dogs) with the signs of corneas infections. The extended study was also carried out against the environmental samples in order to investigate the variations of *Acanthamoeba* genotypes that distributed in Malaysian domestic environments, of which may have possibility associated with corneas infected in both dog and cat.

The results of this research could be added to the new knowledge on the potential pathogenic free living *Acanthamoeba* that was occurred in domestic animals and Malaysian environments. Increase awareness among veterinarians and health personnel is an essential requirement in the clinical recognition of the signs and risk factors for *Acanthamoeba* infection especially keratitis.

1.3 SIGNIFICANCE OF THE STUDY

The findings from this study will be generated an important information on aspects such as possible environmental source that contributing to the *Acanthamoeba* infections in animals as well as humans. Furthermore, the identification of *Acanthamoeba* genotypes can provide more information about the dynamics of transmission and pathogenicity among *Acanthamoeba* strains for the interest of public health and the need to enforce the routine diagnosis of *Acanthamoeba* sp. in all clinical laboratories in Malaysia.

1.4 OBJECTIVES OF THE STUDY

1. To determine the occurrence of *Acanthamoeba* isolated from naturally infected corneas of cats and dogs as well as in environmental samples.

2. To identify the genotypes of *Acanthamoeba* isolates

3. To analyse the distribution of *Acanthamoeba* genotypes in environmental samples.

CHAPTER 2

LITERATURE REVIEW

2.1 FREE-LIVING AMOEBA

Free-living amoeba (FLA) in the amoebozoa group encompasses the largest group of protists, and its important was known ecologically and medically. FLA was first discovered using the microscope by August Johann Rösel von Rosenhof in 1757 (Joseph, 1878). The origins of the name "Amoeba" is derived from the Greek word referring to their common amoeboid motion (i.e. crawling-like movement) and was described into several different groups. The FLA is a single living cell with the organelles and cytoplasm enclosed by a cell membrane with no definitive shape. They usually produced uninucleate and their trophozoites have pseudopodia (false feet) at the anterior end, with several secondary that branching to the sides which are extended from the cell surface to achieve the distinctive amoeboid locomotion and phagocytosis. Trophozoites consume varieties of food sources include bacteria, other protists, some detritivores and organic material. Phagocytosis occurrs when amoebae extend the pair of pseudopodia around the food. They fuse to make a food vacuole, then fuses with a lysosome to add digestive chemicals. The undigested food will be expelled out from the cell membrane. Pseudopodia extend and contract by the reversible assembly of actin subunits into microfilaments (Allen,

1961; Taylor, 1977) which results in the flow of organelles through the cytoplasm within the cell. It has ability to detect concentration gradients of nutrients and other substances in the environments such as a chemo toxic response.

Like other unicellular eukaryotic organisms, FLA reproduce asexually via mitosis and cytokinesis, which difference from prokaryotes (bacteria) that via binary fission. The majority of amoeboid stage showed lineage sexual reproduction, in which contrary to the popular belief that in the past reproduce sexually and currently a new group reproduce asexual independently (Hurst *et al*. 1992; Lahr *et al*. 2011). In certain cases where the amoebas are forcibly divided, the portion that retains the nucleus will survive and form a new cell and cytoplasm, while the other died.

FLA is placed under the protozoa phylum that divided into six supergroups and FLA is placed in amoebazoa (Adl *et al*., 2005). Indeed, FLA has seen many changes and continuously being studied until this day when the scientists discover new data through genomic sequencing (De Jonckheere, 2004; Nassonova *et al*., 2010; Smirnov *et al*., 2007). The current classification scheme of FLA is shown in Figure 2.1 (Khan, 2006).

FLA species are freely living in all environments, but they do not have a very good adaptation in their hosts compared to other classic parasitic protozoa such as *Plasmodium* sp. and *Leishmania* sp. In the hosts, several FLA have the ability to produce fatal diseases but they do not evolve to survive for a long time. Due to the free living ability, FLAs are widely distributed in the environments worldwide and have very high potential to be transmited to human, but rarely happened and most species are limited to immunocompromised hosts. Most of infected cases were due to natural transmission via contaminated water or soil and recently due to contaminated contact lenses. FLA genus/species such as *Acanthamoeba* sp., *Balamuthia mandrillaris*, *Naegleria fowleri*, *Sappinia pedata*, *Vahlkampfia* sp. and *Hartmannella* sp are capable to survive in

mammalian hosts by virtue of their ability to adapt as a pseudoparasite and potentially pathogenic behaviour of producing diseases in humans and animals (Aitken *et al*., 1996; Qvarnstrom *et al*., 2009). Disease in immunosuppressed such as granulomotous amoebic encephalitis (GAE) is caused by *Acanthamoeba* or *Balamuthia*, while eye keratitis in immunocompromised was caused by *Acanthamoeba* (Marciano-Cabral, 2003).

In *Acanthamoeba* keratitis cases, co-infections with *Vahlkampfia* and *Hartmannella* were also found (Aitken *et al*., 1996). Several species of FLA have the potential to live as parasites called amphizoic in recognition of their hosts, yet they are capable as a free-living existence (Page, 1988). *Naegleria fowleri* can invade the central nervous system causing primary amoebic meningoencephalitis (PAM), even though the cases are rare [The Centers of Disease Control and Prevention, Division of Parasitic Diseases, (2012)] but fatality rate is greater than 95% (Cetin & Blackall, 2012). Recently, *Sappinia pedata* was also reported in encephalitis in a healthy young man (Qvarnstrom *et al*., 2009), therefore, there are possiblities that many more genus of FLA causing fatal infections in humans. Cases due to FLA infections were noted to be significantly increased over the years and gained attention from the scientific community due to their diverse roles in causing serious and sometimes fatal infections (Khan, 2006).

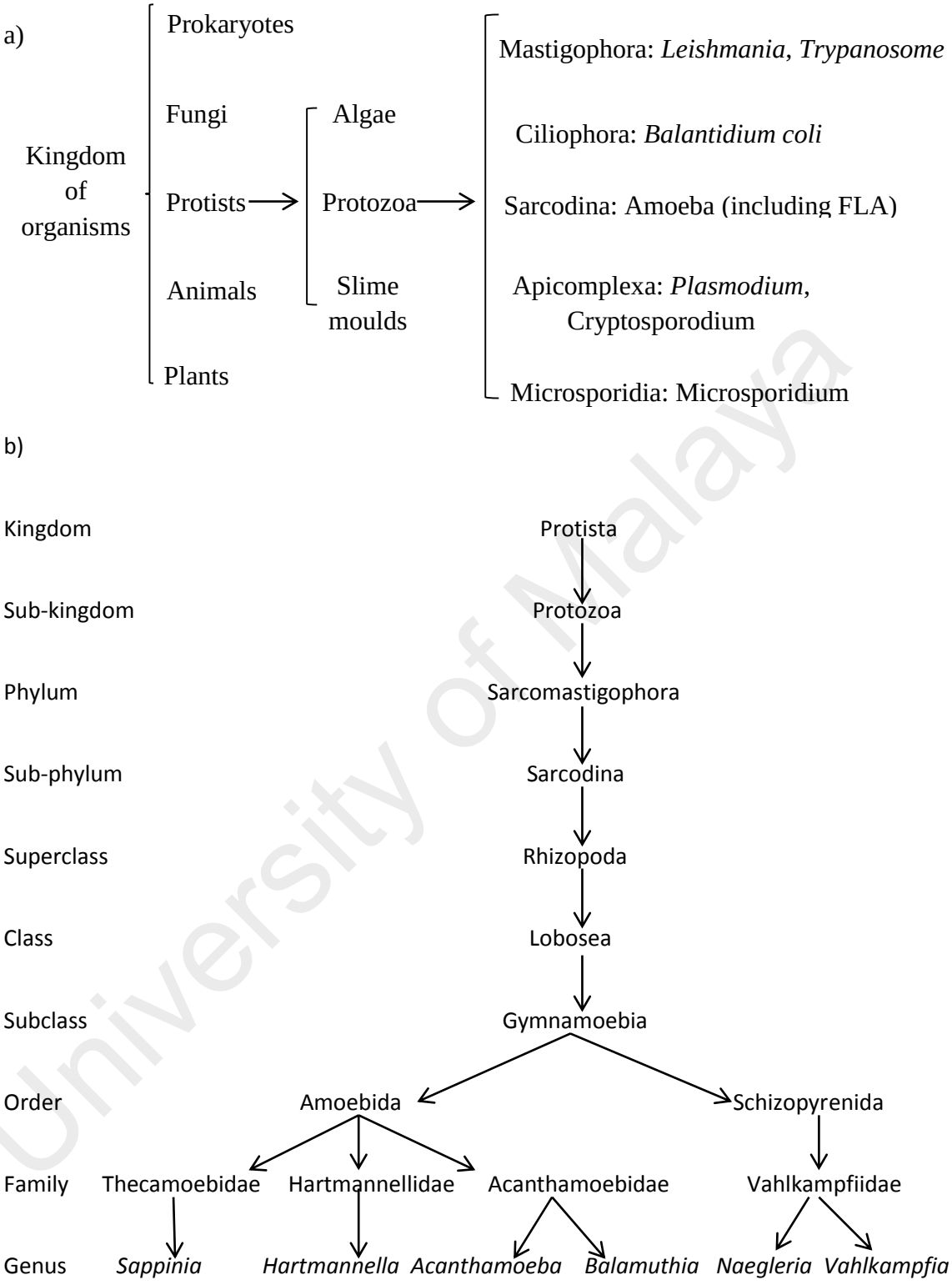


Figure 2.1: The classification scheme of free-living amoeba (Khan, 2006).

2.2 HISTORY OF *ACANTHAMOEBA*

Acanthamoeba genus was first isolated from dust and was named *Amoeba polyphagus* (Puschkarew, 1913) but was later renamed as *Acanthamoeba polyphaga* (Page, 1967). In 1930, Castellanii reported the presence of an amoeba in yeast (*Cryptococcus pararoseus*) cultures (Castellanii, 1930) which later named this amoeba as *Hartmannella castellanii* (Douglas, 1930). In 1931, the genus of *Acanthamoeba* was identified by Volkonsky and ultimately distinguished from *Hartmannella*, in which *Hartmannella castellanii* was reclassified as *Acanthamoeba castellanii* (Volkonsky, 1931). The classification of *Acanthamoeba* genus was than revised and placed under Family Acanthamoebidae, that capable of causing diseases in humans and animals (Booton *et al*., 2002: Byers *et al*., 1991; Gast, 2001; Kong & Chung, 2002; Schroeder *et al*., 2001).

In the late 1950's, *Acanthamoeba* was broke it silent after it was again discovered as a tissue culture contaminants. In culture, *Acanthamoeba* showed pathogenic potential by exhibiting cythopathic effects against the trypsinised monkey-kidney cells. Furthermore, inoculation of *Acanthamoeba*-contaminated monkey-kidney cell was resulted in fatal encephalitis or encephalomyelitis diseases in experimental immunosuppressed monkeys and mice. This result was initially thought to be caused by an unknown virus. However, upon histological examinations of the monkeys and mice tissue couples with microscopic observation of the contaminants culture fluids, it was indeed caused by *Acanthamoeba* genus (Culbertson *et al*. 1958; Culbertson *et al*. 1959).

In 1972, the first case of granulomatous amoebic encephalitis in human due to *Acanthamoeba* infection was reported (Jager & Stamm, 1972). Followed case was *Acanthamoeba* keratitis reported in 1974 (Nagington *et al*., 1974). After almost a decade later, *Acanthamoeba* was described as an opportunistic organism, which infects humans with debilitated antibody or chronically ill patients. Since that time, a large number of

reports have been presented by various workers about the morphological characteristics, cultivation, epidemiology and pathogenicity of *Acanthamoeba* species.

2.3 TAXONOMY AND CLASSIFICATION OF *ACANTHAMOEBA*

The taxonomy of *Acanthamoeba* genus was classified into three morphological groups (I, II and III) based on cysts features including diameter and shape of both endocyst and ectocyst walls (Pussard & Pons, 1977; De Jonckheere, 1987; Page, 1988). The Group I consists of four species which are *Acanthamoeba astronyxis*, *Acanthamoeba comandoni*, *Acanthamoeba echinulata* and *Acanthamoeba tubiashi*. These species exhibit large trophozoites and cyst forms have ectocyst that is widely separated from endocyst with the addition characteristics, e.g. (i) *Acanthamoeba astronyxis* trophozoites are exhibited > 6 pseudopodia arms and average of cysts diameter is ≥18 µm; (ii) *A. comandoni* trophozoites are exhibited 6–10 pseudopodia and average of cysts diameter is ≥25.6 µm; (iii) *A. echinulate* trophozoites are exhibited 12–14 pseudopodia and average of cysts diameter is ≥25 µm; (iv) *A. tubiashi* only exhibited average of cysts diameter of ≥22.6 µm. Group II consists of 11 species and the most commonly found widespread in environment. They were *A. mauritaniensis*, *A. castellanii*, *A. polyphaga*, *A. quina*, *A. divionensis*, *A. triangularis*, *A. lugdunensis*, *A. griffini*, *A. rhysodes*, *A. paradivionensis* and *A. hatchetti*. Their ectocyst and endocyst either close together or widely separated. Ectocysts could be thick or thin whereas endocysts maybe polygonal, triangular or round with an average diameter of >18 µm. Group III consists of five species include *A. palestinensis*, *A. culbertsoni*, *A. royreba*, *A. lenticulata* and *A. pustulosa*. Ectocysts in this group are thin and endocysts may have 3–5 gentle corners with the mean cyst diameter of <18 µm. However, *A. tubiashi* (in group I) and *A. hatchetti* (in group II) were added later in 1991 by Visvesvara.

Most of the pathogenic species were accumulated under Group II, although *A. culbertsoni* from Group III is also recognized as a pathogen (Pussard & Pons, 1977). This morphological taxonomy was the most comprehensive and was used up to 20 years (Page, 1988). The classification of *Acanthamoeba* based on morphological characteristics was later realized to be unreliable because the morphology of cysts can be change during *in vitro* cultivation (Armstrong, 2000). Many studies have demonstrated inconsistencies and/or variations in the cyst morphology of the same isolate/strain (Khan, 2006). The ionic strength of the growth medium can alter the cyst morphology and interfere with the identification of pathogenic species (Sawyer, 1971). After years with unsatisfactory classification, the spindle shaped of the cyst was finally considered as a distinctive morphology used for a decisive character at the generic level to recognize the *Acanthamoeba* genus, which was differentiated from *Hartmannella* genus (Singh, 1950; Pussard, 1966; Page, 1967). In 1975, Sawyer and Griffin established family Acanthamoebidae and Page (1988) established *Hartmannella* under family Hartmannellidae.

Acanthamoeba trophozoite is recognized by the presence of special structures of spike-like (acanthopodia) on the membrane surface, and the term was introduced from the Greek word "acanth" means "spike". Besides morphology, other characteristics such as immunological, biochemical and physiological were applied to classify the differentiation of *Acanthamoeba* species (Alves *et al*., 2000; Walochnik *et al*., 2000). Currently, approximately 24 species from both pathogenic and non-pathogenic *Acanthamoeba* have been classified using morphological characteristics (Schuster & Visvesvara, 2004, Visvesvara *et al*., 2007b). The classification of *Acanthamoeba* is as follows,

Kingdom: Protista

Phylum: Rhizopoda

Class: Lobosea

Subclass: Gymnamoebia

Order: Centramoebida

Family: Acanthamoebidae

Genus: *Acanthamoeba*

Figure 2.2: A classification of *Acanthamoeba* spp.

The pathogenic species are noted as *A. polyphaga*, *A. rhysodes*, *A. quina*, *A. griffini*, *A. lugdunensis*, *A. castellanii*, *A. culbertsoni*, *A. healyi* and *A. hatchetti* (Schuster and Visvesvara, 2004, Yu *et al*., 2004).

Recently, a new system of molecular method has been proposed to classify the genotype of *Acanthamoeba* based on 18S rRNA gene (Gast *et al*., 1996, Schroeder-Diedrich *et al*., 1998). The 18S ribosomal RNA (18S rRNA) is a structure of a small component of the eukaryotic cytoplasmic ribosome. It also shows a slow rate of evolution, thus making it a suitable candidate to study and reconstruction of the basic different ancestors. *Acanthamoeba* is compiled based on the sequence variation of evolutionary clades. Based on 18S rRNA sequences, 19 clades have been recognized to have closely related species namely T1-T12 (Gast *et al*., 1996, Schroeder-Diedrich *et al*., 1998), T13 (Horn *et al*., 1999), T14 (Gast, 2001), T15 (Hewett, 2003), T16 (Corsaro & Venditti, 2010), T17 (Magliano *et al*, 2012), T18 (Qvanstrom *et al*, 2013) and a newly discovered strain T19 (Magnet *et al*., 2014) (Table 2.1). Every genotype exhibits 5% or more sequence divergence between different genotypes (Khan, 2006). Based on this classification, the majority of human infections due to *Acanthamoeba* were seen associated with genotype T4. This genotype was detected approximately 90% of *Acanthamoeba* keratitis cases (Khan, 2009; Stothard *et al*., 1998). It is also found to be

the most genotype that associated with granulomatous amoebic encephalitis (GAE), skin lesions and sinusitis (Walochnik *et al*., 2000).

Table 2.1: Group of *Acanthamoeba* designated by T-genotye and cyst morphology characteristics

T-genotye	Species	Strain ID	Reference	Cyst group
T1	*A.castellanii*	ATCC 50494	Dudley *et al*., 2005	II
T2	*A.palestinensis*	ATCC 30870	Gast *et al*., 1996	III
T3	*A. griffini*	ATCC 30731	Gast *et al*., 1996	II
T4	*A. castellanii*	CDC:0981:V006	Gast *et al*., 1996	II
	A. polyphaga	Page-23	Stothard *et al*, 1998	II
	A. rhysodes	ATCC 50368	Gast *et al*., 1996	II
	A.lugdunensis	L3a	Kong, 2009	II
T5	*A. lenticulata*	ATCC 50428	Stothard *et al*, 1998	III
T6	*A. palestinensis*	ATCC 50708	Stothard *et al*., 1998	III
T7	*A. astronyxis*	ATCC 30137	Stothard *et al*., 1998	I
T8	*A. tubiashi*	ATCC 30867	Stothard *et al*., 1998	III
T9	*A. comandoni*	ATCC 30135	Stothard *et al*., 1998	I
T10	*A. culbertsoni*	ATCC 30171	Stothard *et al*., 1998	III
T11	*A. hatchetti*	BH-2	Stothard *et al*., 1998	III
	A. stevensoni	RB:F1	Kong, 2009	II
T12	*A. healyi*	CDC 1283:V013	Stothard *et al*., 1998	III
T13	*Acanthamoeba* sp.	UWC9	Horn, M. *et al*, 1999	_
T14	*Acanthamoeba* sp.	PN15	Horn, M. *et al*, 1999	_
T15	*A. jacobsi*	ATCC 30732	Hewett, 2003	III
T16	*Acanthamoeba* sp.	cvX	Corsaro & Venditti, 2009	_
T17	*Acanthamoeba* sp.	TSP07/T17	Magliano *et al*., 2011	_
T18	*Acanthamoeba* sp.	CDC:V621	Qvarnstorm *et al*., 201	_
T19	*Acanthamoeba* sp.	USP-AWW-A68	Magnet *et al*., 2014	_

2.4 GENETIC DIVERSITY OF *ACANTHAMOEBA*

Acanthamoeba species exhibits high level of genetic diversity that has been observed among numerous isolates from both humans and animals. Various molecular methods have been used to characterize *Acanthamoeba* isolates genetically. Due to the increasing in *Acanthamoeba* keratitis cases and the awereness among the researchers that *Acanthamoeba* can also be a carrier for pathogenic bacteria infection in human, therefore, the study of *Acanthamoeba* have greatly gain an attention wordwide.

Acanthamoeba is able to reproduce by mitosis via asexual reproduction (Jantzen *et al*., 1990). The nuclear membrane will be removed to clone itself and splits into two identical cells during the process of division (Ma *et al*., 1990). Its cell consists of

numerous sizes of nuclear chromosomes ranging from 200 Kb to > 2 Mb (Byers, 1986, Byers *et al*., 1990, Rimm *et al*., 1988) and having a large nucleus approximately 16.6% of the size of trophozoite (Khan, 2006) with significant morphological features of large centrally located nucleolus without kromatin (or any membrane bound components).

Experiment on *A. castellanii* Neff, 60% of genomic DNA consists of GC bases which approximately 33MB (Byers, 1986), and this showed much higher as compared to other amoeba that normally consists around 1.28 pg / amoeba (Adam *et al*., 1969). Nuclear DNA content varies throughout the growth and decline to about 50% (in unagitated cell) during changes in the cell in preparation for encystment (Byers, 1986). The mitochondrial DNA content is also reduced during *in vitro* encystment (Byers, 1986). *Acanthamoeba* mitochondrial genome is a circular molecule consisting of 41.6 Mb with AT (adenine and thymine) content of 70.6%. It contains the genes that code for both large and small subunit rDNA, 16 tRNA, 8 open reading frames (ORF) of undetermined function and 33 proteins. All of these are found in the same transcriptional orientation and make up 93.2% of the total sequence (Burger *et al*., 1995).

Based on nuclear rDNA sequences, *Acanthamoeba* was initially placed as an outer group in the phylogenetic tree of the green algae and land plants. However, when further study of the small subunit rDNA SSU, *Acanthamoeba* was transferred to the animals and fungi clade (Wainright *et al*., 1993) which perfectly assemblage under the multicellular ancestry (Cavalier-Smith, 1998). In 1994, Lonergan and Gray discovered evidence of horizontal gene transfer between algal chloroplasts and *Acanthamoeba* mitochondria. Three groups of I intron were found in the LSU (large subunit) rRNA (Rnl) and identified through mitochondrial of *Acanthamoeba* sequence with copy 7778 bp LSU and small subunit (SSU) rRNA. The introns are placed within highly conserved regions with freestanding open reading frame (ORF) and were found to be identical to the single group

I intron in the chloroplast Rnl of green algae *Chlamydomonas reinhardtii*, and structurally homologous within the core region and the ORF they encode. Suggesting intron movement has occurred between mitochondria and chloroplasts, either intracellularly in a photosynthetic, remote common ancestor of *Acanthamoeba* and *C. reinhardtii* or, more recently as a result of an intercellular exchange of genetic material (Lonergan & Gray, 1994).

Complete sequencing of *A. castellanii* mitochondrial DNA revealed it most closely resembles the chlorophycean algae *Prototheca wickerhamii*, than *C. reinhardtii*. Comparison of the mitochondria from all three organisms shows *A. castellanii* and *P. wickerhamii* have almost identical respiratory and ribosomal protein genes, while *C. reinhardtii* does not encode any ribosomal proteins and lacks several standard respiratory genes. These results interpreted in two possible scenarios, either *C. reinhardtii* does not share a common ancestor with land plants and *A. castellanii*, or more likely, that they do all share a common ancestor, and *C. reinhardtii* has diverged radically and lost much of its former gene content (Burger *et al*., 1995).

Next, the ribosomes DNA and their associated sequences have been extensively studied throughout biology as a means to better understand evolutionary origins. One of the most studied and frequently used genes throughout eukaryotic biology is a section of the small ribosomal subunit 40S, recognize as the small subunit (SSU) 18S rRNA. SSU 18S is the structural RNA for the small section of the cytoplasmic ribosomes, and is therefore integral to protein synthesis in all living cells. Molecular analysis using 18S data to understand evolutionary divergences has become extremely popular. SSU 18S is a prime target for molecular analysis because, it is slow evolutionary rate and repetition throughout the genome, thus provide an ample template for PCR. In addition, this gene is

usually flanked with highly conserved regions, therefore making it relatively easy to locate at the outset of the studies, and readily accessible.

Amoebae coding sequences (rDNA) is arranged typical for eukaryotic ribosomal gene repeat units, in which they contain one set of 5' 18S, 5.8S and 28S 3' genes. Between these genes and neighbouring sets are spacer regions or internally transcribed spacers (ITS). *Acanthamoeba* rRNA repeats unit is 12 Kb, containing approximately 600 copies (Byers *et al*., 1990), while the coding sequence for the 18S rRNA gene is 2,303 bp long.

Acanthamoeba nuclear SSU 18S rRNA genes (18S rDNA coding sequence; *Rns*) have been extensively studied and are at present receiving much attention, in the hope of understanding why some strains appear pathogenic and others do not. Group I intron within the rRNA sequence have been found in *A. griffini* and *A. lenticulata* (Gast *et al*., 1994, Schroeder-Diedrich *et al*., 1998), increasing the size of their nuclear rRNA genes of approximately 2,800 bp, compared with 2,300 bp. Sequence analysis of the *Rns* has allowed the development of a classification scheme, allowing *Acanthamoeba* to be typed into one of a possible 15 *Rns* genotypes known as T-groups (Booton *et al*., 2005; Gast *et al*., 1994; Hewett, 2003; Stothard *et al*., 1998).

Many amino acid sequences have been studied and published, these include complete and/or partial, mRNA, RNA and genomic sequences. The literature and GenBank are dominated by 18S sequences from many *Acanthamoeba* species, but other sequences available include: actin I (Nellen and Gallwitz, 1982); myosin heavy chains I (Brzeska *et al*., 1999); 26S (Lai & Henney, 1993); 5S (Zwick *et al*., 1991); lactate dehydrogenase-like (Watkins & Gray, 2006); mannose-binding protein (Garate *et al*., 2004); polyubiquitin (Hu & Henney, 1997) and profilin I and II (Pollard & Rimm, 1991).

Recently, research on *Acanthamoeba* molecular biology has moved forwards rapidly. Evidence has recently been presented to include a new genotype of 18S, T19

(Magnet *et al*., 2014). While data also suggests an analysis of microsatellites found within the ITS located between 18S and 5.8 S, could be a potential candidate to further distinguish within the clades, especially T4 (Kohsler *et al*., 2006).

2.5 *ACANTHAMOEBA* BIOLOGY

The life cycle of *Acanthamoeba* consists of two stages (Figure 2.2), the trophozoite (an active feeding and dividing) and cyst (dormant). Under favourable conditions, the *Acanthamoeba* cyst would transform into feeding trophozoite (diameter range 12–35 μm) and proliferate continuously, but the size varies significantly between isolates belong to different species or genotypes. The trophozoites possess a nucleus containing a distinctive centrally located nucleolus approximately one-sixth the size of the trophozoite. The acanthopodia are protruding specific spiny pseudopodia at the cell surface and most likely important in adhesion to surfaces (biological or inert), cellular movements or capturing prey (Figure 2.3). During the trophozoite stage, *Acanthamoeba* actively feeds on bacteria, algae, yeasts or other small organic particles. It has many food vacuoles that can be seen in the cytoplasm of the cell. The contractile vacuole can also be found in the cytoplasm integral to maintain osmotic equilibrium by expelling water. The cytoplasm is granular and contains many mitochondria, lysosomes and ribosomes. The important role of mitochondria is to generate the energy for the use of cell activities such as feeding, movement, reproduction and other cellular function. Furthermore, *Acanthamoeba* possesses a multi-layered microtubule-organizing centre (Patterson, 1999).

The trophozoite (Figure 2.3) of *Acanthamoeba* performs asexual cell division which occurs by binary fission. *Acanthamoeba* can sustain in the trophozoite stage with an abundant food supply, neutral pH, appropriate temperature (i.e. 30°C) and osmolarity between 50-80 mOsmol. Under the harsh environmental conditions, the trophozoite becomes metabolically inactive, in which their food, water and particle matter are

expelled. The trophozoite will then transform itself into a spherical structure shielded with a double-walled (cyst) that acts as a protection to help survive in hostile conditions. During the encystment process, cellular levels of RNA, proteins, triacylglycerides and glycogen decline substantially resulting in decreased cellular volume and dry weight (Weisman, 1976). The double-walled cyst is wrinkled with diameter 5-20 µm and variable in shapes among isolates belonging to different species as well as genotypes. They are resistant to the environmental changes, chemicals and therapeutic drugs and survive for years under adverse conditions, such as extreme pH, desiccation and chemical exposure (Byers *et al*., 1991; Ma *et al*., 1990).

Cysts (Figure 2.3) of *Acanthamoeba* are distributed in the environment probably by airborne and this will enhance possibility to infect in the susceptible human and animal host. Several studies have reported that cysts can remain viable and maintaining the pathogenicity for several years (Mazur *et al*., 1995). Cyst is a resting or dormant stage. After received a good environmental changes, the dormant young amoeba emerges through a pore (ostiole) and the cyst wall is removed at the junction of the ectocyst and endocyst (Martinez & Visvesvara, 1997), and further continue with the feeding and dividing trophozoite stage. Both of the processes encystment and excystment require active macromolecule synthesis and can be blocked by cycloheximide (a protein synthesis inhibitor) (Khan, 2006).

Free-living *Acanthamoeba* trophozoite feeds on microorganisms and organic debris that present on the moist surfaces, and utilizing acanthopodia that arise from the surface of *Acanthamoeba* trophozoites to capture food particles customarily bacteria (Weekers *et al*., 1993), algae, yeast (Allen & Dawidowicz, 1990) and other protists or debris materials. The food uptake is occurs by using phagocytosis (receptor-dependent process) and pinocytosis (nonspecific process) through a membrane invaginations and is used to take

up large volumes of solutes or food particles (Bowers & Olszewski, 1972; Allen & Dawidowicz, 1990; Alsam *et al*., 2005).

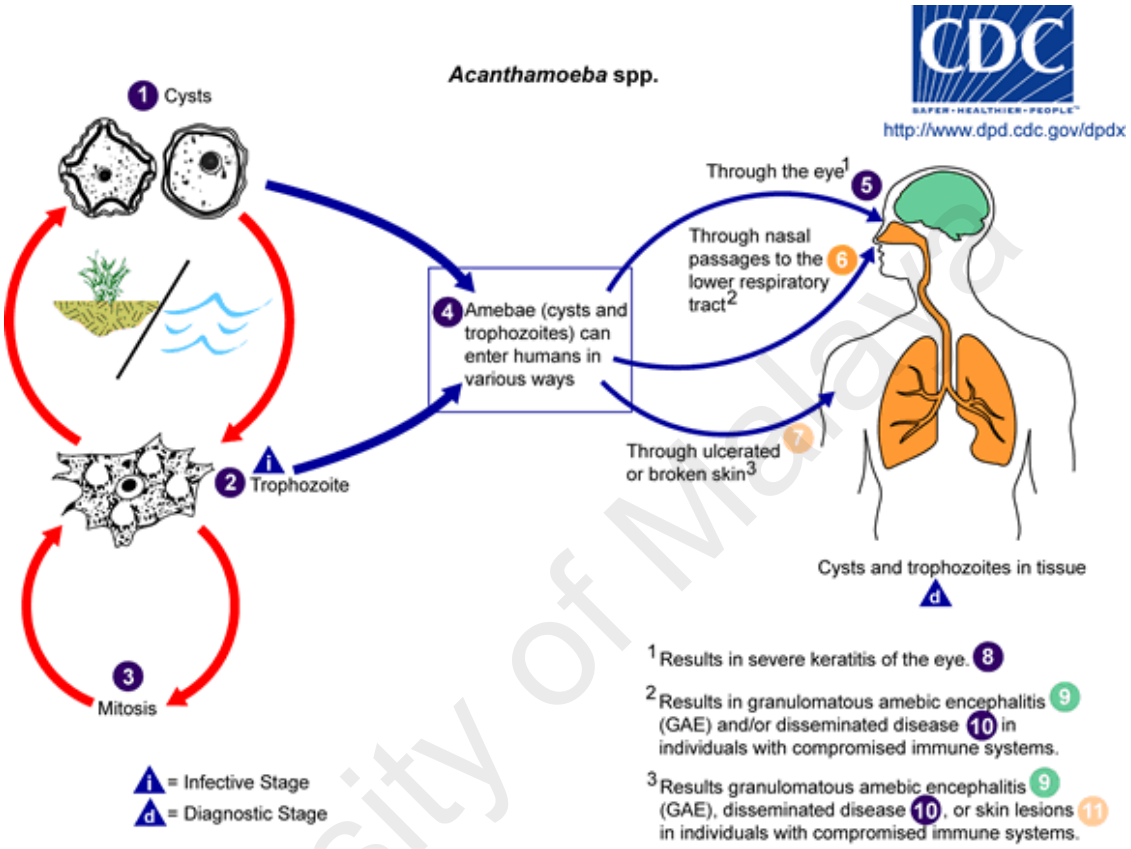
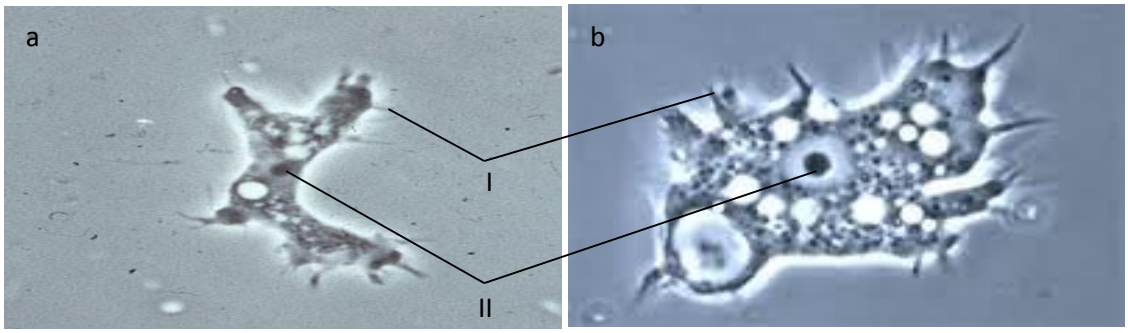
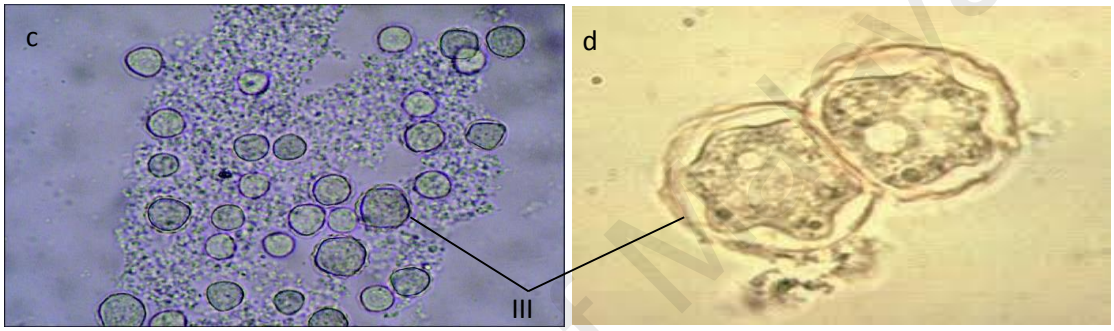


Figure 2.3: Life cycle and transmission mode of *Acanthamoeba* in human (Centers for Disease Control Parasites and Health website).

Trophozoites



Cysts



I) nucleus

II) acanthopodia

III) wrinkled double walled

Figure 2.4: Cysts and trophozoites forms of *Acanthamoeba*.
a) http://www.cdc.gov/dpdx/freeLivingAmebic/gallery.html)
b) http://www.menicon.com/pro/soft/soft-lens-care/menicare-soft/acanthamoeba-and-viruses
c) http://www.artisanoptics.com/services/eye_diseases___conditions/*acanthameoba*_keratitis4)
d) http://ruby.fgcu.edu/courses/davidb/50249/web/acanth41.htm

2.6 *ACANTHAMOEBA* ECOLOGY

Acanthamoeba is widespread in many types of habitats due to their, tolerancy toward extremes temperature, osmolarity, oxygen availability, water potential and pH. It has the ability to survive throughout nature and is found everywhere. It was detected from recreational areas (rivers, streams and lakes), thermally polluted waters, sediment, baths, heating/ventilation, cold water storage tanks, swimming pools, soil, dust, air conditioning vents, air, domestic water supply cooling towers of electric and nuclear power plants, humidifiers, Jacuzzi tubs, dental treatment units, dialysis units, eye wash stations, hospital hydrotherapy pools, bottled water, contact lenses and lens cases, cell cultures (included bacterial, fungal and mammalian cell cultures) medical equipment and resistant to most environmental conditions, viable in dry area as well as highly chlorinated water (Barbeau & Buhler, 2001; De Jonckheere, 1991; Init *et al*., 2010; Marciano-Cabral, 2003 Martinez, 1985; Martinez & Visvesvara, 1997; Mergeryan, 1991; Rodriquez-Zaragoza 1994; Szenasi *et.al*., 1998; Schuster & Visvesvara, 2004; Visvesvara & Stehr-Green, 1990; Visvesvara *et al*., 2007b). In clinical speciments, *Acanthamoeba* was isolated from ear discharge, skin lesions, corneal biopsies, lungs tissues, cerebrospinal fluid (CSF), pulmonary secretions, mandibular autografts, brain necropsies, human nasal cavities and nasopharyngeal mucosal swabs (Khan, 2003; Marciano-Cabral & Cabral, 2003; Schuster & Visvesvara, 2004). In addition, the majority of healthy individuals have shown *Acanthamoeba* antibodies, indicating exposure to these pathogens (Chappell *et al*., 2001; Cursons *et al*., 1980).

They have the ability to encyst when conditions become unfavourable. Studies show that the cyst can live through cryotherapeutic technique under -50 to -130 °C (Meisler *et al*., 1986). The cysts can also endure periods of up to 8 months at low temperatures of –10, 4, 10 and 15°C (Biddick *et al*., 1984), pH 2.0, moist heat of 60°C for 60 min

(Kilvington, 1991), gamma irradiation (250 K rads) and ultraviolet radiation (800 mJ/cm^2) (Aksozek *et al*., 2002). *Acanthamoeba* has also been shown to remain viable when it cystic stage stored in a state of desiccation for 20 years, have excysted and resumed trophic growth (Sriram *et al*., 2008).

Several environmental factors such as the presence of organic material help in the distribution of *Acanthamoeba*. The presence of bacteria as a food source is very important in ensuring survival of *Acanthamoeba*. In addition, *Acanthamoeba* can also grow in the biofilm, where the food supply is continuous and can withstand a broad range of temperature. Thermotolerant strains has been isolated in soil sample taken from the island of Tenerife, Canary Islands, Spain, which showed 90.6% (39 out of 43 isolates) were thermotolerance at 37°C (Lorenzo-Morales *et al*., 2005a). Meanwhile the soil samples from Talbot County, Maryland, United States of America shows 81.9% (17 of 21 isolates) were thermotolerance at 37-39°C (Sawyer, 1989). Eventually, strains from clinical isolates showed good growth at warmer temperatures at approximately 37°C, in order to survive in mammal body. However, many pathogenic isolates were also showed good growth at lower temperature around 32-35°C, as such those strains that capable to infect and colonize the surface of the cornea causing *Acanthomoeba* keratitis (Schuster & Visvesvara, 1998).

2.7 ACANTHAMOEBA KERATITIS

Acanthamoeba keratitis (AK) discovered in patient with a history of one eye trauma and was first documented from United Kingdom (Nagington *et al*., 1974) followed by the South Texas, United States (Jones *et al*., 1975). Since then, the number of reported cases were continues to increase although initially *Acanthamoeba* has been known to cause a rare infection. Recently, increasing cases of AK were documented to be parallel with the

contact lens wearers that contributed up to 90% of cases worldwide (Patel & Hammersmith 2008), in both immune-competent and immune-suppressed individuals.

The incidence of AK in contact lens wearers were estimated 1-2 cases per million in US (Schaumberg *et al*., 1998), one in 30,000 in UK (Seal, 2003), 0.33 per 10,000 in Hong Kong, 0.05 per 10,000 in the Netherlands (Stehr-Green *et al*., 1989), 0.19 per 10,000 in England (Radford *et al*., 2002) and 1.49 per 10,000 in Scotland (Lam *et al.*, 2002; Seal *et al.*, 1999). The reports also were from Sweden (Nilsson & Montan, 1994), Scotland (Seal *et al*., 1999) and the Netherlands (Cheng *et al*., 1999). The variations of documented incidents were due to extended wear of contact lens, lack of awareness and hygiene, water hardness and salinity that promote the growth of *Acanthamoeba*. The used of contaminated contact lens have caused outbreak in Singapore (Por *et al*., 2009) and US (Yoder *et al*., 2012). Concurrently, about 70 million people around the world were wearing contact lens (Barr, 1998) with the application of vision correction (such as UV light protection) or cosmetic purpose and eventually the soft contact lens users were noted to be at higher risk of acquiring AK (Stehr-Green *et al*., 1989).

The exact mechanisms that associated with AK are not fully understood but the predisposing factor is strongly believed due to the used of contact len that was exposed to contaminated water Several possible factors that may be associated with *Acanthamoeba* infections were documented by Khan (2006), which included: (1) rubbing the eyes and lack of hygiene while handling contact lenses (without proper hand washing after working with soil), (2) contact lens are used more than the allowable intervals, (3) inappropriate cleaning of contact lens, (4) washing eyes during or immediately after contact lens wear, (5) exposure to contaminated water during swimming or water activities and (6) the used of homemade saline for contact lens cleaning contributes to

formation of biofilm on contact lens. AK has been associated with individual behaviour, especially in young males (Niederkorn *et al*., 1999).

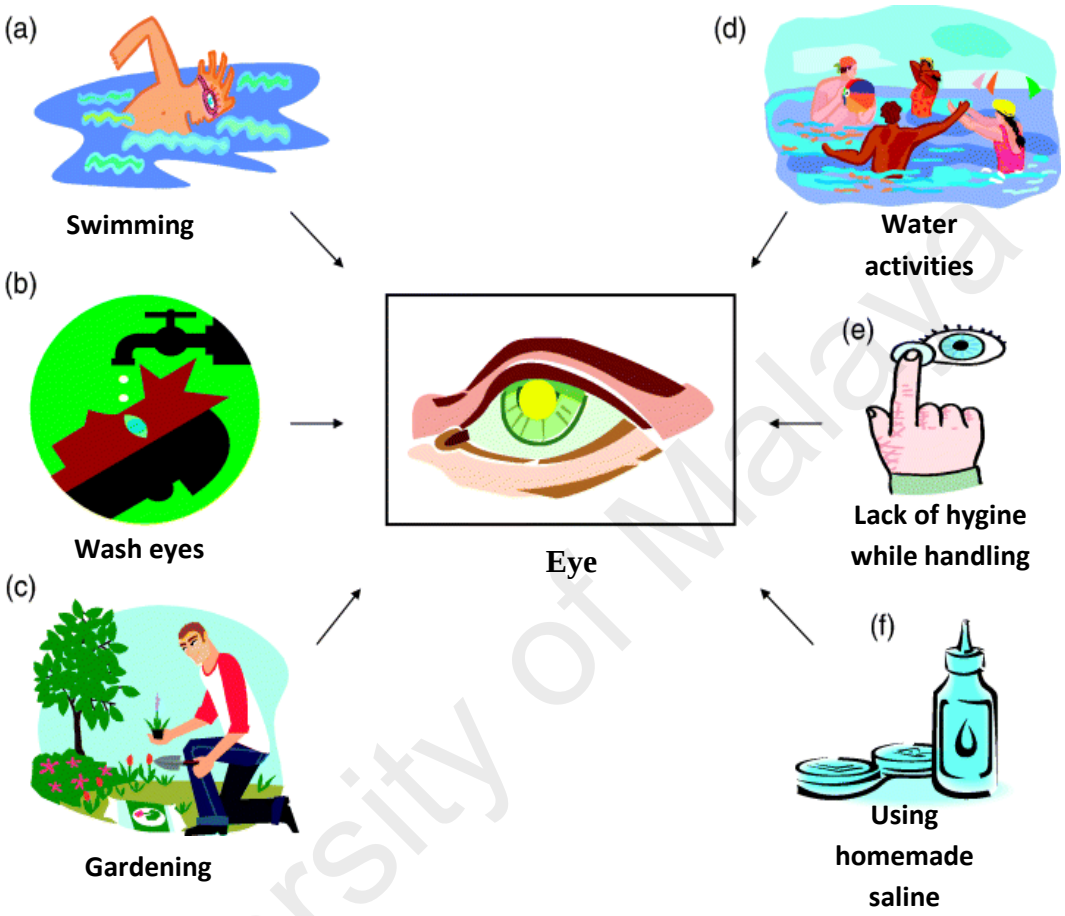


Figure 2.5: The risk factors contributing to *Acanthamoeba* keratitis (Khan, 2006).

The used contact lens was indicated with the presence of mannose, glucose, galactose, fucose, N-acetyl- D-glucosamine, N-acetyl-D-galactosamine, N-acetyl neuraminic acid (sialic acid), and proteins, glycoproteins, lipids, mucins, polysaccharides, calcium, iron, silica, magnesium, sodium, lactoferrin, lysozyme and immunoglobin molecules on the surface of contact lens after 30 minutes being worn. (Tripathi & Tripathi, 1984; Gudmundsson *et al*., 1985; Klotz *et al*., 1987). These molecules may be acted as receptors for *Acanthamoeba* trophozoites to express a mannose binding protein (MBP) on the contact lens surface (Beattie *et al*., 2003). *Acanthamoeba* trophozoites

could also have a good binding ability with biofilms coated lenses as compared to contact lenses without biofilms (Beattie *et al*., 2003; Simmons *et al*., 1998; Tomlinson *et al*., 2000). Biofilms provide required nutrients to enhence the increase of *Acanthamoeba* trophozoites on contact lens during storage or cleaning. Their trophozoites were also showed to have binding ability with the corneal epithelial cells (Dudley *et al*., 2005; Garate *et al*., 2006)

In the cases of non-contact lens, the *Acanthamoeba* infection occurred after having a corneal trauma of minor abrasions caused by inserted object or rubbing with dirty hands after exposed to contaminated water, soil or other particles (Sharma *et al*., 1990; Chang & Soong, 1991). The requirement for traumatic corneal infections can be explained by the fact that expression of *Acanthamoeba*-reactive glycoprotein (S) on damaged cornea is 1.8 times higher than the healthy cornea, thus contributing to the higher rate of infection in the injured cornea (Jaison *et al*., 1998).

The onset of symptoms of *Acanthamoeba* in corneal invasion can take from several days to weeks, depending on the inoculum number of *Acanthamoeba* and the level of corneal trauma. Once the *Acanthamoeba*-contaminated contact lens is placed on the cornea, the amoebae will invade cornea and show non-specific signs and symptoms such as eye pain, eye redness, photophobia, blurred vision and a sensation of something in the eye, along with excessive tearing. The corneal will then showed acute ulcer leading to the formation of a ring-like stromal infiltrate, corneal oedema and erosion of the corneal epithelial cells (Figure 2.6). AK usually occurs in one eye, but can be occurred bilaterally and had been documented as a complication of the initial infection (Lee & Gotay, 2010). Patients with AK usually experience severe eye pain, due to the invasion of trophozoites along the corneal nerves (keratoneuritis) which leads to thickening and distortion of the nerve (Yoo *et al*., 2004). The next infection step would cause denudation of epithelial

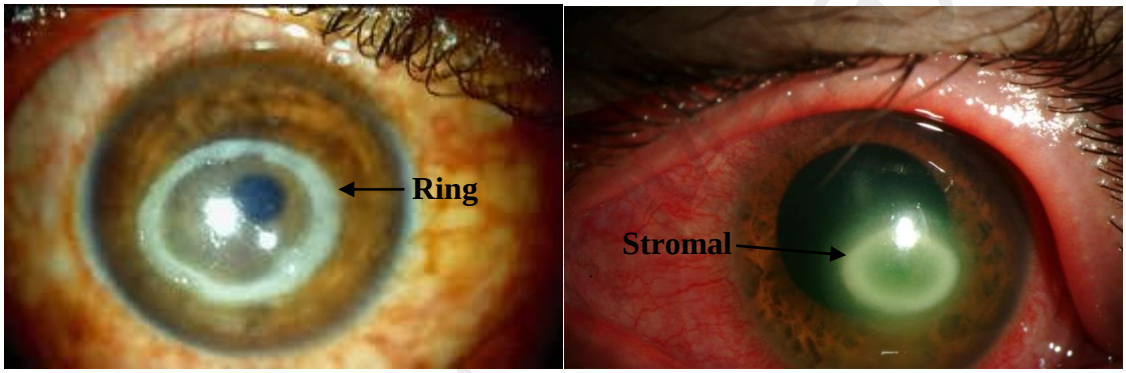
cells, and stromal necrosis (da Rocha-Azevedo *et al*., 2009). Glaucoma is commonly reported, and occasionally posterior segment signs such as nerve oedema, optic atrophy and retinal detachment. Secondary infections caused by bacteria increase the complexity of clinical management of AK. Misdiagnosis or delay in treatment, can eventually lead to blindness (Niederkorn *et al*., 1999).

Most of AK cases were always misdiagnosed, as such similarity in most of the signs and symptoms that caused by the herpes simplex virus, adenovirus (Martinez & Visvesvera, 1997) and bacterial infection caused by *Pseudomonas aeruginosa* (Clarke & Niederkorn, 2006) and *Staphylococcus* (Giese & Weissman, 2002). Depites, keratitis cases were also due to the filamentous fungi infections, including *Aspergillus flavus*, *Aspergillus fumigatus*, *Fusarium* spp., *Alternaria* spp. as well as a yeast, *Candida* (Halde, 1986). AK can also present as a mixed infection with bacteria or fungi that complicates diagnosis and treatment (Goodall *et al*., 1996). On the other hand, *in vivo* studies have reported that AK is host specific, in which the disease is only produced in pigs and Chinese hamsters, but not in rats, mice or rabbits. In dditionally, AK will not develop in animal corneas that have intact epithelial layers (Niederkorn *et al*., 1999).

Regarding diagnosis, the confirmation of AK should be carried in all possible samples such as swabbing from corneas, contact lens, contact lens storage and corneal biopsy by using microscopic observations (Epstein *et al*., 1986) and confocal microscopy (Winchester *et al*., 1995). The examiners must be able to differentiate the characteristics of *Acanthamoeba* based on identification keys. Currently, PCR-based methods have been used for specificity and rapid detection (Khan *et al*., 2001; Schroeder *et al*., 2001).

Indeed, confirmation of *Acanthamoeba* infection at the early diagnosis play an important role for further aggressive treatment with the suitable therapeutic drugs that essential for a succesfull recovery (Perez-Santonja *et al*., 2003). The recommended and

succesfull treatment of AK is by using Brolene (0.1% propamidine isethionate), PHMB (0.02% polyhexamethylene biguanide) or CHX (0.02% chlorhexidine digluconate) or Desomedine (0.1% hezamidine) (Bacon *et al*., 1993; Draulans & Maudgal, 1992; Moore & McCulley, 1989; Seal *et al*., 1996; Wright *et al*., 1985). In addition, supplemented with antibiotics in the infection that is suspected or associated with bacteria. More recently, treatment of AK patients with antimicrobial and biocides using alkylphosphocholines showed a faster recovery and safer for patients (Hiti *et al*., 2002; Murdoch *et al*., 1998; Turner *et al*., 2000) (Figure 2.5).



a) Ring infiltrate **b) Stromal infiltration**

Figure 2.6: *Acanthamoeba* infected eye exhibiting the severity of acanthamoeba keratitis
a) http://www.eyecalcs.com/DWAN/pages/v5/ch061/004f.ht
b)http://www.eyeworld.org/article

2.8 GRANULOMATOUS AMOEBIC ENCEPHALITIS

Granulomatous amoebic encephalitis (GAE) is a central nervous system (CNS) disease caused by *Acanthamoeba* infections in immunosuppressed and was first documented in 1972 (Jager & Stamm, 1972). GAE normally a secondary, rare infection but often fatal that is increasingly important in immunocompromised individuals due to HIV positive, diabetes mellitus, lymphoproliferative disorders, haematological disorders, pneumonitis, renal failure, liver cirrhosis, rhinitis, pharyngitis, gammaglobulinaemia, systemic lupus erythematosus, malignancies, glucose 6-phosphate dehydrogenase deficiency, tuberculosis and malnutrition, pregnant woman, alcoholism, chronic illness, undergoing radiotherapy, organ/tissue transplant, steroids, excessive antibiotics, immunosuppressive therapy or other complications may all contribute to GAE infections (Khan, 2006).

Acanthamoeba enters the body through the lower airways that lead to the invasion of the intravascular space, followed by haematogenous spread reaches into the CNS (Martinez & Visvesvara, 1997). Infection in the CNS may present with several signs and symptoms within weeks to months. The signs and sypmtoms for chronic progressive GAE were documented as headaches, slight fever, behavioural abnormalities, personality changes, lethargy, stiff neck, aphasia, ataxia, vomiting, nausea, hemiparesis, cranial nerve palsies, increased intracranial pressure, seizures, typical signs of localised encephalopathy and death (da Rocha-Azevedo *et al*., 2009; Marciano-Cabral, 2003; Schuster & Visvesvara, 2004; Walochnik *et al*., 2008). These symptoms were all due to the haemorrhage necrotizing lesions with severe meningeal irritation and encephalitis (Martinez, 1985). Lesions caused by GAE were commonly found in basal ganglia and midbrain. These lesions are believed to be caused by actively feeding *Acanthamoeba* trophozoites or inflammatory process with the release of cytokines. Other than CNS, tissue and organs such as subcutaneous tissue, skin, liver, lungs, kidneys, adrenals,

pancreas, prostate, lymph nodes and bone marrow may also be affected and show lesions. Symptoms such as skin lesions can be seen with a variety of hard erythematous nodules, papules, or ulcers, on the whole surface of the body (May *et al*., 1992). These symptoms are similarity with bacterial meningitis, viral encephalitis, neurocysticercosis, tuberculosis meningitis and brain tumors, which causing confusion in the identification of the real cause of GAE by a medical practitioner (da Rocha-Azevedo *et al*., 2009; Matson *et al*., 1988; Ofori-Kwakye *et al*., 1986). Therefore, most of GAE diagnosis was done against biopsy of brain tissue, cerebrospinal fluid (CSF), bronchoalveolar lavage (BAL), skin and lung tissue to detect the presence of *Acanthamoeba* by microscopic observation. Identification of *Acanthamoeba* can be performed by using the specific PCR technique on the biopsy specimens because the cultivation techniques may show negative growth (Walochnik *et al*., 2008). Both trophozoites and cysts could be identified in fresh of fixed biopsy samples (da Rocha-Azevedo *et al*., 2009).

Early detection of GAE cases were documented to have better prognosis (Lackner *et al*., 2010; Seijo-Martinez *et al*., 2000; Walia *et al*., 2007; Walochnik *et al*., 2008). Patients were usually treated with different anti-microbial due to the differences in each individual responses, a drug treatment used successfully in one patient may not be effective for other patients. Several factors which influence the prognosis status includes how early the treatment is given, immune status of the host, infective dose of the amoeba, antimicrobial sensitivity against the strains and its virulence (Schuster & Visvesvara, 2004). Many drugs have been used in treating GAE including amphotericin B, azithromycin, ketoconazole, intraconazole, fluconazole, 5-fluorocytosine (flucytosine), pentamidine isethionate, meropenem, linezolid, rifampicin, moxifloxacin, miltefosine, amikacin, voriconazole or sulfadiazine (Lackner *et al*., 2010; Schuster & Visvesvara, 2004; Seijo-Martinez *et al*., 2000; Walia *et al*., 2007; Walochnik *et al*., 2008). These drugs

may produce severe side effects, in which survivors might develop disability such as hearing loss and vision impairment.

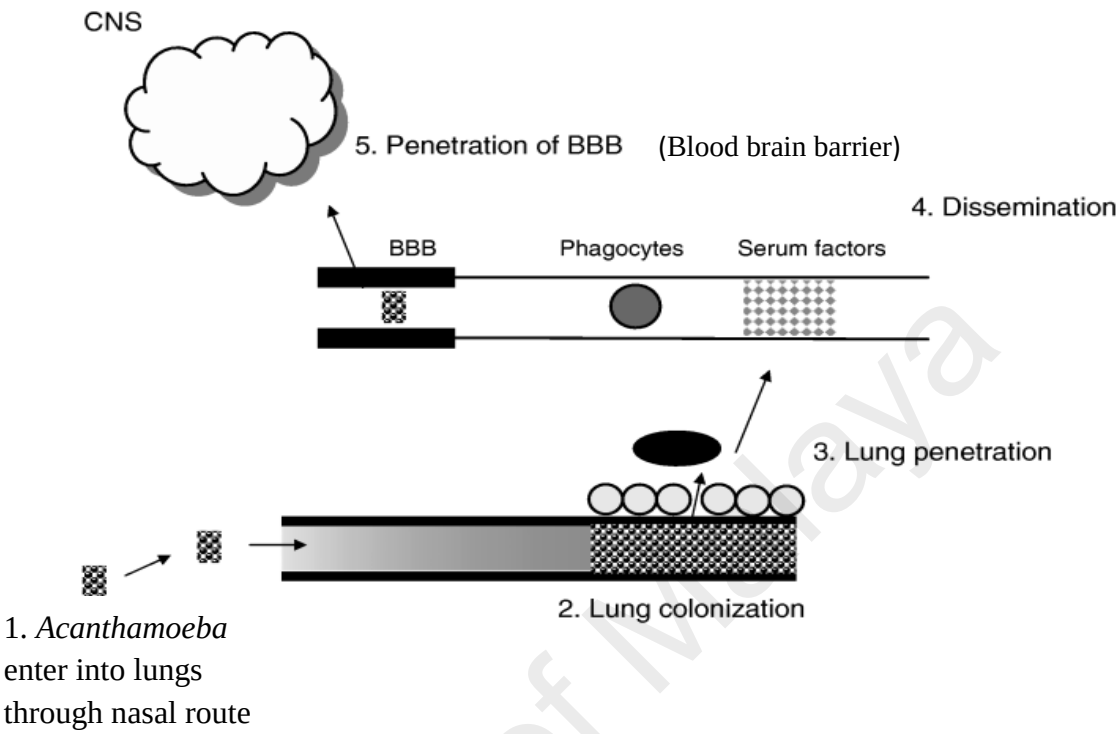


Figure 2.7: The route model of *Acanthamoeba* species entering human body and cause granulomatous amoebic encephalitis (Khan, 2006).

CHAPTER 3

MATERIALS AND METHODS

3.1 ETHICAL CONSIDERATION

The protocol of this study was approved by the Institutional Animal Care and Use Committee of the University of Malaya (UM IACUC), Kuala Lumpur (Ethics Reference number: PAR/29/06/2012/II (R)). Prior to the collection samples from animals (cats/dogs), the objectives and protocols of the research were discussed with the authorities in charge or with the owners of these animals. For collection of the environmental samples, the letter entitled 'request for permission to collect samples' from the Head of Department of Parasitology, Faculty of Medicine, University of Malaya (or first investigator of this project) was obtained and the samples were only collected after getting permission from the authorities or the security in charge.

3.2 STUDY AREAS AND SELECTED SAMPLES

These study areas were selected based on several criteria such as no history of previous similar study, easy to access and convenience to collect the specimens, including the environmentals materials such as dust, debris, water and soil.The environmental samples were collected in the vicinity of Kuala Lumpur included the PAWS (PAWS Animal

Welfare Society) shelter, recreational natural rivers, children's playgrounds and Faculty Medicine of University Malaya.

As for the corneal swabs, it's were collected from stray cats and dogs with the signs of naturally eye infection such as watery eyes, grey eye discharge and red eye (Appendix C.1). The animals were from all gender and ages, male, female, adult, kitten/puppy of stray and pets. The main sampling site was from PAW animal shelter after obtained the written permission from PAWS management. The sites of the PAWS were targeted at the animal bedding areas (dust), food and drink containers (moist debris) (Appendix C.2 and Appendix C.3). Concerning PAWS Animal Welfare, it is located in Subang, approximately 15 km from Kuala Lumpur city. PAWS is a social house for unwanted, abandoned, injured and stray cats/dogs in the vicinity of Klang Valley (Kuala Lumpur and its adjoining areas in the state of Selangor). Most of these stray cats and dogs were brought in by the workers (dog catchers) of the Kuala Lumpur City Council to the PAWS. They were brought in twice a week, at the morning (~ 8.00 ─ 9.00 am) and approximately 10-15 animals each time. Most of these newly sent animals were with several signs of skin diseases as well as ocular infections. However, they were then treated accordingly and taken care by the volunteer personals including the veterinarian and animal lovers. Therefore, most of the eye swab samples were collected in newly sent stray animals (had not been treated) as compare to long time PAWS pets. There are also stray cats from residential areas and street were obtained (Appendix C.4).

The infected corneal swabs from stray animals were carried out with assistance from the veterinarian, workers in the PAWS and experience dog catchers of the Kuala Lumpur City Council. The stray dogs were fierce and needed experience assistant to handle, therefore the corneal swabbing from infected dogs were only carried out from PAWS that facilitated by experience workers. The corneal swabs from PAWS were

carried out twice a month for twelve (12) months continuously, starting from May 2013 to April 2014. Due to team behaviour of cats, the corneal swab was also carried out occasionally in other sampling sites, where ever we found the infected cats. There sampling sites are summarised in Figure 3.1 and Table 3.1.

In the recreational rivers (Appendix C.5 and Appendix C.6), the sites were targeted at the riverside (wet soil), the surface of rocks or stones (wet debris), and the middle (surface water) of each of these 15 rivers. At the children playground and primary school field (Appendix C.7), the naturally dry soil samples were collected from the active areas where children were seen playing. Samples from Medical Faculty of University Malaya (Appendix C.8) were targeted at indoor and outdoor dust. The indoor dust samples were collected from the close environments, as such at the wall surface inside the lecture halls, seminar rooms and undergraduate laboratories. While for the outdoor dust, the selected sites were at the wall surfaces of the buildings adjacent to the car parking areas.

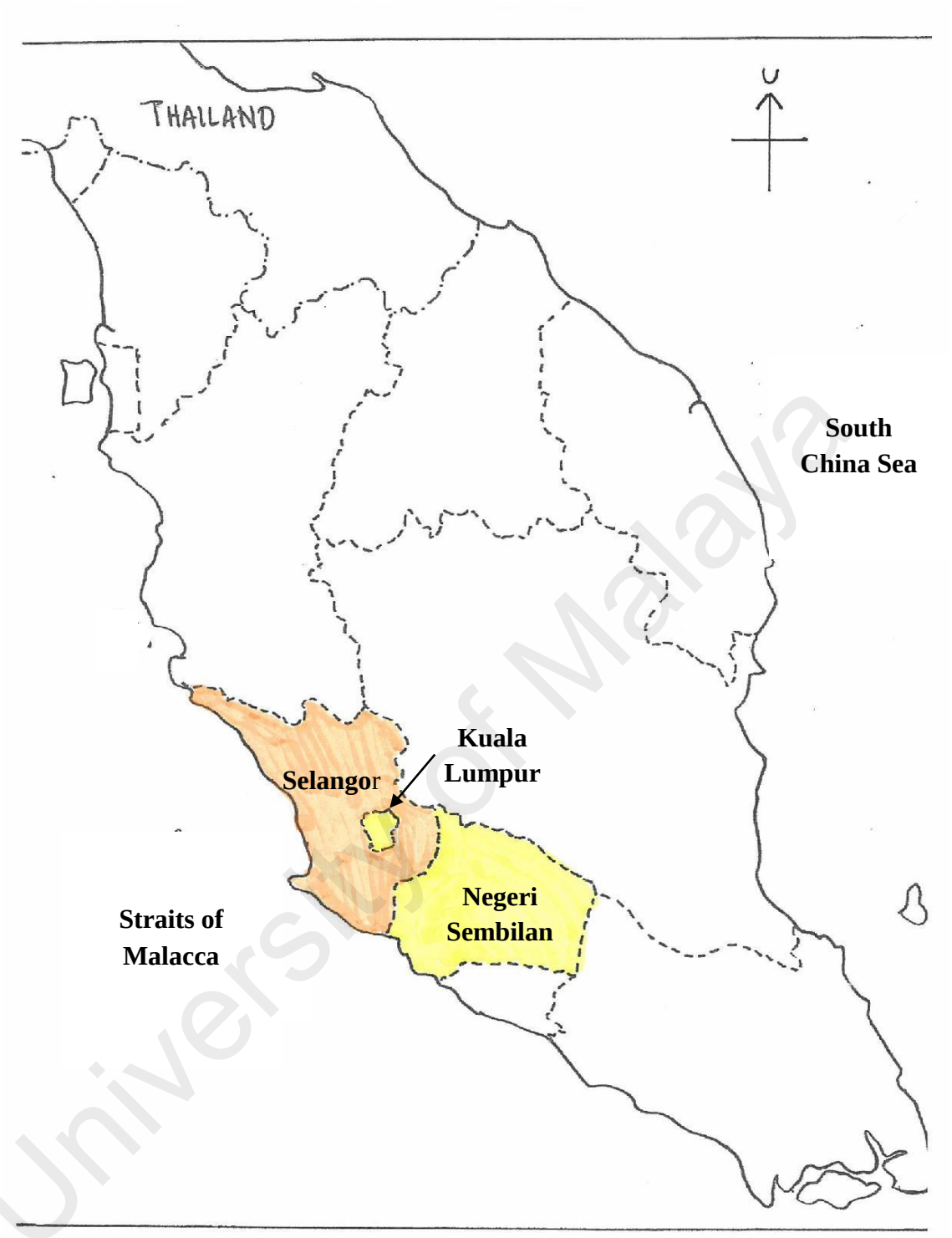


Figure 3.1: Map of Peninsular Malaysia showing locations of study areas.

Table 3.1: Selected locations for collection of animal corneal swabs and environmental samples.

Latitude (ºN)	Longtude (ºE)	LOCATION / SAMPLE
3.13044	101.55183	**PAWS animal shelter:** 1. Infected corneal swabs samples from cats and dogs. 2. Environmental samples from bedding, drinking and food containers.
		Residential areas/streets: infected corneal swabs from cats
3.16046	101.69488	1. Chow Kit, Kuala Lumpur
3.16361	101.70967	2. Kampong Baru, Kuala Lumpur
3.18767	101.70469	3. Setapak, Kuala Lumpur
3.19897	101.73957	4. Wangsa Maju, Kuala Lumpur
3.22376	101.72533	5. Taman Melati, Kuala Lumpur
3.19834	101.68690	6. Sentul, Kuala Lumpur
3.15880	101.75745	7. Ampang, Selangor
3.10709	101.60825	8. Petaling Jaya, Selangor
3.09060	101.52958	9. Shah Alam, Selangor
3.04390	101.58065	10. Subang Jaya, Selangor
2.71861	101.94055	11. Ampangan, Negeri Sembilan
2.53642	101.80681	12. Port Dickson, Negeri Sembilan
2.73892	101.14114	13. Kuala Pilah, Negeri Sembilan
2.54354	10216913	14. Rembau, Negeri Sembilan
2.86441	102.09411	15. Jelebu, Negeri Sembilan
2.84752	101.82610	16. Taman Mutiara, Negeri Sembilan
2.69029	101.90771	17. Seremban 2, Negeri Sembilan
		Recreational areas-water, dust, debris, and soil
3.11164	101.81316	1.Sungai Pangsun, Selangor
3.02607	101.48040	2.Sungai Congkak, Selangor
3.21842	101.86485	3.Sungai Lopo, Selangor
3.29973	101.61925	4.Sungai Kanching, Selangor
3.43173	101.65667	5.Sungai Sendat, Selangor
3.24277	101.61101	6.Sungai Rumput, Selangor
3.05847	101.87191	7.Sungai Tekala, Selangor
3.36603	101.63678	8.Sungai Serendah, Selangor
3.45140	101.64208	9.Sungai Kedondong, Selangor
3.59907	101.73736	10.Sungai Chiling, Selangor
3.33183	101.70248	11.Sungai Tua, Selangor
3.15528	101.89915	12.Sungai Gabai, Selangor
3.21842	101.76676	13.Sungai Kemensah, Selangor
3.29875	101.62025	14.Sungai Templer, Selangor
3.23694	101.63777	15.Sungai FRIM, Selangor
		Children Playground-soil: natural dry soil
3.11814	101.63216	1.Section 4, Petaling Jaya, Selangor
3.11814	101.63216	2.Section 5, Petaling Jaya, Selangor
3.11814	101.63216	3.Section 12, Petaling Jaya, Selangor
3.08500	101.64405	4.SJKC Chen Moh, Petaling Jaya, Selangor
3.11078	101.61733	5.SMK Petaling, Petaling Jaya, Selangor
3.31770	101.27431	6.Taman Mawar Putih, Kuala Selangor, Selangor
3.09141	101.72390	7.SK Seri Bahagia, Sabak Bernam, Selangor
3.74769	101.05555	8.SK Bagan Terap, Sabak Bernam, Selangor
3.74463	101.05549	9.SRA Bagan Terap, Sabak Bernam, Selangor
3.72561	101.08083	10.Kampung Bagan Terap, Sabak Bernam, Selangor
3.74769	101.05555	11.SMK Bagan Terap, Sabak Bernam, Selangor
3.72846	101.07302	12.SK Tok Kharifah, Sabak Bernam, Selangor
3.14775	101.77386	13.SK Taman Kosas, Ampang, Selangor
3.14795	101.77224	14.SMK Taman Kosas, Ampang, Selangor
3.09337	101.68302	15.SMK Sri Sentosa, Kuala Lumpur
3.11363	101.65314	**Faculty Medicine of University Malaya** 1. Dust from indoor wall surfaces 2. Dust from outdoor wall surfaces

3.3 COLLECTION OF ENVIRONMENTAL AND INFECTED CORNEAL SWABS SAMPLES

First, the cotton-bud packaging (Appendix C.9.A) was prepared in a sterile 15-mL test tube that consisted 0.5 mL of sterile normal saline 0.9%, cotton-bed and cotton-bud. The packaging was then autoclaved at 15 lbs pressure, 121°C for 15 minutes and followed by stored at 4°C until used.

The sterile wetted-cold cotton bud from packaging was carefully withdrawn and swabbed on the particular sites to collect the dust, debris and soil samples, respective. The swabbed cotton bud was placed back into its original packaging, labelled accordingly and transported to our laboratory.

As for water samples, 5 litres of the surface water sample was collected from the middle of the river by using sterile polypropylene bottle and was the capped tightly. The wet debris was collected at the water-magin on the surface of rocks/stones that scattered in the recreational sites while the muddy wet soil was collected from the riverside by using sterile wetted cold cotton bud as mentiond above. The collections of these samples were carried out in 15 natural rivers between October and December 2013.

In the children playground, the naturally dry soil samples (Appendix C.9.B) were collected from 15 playgrounds (at primary school field and recreational area), at active sites where many children are playing. The collection of these soil samples were carried out during September 2013. While the dust samples from the Faculty of Medicine, University of Malaya were collected at February 2014.

The corneal swabs were collected from stray cats and dogs with the signs of naturally eye infection such as watery eyes, grey eye discharge and red eye (Appendix C.1). The animals were from all gender and ages, male, female, adult, kitten/puppy of stray and

pets. The main sampling site was from PAW animal shelter after obtained the written permission from PAWS management. The sterile wetted-cold cotton bud from packaging was carefully withdrawn and swabbed on the surface of infected corneas. Fingers were used to open the eyelids broadly and avoided from the eye blinking while swabbed the infected corneas. The swabbed cotton bud was placed into its original packaging, labelled accordingly and placed in a cold (10±2ºC) polystyrene box. It was then transported to the laboratory at the Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur.

3.4 CULTIVATION AND DETECTION OF FREE-LIVING AMOEBA

3.4.1 Preparation of Page's amoeba saline (PAS) solution

PAS solution was prepared by dissolving the following chemicals; 0.004 g magnesium sulfate ($MgSO_4$), 0.12 g sodium chloride (NaCl), 0.142 g dibasic sodium phosphate (Na_2HPO_4), 0.004 g calcium chloride ($CaCl_2$) and 0.136 g potassium phosphate (KH_2PO_4) in 900-mL distilled water (dH_2O) and later made up to 1000-mL. The solution was then transferred into Schott bottle and autoclaved at 15 lbs pressure, 121°C for 15 minutes. The sterile PAS solution was then stored at room temperature until used.

3.4.2 Preparation of nutrient agar (NA) and *Escherichia coli* culture

The nutrient agar was prepared by mixing 10 g nutrient agar powder (Oxoid) with 500-mL of distilled water in Schott bottle. The mixture was heated in an oven for a minute (to dissolve the nutrient agar) and then autoclaved at 15 lbs pressure, 121°C for 15 minutes for sterilization. This nutrient agar medium was left to cool down to 45°C, then poured approximately 25-mL into petri dish, aseptically, covered and left at room temperature to solidify. The solidified nutrient agar plates were ready for inoculation of *E. coli* or storage at 4°C until used. *E. coli* inoculated plates were incubated at 37°C for at least two (2) days

to propagate. The growing phase of *E. coli* can be harvested and used as a food source in *Acanthamoeba* culture. Its also can be kept (up to 14 days) in 4°C for future propagated when needed.

3.4.3 Preparation of non-nutrient agar with *E. coli* lawned

A total of 15 g of non-nutrient agar powder (Oxoid) was mixed with 1000 mL of PAS in Scott bottle. It was then autoclaved, left to cool down and poured into petri dish as steps mentioned in section 3.5.2. The solidified agar plates were sealed with parafilm and kept at 4°C or ready to use for *E. coli* lawned. The harvested *E. coli* cells from section 3.5.2 (by adding 5 mL cold PAS) were resuspended by pipetting repeatedly. One (1) mL of suspension was transferred onto a surface of non-nutrient agar (from section 3.5.2), followed by evenly spreading and left at room temperature for 45 minutes. The *E.coli* plates are ready to use for *Acanthamoeba* culture or kept at 4°C until used.

3.4.4 Cultivation of *Acanthamoeba* isolates

Swabbed cotton-buds (also cotton-beds) from section 3.4 were placed individually on the surface of each of the nonnutrient-*E.coli* agar plates (Appendix C.9). The plates were then covered, sealed with parafilm and incubated at 28-30°C (room temperature) for 14 days. The negative control was included in every cultivation batches, in which it was carried out by replacing with a clean non-swab cotton-bud (and cotton-bed). As for water samples, 5 litres of water was filtered through a nitrocellulose membrane (47 mm diameter, 1.2 µm pore size, Millipore, USA) held in a filter holder to trap the sediment. The sediment on the membrane was rince, transferred into a 50-mL tube and centrifugated at 3000 rpm for 15 minutes. Discarded the supernatant up to about 6 mL left and consequently resuspended the pallet. Approximately 2 mL of this suspension was placed onto nonnutrient-*E. coli* agar plate, and there were triplicate plates per water sample. The culture plate was then sealed with parafilm and incubated at room temperature (26±2°C)

for up to 14 days. A negative control was included in every test sets by inoculated with sterile distilled water.

3.4.5 Morphological detection of free living amoeba and isolation of *Acanthamoeba*

The culture plates were observed daily for up to 14 days by using light inverted microscope (NIKON) before all negatives plates were discarded. The trophozoite and cyst stages of free living amoeba (FLA) such as *Acanthamoeba*, *Naegleria*, *Vahkamphia* and *Harmannella* were identified under x200, followed by x400 magnification. The FLA-positive plates were also observed co-existed with bacterial and fungal. The Positive plates were further subcultured by cutting out a small piece of agar (either colonized by *Acanthamoeba* or *Naegleria*) and placed onto newly fresh nonnutrient-*E. coli* agar plates. Subcultivation was repeated at least ten (10) times to select the dominant cultivable *Acanthamoeba* or *Naegleria* isolates.

3.5 MOLECULAR AND PHYLOGENETIC ANALYSES

3.5.1 Harvesting of *Acanthamoeba* trophozoites cells

The dominant cultivable *Acanthamoeba* isolates were subcultured and approximately 3-4 plates of log phase trophozoites were harvested by detaching their cells from the surface of agar by adding 5 ml of sterile cold PAS, pH 7.2. The suspension was then transferred into 4 microcentrifuge tube (volume 1.5 ml) and centrifuged for 5 minutes at 3500 rpm. Supernatant was discarded and the pellet containing the trophozoites was further washed at least twice with PAS solution. The resuspended pellet was accumulated in one microcentrifuge tube followed by adjusted to a final volume of 200 µl prior subjected to the DNA extraction kit.

3.5.2 Extraction of *Acanthamoeba* genomic deoxyribonucleic acid (DNA)

Extraction of DNA was performed using the manufacturer's protocol of QIAamp® DNA blood mini kit (QIAGEN, Hilden, Germany). Approximately, $1x10^6$ of cells were used for each extraction process. Harvested trophozoites from section 3.6.1 were first lysed with 20 µl QIAGEN protease followed by 200 µl of buffer AL (lysis buffer) to provide optimal DNA-binding conditions. In order to ensure efficient lysis, it was essential that the sample and buffer AL (lysis buffer) were mixed thoroughly by vortexing for 15 seconds. The sample was then incubated at 56°C for 10 minutes. Two hundred (200) µl of absolute alcohol was added to the sample and mixed again by pulse-vortexing for 15 seconds. Carefully the mixture was transferred into QIAamp silica-gel-membrane spin column in a 2-ml collection tube and centrifuged at 8000 rpm for 1 minute to selectively bind DNA to the membrane.

The collection tube containing the filtrate was discarded and replaced with a new 2-ml collection tube. Washing steps were carried out to remove contaminants and enzyme inhibitors by adding 500 µl buffer AW1 (wash buffer 1) to the column followed by centrifugation at 8000 rpm for 1 minute. The collection tube was discarded and 500 µl buffer AW2 (wash buffer 2) was added into the column and centrifuged at full speed (13,000) for 3 minutes to remove the residual ethanol that may interfere with subsequent reactions. The column was then replaced into a new 2-ml collection tube and centrifuged at full speed for another 1 minute to completely dry the membrane. A volume of 100 µl buffer AE (elution buffer) was added to the column with a new 1.5-ml microcentrifuged tube and incubated at room temperature for 3 minutes. Finally, the column was then centrifuged again for 1 minute at 8000 rpm to elute the genomic DNA.

3.5.3 Polymerase chain reaction (PCR) amplification

DNA quantity of *Acanthamoeba* isolates were determined by PCR method since spectrophotometer device was not available. Confirmation of *Acanthamoeba* genus was performed by *Acanthamoeba* specific primer pair JDP1 (5'- GGG CCC AGA TCG TTT ACC GTG AA -3') and JDP2 (5'- CTC ACA AGC TGC TAG GGG AGT CA -3') which amplified the ASA.S1 amplicon or diagnostic fragment 3 region of 18S rRNA gene (Schroeder *et al*., 2001). These primers yield a fragment ranges from 400-470 bp depending on the genotype. PCR was performed in a volume of 20 µl containing:

10x PCR buffer with $(NH_4)_2SO_4$	2.0 µl
dNTP Mix (10 mM)	0.4 µl
Forward primer (10 pmol/µl)	1.0 µl
Reverse primer (10 pmol/µl)	1.0 µl
Taq DNA Polymerase (1U/µL)	0.5 µl
$MgCl_2$ (25 mM)	1.2 µl
DNA template	5.0 µl
Water, nuclease-free	8.9 µl

The thermal cycling condition was performed as follows: a pre-PCR cycle at 94°C for 5 minutes, followed by 40 cycles at 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute and an extension at 72°C for 5 minutes (Schroeder *et al*., 2001). As for negative control, the DNA template was replaced with the same volume of distilled water.

3.5.4 Agarose gel electrophoresis of PCR product

Agarose gel electrophoresis was carried out to observe the DNA that has been amplified after PCR. Firstly, agarose, (ultra-pure agarose, BRL-Bethesda Research Lab) was weighed 1.5 g and transferred into an Erlenmeyer flask containing 100 ml of 1xTAE buffer. The mixture was then heated in a microwave oven until the agarose has dissolved. Subsequently, the dissolved agarose was allowed to cool at 55°C by placing the flask under running tap water. Ethidium bromide (10 mg/ml, Promega) was then added to produce a final concentration (0.2 µg/ml). The mixture was mixed thoroughly with gentle swirling and poured into a horizontal gel casting tray (Owl) with the appropriate comb in placed.

The agarose gel should only cover about one-third the height of the comb teeth or a depth of about 5 mm. Pipette tip was used to remove bubbles or solid debris while the gel was still in liquid form. The agarose gel was then allowed to solidify at room temperature and the comb was removed carefully. The tray was placed in the horizontal tank with the walls oriented at the cathode end. A sufficient volume of 1xTAE buffer was poured into the tank until it covered the whole agarose gel.

A droplet (2 µl each) of 6X bromophenol blue (Fermentas) loading dye solution was placed onto a small square of Parafilm. A volume of 10 µl PCR product was withdrawn from microcentrifuge tube and mixed with the loading dye droplet by pipetting in and out to produce a mixture with a total volume of 12 µl each. Subsequently, the mixture was loaded into the well of the agarose gel prepared earlier. A new sterile pipette tip was used for loading individual PCR product. DNA ladder was loaded on both right and left wells of the agarose gel as a standard marker. The ASA.S1 amplicons size was estimated by comparison with the GeneRuler 100 bp DNA ladder Plus (Fermentas Life Science, Canada).

The lid of the gel tank was closed and a 100V voltage of electrophoresis was applied. Bubbles were seen at the anode and cathode and within a few minutes, the bromophenol was seen migrating from the wells into the body of the agarose gel with the direction from the cathode towards the anode end.

After the DNA samples with dyes have migrated to a sufficient distance through the gel, approximately after 45 minutes, the electrophoresis was stopped. The gel was removed from the tank and examined under medium-wavelength ultraviolet (UV) light (UV-B, 302 nm). Exposure of DNA to UV light was kept to a minimum in order to avoid the formation of pyrimidine within the DNA molecules. Images of the gel were photographed using gel documentation device (DOC-088.XD, UVItech) and saved in a digital TIFF format.

3.5.5 Extraction and purification of PCR product from agarose gel

QIAquick gel extraction kit (QIAGEN) was used to extract and purify the amplicon. All centrifugation steps were carried out at 13,000 rpm. Firstly, the DNA fragment (amplicon) from agarose gel was excised by using a scalpel and the gel slice was weighed before transferred in a 1.5-ml microcentrifuge tube. Then, 3 volumes of buffer QG (solubilization buffer) were added to 1 volume of gel (100 mg ~ 100 µl) and the mixture was incubated at 50°C for 10 minutes until the gel was completely dissolved.

Subsequently, 1 gel volume of isopropanol was added to the sample and mixed well. The mixture was transferred into a QIAquick spin column in a 2-ml collection tube and centrifuged for 1 minute to bind the DNA. The supernatant was discarded and the column was placed back into the same collection tube.

Then again, 0.5 ml of buffer QG (solubilization buffer) was added to remove all traces of agarose gel as an optional step and centrifuged for 1 minute. Washing step was

carried out by adding 0.75 ml of buffer PE (wash buffer) into the column and incubated for 2 minutes before centrifuged for another 1 minute. The supernatant was discarded and the 2-ml collection tube was replaced back into the column and centrifuged for an additional 1 minute to remove all residual ethanol from the buffer PE (wash buffer). The column was then placed into a sterile 1.5-ml microcentrifuge tube. Lastly, 30 µl of buffer EB (elution buffer) was added to the center of the Qiaquick membrane column and incubated for 1 minute at room temperature before being centrifuged for 1 minute to elute the DNA.

3.5.6 Cloning of PCR product

The representatives of the PCR products which were positive for *Acanthamoeba* were cloned by using pGEM®-T Vector system II product cloning kit (Promega).

3.5.6.1 Preparation of Luria-Bertani (LB) broth and media

Firstly, 10 g of Bacto-tryptone, 5 g of Yeast extract and 10 g of NaCl were mixed and dissolved in 800-mL of distilled water. The pH of the mixture was adjusted to 7.0 with NaOH and later made up to 1000 ml. Before autoclaved, the mixture was divided into 500 ml in each of two Schott bottles. The mixtures were then steriled by autoclaving at 15 lbs pressure, 121°C for 15 minutes. The sterile LB agar was stored in 4°C until used.

The 500 ml steriled LB media was added with 7 g of agar and proceed for further autoclaving at 15 lbs pressure, 121°C for 15 minutes. The autoclaved LB medium was left to cool down to about 55°C, prior adding with antibiotic. Later, 100 µl ampicillin was added and mixed gently before pouring approximately 30 ml onto each petri dish, aseptically. The LB/ampicillin agar medium was covered and left at room temperature to solidify. Subsequently, 40 µl of X-gal (20 mg/ml) and 40 µl IPTG (100 mM) was added

onto LB/ampicillin agar plates with steriled L-shape spreader. Then, the agar mediums containing LB/ampicillin/X-gal/IPTG were stored at 4°C until used.

3.5.6.2 Ligation

The primary step in cloning is the ligation. In this process, the pGEM®-T Vector and Control Insert DNA tubes were briefly centrifuged and contents at the bottom of the tubes were collected. The 2X rapid buffer was vortexed vigorously before each use. The fragment of DNA (PCR product) containing gene to be cloned was inserted into plasmid pGEM®-T Vector. This combination produced a recombinant DNA molecule. The following components were added into a 1.5-mL microcentrifuge tube:

2X Rapid Ligation Buffer	5.0 µl
pGEM®-T Vector (50 ng)	1.0 µl
PCR product	3.0 µl
T4 DNA Ligase (3 Weiss units)	1.0 µl

The reactions was mixed by pipetting and incubated for 1 minute at room temperature. However, the incubation time can also be extended overnight at 4°C for a maximum yield of recombinants.

3.5.6.3 Transformation

In this step, the tube containing the ligation reaction was centrifuged and 5 µl of reaction was collected from the bottom, followed by transferring into a 1.5-ml microcentrifuge tube on ice. Later, the ligation reaction was mixed with 50 µl *E. coli* strain JM109 High Efficiency Competent Cells and gently flicking the tube before incubating 20-30 minutes

in ice. Then, the tube was heat-shock for about 45-60 seconds in water bath at exactly 42°C and immediately returned the tube to ice for 2 minutes incubation.

Subsequently, 950 µl LB broth was added into the tube and incubated in shaking incubator for 1.5-2 hours in 37°C at 200-250 rpm. After the incubation, the tube was centrifuged at 5000 rpm for 5 minutes and the supernatant was discarded leaving 100 µl of solution and pellet at the bottom of the tube. Lastly, the pellet was placed onto the surface of LB/ampicillin/X-gal/IPTG plate and incubated overnight (16-24 hours) at 37°C.

3.5.6.4 Analyses of the recombinant clones

The plates from section 3.6.6.3 were removed from the incubator and stored at 4°C for several hours to facilitate the blue/white colonies screening. The colonies with no recombinant plasmids showed blue in colour as X-gal was broken down by β-galactosidase, while the colonies carrying recombinant plasmids showed white in colour.

The recombinant colonies (white) were numbered using permanent marker at the bottom of the LB/ampicillin/X-gal/IPTG plates. A new LB/ampicillin plates from section 3.6.6.1 were drawn with 24-square grids using permanent marker. Each colony which has been labelled was streaked onto LB/ampicillin plate according to the respective numbers using different sterile pipette tips for each colony. The plates were incubated upside down in a 37°C for 12-24 hours.

3.5.6.5 Selection and identification of recombinant clones

The recombinant colonies were selected and identified using the boiling method followed by PCR. Firstly, each of the white colony were selected as in section 3.6.6.4 and was transferred into a 1.5-ml microcentrifuge tube containing 20 µl distilled water. The cell suspension was boiled for 10 minutes, followed by centrifugation at 13000 rpm for 10

minutes. The supernatant was then used for PCR amplification. Lastly, 5 µl of supernatant containing the released plasmids from each clone was subjected to PCR using the respective primers as mentioned in 3.6.3.

3.5.6.6 Purification of plasmid DNA

Purification of plasmid DNA was performed by using a QIAprep® Spin Miniprep kit (QIAGEN). A single colony that was confirmed with a presence of DNA from section 3.6.6.5 was selected and cultured onto freshly prepared LB/ampicillin medium followed by incubation for 12-16 hours at the 37°C shaking incubator (150 rpm) in 15-ml test tube. The incubation was not more than 16 hours to avoid the cells from lysing and plasmid yields may be reduced. The cells were then harvested by centrifugation at 3000 rpm for 5 minutes at room temperature. The pellet was mixed with 250 µl buffer P1 (resuspension buffer) and transferred to a 1.5-ml microcentrifuge tube followed by vortexing or pipetting up and down until there was no cell clumps.

Subsequently, 250 µl of buffer P2 (lysis buffer) was added and mix thoroughly by inverting the tube gently until the solution becomes viscous and slightly clear. Later, 350 µl buffer N3 (neutralization buffer) was added and immediately mixed by inverting 4 to 6 times. The tube was then centrifuged for 10 minutes at 13,000 rpm for a compact white pellet. The supernatant was transferred into QIAprep spin column by pipetting and centrifuged for 30 to 60 seconds at 13,000 rpm. The bound plasmid DNA was washed twice; first with 500 µl of buffer PB (binding buffer), followed by 750 µl buffer PE (wash buffer) and performed centrifugation at 13,000 rpm for 1 minute. An additional centrifugation (13,000 rpm) of the column was run for another 1 minute to completely remove any residual ethanol present in buffer PE (wash buffer). Finally, the QIAprep column was placed in a sterile 1.5-ml microcentrifuge tube and 50 µl buffer EB (elution

buffer) was added in the column. Finally, the column was incubated for 1 minute followed by centrifugation at 13,000 rpm for 1 minute.

3.5.6.7 Maintenance and preservation of recombinant *E. coli* cells, (JM109)

The bacteria *E. coli* strain JM109, from section 3.6.6.5 containing plasmids (recombinant *E. coli* cells) was maintained by sub-culturing to fresh LB/ampicillin plates every 2 weeks. For long-term storage, the recombinant clones of interest were grown in 5-ml of LB/ampicillin broth (section 3.6.6.1) for 16 to 24 hours with 150 rpm agitation. Subsequently, 1.6-ml of 60% (v/v) solution of glycerol was added to the overnight broth culture. The mixture was mixed thoroughly by pipetting and inverting the tubes for several times. Finally, the mixture was dispensed about 500 µl each into several sterile, screw-vials (Nalgene) and placed at -20°C for overnight before being transferred to -80°C freezer. The preserved clones can be recovered by partial thawing of the mixture and spreading of the aliquots onto free LB/ampicillin plates.

3.5.7 Sequencing of recombinant DNA

Sequencing of DNA was carried out in Taiwan using the ABI Big Dye® Terminator Cycle Sequencing Ready Reaction Kit v3.1, using the ABI PRISM® 3730xl DNA Analyzer (Applied Biosystems, USA). Three to four clones containing inserts of approximately the expected size was arbitrarily selected for each sample and sequenced on both strands using a specific primer pair JDP1 forward primer (5'- GGG CCC AGA TCG TTT ACC GTG AA -3') and JDP2 reverse primer (5'- CTC ACA AGC TGC TAG GGG AGT CA -3').

3.5.8 Phylogenetic analyses

DNA chromatograms were examined and the forward and reverse sequences were pair-wised aligned using Bioedit software ClustalW2 (Labarga *et al*., 2007), followed by

manually refined to obtain a better consensus sequence. The sequences were subjected to BLASTn searches against all nucleotide sequences archived in the GenBank database to confirm the of *Acanthamoeba* genotypes (Altschul *et al*., 1990). The *Acanthamoeba* genotypes were then identified by determining the exact match or closest similarity. Sequences were achieved from Genbank as well as those obtained from this study were aligned using ClustalW of Bioedit software.

Phylogenetic tree was then constructed using maximum likelihood and neighbor-joining methods using Mega version 4 software (Tamura *et al*., 2007) based on the selected sequences of both the tests and references isolates with published genotypes and followed by Kimura-2 parameter algorithm and constructed tree by a bootstrap analyses of 1000 replicated. Nucleotide differences between the genotype and clusters were calculated using BioNJ on the basis of the total SSU-rDNA with the MEGA program. The calculation was based on the p-distance with pairwise deletions and without selected sites.

3.6 LIMITATIONS OF THE STUDY

Collection of infected corneal samples from cats and dogs was a big challenge. Although, there were an abundance of stray cats and dogs, but those with infected cornea was hard to find. Furthermore, the stray animals were not tame, scared and some were too fierce to approach, obviously the stray dogs on the streets. Therefore, dog corneal samples were only collected from PAWS, with experience workers (at least 2-3 persons) used to assist during collection of the infected dog corneals. After cultivation of infected corneal samples, only few samples were showed positive growth in non-nutrient agar with *E. coli* lawned (the culture medium for FLA especially *Acanthamoeba*). Thus, the higher percentage of infected corneal might be due to others organisms such as bacteria, fungi

or virus which could not carried out in this study due to limitation in budget, facilities and expertist.

3.7 OTHER FREE-LIVING AMOEBAE

Other FLAs such as *Naegleria, Vahlkampfia* and *Hartmannella* were also detected in on the agar surface during cultivation of environmental samples. Therefore, several subcultures into newly fresh non-nutrient agar plates with *E. coli* lawned were carried out to isolate the *Acanthamoeba* from other FLA, especially *Naegleria* species. In addition, several *Naegleria* isolates were also success to culture. Identified by PCR amplification using two sets of internal transcribed spacers (ITS) primer (forward primer, 5'- GAA CCT GCG TAG GGA TCA TTT -3' and reverse primer, 5'- TTT CTT TTC CTC CCC TTA TTA -3') for the detection of all FLA species as well as specific primers (forward primer, 5'- GTG AAA ACC TTT TTT CCA TTT ACA -3' and reverse primer, 5'- AAA TAA AAG ATT GAC CAT TTG AAA -3' (Pélandakis *et al*., 2000)) for the detection of the pathogenic *Naegleria fowleri* were also carried out for addition finding. The amplication reactions were performed in a volume 20 µl as described in section 3.6.3 with adjustment of the thermal cycling conditions. All the positive amplicons were cloned and sequenced. The subjected sequence (ITS1, 5.8S and ITS2) was BLASTn searches against all nucleotide sequences archived in the GenBank database and were aligned using ClustalW of Bioedit software.

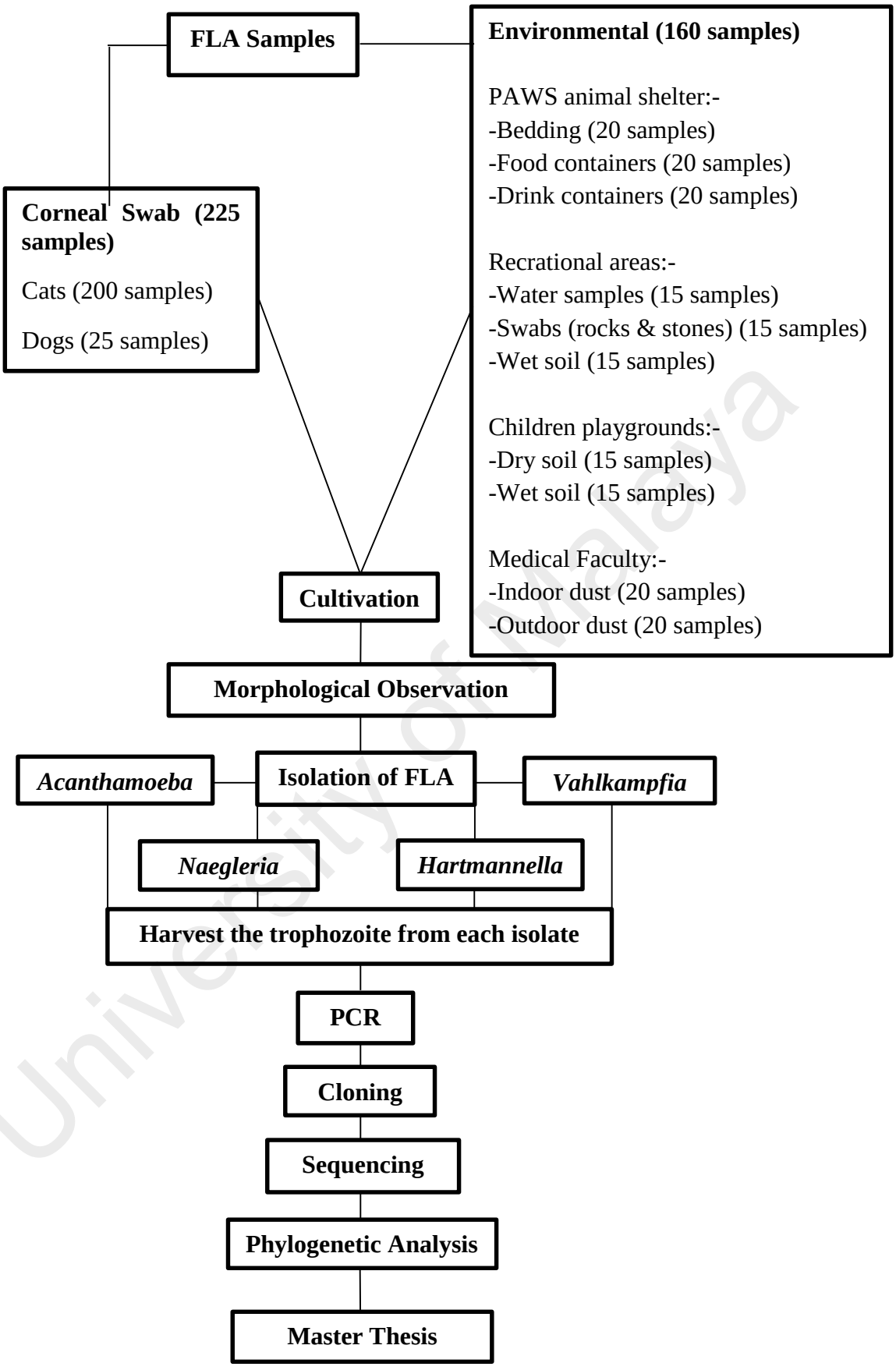


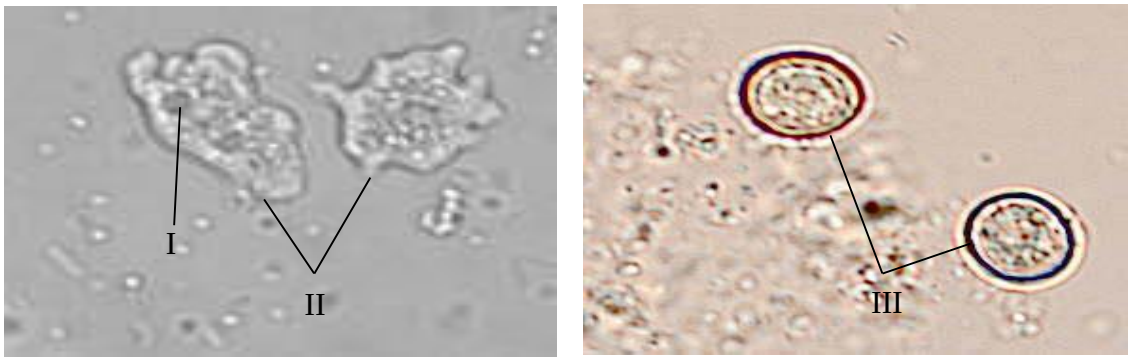
Figure 3.2: Flow chart of the research

CHAPTER 4

RESULTS

4.1 TROPHOZOITE AND CYST OF *ACANTHAMOEBA*

The trophozoite and cyst stages (Figure 4.1) were observed as early as the second and fifth days of cultivation, respectively. The trophozoites showed a specific characteristic of having spike-like pseudopodia called acanthopodia which protruded around the cell surface. Trophozoites possess a single nucleus, many contractile vacuoles, vary in size with diameter ranges between 12-35 μm and proliferate continuously. With prolonged cultivation, the trophozoites become stagnant and slowly transformed to cysts with wrinkled double thick walled. The dormant cysts vary in diameter which ranges between 5-20 μm and shapes depend on the different in species or genotypes.



(a) Trophozoites **(b) Cysts**

(I) Nucleus, (II) Acanthopodia, (III) Wrinkled Double Walled

Figure 4.1: Characteristics of trophozoite and cysts:

4.2 OCCURRENCE OF *ACANTHAMOEBA*

4.2.1 Infected corneal samples of stray cats and dogs

A total occurrence of *Acanthamoeba* in infected corneal samples of stray animals was 24.8% (56/225), with 24% (48/200) in cats and 32% (8/25) in dogs. All *Acanthamoeba* isolates showed good growth at 26±2°C on the surface of non-nutrient agar with *Escherichia coli* lawned (NNA-*E. coli*). The isolates were from infected corneal swab amples of stray cats at PAWS animal shelter (10), Chow Kit (7), Kg.Baru (2), Setapak (1), Wangsa Maju (2), Taman Melati (3), Sentul (2), Ampang (2), Petaling Jaya (4), Shah Alam (6), Subang Jaya (1), Ampangan (1), Port Dickson (3), Kuala Pilah (2) and Pulau Pangkor (2). They were from 5 adult males (10.4%), 14 adult females (29.2%), 10 male kittens (20.8%) and 19 female kittens (39.6%). As for stray dogs, 32% (8/25) of infected corneal swab samples were positive for *Acanthamoeba*. The isolates were detected in adult (male, 12.5% and female, 12.5%) and puppies (male, 25% and female, 50%). The cultivable *Acanthamoeba* isolates from these infected corneas were summarized in Table 4.1. Culture plates without FLA growth by day 14 were discarded and recorded as negative.

Table 4.1: Occurrence of cultivable *Acanthamoeba* in natural infected of animals corneal samples

		Infected samples, n (%)		
Animal corneal sample	**Gender**	**Adult**	**Kitten**	**Total**
Cats (N=200)	Male	5 (2.5)	10 (5.0)	15 (7.5)
	Female	14 (7.0)	19 (9.5)	33 (16.5)
Total		19 (9.5)	29 (14.5)	48 (24.0)
			Puppy	
Dogs (N=25)	Male	1 (4.0)	2 (8.0)	3 (12.0)
	Female	1 (4.0)	4 (16.0)	5 (20.0)
Total		2 (8.0)	6 (75.0)	8 (32.0)
Grand Total (N=225)		21 (9.3)	35 (15.6)	56 (24.8)

n = number of positive samples

4.2.2 Environmental samples

The occurrence of *Acanthamoeba* detected in environmental samples was 85% (136/160) (Table 4.2). In PAWS animal shelter, the isolates were detected in dust/debris samples from bedding (65%, 13/20), food containers (85%, 7/20) and drinking containers (75%, 15/20). In recreational rivers, all the water and soil samples were showed 100% positive but swab debris samples from the rocks/stones were found lesser (73.3%, 11/15) occurrence. From the playgrounds sites, the dry-soil samples showed 100% occurence. Subsequently, the indoor and outdoor dust samples from Medical Faculty, University of Malaya showed 75% (15/20) and 100% positive growth, respectively.

Table 4.2: Occurrence of cultivable *Acanthamoeba* in environmental samples

Location	Samples	Cultivable *Acanthamoeba* (%)
PAWS animal shelter		
i. Bedding	20	14 (70%)
ii. Food containers	20	17 (85%)
iii. Drink containers	20	9 (45%)
Recreational rivers		
i. Water samples	15	15 (100%)
ii. Swabs (rocks & stones)	15	11 (73.3%)
iii. Wet soil	15	15 (100%)
Children playgrounds		
i. Dry soil	15	15 (100%)
Medical Faculty, UM		
ii. Indoor (dust)	20	20 (100%)
iii. Outdoor (dust)	20	20 (100%)
Total	160	**136 (85%)**

4.2.3 Occurrence of other free-living amoebae (FLA)

In infected cornea spesimens, no FLA was detected accept *Acanthamoeba* isolates. In environmental samples, despite *Acanthamoeba*, there were several FLA such as *Naegleria*, *Vahlkampfia* and *Hartmannella* species were also occurred in the culture plates (Table 4.3). *Naegleria* isolates were detected in river water (100%, 15/15), swab debris from rocks (6.6%, 1/15), wet-soil from river-side (20%, 3/15) as well as dried-soil from playground (26.7%, 4/15). *Hartmannella* isolates were detected in dried-soil from children's playground (47%, 7/15). *Vahlkampfia* isolates were detected in indoor dust (10%, 2/20) samples. All these FLAs were detected as coinfection in the same culture plates especially at the early stage of cultivation. The flagellation test (by adding sterile distilled water) was carried out to differentiate between *Naegleria* and *Vahlkampfia* isolates.

Table 4.3: Occurrence of *Naegleria* and *Hartmannella* in environmental samples

Location	Samples	Cultivable *Acanthamoeba* (%)
Recreational areas		
i. Water samples (*Naeglaria* sp.)	15	15 (100%)
ii. Swabs (rocks & stones) (*Naeglaria* sp.)	15	1 (6.6%)
iii. Wet soil (*Naeglaria* sp.)	15	3 (20%)
Children Playground		
i. Dry soil (*Naeglaria* sp.)	15	3 (20%)
ii. Dry soil (*Hartmannella* sp.)	15	4 (26.7%)
Medical Faculty, UM		
i. Indoor dust (*Vahlkampfia* sp.)	20	2 (10%)
ii. Outdoor dust (-)	20	
Total	115	**28(24%)**

4.3 MOLECULAR DETECTION OF *ACANTHAMOEBA* GENOTYPES

4.3.1 PCR products using JDP1 and JDP2 primers

A total of 192 *Acanthamoeba* isolates were subjected for PCR amplification produced amplicons with expected size ranges between 400-500 bp known as ASA1 (or DF3) (Figure 4.2) against all tested isolates. The amplicons (bands) were seen in isolates from the corneal specimen (AC) from stray cats (48), dogs (8), environmental dust from PAWS animal shelter (45), recreational rivers (15 isolates from water, 11 stone debris, 15 wet soil), playgrounds (15 dry soil), Faculty of Medicine, University of Malaya (15 indoor dust, 20 outdoor dust). This set of primers did not yield any PCR products against negative template.

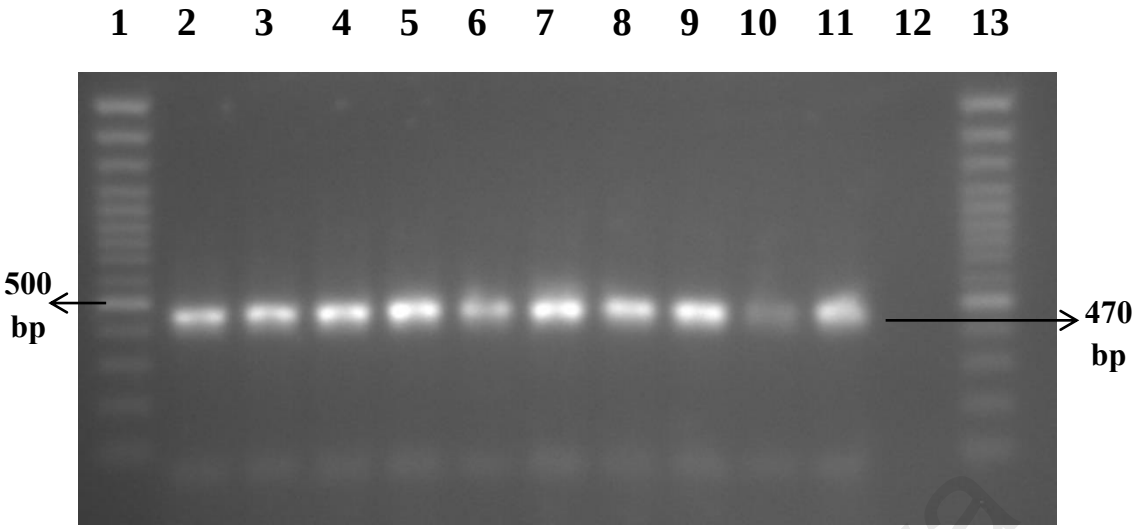


Figure 4.2: PCR amplicons of 460 – 500 bp revealed by *Acanthamoeba* isolates

Lanes 1 & 13= 100-bp DNA ladder
Lane 2 = isolate from GD1
Lane 3= isolate from GD2
Lane 4= isolate from GD3
Lane 5= isolate from GD4
Lane 6= isolate from GD5
Lane 7= isolate from GD6
Lane 8= isolate from GD7
Lane 9= isolate from GD8
Lane 10= isolate from GD9/GD10
Lane 11= isolate from GD11
Lane 12= Negative control

4.3.2 Analyses of DNA sequences of *Acanthamoeba* amplicons

The DNA sequences of amplicons were analyses by using Clustal W of Bioedit software. The sequence were individually BLASTn with reference sequences (retrieved from Genbank) to obtain the identical nucleotide sequences and were grouped according to their species and genotypes. All test sequences obtained in this study showed high homology (98-100%) with their most matched reference of *Acanthamoeba* from Genbank (Table 4.5).

The 18S rDNA sequence of GD1 (39 isolates, Appendix B.1) and GD2 (46 isolates, Appendix B.2) showed 100% and 99% homology with human keratitis isolate from USA, *Acanthamoeba castellanii* CDC:0184:V014 (U07401), genotype T4 after blasting in Genbank data base, respectively. GD1 comprises subgroup samples from animal cornea (AC1), dry soil (PS1), indoor (UID2) and outdoor (UOD2) debris while GD2 comprises

subgroup samples from animal cornea (AC2), dry soil (PS2) and wet soil (RS2). The difference between GD1 and GD2 is due to the presence of bases G^{354} and A^{354} respectively. Respectively, GD1 and GD2 also showed 99% and 100% identity with human keratitis isolate from France (DQ087314). Furthermore, GD1 and GD2 showed 100% and 99% identity respectively with human keratitis (GU808323) and GAE in human CSF (GU808321) from Thailand. In this study, GD1 and GD2 or *Acanthamoeba castellanii* genotype T4 was the only species found in infected corneal swab samples of stray cats and dogs. Subsequently, the 18S rDNA sequence of GD3 (42 isolates, Appendix B.3) showed 100% identical with *Acanthamoeba castellanii* genotype T4 (U07409-keratitis, Houston, USA). While in GD3 group consist subgroup samples from PAWS environment (P2- animal bedding, drink containers and food containers), wet soil (RS1), indoor (UID3) and outdoor (UOD3) debris. Besides, GD4 (8 isolates, Appendix B.4) comprise subgroup samples from swab (RD4-wet debris) and outdoor (UOD1) debris were 100% identical with *Acanthamoeba polyphaga* genotype T4 (AY026243).

Subsequently, the DNA sequences of isolate clones under subgroup GD5 (1 isolate, Appendix B.5) showed 100% homology with their closest match reference, *A.griffini* (KJ446979) under genotype T3. GD5 consist only one subgroup sample from swab (RD2-wet debris) in recreational area environment. Two of the groups (GD6 and GD7) were showed 99% homology with *A. lenticulata* (U94741), genotype T5, due to the 3 insertion bases ($-^{30}$→C, $-^{55}$→C and $-^{120}$→C) and 2 substitution bases (G^{12}→T and G^{54}→A), respectively. GD6 (16 isolates, Appendix B.6) comprise subgroup debris from PAWS animal shelter environment (P1-animal bedding, drink containers and food containers), GD7 (12 isolates, Appendix B.7) consist subgroup samples from recreational water (RW1) and wet debris from recreational area (RD1). While group GD8 (11 isolates, Appendix B.8) consist subgroup samples from recreational water (RW2) showed 98% homology with *A. lenticulata* (U94741), genotype T5, due to 7 bases substitution.

Similarly, groups GD9 (9 isolates, Appendix B.9), GD10 (1 isolate, Appendix B.10) and GD11 (9 isolates, Appendix B.11) were respectively showed 99% homology with *A. jacobsi* (KC164249, genotype T15) but were different in the base substitutions. The group of GD9, GD10 and GD11 consisting of different subgroup that respectively isolated from debris from PAWS animal shelter (P3-animal bedding, drink containers and food containers), wet debris from recreational rivers (RD3) and indoor debris (UID1).

Multiple sequence alignment (Appendix B.12) was done on 18S rDNA of all the test isolates and has been confirmed as *Acanthamoeba* species, although there are great sequences polymorphism due to occurrence of base substitutions, insertions and deletions. All obtained DNA sequences were organized by multiple alignments and were grouped up according to close-matched sequences as in Table 4.5 and were designated as GD1, GD2, GD3, GD4, GD5, GD6, GD7,GD8, GD9, GD10 and GD11.

Table 4.4: Groups and isolates of cultivable *Acanthamoeba* according to their source and DNA sequence similarity

Group	Sources and *Acanthamoeba* isolates
AC1	Animal cornea (56 isolates): CAM5, CAM30, CAF7, CAF22, CAF32, CKM17, CKM39, CKM40, CKM44, CKF1, CKF16, CKF28, CKF35, CKF41, CKF45, DAF1, DPF3, DPM4 = 18
AC2	CAM3, CAM9, CAM23, CAF2, CAF6, CAF8, CAF10, CAF11, CAF21, CAF24, CAF29, CAF31, CAF33, CAF36, CKM14, CKM15, CKM19, CKM20, CKM27, CKM43, CKF4, CKF12, CKF13, CKF18, CKF25, CKF26, CKF34, CKF37, CKF38, CKF42, CKF46, CKF47, CKF48, DAM6, DPF2, DPF5, DPF8, DPM7= 38 [C=cat, A=adult, M=male, F=female, K=kitten, D=dog]
	PAWS (40 isolates):
P1	B1, B2, B3, B10, B11, B15, DC1, DC9, DC10, DC13, DC16, DC18, DC19, FC2, FC3, FC11 = 16
P2	B4, B8, B13, B17, B18, B19, DC15, DC16, FC6, FC7, FC8, FC9, FC14, FC17, FC18 = 15
P3	B12, B14, FC4, FC5, FC10, FC12, FC15, FC16, FC19 = 9 [Debris from B-bedding, FC-food container, DC-drinking container
	15 Recreational rivers (41 isolates):
RW1	Water: w1, w2, w12, w14 = 4
RW2	: w3, w4, w5, w6, w7, w8, w9, w10 ,w11, w13, w15 = 11
RD1	Swab (Debris): d1, d2, d6, d7, d8, d9, d14, d15 = 8
RD2	: d3 = 1
RD3	: d4 = 1
RD4	: d5 = 1
RS1	Wet soil: s1, s2, s3, s4, s7, s8, s9, s10, s11, s12, s13, s14, s15 = 13
RS2	: s5, s6 = 2 [w=water, d=debris, s=soil from river 1-15]
	15 Children playground (15 isolates):
PS1	Dry soil: S1, S2, S4, S7, S8, S10, S12, S13, S14 = 9
PS2	: S3, S5, S6, S9, S11, S15 = 6
	Medical Faculty, University Malaya (40 isolates):
UID1	ID1, ID2, ID3, ID5, ID6, ID12, ID14, ID15, ID20 = 9
UID2	ID4, ID7, ID10, ID13, ID16 = 5
UID3	ID8, ID9, ID11, ID17, ID18, ID19 = 6
UOD1	OD1, OD2, OD3, OD4, OD15, OD18, OD20 = 7
UOD2	OD5, OD7, OD8, OD 9, OD10, OD12, OD19 =7
UOD3	OD6, OD11, OD14, OD13, OD16, OD17 =6 Dust from ID=indoor, OD=outdoor

Designation: P (samples from PAWS), RW (river`s water), RD (river`s debris), RS (river`s wet-soil), PS (playground`s dry-soil), UID (University`s indoor-dust), UOD (University`s outdoor-dust), AC (animal corneal)

Table 4.5: Homo- and hetero-geneity of cultivable isolates against reference *Acanthamoeba* species retrieved from Genbank.

No	Group (n isolates)	Closes homology with reference, bp number, (total isolates detected)	Genotype	BI	BS	BD	No. of BI/ BS/BD
GD1	PS1 (9) UID2 (5) UOD2 (7) AC1 (18)	100%, *A.castellanii* (U07401), 459 bp, (39)	T4				
GD2	RS2 (2) PS2 (6) AC2 (38)	99%, *A. castellanii* (U07401), 459 bp, (46)	T4		$A^{354}\rightarrow G$		1BS
GD3	P2 (15) RS1 (13) UID3 (6) UOD3 (6)	100%, *A.castellanii* (U07409), 463 bp, (42)	T4				
GD4	RD4 (1) UOD1 (7)	100%, *A.polyphaga* (AY026243) 455 bp, (8)	T4				
GD5	RD2 (1)	100%, *A.griffini* (KJ446979), 466bp, (1)	T3				
GD6	P1 (16)	99%, *A.lenticulata* (U94741), 424 bp, (16)	T5	$-^{30}\rightarrow C$ $-^{55}\rightarrow C$ $-^{120}\rightarrow C$			3B1
GD7	RW1 (4) RD1 (8)	99%, *A.lenticulata* (U94741), 421 bp, (12)	T5		$G^{12}\rightarrow T$ $G^{54}\rightarrow A$		2BS
GD8	RW2 (11)	98%, *A.lenticulata* (U94741), 421 bp, (11)	T5		$A^{9\rightarrow}C$ $G^{10\rightarrow}A$ $C^{15\rightarrow}T$ $A^{16\rightarrow}C$ $G^{54\rightarrow}A$ $G^{346\rightarrow}A$ $A^{368\rightarrow}G$		7BS
GD9	P3 (9)	99%, *A jacobsi*, (KC164249), 459 bp, (9)	T15		$C^{372}\rightarrow G$ $T^{393}\rightarrow C$		2BS
GD10	RD3 (1)	99%, *A. jacobsi*, (KC164249), 459 bp, (1)	T15		$C^{381}\rightarrow T$ $T^{393}\rightarrow C$		2BS
GD11	UID1 (9)	99%, *A. jacobsi*, (KC164249), 459 bp, (9)	T15		$G^{367}\rightarrow C$		1BS

Designation: BI (base insertion), BS (base substitution), BD (base deletion), G (guanine), C (cytosine), A (adenine), T (thiamine), - (base deletion/insertion). *Acanthamoeba* groups are designated as in Table 4.4.

4.3.3 Phylogenetic analyses

Phylogenetic trees which are based on the 18S rDNA gene sequences of *Acanthamoeba* isolates were constructed to illustrate the relationships between the test and reference sequences from Genbank. The relationship between the reference sequences of genotypes T1-T19 (retrived from Genbank) and test isolates sequences was depicted in phylogenetic trees. The test isolate sequences were seen clustered under 4 groups of genotypes (T3, T4, T5 and T15) in the phylogenetic trees (Figures 4.3 to 4.5).

Phylogenetic relationships among test isolates were examined by neighbor-joining (NJ) (Figure 4.3), minimum evolution (ME) (Figure 4.4) and maximum parsimony (MP) (Figure 4.5) analyses. The comparisons were then made of to form a bootstrap value and tree topography. Bootstrap values have been included to help determine groups, as it is generally considered that values of greater than 50% are evidence to support the distinction of the clade. Phylogenetic trees produced by NJ, ME and MP algorithms, do vary from each other resulting in a greater number of higher bootstrap values. However the clusters of taxa remain largely the same, although their positions within the tree vary depending on the analysis used.

The constructed phylogenetic trees revealed 11 clades that corresponded to genotypes of T1 to T19 and each genotype is strongly supported by moderate to high bootstrap values (bootstrap values from 56 to 100% in NJ tree, 52 to 100% in ME tree and 50 to 99% in MP tree). Additionally, the neighbour-joining tree (NJ) and minimum evolution tree (ME) showed some multiple genotypes (T3/T4/T11, T2/T6, T10/T12 and T9/T17) clumped together in same clade, and some genotypes (T1, T5, T7, T8, T13, T14, T15, T16, T18 and T19) were placed in their own clade. However, maximum parsimony (MP) showed different result with genotypes T3/T4/T11, T1/T10/T12/T14, T15/T19,

T2/T5/T6 and T8/T18 clumped together in same clade while genotypes T7, T9, T13, T16 and T17 were placed in their own clade.

Genotypes T3 *A. griffini* (AD2) and T4 *A. castellanii* (AC1,AC2, AP2, AOD2, AS1, AS2A and AS2B) and T4 *A. polyphaga* (AOD1 and AD4) were seen as polyphyletic group as they grouped in the same clade clustered with each other with high bootstrap value. The 18S rRNA tree (NJ, ME and MP) highlights the close relationship between T3, T4 and T11 with high bootstrap value between three clades more than 80% in NJ and ME while MP moderate value of 65% (Figures 4.3 to 4.5). On the other hand, *A. castellanii* and *A. polyphaga* groups were both essemblaged under genotype T4 and had a very close relationship with the bootstrap value more than 90% in NJ and ME while MP of 82%. Furthermore, *A. castellanii* strain T4 was divided into two groups (animal corneal and environmental) with similarity value of 100% in NJ, 98% in ME and 92% in MP. *Acanthamoeba* isolates from animal corneas (AC1 and AC2) were also divided into groups with moderate similarity value at all phylogenetic tree analysis. Other differences seen in the phylogenetic are in ME tree genotype T11 which were located between genotypes T4 and T3, while in NJ and MP genotype T11 was placed under both genotypes, respectively

Subsequently, *Acanthamoeba* groups AP1, AW1, AW2 and AD1 from *A. lenctiulata* genotype T5 were clearly separated to form two clades but with high bootstrap value of 99% in NJ and ME. While, the genotype T5 was clumped together with genotypes T2 and T6 (U07411 and AF019063) in MP only with moderate bootstrap values of 59% by maximum parsimony but not in NJ and ME phylogenetic tree. Despites, genotype T5 also have closest relationship with genotypes T10 (AF019067), T12 (AF019070) and T14 (AF333607) as seen in NJ and ME with bootstrap values of 99%. Furthermore *Acanthamoeba* groups AP3, AID1, and AD3 from *A. jacobsi* genotype T15 showed

closest relationship with genotypes T1 (U07400), T13 (AF132134), T16 (GQ380408) and T19 (KJ413084) as seen in NJ and ME with high bootstrap values of 99%. MP analysis showed genotype T15 was clumped together with T19 with moderate bootstrap values of 54%. The respective group of genotypes T5 and T15 were clustered independently in their own clade in NJ and ME and it's known as monophyletic groups.

The genotypes T7, T8, T9, T17 and T18 were found to have a more distant relationship from other genotypes, in which T7 (AF019064), T8 (AF019065) and T9 (AF019066) are in morphological group I, but T17 (AF019064) and T18 (KC822469) are in an unknown morphological group. When comparing average pairwise distances (Table 4.6) of all the T-groups, maximum values occur between genotypes T9 verses T13 (0.280) and minimum between genotypes T3 verses T4 (0.042). Thus in this study, the differences between the closely related sequence types T3, T4 and T11, are less than in previously recorded value of at least 5% (which is an arbitrary value) (Stothard *et al*., 1998) in which the dissimilarity values are 4.2% (T3 verses T4), 5.0% (T3 verses T11) and 4.5% (T4 verses T11). The differences between genotypes were always greater than within sequence types. Furthermore, the clades containing all genotypes from strain T1 until T19 were clustered and derived from the same common ancestors.

Table 4.6: Genbank reference sequences used in the construction of phylogenetic trees.

Genotype	Name of Isolate/strain	Accession no.	Source/Location
T1	*A. castellanii*	U07400	Granulomatous amoebic encephalitis, Human, Georgia, USA.
T2	*A. palestinensis* Reich ATCC 30870	U07411	Soil, Israel.
T3	*A. griffini* Adana	KJ446979	Acanthamoeba keratitis, Spain.
T4	*A. castellanii* CDC: 0981:V006	U07401	Acanthamoeba keratitis, USA.
	A. castellanii S22	DQ087314	Acanthamoeba keratitis, France.
	A. castellanii ATCC 50369	U07409	Acanthamoeba keratitis, Houston, USA.
	A. polyphaga	AY026243	Environmental sample, Brazil
T5	*A. lenticulata* PD2S ATCC 30871	U94741	Swimming pool, France.
T6	*A. palestinensis* 2802	AF019063	Swimming pool, France.
T7	*A. astronyxis* Ray & Hayes ATCC 30137	AF019064	Lab water, Washington, USA.
T8	*A. tubiashi* OC-15C ATCC 30867	AF019065	Freshwater, Maryland, USA.
T9	*A. comandoni* Comadon & de Fanbrune	AF019066	Soil, France.
T10	*A. culbertsoni* Lilly A-1 ATCC 30171	AF019067	Human cell culture, Indiana, USA.
T11	*A. hatchetti* BH-2	AF019068	Brackish water, Maryland, USA.
T12	*A. healyi* CDC:1283:V013	AF019070	Granulomatous amoebic encephalitis, Barbados.
T13	*Acanthamoeba* sp. UWC9	AF132134	Contact lens case.
T14	*Acanthamoeba* sp. PN15	AF333607	Clinical sample, Pakistan.
T15	*A. jacobsi* CF1-219	KC164249	Soil, Switzerland.
T16	*Acanthamoeba* sp. cvX	GQ380408	Environmental sample
T17	*Acanthamoeba* sp. TSP07	JF325889	Soil samples, Brazil.
T18	*Acanthamoeba* sp. CDC:V621 clone 9	KC822469	Granulomatous amoebic encephalitis, US.
T19	*Acanthamoeba* sp. USP-AWW-A68	KJ413084	Water treatment plant.

Table 4.7: Pairwise distance estimate of 18S sequence pairs between T-group genotypes (Maximum Composite Likelihood model) involved 38 nucleotide sequences using MEGA version 4 software (Tamura *et al*., 2007, 2004).

	T11	**T3**	**T4**	**T5**	**T15**	**T13**
T3	0.050	-	-	-	-	-
T4	0.045	0.011	-	-	-	-
T5	0.079	0.080	0.087	-	-	-
T15	0.065	0.078	0.078	0.083	-	-
T13	0.090	0.078	0.083	0.083	0.076	-
T9	0.268	0.242	0.269	0.218	0.260	0.284

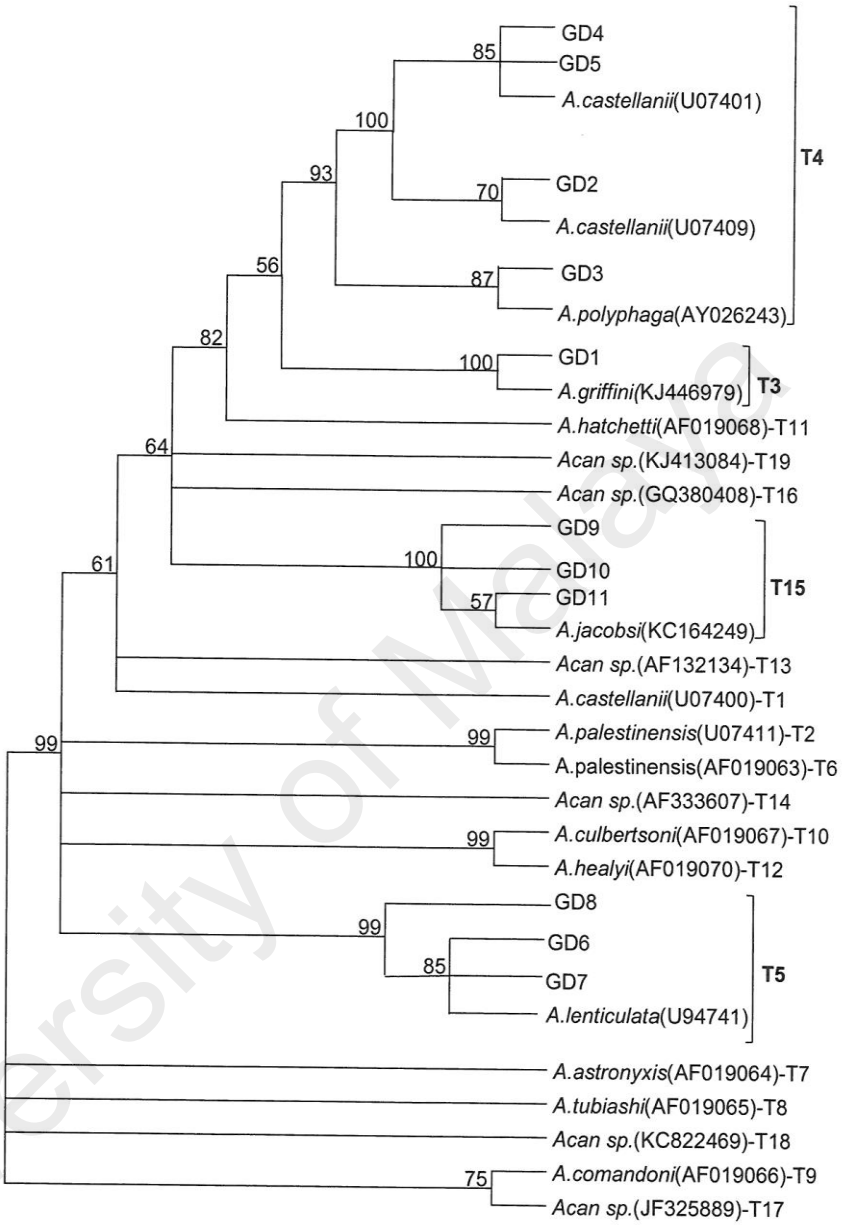


Figure 4.3: Neighbour joining tree depicting the relationships between test isolates and reference strains representing genotypes of *Acanthamoeba*. Numbers at the nodes are percentage-bootstraping values on 1000 replicates. Genbank accession numbers and genotypes for reference sequence are indicated at the ends of the *Acanthamoeba* isolates designations.

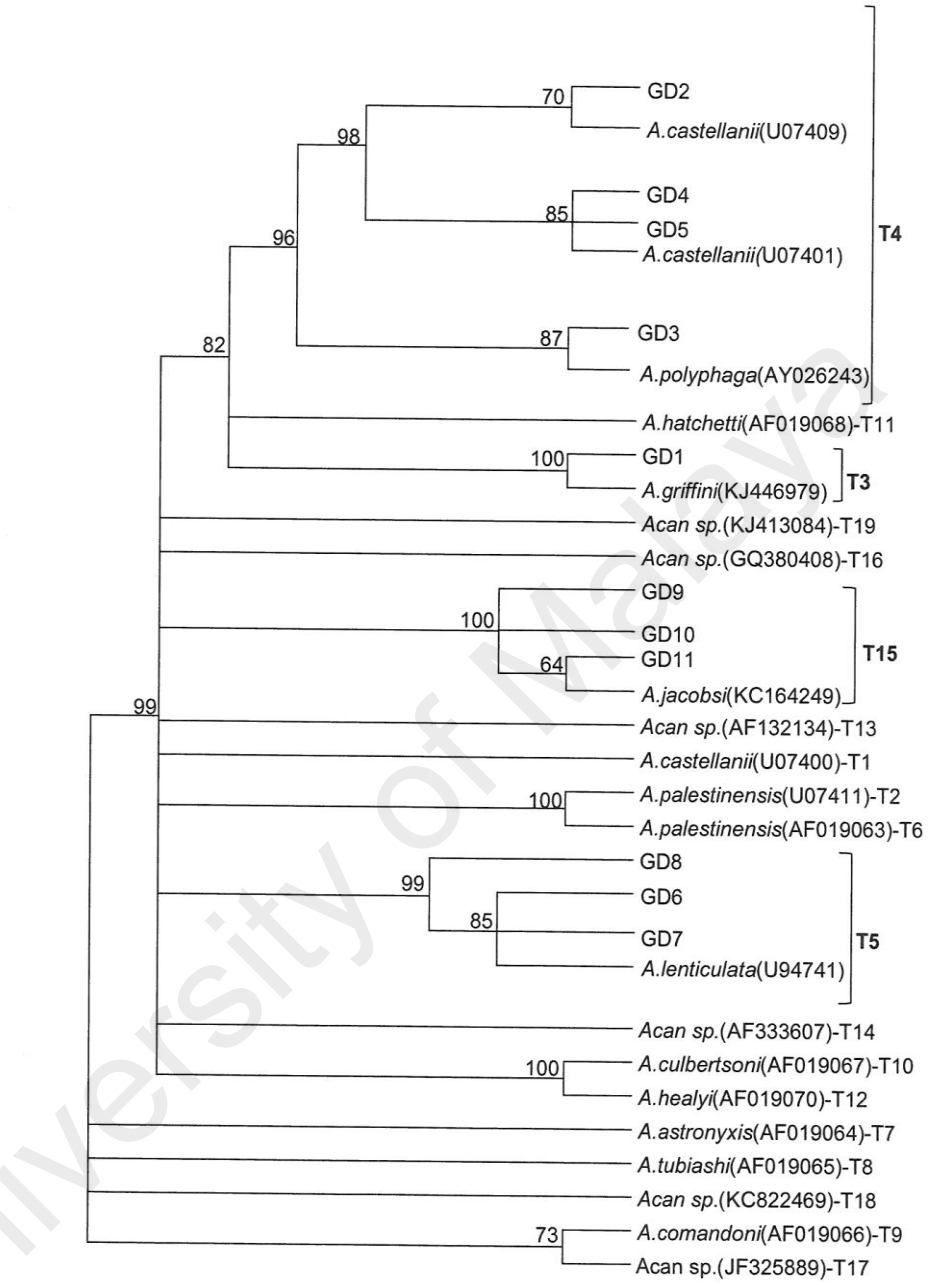


Figure 4.4: Minimum evolution tree depicting the relationships between test isolates and reference strains representing genotypes of *Acanthamoeba*. Numbers at the nodes are percentage-bootstraping values on 1000 replicates. Genbank accession numbers and genotypes for reference sequence are indicated at the ends of the *Acanthamoeba* isolates designations.

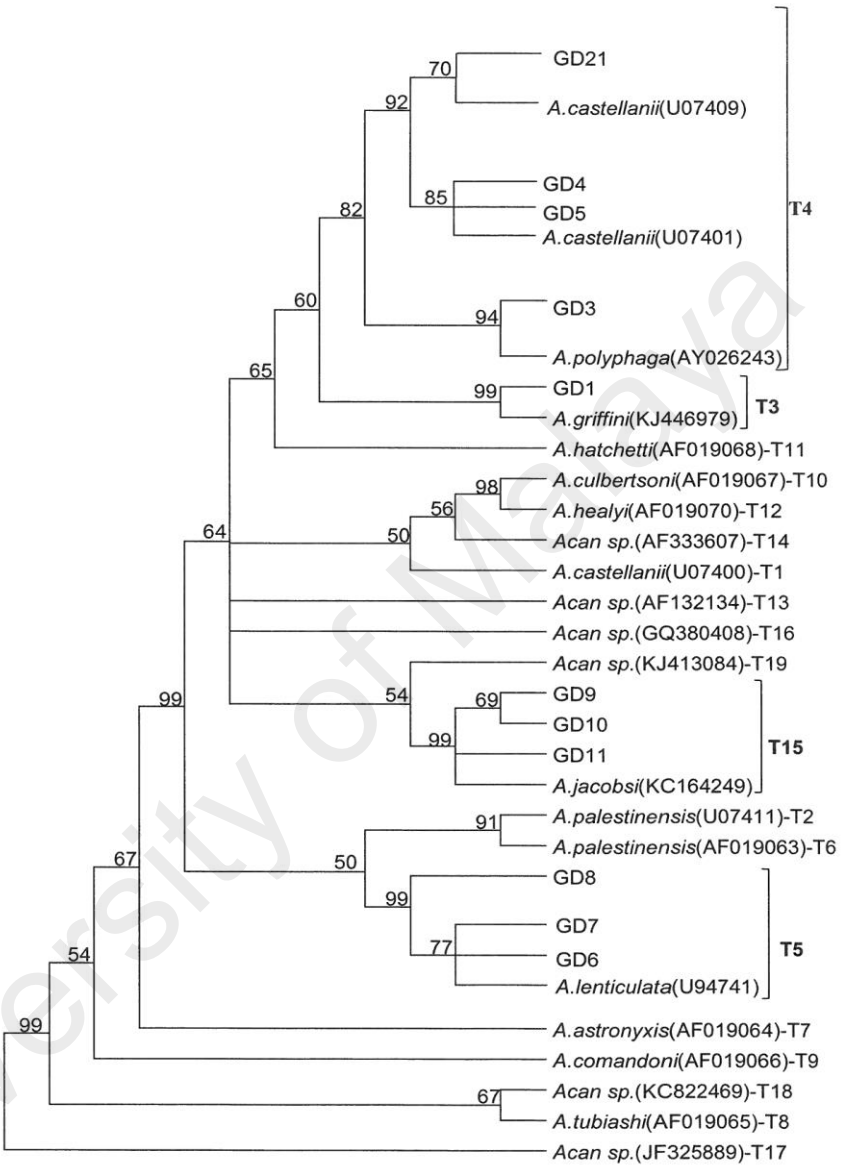
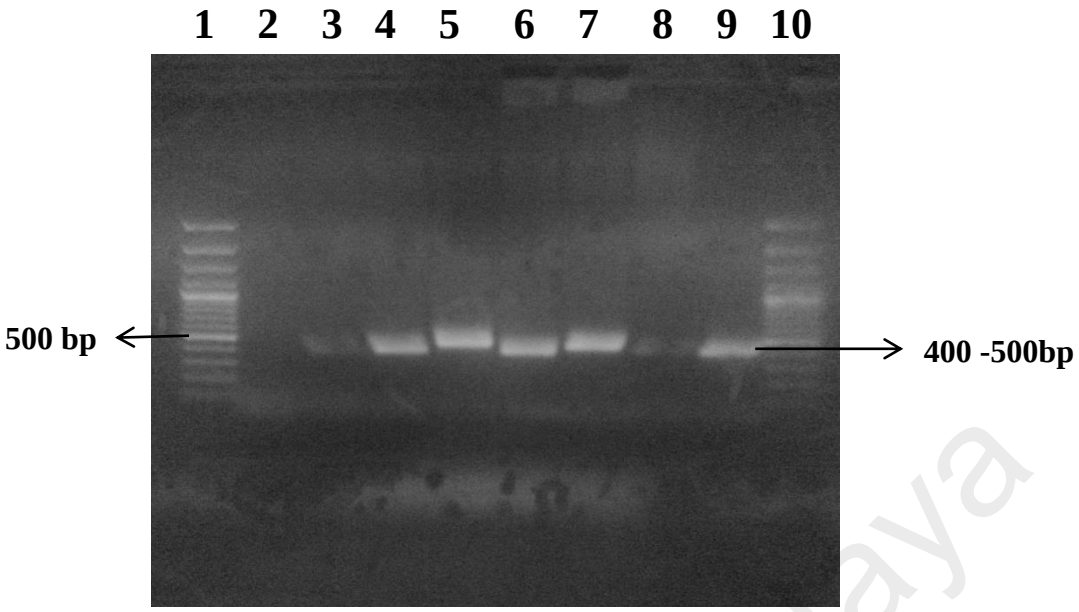


Figure 4.5: Maximum parsimony tree depicting the relationships between test isolates and reference strains representing genotypes of *Acanthamoeba*. Numbers at the nodes are percentage-bootstraping values on 1000 replicates. Genbank accession numbers and genotypes for reference sequence are indicated at the ends of the *Acanthamoeba* isolates designations.

4.4 IDENTIFICATION OF OTHER FLA GENUS BY USING INTERNAL TRANSCRIBED SPACERS (ITS) PRIMERS

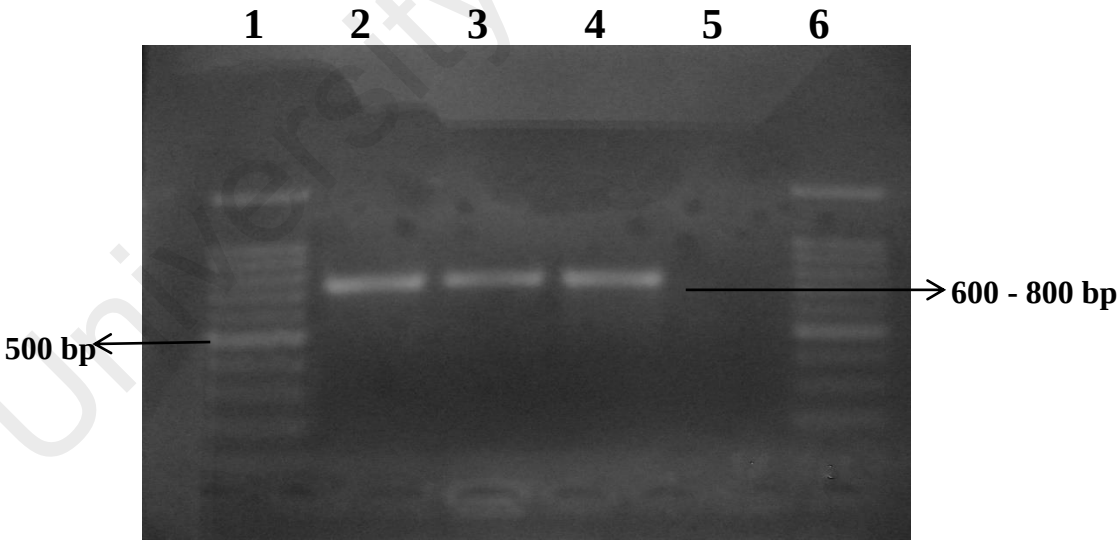
PCR detection using a set of ITS primers was also carried out to identify the genera of FLA that have been isolated from environmental samples collected from various sampling sites. Several sizes of PCR amplicons (Figure 4.6 and Figure 4.7) were obtained and were differentiated into eight (8) types designated as Ty-1 (363 bp), Ty-2a (315 bp), Ty-2b (315 bp), Ty-3 (315 bp), Ty-4a (310 bp), Ty-4b (370 bp), Ty-5a (667 bp) and Ty-5b (553 bp). The amplicons expected size ranges for *Naegleria* DNA were approximately 400-500 bp, while *Hartmannella* between 600-800 bp.

Ty-1 amplicon (363 bp) was yielded by *Naegleria* DNA isolates origins from environmental samples included the river water (nw1), maddy soil (ns11, ns13 and ns14) and dry soil (S6) from playground. Ty-2a (315 bp) was yielded by isolates from water (nw4, nw5 and nw7) and debris (nd6). Ty-2b (315 bp) from water samples (isolates nw9 and nw14). Ty-3 (310 bp) from water (nw2, nw8, nw11, nw12) and dry soil (S15). Ty-4a (370 bp) from water (nw3 and nw15). Ty-4b (310 bp) from water (nw6, nw10 and nw13) and dry soil (S4 and S9). Ty-5a (667 bp) from indoor dust (ID9 and ID13), and Ty-5b (553 bp) from dry soil (S6, S7 and S8) samples (Table 4.8).



Lanes 1 and 10= 100-bp DNA ladder
Lane 2= Negative control
Lanes 3 and 8= Ty-2b
Lane 4= Ty-1
Lane 5= Ty-2a
Lane 6= Ty-3
Lane 7= Ty-4b
Lane 9= Ty-4a

Figure 4.6: PCR amplicons revealed by *Naegleria* genus using ITS primers.



Lanes 1 and 6= 100-bp DNA fragment
Lane 2= Ty-5b
Lanes 3 and 4= Ty-5a
Lane 5= Negative control

Figure 4.7: PCR amplicons revealed by *Hartmannella* genus using ITS primers.

4.4.1 Analyses of DNA sequences of PCR products yielded by *Naegleria* and *Hartmannella* genera.

Recombinant PCR products were individually clones in *Escherichia coli* JM109 were successfully sequenced and produced readable DNA sequences of approximately 400-500 bp for *Naegleria* and 600-800 bp for *Hartmannella* species. After multiple alignments using Clustal W of Bioedit software, these sequences were BLASTn with Genbank references and were found all showed high homology (98-100%) (Table 4.7)

The ITS1-5.8S-ITS2 sequences from Ty-1 (363 bp) showed 100% homology with *Naegleria* sp. (JN034054) from the environment in Peninsular Malaysia (Appendix B.13). Ty-2a (315 bp) and Ty-2b (315 bp) were respectively 100% and 98% identical with *Naegleria fultoni* (DQ768742), isolated from fish gills, Czech Republic (Appendix B.14 and Appendix B.15). The heterogenesity in Ty-2b was due to five (5) substitutions bases included (G^{283}→T), (C^{289}→T), (T^{294}→A), (T^{297}→C), (A^{298}→G) and a deletion base (G^{291}→-). The length of both ITS1 (33 bp) and 5.8S (175 bp) sequences were identical to to those of *Naegleria fultoni*, however the differences occurred within ITS2 with one (1) base shorter in Ty-2b. The ITS1-5.8S-ITS2 sequences from Ty-3 (310 bp) revealed 100% homologous with *Naegleria philippinensis* (AB332225, tap-water sources, Japan) (Appendix B.16). Ty-4a (370 bp) was found to be 100% identical to *Naegleria canariensis* (FJ475125, environmental water, Brazil) (Appendix B.17), while Ty-4b (310 bp) was 100% homologous with *Naegleria australiensis* (GU597038, environmental water, Taiwan) (Appendix B.18). Multiple sequence alignments ofTy-1 to Ty-4b amplicons sequences were confirmed as *Naegleria* sp. after BLAST analysis (Appendix B.21) with two (2) base substitutions (T^{164}→C) and (C^{205}→A). Furthermore, the ITS1-5.8S-ITS2 sequences from Ty-5a (667 bp) was 100% identical with *Hartmannella* sp. (AB330065, tap water sources, Japan) (Appendix B.19), while Ty-5b (553 bp) was 100% homologous with *Hartmannella* sp. (HE617188, environment, Ghana) (Appendix B.20).

Table 4.8: Heterogenesity of other free-living amoebae isolated from environmental samples and their closest relatives match from Genbank.

Amplicon type	Sources and isolates of *Acanthamoeba*	Sequence homology, Reference isolates (Accesion no), Base	BI	BS	BD	No. of BI/ BS/BD
Ty-1	Recreational rivers: Water: nw1 Soil: ns11, ns13, ns14 Children's playground: S6	100%, Unclassified *Naegleria* sp. (JN034054), 363 bp				
Ty-2a	Recreational rivers: Water: nw4, nw5, nw7 Swab (Debris): nd6	100%, *N. fultoni* (DQ768742), 315 bp				
Ty-2b	Recreational rivers: Water : nw9, nw14	98%, *N. fultoni* (DQ768742), 315 bp		$G^{283}\rightarrow T$ $C^{289}\rightarrow T$ $T^{294}\rightarrow A$ $T^{297}\rightarrow C$ $A^{298}\rightarrow G$	$G^{291}\rightarrow$-	5BS, 1BD
Ty-3	Recreational rivers: Water: nw2, nw8, nw11, nw12 Children's playground: S15	100%, *N. philippinensis* (AB332225), 310 bp				
Ty-4a	Recreational rivers: Water: nw6, nw10, nw13 Children's playground: S4, S9	100%, *N. canariensis*, (FJ475125), 370 bp				
Ty-4b	Recreational rivers: Water : nw3, nw15	100%, *N. australiensis* (GU597038), 310 bp				
Ty-5a	Indoor dust: ID9, ID13	100%, *Hartmannella* sp. (AB330065),667bp				
Ty-5b	Children's playground: S6, S7, S8	100%, *Hartmannella* sp. V26 (HE617188), 553 bp				

Designation: nw (surface water from river), nd (debris from stone surface), ns (wet soil from riverside), ID (dust from indoor wall surface), S (dry soil from children's playground), base insertion (BI), base substitution (BS), base deletion (BD), guanine (G), cytosine (C), adenine (A), thiamine (T), base deletion position (-), no 1-15 are the respective selected location as designated in Table 3.1

CHAPTER 5

DISCUSSION

5.1 Occurrence and genotyping of *Acanthamoeba* isolates based on 18S rDNA sequences

In this study, the selected stray cats and dogs were abandoned by their owners or being born from stray parents. They grow and live without shelter and some of them were injured due to fighting among themselves. Some of these stray animals were brought to PAWS animal shelter by City Council of stray animal catchers for treatment and adoption. As for our study, a newly catch and infected animals (before treated by veterinarian) in PAWS shelter were selected for corneal swabs. Due to the fierce character of dogs, the infected corneal swabs were only carried out in PAWS shelter of which 2-3 experience dog catchers were needed to catch hold during the procedure. As for stray cats, they were more tameable and much easier to catch hold, thus the collection of corneal samples were also carried out in many other sampling sites such as at the road-side, food stalls or restaurants, back alleys and residential areas.

As noted in the methodology, the infected corneas of stray cats and dogs were recognized by several eye signs such as watery, discharge, redness, swollen and white membrane on the eye surface. These signs are usually caused by several microorganisms

such as herpes simplex virus (Martinez & Visvesvara, 1997), bacterial *Pseudomonas aeruginosa* (Clarke & Niederkorn, 2006) and *Staphylococcus* (Giese & Weissman, 2002) or fungi *Aspergillus flavus*, *Aspergillus fumigatus*, *Fusarium* spp., *Alternaria* spp. and yeast *Candida* (Halde, 1986). In this study, the presence of herpes simplex virus, bacteria and fungi were not detected due to limitation of facility in our laboratory. The presence of Acanthamoerba associated with the above eye signs and their distribution in environmental samples were carried out in this study.

As for environmental samples, the location of sampling sites were selected based on the human activities and also accessed by stray animals (cats and dogs). Besides, the main characteristic of *Acanthamoeba* itself is ubiquitous and can live in diversity of environment. As in recreational rivers, samples of water, debris (rocks and stones) and wet-soil were collected as they were related to the outdoor activities such as resting, playing, swimming, camping, etc. While in the playgrounds, dry soil samples were collected where the children were seen playing on the soil. The indoor and outdoor dust samples were collected from the Medical Faculty, University of Malaya, Kuala Lumpur. Internal environment at PAWS animal shelter such as animal bedding, food and drinking containers were also checked for the possible pathogenic *Acanthamoeba* genotype.

After cultivation of samples, the cultivable, *Acanthamoeba* species were identified by morphological characteristics of the trophozoites and cysts under the inverted and light microscopes. Under the microscope of positive surface agar smear. The trophozoites showed specific characteristics of protruding spike-like acanthopodia on the cell surface, while the cysts vary in shapes with a wrinkled double thick walled. These characteristics are usually used for identification of *Acanthamoeba* genus which can be differentiated from other free-living amoebae (FLA) such as *Naegleria*, *Vahlkamphia* and *Hartmannella*. Previous report noted that, the morphology of cysts was used in

Acanthamoeba classification into three groups (Pussard & Pons, 1977). However, the classification method was not convincing due to alternating cyst appearance. Thus, molecular approaches based on 18S rDNA (Rns) gene phylogeny (Gast *et al*., 1996, Schroeder-Diedrich *et al*., 1998) were used in order to identify the species, strains and genotype of *Acanthamoeba*, accurately. The molecular methods have been widely used in organisms that share the features structure of RNA sequence that linked to the establishment of the phylogenetic tree (Woese *et al*., 1990).

Cultivable *Acanthamoeba* was detected high prevalence in both samples from the environment (85%, 136/160) and infected corneas (24.8%, 56/225). The environmental samples were included dry soil (100%), wet soil (100%), surface water (100%), wet debris on the rocks surface (73.3%), moist debris on animal's food containers (85%) and drinking containers (75%), indoor dust (75%), outdoor dust (100%) and dust at animal bedding (65%). *Acanthamoeba* species have an ability to survive in nature, thus reported occurring worldwide. They were found highly associated with water (either fresh or salty) in aquatic environments (lake, river, pond and ocean). Init *et al*. (2010) detected 100% of *Acanthamoeba* in water samples from treated swimming pools in Petaling Jaya and Kuala Lumpur, Malaysia. Other workers reported from Spain with 37.7% in soil, 42% in sand beach, 40.0% in sea water, 59.5% in tap-water and 40.0% in spring water samples (Lorenzo-Morales *et al*., 2005a&b). In Taiwan, 21.2% of *Acanthamoeba* was detected in spring recreation areas (Huang & Hsu, 2010), 43.2% in Nile Delta of Egypt (Lorenzo-Morales *et al*., 2006), 3% in James River of Virginia (Ettinger *et al*., 2003) and 100% in soil and water samples of Turkey (Kilic *et al*., 2004). In infected corneal swabs, the cultivable *Acanthamoeba* was detected high in stray dogs (32%, 8/25) as compared to stray cats (24%, 48/200). However the prevalence of *Acanthamoeba* in dog corneas in this study is not conclusive due to small sampling size and this is our limitation especially in getting specimen from infected dog.

Molecular method based on 18S rDNA (Rns) gene phylogeny (Gast *et al*., 1996, Schroeder-Diedrich *et al*., 1998) was successfully recognized 19 clades. These clades were closely related and recognized as genotypes. Every genotype exhibits 5% or more sequence divergence between different genotypes (Khan, 2006). Thus, the recognized 19 clades were identified as genotypes which were named T1-T12 (Gast *et al*., 1996, Schroeder-Diedrich *et al*., 1998), T13 (Horn *et al*., 1999), T14 (Gast, 2001), T15 (Hewett, 2003), T16 (Corsaro & Venditti, 2010), T17 (Magliano *et al*, 2012), T18 (Qvanstrom *et al*, 2013) and newly discovered strain T19 (Magnet *et al*., 2014). All these genotypes were based on the entire dataset of diagnostic fragment 3 (DF3) region of *Acanthamoeba* 18S rRNA gene. The DF3 is one out of the three highly variable portions of the 18S rDNA (Gast, 2001; Stothard *et al*., 1998). The DF3 region sequence was reported to be the best for use in contruction of the phylogenetic tree which depictured the genetic variability among the isolates. The topology of the phylogenetic trees revealed that the DF3 region is a good target to locate intra-genotype differential variation rather than as a tool for genotype assignment (Gast, 2001; Stothard *et al*., 1998; Cave *et al*., 2009).

Similarly in this study, the readable DNA sequences of DF3 region were analysed to identify the genotypes of *Acanthamoeba* isolates from infected corneal swabs and environmental samples. Initially, each of the *Acanthamoeba* culture was subcultured continuously for at least 10 times before subjected to molecular procedures. The purpose of repeated subculture was to homogenise the *Acanthamoeba* cells clone and to dispose the potential existence of Taq polymerase inhibitors (Orlandi & Lampel, 2000). Subsequently, the cold PAS solution was used to detach the trophozoite cells from the surface of non-nutrient agar and were then washed at least three times (or more) with sterile PAS solution to remove the excessive bacteria cells before subjected to DNA extraction (approximately 10^6). Precaution during PCR procedure was taken because too high concentration of DNA template may result in smearing of the amplicon bands or

undetectable products can be observed (Grunenwald, 2003). Thus dilution of the eluted DNA template at 100-2400 times was suggested to overcome this problem. Subsequently, the optimum concentration of $MgCl_2$ at 1.5 mM was needed. Inadequate magnesium concentration will result in low yield and no band will appear. The best result of DNA is obtained if the $MgCl_2$ concentration exceeds the total dNTPs concentration in each PCR mixture (Roux, 1995).

Construction of phylogenetic tree using sequences from our test isolates and Genebank references of genotypes T1 to T19 revealed 4 genotypes which were T3, T4, T5 and T15. Of these, only one genotype (T4) was detected in all infected corneal swabs and four genotypes (T3, T4, T5 and T15) were detected in environmental samples.

The DNA sequences of DF3 region in the 18S rRNA gene of all cultivable *A. castellanii* T4 (U07401) strains were divided into two homologous groups designated as GD1 (39 isolates) and GD2 (46 isolates) due to the presence of bases G^{354} and A^{354}, respectively. As in group GD1 includes corneal swab samples (18 isolates under group AC1) dry soil from playground (9 isolates under group PS1), the dust from wall's surface in the indoor (5 isolates under group UID2) and outdoor (7 isolates under group UOD2), while group GD2 includes corneral swab samples (38 isolates under AC2), wet soil from recreational rivers (2 isolates under RS2), dry soil from playground (6 isolates under PS2). The GD1 and GD2 were detected to be homologous with *Acanthamoeba castellanii* (U07401) T4 CDC:0184:V014 source from human keratitis. These isolates also showed 99-100% homologous with human keratitis isolates (DQ087314) from France, human keratitis (GU808323) and GAE in human CSF (GU808321) from Thailand. On the other hand, the genotype T4 strain was reported to have high chances of causing acanthamoeba keratitis (AK) and granulomatous amoebic encephalitis (GAE) in human worldwide (Booton *et al*., 2005). Thus, *Acanthamoeba* genotype T4 which infected animal corneas

may have similar scenario as in human in causing amoebic keratitis. In this study, the infection of *Acanthamoeba* in cats/dogs corneas may possibly be due to entering of particles such as soil/dust contaminated with *Acanthamoeba* T4 cyst, which was also found to be dominant in soil/dust.

The occurrence of *Acanthamoeba* keratitis (AK) cases in humans were rare and usually accidental findings (Christopher & Stuart, 1998; Ledbetter, 2011). In the recent years however, AK was reported to increase parallel with contact lens wear contributing to the rise in AK cases worldwide. *Acanthamoeba* keratitis in animals was noted but no conclusive reported case up to date (Ithoi *et al*., 2013). Most veterinarians believe that eye keratitis occurred in animals (especially in cats and dogs) were caused by viral, bacteria or fungal infections (Doyle, 2009), forgetting free-living amoebae. On the other hand, *Acanthamoeba* was detected in brain outopsy specimens of several animals that died due to granulomatous amoebic encephalitis (GAE), including gorillas, monkeys, dogs, ovines, bovines, horses, kangaroos as well as birds, reptiles, amphibians, fishes and even invertebrates (Visvesvara & Stehr-Green, 1990; Dykova´ et al., 1999; Visvesvara & Maguire, 2006). *Acanthamoeba* genotype T4 (DQ451161, 95% resemblance to *A. castellanii*) showed growth capability at 44ºC was isolated from a bird (toucan) liver due to dissemination, was previously reported by Visvesvara *et al*. (2007a).

Consequently, genotype T4 comprises several species and strains and in this study we detected the strains of *A. castellanii* (U07401) (11%, 15/136), *A. castellanii* (U07409) (28.7%, 39/136) and *A. polyphaga* (AY026243) (8%, 11/136) in environmental samples. In addition, the strains belong to genotype T3 of *A. griffini* (KJ446979) (0.7%, 1/136), T5 of *A. lenticulata* (U94741) (33%, 45/136) and T15 of *A. jacobsi* (KC164249) (18.4%, 25/136) were also detected from our environmental isolates.

A. castellanii T4 (U07409) strains were designated as group GD3 (42 isolates) were found in debris from PAWS animal shelter environment (15 isolates under P2), dry soil from playground (13 isolates under RS1), indoor (6 isolates under UID3) and outdoor dust (6 isolates under UOD3) from Medical Faculty, University of Malaya. Meanwhile GD4 (8 isolates) *A. polyphaga* T4 (AY026243) strains were found in wet debris from recreational areas (1 isolate under RD4) and outdoor dust (7 isolates under UOD1) from Medical Faculty, University of Malaya. Lastly, the GD5 group genotype T3 of *A. griffini* (KJ446979) strain was only found in wet debris (1 isolate under RD2) from recreational areas.

However, group GD6 (16 isolates), GD7 (12 isolates) and GD8 (11 isolates) were 99%, 99% and 98% resembles with *A. lenticulata* T5 (U94741) strains, of which their differences are due to base insertion (BI) and base substitution (BS), respectively. Group GD6 were found in PAWS animal shelter environment (16 isolates under P1 group-animal bedding, food and drink containers), GD7 were found in recreational water (4 isolates under RW1 group) and wet debris from recreational water (8 isolates under RD1) and GD8 were found in recreational water (11 isolates under RW2).

In addition, genotype T15 of *A. jacobsi* (KC164249) strains were also detected and were groups in GD9 (9 isolates), GD10 (1 isolates) and GD11 (9 isolates) due to their differences in bases substitution (BS). The GD9 were found in environmental samples from PAWS animal shelter (9 isolates under P3 group), group GD10 were found in wet debris from recreational areas (1 isolate under RD3), and group GD11 were found in indoor dust (9 isolates under UID1) from Medical Faculty, University of Malaya. Lastly, the result showed that genotype T4 (*A. castellanii* and *A. polyphaga*) appears to be the most prevalence geographically followed by genotype T5 (*A. lenticulata*), T15 (*A. jacobsi*) and T3 (*A. griffini*).

The distribution of the *Acanthamoeba* species is dependent on their cysts resistance level enhancing them to disperse by airborne (Chan *et al*., 2011; Lemgruber *et al*., 2010) to grow, multiply and colonize in a suitable environment. Genotype T4 of *A. castellanii* strains were reported to be commonly occurring in wet and dry environments worldwide (Fuerst *et al*., 2003; Lorenzo-Morales *et al*., 2006; Maghsood *et al*., 2005; Niyyati *et al*., 2009). This study, suggested that the most resistant and dominant cysts were the genotype T4 of the strain *A. castellanii* (U07409), which were mostly detected in dried samples of soil and dust. It is concurred that the cysts of *A. castellanii* (U07409) T4 strain have the capability and suitability to live in the soil ecosystem that consists of bacteria, fungi and other protozoa as a source of nutrient (Allen & Dawidowicz, 1990; Weekers *et al*., 1993). The cysts were still viable under dry, in which subsequently accumulated together with the dust particles and placed themselves at the wall surface of the outdoor and indoor environments. Therefore, genotype T4 of *A. castellanii* (U07409) strains were abundantly detected in dried accumulated particles of dust as well as dry soil.

As for *A.castellanii* (U07401), it was the only species detected in all 56 cultivable isolates from animal corneas. Their strains was also found more frequent in dust and dry soil samples collected from playgrounds. There genotype T4 (*A. castellanii*, U07401) is suggested to be the most susceptible strain to infected the corneal of stray cats and dogs. The transmission of *Acanthamoeba* is usually not correlated with vector, poor hygiene or dirty environment. The infection occurs in low immunity or injury/trauma to the corneas. Thus, any activities or behaviour that caused injury to the eye could trigger *Acanthamoeba* infection. The dogs with aggressive behaviour, robust, fierce fighting and less hygienic may have more chances for foreign body (contaminated soil/dust particles) to enter the eye. Younger animals (kittens and puppies) may have less immune protection but they are more active in movement (playing) that enhance the contaminated soil/dust particles to enter their eye. As in cats, the used of their paws to cover their faeces with

soil after defecation (that would probably be contaminated with *Acanthamoeba* cysts/trophozoites) followed by clean their face may influent the *Acanthamoeba* infection. In this study all the corneas infections were occurred in one eye but there is the possibility of bilateral eye infection (Lee & Gotay, 2010).

Generally, genotype T4 is always reported in patients with keratitis (AK) and granulomatous amoebic encephalitis (GAE). Previous review showed that 90% of humans AK were associated with genotype T4 (Khan, 2009). While Booton *et al*. (2005) reported that 72% (179/249) of AK cases were due to genotype T4 and it was detected in 53% of environment samples. The evidence of *Acanthamoeba* genotype T4 contributing to humans AK were reported in many countries such as Greece (4 out of 5 AK cases) (Spanakos *et al*., 2006), Hong Kong (12/13) (Booton *et al*., 2002), France (8/11) (Yera *et al*. 2007, 2008), UK and Iran (17/24) (Maghsood *et al*., 2005). Despite eye infection causing AK in healthy subjects, it was in fact predominantly detected in central nervous systems (CNS) of immunocompromised patients, causing granulomatous amoebic encephalitis (GAE) (Martinez, 1985, 1991). It was reported that 103 out of 169 GAE cases were caused by *Acanthamoeba* worldwide (Martinez & Visvesvara, 1997).

Following the predominance of *Acanthamoeba* T4 in environments and infected subjects, our findings suggested that in the genotype T4, the strain of *A. castellanii* (U07409) was dominant and abundant in non-aquatic environments, but *A. castellanii* (U07401) strain was the most susceptible to infect the animal corneas. However, the virulence and pathogenicity of *A. castellanii* (U07401) T4 strain causing *Acanthamoeba* keratitis alone could not be ruled out due to limitation of not detecting the other pathogens (bacteria, virus and fungi) in these infected animals. Subsequently, *A. polyphaga* T4 (AY026243) was found in wet debris and dried dust, suggesting of it resistant to dryness. The capability of *Acanthamoeba* causing keratitis (Maghsood *et al*., 2005) and GAE

(Walochnik *et al*., 2000) were also reported from other genotypes such as T3, T5, T6 and T11. *Acanthamoeba lenticulata* genotype T5 was proven to cause keratitis and the first case in the world was found in Greece (Spanakos *et al*., 2006) and first in USA (Leede *et al*., 2009), thus confirming that genotype T5 can also be pathogenic (Booton *et al*., 2004, 2005). These reported cases were long time suspected, since genotype T5 was found to be cytopathogenic to human tissue culture cells and virulent in mice (De Jonckheere, 1987; Niszl *et al*., 1998). There was also a reported case of *A. lenticulata* genotype T5 causing fatal disseminated infection in a heart transplant patient (Barete *et al*., 2007).

In this study, genotype T5 was the second most commonly detected after T4, and followed by T15 and T3 from environmental samples. Genotype T5 (*Acanthamoeba lenticulata*, U94741) was found to be predominant in watery areas, of which in this study were the samples of water and wet debris from recreational rivers and wet debris of animals food/drink containers in PAWS. None of T5 was detected in dry soil or dust samples suggesting that this genotype is less resistant to dryness or sun heat resulting in lesser dispersal by airborne, and less possibility of contaminating contact lens as compared to T4 strain. With high occurrence of genotype T5 in aquatic environments it may play an important role as a potential source of *Acanthamoeba* infection to those involved with water related activities. Review from previous studies noted that preference habitat of T5 is in wet condition including nasal mucosa cavity (Cabello-Vílchez *et al*., 2013; Khan, 2006) and those sites that have high moisture content including central air-conditioners (Chan *et al*., 2011).

In contrast, genotype T15 (*Acanthamoeba jacobsi*, KC164249) was not detected in the wet as well as dry soil (with sun heat) samples but was found in dry indoor dust and moist debris from food/drinking containers in PAWS. This result may support that T15 cyst is resistant to dryness but less viable under continuous sun heat. The T15 cyst-dust

particles that accumulated in indoor environments are viable and will be transformed to trophozoites stage when meet its growth requirements in the moist condition (such as food/drinking containers of cats/dogs that may contain bacteria as food source for *Acanthamoeba*). In contrast, the excysted trophozoite of T15 may not be viable in water environment that is lacking in the growth requirements. *A. jacobsi,* T15 was previously reported to be non-keratitis causing strain (Flint *et al*., 2003) but recently, it was reported in Italy as a first to be associated with keratitis in human (Cave *et al*., 2009).

As for *A. griffini* genotype T3 (KJ446979), it was rarely detected in our environment and was only found in debris (stone surface) from a recreational river. It was reported to be commonly found in tap-water sources (Edagawa *et al*., 2009) and freshwater environment (Lorenzo-Morales *et al*., 2006). *A. griffini* T3 was detected in many human keratitis worldwide (Maghsood *et al*., 2005). Several studies have determined that genotype T3 contributed to human AK by contaminating the contact lens/cases (Booton *et al*, 2002; Booton *et al*, 2005; Yera, *et al*, 2008).

In this study, the findings of 4 genotypes which were T3 (0.74%), T4 (48.15%), T5 (32.60%) and T15 (18.52%) in environmental samples is believed to be under estimated, which dependent on cultivable *Acanthamoeba* isolates. Other limitations were the amount of samples collected and sampling sites. However, this result give an information of the predominant genotypes, T4 and T5 in our domestic environment. Positive detection of genotype T4 (*A.castellanii*, U07401) in animals corneas may possibly be due to the susceptibility of *A.castellanii* (U07401) strain that is vulnerable to infect the injured corneas. Thus the possibility of this strain infecting and causesing AK in human cannot be ignored. As a matter of fact, *Acanthamoeba* strains of genotype T4 (*A.castellanii*, U07401, U07409), T3 (*A.griffini*, KJ446979), T4 (*A. polyphaga*, AY026243), T5 (*A. lenticulata*, U94741) and T15 (*A. jacobsi*, KC164249) were all reported to cause human

AK (Maghsood *et al*., 2005) and GAE (Walochnik *et al*., 2000). Thus, the presence of these genotypes in our domestic environment can be considered as one of the public health issue that is associated with *Acanthamoeba* infection in traumatic corneas of humans and animals during their outdoor activities. Therefore, the distribution of potential pathogenic *Acanthamoeba* species in the environment should be made known to the public, especially to those who are under the high risk groups such as HIV/AIDS patients, contact lens wearers and those involved with activities in natural water.

Health education should be also given to the public on environmental (water, soil and dust) and physical (corneal trauma and immunosuppressed) risk factors of *Acanthamoeba* infection especially when they choose to wear contact lens. As for pet cats or dogs, *Acanthamoeba* along with virus, bacteria and fungi should be considered in the diagnosis of pets with eye/brain infection by all veterinarians. The owners of these pets must always keep their pets in good and clean environment in their homes, with appropriate cages to minimize the exposure to the infected stray animals. In addition, it is also to ovoid the pets owners especially children from getting any potential infection from their pets.

The clinicians and veterinarians should be aware that *Acanthamoeba* infection can cause AK and GAE. In addition, the awareness should also be towards other pathogenic microorganisms such as *Legionella pneumophila*, *Parachlamydia* and mimivirus that live as endosymbionts in the same environment or *Acanthamoeba* which can be transmitted and caused diseases in infected subjects. Unawareness clinicians will lead to mistaken and wrong treatment of it infection for bacteria, virus or fungi which would affected the infected patients. Besides that, there were few reports of successfully treatment with biguanide and diamidine in patients with correct diagnosis at an early stage of

Acanthamoeba infection with long term (6 to 9 months) treatment regiment (Perez-Santonja *et al*., 2003).

5.2 Other free-living amoebae (FLA) by using ITS1-ITS2 primers

Except for *Acanthamoeba*, there was no other FLA found in stray animal corneal samples. However in environmental samples, other free-living amoebae (FLA) such as *Naegleria* and *Hartmannella* were detected during the early stage of *Acanthamoeba* cultivation. Approximately 17.5% (28/160) of these FLAs were found growing together with *Acanthamoeba* in origin and sub-culture plates. Most of other FLAs were detected in water or wet/moist rather than dry samples. *Naegleria* species were found highest in water samples (100%) but negative for others FLA species. In wet soil, *Naegleria* showed high (26.7%) prevalence as compared to *Hartmannella* species (20%). On the other hand, the species of *Naegleria* and *Vahlkampfia* (not found in this study) were showed similarity in their trophozoite stage but however, only *Naegleria* showed transition flagellate stage. Further DNA sequence analysis revealed that the transition flagellate stage method is not sufficient to identify the *Naegleria* because not all (only 45%) of their species could transformed into flagellate stage (De Jonckheere, 2006). The molecular method by using ITS gene to detect the genetic heterogeneity in *Naegleria* (De Jonckheere, 1998), *Vahlkampfia* (Garstecki *et al*., 2005) and *Hartmannella* (Glücksman *et al*. 2011) species were documented by many researchers.

In this study, 28 isolates of the other FLA were identified by PCR using a set of ITS primers (ITS1, ITS2) designated by Pélandakis *et al*. (2000). This set of primers was reported to produce amplicons 400-500 bp for *Naegleria* species, 800 bp for *Hartmannella* species and 600 bp for *Vahlkampfia* species (Pélandakis & Pernin, 2002). On the other hand, there was no detection of pathogenic *Naegleria fowleri,* in this study which may due to the absent or under detection of this species in our environmental

samples. Consequently, there were 6 types of non-pathogenic *Naegleria* species sequences were detected and were designated as Ty-1 (363 bp), Ty-2a (315 bp), Ty-2b (315 bp), Ty-3 (310 bp), Ty-4a (370 bp) and Ty-4b (310 bp). Ty-1 was 100% matched with *Naegleria* species (JN034054) and was also found in Malaysian environment previously (Init *et al*., 2011) which was believed to be a new species of *Naegleria*. Both Ty-2a and Ty-2b were respectively most matched up to 98%-100% with *Naegleria fultoni*. Subsequently, Ty-3, Ty-4a and Ty-4b were most matched with *Naegleria philippinensis* (AB332225), *Naegleria canariensis* (FJ475125) and *Naegleria australiensis* (GU597038), respectively. In *Naegleria* ITS gene, the ITS1 (33 bp) and 5.8S (175 bp) regions for Ty-1 to Ty-4b showed either identical or very low heterogenesity in sequences and lengths with the Genebank reference isolates. While for the ITS2 region, the sequences were highly showed heterogenesity which is more suitable in detection of *Naegleria* intra- and interspecies variation of these non-pathogenic species of *Naegleria*. This finding is concurred with the previous report by De Jonckheere, (2004), that the differences in ITS2 sequences is a key for detection of *Naegleria* species.

As for the larger amplicons (600-800 bp), they were reported to be *Hartmannella* genus (De Jonckheere, 2002; Behets *et al*., 2006; Pélandakis & Pernin, 2002) In this study, two larger fragments were identified and designated as Ty-5a (667 bp) and Ty-5b (553 bp) which were both most matched with the strain of *Hartmannella* (AB330065) and (HE617188), respectively.

Concerning pathogenicity, all *Naegleria* species found in this study were not pathogenic to human, although *N. australiensis* was previously reported to be pathogenic in animals (Scaglia *et al*., 1989; John & Howard, 1995). In addition, there was no report case of primary amoebic meningoencephalitis (PAM) in Malaysia up to date, although many *N. fowleri* and PAM cases were reported from our neighbour country Thailand

(Jariya *et al.*, 1983 and 1997; Poungvarin & Jariya, 1991; Sirinavin *et al.*, 1989; Somboonyosdech *et al.*, 1987). As a matter of fact, Peninsular Malaysia is bordered with Thailand at the northern regions (Perlis, Kedah, Perak and Kelantan), and the possibility of contamination with *N. fowleri* at these regions especially during flood seasons cannot be ignored. The risk of *N. fowleri* infection must be made aware when dealing with water activities such as swimming in recreational natural rivers. Muslims who use contaminated water for ablution before praying may also be exposed to this FLA that entered via the nostrils.

As for *Hartmannella* infection, there is not report case in both human or animal in Malaysia up to date. Furthermore, *Hartmannella* genus is still no proof of causing human disease as a single infection (Kinnear, 2003). *Hartmannella vermiformis* was reported to be a carrier for *Legionella pneumophila* that causes lung infection (Brieland *et al*., 1997). In addition, *Hartmannella* also found together with *Acanthamoeba* on agar culture of corneal swab, contact lens and lens case samples from a patient with stromal keratitis (Lorenzo-Morales, 2007b).

Vahlkampfia species which was not found in this study was documented to cause amoebic keratitis in contact lens and non-contact lens wearer. The first case occurred in a contact lens wearer where *Vahlkampfia* was found in the corneal biopsy tissue, contact lens, lens case, and home water supply (Alexandrakis *et al*., 1998). Meanwhile, the second case was a first non-associated with contact lens usage. The patient suffered severe pain and irritation in the right eye for a month with corneal abrasion in the right eye due to fiberglass while working on his boat and subsequently self-irrigated the eye with tap water (Aitken *et al*., 1996). Occasionally, amoebic keratitis occurred due to mixed infection of *Vahlkampfia* with *Acanthamoeba* (Niyyati *et al*., 2009). However, two cases of encephalitis presumably due to *Vahlkampfia* have been reported (John & Howard, 1995).

Although FLA infections are rarely reported in Malaysia, investigations of potential pathogenic FLA *(Acanthamoeba*, *Naegleria*, *Balamuthia* and *Vahlkampfia*) should be carried out in order to know the current status of their existence in our local environment and their ability to associate with other pathogens and cause diseases to humans and animals. Clinicians should be aware of primary amoebic meningoencephalitis (PAM) that is caused by *Naegleria fowleri* and how to differentiate the signs, symptoms and diagnosis of meningoencephalitis (GAE) that is caused by other pathogens including *Acanthamoeba* species. Continuous health education about preventive and control measures on FLA must be given to the public especially to those dealing with water activities as well as contact lens wearers. Awareness among the medical practitioners and adequate treatment of public water supplies must be emphasized.

CHAPTER 6

CONCLUSION

In environmental samples, *Acanthamoeba* was detected with high occurrence of 85% (136/160), including samples from PAWS animal shelter, recreational rivers, children playgrounds and Medical Faculty, University of Malaya. This concurrence with a high level of their cyst resistant and has capability to grow in various types of environments. On the other hand, *Acanthamoeba* was found less occurrence (24.8%) in stray animal corneas (cats and dogs) which may be due to several factors such as needed a mild corneal injury and virulent strain in order to infect the eye corneal. Further DNA analysis showed that only genotype T4 of *A. castellanii* (U07401) strains were detected in animals corneal. Beside, *A. castellanii* (U07401) strains were also detected environmental samples especially in dry materials [soil (15), dust (12)] and wet soil (2) samples. Therefore, the source of infection in the stray animals is highly from dry soil, dust and wet soil.

Subsequently, other *Acanthamoeba* genotypes such as T4 *A. castellanii* U07409 and *A. polyphaga* AY026243, T5 *A. lenticulata* U94741, T15 *A. jacobsi* KC164249 and T3 *A. griffini* KJ446979 were also detected in environmental samples. *A. castellanii* (U07409) strains were was frequently in dry materials (soil and outdoor dust). Meanwhile *A. polyphaga* (AY026243) strains were commonly found in swab wet debris from recreational rivers.

Genotype T5 of *A. lenticulata* (U94741) strain was frequently found in aquatic environment [water (15), wet debris from recreational rivers (8)] followed by moist debris from food/drinking containers (10) and dust from animal bedding (6). Genotype T15 of *A. jacobsi* (KC164249) strains were found more frequent in dry material (dust, soil) followed by moist debris and the least in aquatic environmental. Genotype T3 of *A. griffini* (KJ446979) strain was found in very low percentage (0.74%), only in wet debris from recreational rivers.

All the 4 existing genotypes (T3, T4, T5 and T15) have a capability to infect and cause diseases in human (Maghsood *et al*., 2005; Walochnik *et al*., 2000). Therefore, exposure to these genotypes may contribute to the risk of *Acanthamoeba* infections, especially to the high risk groups such as contact lens wearers and immunocompromised individuals. Therefore, health education shall be given to the public on the environmental risk factors such as natural water, soil and dust particles especially to those in high risk groups. The pet owners must always keep their pets in good care to minimize them from exposure with the infected strays. Awareness of *Acanthamoeba* infections among clinicians and veterinarians are also important in order to achieve an early diagnosis and correct treatment regiments in patients with AK or GAE. Awareness of the distribution of *Acanthamoeba* genotypes in the environment shall be made known to the public especially to those with high risk of acquiring it.

As for other FLA, only *Naegleria* and *Hartmannella*, were detected in environmental samples from water, soil, debris and dust. However, there was no pathogenic *Naegleria fowleri* is detected in this study. The nonpathogenic *Naegleria* species were identified as unclassified *Naegleria* (new species), *N. fultoni*, *N. phillippinensis*, *N. canariensis* and *N. australiensi.* Furthermore, two types of

Hartmannella species were also identified. Both *Naegleria* and *Hartmannella* species found in this study are non-pathogenic.

RECOMMENDATIONS

The following suggested recommendations are important aspects that have not been covered in this study:

i. This study was carried out randomly on stray cats and dogs that found in Selangor, Federal Territory, Negeri Sembilan and Perak states. Similar study is suggest to carry out in the urban and rural animals and involve a greater sample size. Furthermore, other related organisms such as virus, bacteria and fungi should be included in order to comfirm the infection status of *Acanthamoeba* in animals corneal.

ii. The similar study shoud also be carried out in the humans infected corneal. The DNA sequences of related virulent stains of *Acanthamoeba* were then analize by multiple sequence alignments with the environmental isolates that might be a rick for human infection.

iii. A similar study should also be carried out in the environmental samples from other states such as the Northern, Southern and East Coast States of Peninsular Malaysia, Sabah and Sarawak. The collection of samples could be included during the dry and flood-hit season to observe the emerging and spreading pattern of the FLA.

iv. More research on molecular work is highly recommended for an attempt to upgrade the Genbank record of the different FLA species particularly pathogenic *Acanthamoeba* genotypes.

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PUBLICATIONS

A. Publications from this thesis:

Abdul Basher, M.H, Ithoi, I., Mahmud, R., Abdulsalam, A.M., Foead, A. I., Dawaki, S., Mohsen Atroosh, W. M., Nissapatorn, V. & Abdullah, W. O. (2018). Occurrence of *Acanthamoeba* genotypes in Central West Malaysian environments. *Acta Tropica 178*, 219-228.

B. Publication related, but not directly arising from this thesis

Ithoi, I., Mahmud, R., **Abdul Basher, M.H**., Jali, A., Abdulsalam, A.M., Ibrahim, J. and Mak, J.W. (2013). *Acanthamoeba* genotype T4 detected in naturally-infected feline corneas found to be in homology with those causing human keratitis. *Tropical Biomedicine 30*(1): 131–140.

C. Conference presentations from this thesis

Mohamad Hafiz Abd Basher, Init Ithoi, Rohela Mahmud, Nadiah Md Sarani, Nur Izyan Samsudin Awatif Mohamed Abdulsalam & Wan Omar Abdullah. Prevalence and genotyping of *Acanthamoeba* in natural aquatic and PAWS animal shelter. Poster presentation at the 6th ASEAN Congress of Tropical Medicine & Parasitology 2014, 5-7 March 2014, InterContinental Kuala Lumpur, Malaysia. Poster

D. Conference papers, related, but not directly arising from this thesis

Mohamad Hafiz Abd Basher, Init Ithoi, Rohela Mahmud, Awatif Mohamed Abdulsalam & Wan Omar Abdullah. Phylogenetic study of *Acanthamoeba* isolated from naturally-infected feline corneas and domestic water storage. Poster presentation at the 49th Annual Conference of the Malaysian Society of Parasitology and Tropical Medicine, 19&20 March 2013, Grand Seasons Hotel, Kuala Lumpur, Malaysia. Poster

Rohela Mahmud, Init Ithoi, Rosaidatul Akma Mohamed-Rosdi, Jamaiah Ibrahim, Azman Jali, Awatif M. Abdulsalam and **Mohamed Hafiz Abdul Basher**. (2012). Prevalence of *Acanthamoeba* species in feline corneal disease. Paper presented at the 48th Annual Conference of the Malaysian Society of Parasitology and Tropical Medicine (MSPTM), 27th – 28th March 2012, Grand Seasons Hotel, Kuala Lumpur, Malaysia. Poster

Rohela Mahmud, Init Ithoi, **Mohamad-Hafiz Abdul-Basher**, Awatif Mohamed-Abdulsalam-Salih, Rosaidatul Akma Mohamad-Rosdi, Azman Jali, Romano Ngui and Jamaiah Ibrahim. (2012). Identification of *Acanthamoeba* Species in Feline Corneas from Peninsular Malaysia. Paper presented at the 5TH ASEANCongress of Tropical Medicine and Parasitology, 15th –17th May 2012, University of the Philippines, Manila, Philippines. Poster