1. Introduction

1.1 Overview

Acanthamoeba is a free-living protozoan, which can be found in two morphological forms, trophozoite and cyst. The trophozoite is irregular measuring 15 to 40 µm in length, while the cyst is spherical, 15 to 20 µm in diameter. The dry cyst can survive for several years and can be regularly isolated from dust and even from the air (Gardner *et al*., 1991). *Acanthamoeba* spp. normally do not cause disease but live as phagotrophs in ponds, rivers, streams and lakes, where they feed on bacteria. However, they are amphizoic which have an ability to live in two worlds, as free-living organisms and as endoparasites.

Several species of *Acanthamoeba* spp. such as *A. castellanii, A. culbertsoni* and *A. rhysodes* may produce a chronic central nervous system (CNS) infection known as granulomatous amoebic encephalitis (GAE) and an eye infection as *Acanthamoeba* keratitis.

Before they were recognized as pathogens, *Acanthamoeba* spp. were detected as contaminants in tissue cultures (Jahnes *et al.,* 1957 and Culbertson *et al.*, 1958) and were found to cause a cytopathic effect in monkey kidney cell cultures. The cytopathic effect was similar to that produced by viruses. Therefore these free-living amebae are believed to have a pathogenic potential. Culbertson *et al*. (1958, 1959) observed that a strain of *Acanthamoeba* (*A. culbertsoni*) isolated from a cell culture was able to produce *meningoencephalitis* in monkeys (after it was inoculated intracerebrally and intravenously) and in mice (after inoculated intravenously and intranasally). It showed more pathogenic effect in mice compared to monkeys. This finding suggested that these free-living amoebae were able to invade the intact nasal mucosa, move to the brain and produce a fatal CNS infection. *Acanthamoeba* infection in CNS was first reported in the early 1970s (Kenney, 1971; Jager & Stamm, 1972 and Robert & Rorke, 1973). Invasion of the CNS by *Acanthamoeba* is secondary to infection elsewhere in the body (not associated with swimming). It reaches the brain by way of the bloodstream. The disease tends to be chronic, with a

prolonged course, and occurs most often in debilitated or immunocompromised persons. The term granulomatous amebic encephalitis was proposed by Martinez (1980) to describe the CNS infection caused by *Acanthamoeba* and to distinguish it from the fulminant disease produced by *Naegleria fowleri.* The first isolations of *Acanthamoeba* from human tissue were reported by Visvesvara *et al*. (1983). One isolate was obtained from the lung of a patient who died of GAE and the other was obtained from other tissues. Both of these isolates were identified as *A. castellanii*.

While for the *Acanthamoeba* keratitis, the first two cases were reported from Great Britain by Nagington *et al*. (1974). Both of these cases produced a chronic progressive ulceration of the cornea that, in one patient, required surgical removal of the infected eyeball. Moreover, after cultivation of the patients samples, the amoebae were identified as *A. polyphaga* and *A. castellanii*, respectively (Nagington, 1975). The two following cases of *Acanthamoeba* envolving the eye were then reported from Texas (Jones *et al*.,1975) and were described as keratitis and uveitis and both cases were found to be caused by*A. polyphaga* after corneal scraping cultivation.

Most of the earlier cases of *Acanthamoeba* keratitis were associated with eye trauma and exposure to contaminated water. However, a dramatic increase in the number of cases has been noted since 1985 and has been linked to the wearing of contact lenses, especially soft contact lenses. A total of 208 cases of *Acanthamoeba* keratitis have been identified in the United States. Of the 189 patients for whom information was available, 85% wore contact lenses (Stehr-Green *et al*., 1989).

1.2 Taxonomy

Castellani was the first one to describe *Acanthamoeba* when he reportedthe presence of an amoeba in *Cryptococcus pararoseus* cultures(Castellani, 1930). Volkonsky later established the genus *Acanthamoeba* in 1931, however, the actual classification of organisms withinthis genus is currently under review (Amaral Zettler *et al.,* 2000; Booton *et al* ., 2002 and 2001; Bradley, 1996; Burger *et al* ., 1995; Byers *et al* ., 2001;Gast *et al* ., 1996; Kong *et al* ., 2002; Schroeder *et al* ., 2001 and Stothard *et al* ., 1998). Consequently, *Acanthamoeba* has been placed in the FamilyAcanthamoebidae. *Balamuthia* which was previouslyassigned with amoebae

of uncertain affinities, has recently beenincorporated into this family (Corliss, 1998 and Rogerson and Patterson 2000). Nevertheless, studies suggested that thegenus *Balamuthia* be transferred from the family Leptomyxidaeto Acanthamoebidae on the basis of molecular analysis of 16S-likerRNA genes (Amaral Zettler *et al*., 2000 and Stothard *et al.*,1998). Furthermore, both *Acanthamoeba* and *Balamuthia*have a multilayered microtubule-organizing center andboth can cause disease in humans (Patterson, 1999). Due to the presence ofspiny surface projections (acanthopodia) on trophozoites, identification of *Acanthamoeba*at the genus level is relatively easy. However, using morphological criteria to identify these amoebae at the species level is difficult. Based on cyst size and shape, *Acanthamoeba* spp. have been placed into three morphological groups (I, II, and III) (Page, 1967 and Pussard & Pons 1977). Species ingroup I were designated on the basis of having a large cystin comparison to that of species in the other groups. Speciesin group II were characterized as having a wrinkled ectocystand an endocyst which could be stellate, polygonal, triangular,or oval. Species in group III typically exhibited a thin, smoothectocyst and a round endocyst. Nevertheless, classificationof *Acanthamoeba* based on morphological characteristics of thecyst wall has proved unreliable because cyst morphology canchange depending on culture conditions (Armstrong, 2000; Daggett *et al.,* 1985; Sawyer and Griffin 1975 and Stratford and Griffin 1978).Immunological, biochemical, and physiological criteria alsohave been applied to the identification of different speciesof *Acanthamoeba* (Alves *et al .,* 2000; Costas & Griffiths1985;Howe *et al .,* 1997; Kilvington *et al .,* 1991;Vodkin *et al .,* 1992;Walochnik *et al .,* 2000 and2001). However,many species share antigenic determinants. Therefore, resultsobtained through immunological approaches such as western blottingand immunofluorescence have been inconclusive in identifyingspecies. Isoenzyme electrophoresis of different enzyme systemsalso has been used to compare strains of *Acanthamoeba* (Daggett *et al .,* 1985 andDenney *et al .,* 1997). Although this method has the potential to provide insightinto relationships among species, results have indicated interstrainvariation within species as well as similarities between strainsof separate species. Furthermore, studies have shown that enzymepatterns change when

isolates are grown under different laboratoryconditions (Jacobson & Band1987 andWeekers & Jonckheere1997). The position of *Acanthamoeba* in the taxonomic scheme of the society of protozoologists is below:

Kingdom: Protista Haeckel

Subkingdom: Protozoa (Goldfuss, 1818) emd. (Von Siebold, 1846)

Phylum: Sarcomastigophora (Honigberg and Balamuth, 1963)

Subphylum: Sarcodina (Schmarda, 1871)

Super class: Rhizopodea (Von Siebold, 1845)

Class: Lobosea (Carpenter, 1861)

Subclass: Gymnamoebia (Haeckel, 1862)

Order: Amoebida (Kent, 1880)

Suborder: Acanthapodina (Page, 1976)

Family: Acanthamoebidae (Sawyer and Griffin, 1975)

Genus: *Acanthamoeba*(Volkonsky, 1931; emd. Page, 1967)

There are 23 known *Acanthamoeba* species; *A. astronyxis, A. castellani, A. comandoni, A. culbertsoni, A. griffini., A. hatchitti, A. healyi, A. lenticulata, A. lugdunesis, A. palestinensis, A. pearcei, A. polyphaga, A. postula, A. rhyosodes, A. stevensoni, A. triangularis, A. tubiashi, A. terricola, A. quina, A. royreba, A. mauritaniensis, A. jacobsi* and *A. divionensis* (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/ wwwtax.cgi?id=5754).

1.3 Morphology

The cellular organization of *Acanthamoeba* has been studied usingelectron microscopy (Bowers &Korn , 1968 , 1969; Gonzalez-Robles*et al* ., 2001 and Rondanelli*et al* ., 1987). Organelles typicallyfound in higher eucaryotic cells have been identified in *Acanthamoeba*. Bowers& Korn,(1968) indicated the presence of a Golgicomplex, smooth and rough endoplasmic reticula, free ribosomes,digestive vacuoles, mitochondria, and microtubules in *Acanthamoeba*trophozoites. A trilaminar plasma membrane was found to surroundthe cytoplasmic contents of the trophozoite. Furthermore, distinguishingfeatures of the trophozoite were the presence

of spiny surfaceprojections called acanthopodia, a prominent contractilevacuole in the cytoplasm that controls the water content ofthe cell, and a nucleus with a large central nucleolus. Though multinucleated cells arecommon when *Acanthamoeba* are maintained in suspension culture, the ameba is generally uninucleate.Reproduction occurs by binary fission (Byers, 1979 and Page, 1967).A double-walled wrinkled cyst composed of an ectocystand an endocyst ranges in size from 13 to 20 µm and variesfrom one species to another (Bowers & Korn, 1969). Cysts are formed under adverseenvironmental conditions such as food deprivation, desiccation,and changes in temperature and pH (Bowers & Korn, 1969; Byers *et al* ., 1980 and Chagla and Griffiths 1974). Villemez*et al.* (1985) and Yang &Villemez ,(1994) have reported that antibody binding toa specific membrane protein also causes *A. castellanii* to encyst.Cysts are resistant to biocides, chlorination, and antibiotics(De Jonckheere& Van de Voorde , 1976; Khunkitti *et al* ., 1998; Lloyd *et al* ., 2001 and Turner *et al* ., 2000) and survive at low temperatures 0 to 2°C(Brown *et al* ., 1977). Meisler *et al.* (1985), however, have shown that treatmentwith Freon or methylene oxide or autoclaving destroys cysts.Excystment takes place as trophozoites emerge from the cyst undersuitable environmental conditions. Mazur *et al.* (1995) demonstratedthat cysts retained viable amebae for over 24 years after storagein water at 4°C. Because *Acanthamoeba* trophozoites can be induced to transforminto cysts in nonnutrient media and excystation occurs underfavorable conditions, the amebae have been used as a model system to study eucaryoticRNA transcription, RNA polymerase functions, and cellular differentiationin trophozoites and cysts (Bateman, 1998; Byers, *et al.*,1991;Chen and Bateman, 2000; Huang & Bateman, 1997; Jantzen*et al.*, 1988; Orfeo & Bateman, 1998; Schulze and Jantzen, 1982).

1.4 Life cycle

Acanthamoeba was distributed worldwide in soil, fresh water and marine environments. Life cycles are rather simple, the stages being a feeding trophozoite, and a resting cyst. The trophozoite feeds on bacteria, algae, and yeast in the environment but alsocan exist axenically on nutrients in liquid taken up throughpinocytosis (Bowers, 1977 andBowers & Olszewski, 1983). Uptake of food

by trophozoites can occurby pseudopod formation and phagocytosis or by food cup formation and ingestion of particulate matter. Food cups formedon the amoeba surface are temporary structures used to ingestbacteria, yeast, or cells (Pettit *et al.*, 1996). Locomotion involves the formationof a hyaline pseudopodium and is sluggish in all species of*Acanthamoeba* (Preston & King, 1984). The *Acanthamoeba* cycle of trophozoites and cysts is reflected in human infection where both trophozoites and cysts are seen in tissue. *Acanthamoeba* seems to be truly amphizoic in all respects.

1.5 Epidmiology

Acanthamoeba has a world wide distribution, Table 1 is an example from several countries reported to have cases of GAE and *Acanthamoeba* keratitis. Infections with *Acanthamoeba* often occur in persons who are debilitated or immunosuppressed. Two cases, both fatal, have been reported to have occurred in patients with AIDS (Gonzalez *et al*., 1986 and Wiley *et al*., 1987). Most cases of *Acanthamoeba* keratitis have been reported from the United States. This has been the result of a survey by Stehr-Green *et al*. (1989) in which 208 cases were identified. Ten (10), 7 and 4 cases have been reported from India, the United Kingdom and the Netherlands respectively.

Table 1. Global Distribution of Human Infections Caused by *Acanthamoeba* (1989)

Country	GAE	*Acanthamoeba* keratitis
Australia	2	1
Barbados	1	-
Germany	-	2
Honduras	2	-
India	3	10
Japan	1	3
Korea	1	-
Netherlands	-	4
Nigeria	1	-
Peru	4	-
South Africa	1	-
United Kingdom	-	7
United States	22	208
Venezuela	1	-
Zambia	1	-

The number of persons wearing contact lenses increased up to 59% in the years 1980-1985, to an estimated 23.1 million. The continued increase in the number of persons wearing contact lenses, and of persons not properly caring for them, undoubtedly will result in an increase in the incidence of *Acanthamoeba* keratitis. The majority of these new cases will most probably occur in the developed nations, where the wearing of contact lenses is fashionable and affordable (Stehr-Green *et al*., 1989).

1.6 Occurrence in human environment

The free living *Acanthamoeba* has located in various fresh water habitats, including rivers, lakes, ponds, hot springs and spas, domestic water systems, air-conditioning systems, humidifiers, and cooling towers. They are believed to play a role in a hypersensitivity pneumonitis known as humidifier fever. It's ubiquitous in soils, dust, air, composts; has been found on the surface of vegetables; and has been isolated from sewage sludge and fresh water polluted by domestic or industrial waste. *Acanthamoeba* species have also been isolated from sea water, especially associated with discharges of inadequately treated sewage from hotels and municipal sewage effluents (Mergeryan,1991). It has also been found to be associated with thermally polluted discharges in ponds and lakes connected with electric power plants, nuclear power plants, or thermal polluting factories.

Research by Mergeryan, (1991) showed that *Acanthamoeba* could be isolated from various water samples (Table 2). Swimming ponds water was found to be the most frequently positive for *Acanthamoeba* (54%), followed by tap water (37%), and surface water such as rivers and streams (34%). The frequency of isolation from hospital physiotherapy pools (17%) was higher than that for outdoor swimming pools (16%) and indoor swimming pools (11%). Only 7% of water samples taken directly from water supply were positive, but the rate of isolation from bottled drinking water purchased in the original package was high (17%).

Of the tested samples of sand from sandboxes in playgrounds in the community, 85% were found to be positive. The rate of isolation of *Acanthamoeba* from soil and

street dust samples from 17 different sample sites in 11 different countries also yielded a high incidence of *Acanthamoeba* (90%). In Turkey, *Acanthamoeba* were isolated from 88%-99% of the dust samples collected from apartments, hospital rooms, bedrooms, dining rooms, and sitting rooms.

Table 2. Isolation of *Acanthamoeba* from various water samples (1991)

Type of samples	***No. of Acanthamoeba-positive samples/no. of samples tested (%)***
Water	
Bottled drinking water (France, Belgium Turkey, Germany)	11/65 (17)
Municipal water supply (Göttingen. Germany)	13/187 (7)
Tap water (Göttingen; Istanbul and burdur, Turkey; Zambia)	56/425 (37)
Swimming ponds (Göttingen)	7/13 (54)
Outdoor Swimming pools (Göttingen)	27/170 (16)
Indoor swimming pools (Göttingen)	7/64 (11)
Physiotherapy pools (Göttingen)	20/121 (17)
Surface water (Göttingen and environs)	20/59 (34)
Sand	
Göttingen and Holzminden(Germany)(Sandboxes in children's playgrounds)	89/105(85)
Soil and dust (outdoor)	
Istanbul and burdu (Turkey)	99/104 (95)
Sri lanka, Zambia, Southwest Africa, Tunisia, India	27/29 (93)
Sri lanka, Zambia, Southwest Africa, Tunisia, India	27/29 (93)
Montpelier (France)	29/32 (91)
Göttingen	9/11 (82)
Sylt-Westerland (Germany)	30/33 (91)
Total	198/214 (93)
Dust (home, hospital)	
Istanbul	15/16 (94)
Burdur	59/67 (88)

1.7 Pathogenesis

Human infection caused by *Acanthamoeba* involves granulomatous encephalitis, dermatitis, pneumonia, and keratitis (Martinez& Visvesvara, 1997). Granulomatous amebic encephalitis (GAE) usually occurs in debilitated or chronically ill persons, some of whom may be undergoing immunosuppressive therapy. The underlying diseases that have been reported in GAE were Hodgkin's disease (Jager & Stamm, 1972) systemic lupus erythematosus (Grunnet *et al.*, 1981), diabetes mellitus (Harwood *et al.*, 1988), G6PD deficiency (Hoffmann *et al*., 1978), alcoholism (Rutherford, 1986), and acquired immunodeficiency syndrome (AIDS) (Gonzalez *et al*., 1986 and Wiley *et al.*, 1987). However, not all of the victims of GAE have been debilitated or immunocompromised. Some have been otherwise healthy individuals (Carter *et al*., 1981; Martinez *et al*., 1977 and Sotelo-Avila *et al*., 1974). The course of infection in GAE is sub acute or chronic, lasting from weeks to months and, in some cases prolonged to years (Ringsted *et al.*, 1976), and is characterized by focal granulomatous lesions of the brain. *Acanthamoeba* infection most probably occurs through the lower respiratory tract or through ulcers of the skin or mucosa. Invasion into the central nervous system is by hematogenous spread from the primary focus of infection. Since there are no lymphatic channels in the brain, invasion of the brain must be via the bloodstream (Martinez, 1987). Even though some *Acanthamoeba* isolates are able to produce a central nervous system infection after intranasal instillation in mice, there is no proof that similar invasion occurs in the human disease.

The incubation period in GAE is not known, but probably lasts weeks or months, and during the prolonged clinical course single or multiple-space-occupying lesions develop. An altered mental state is a prominent feature in GAE. Headache, seizures, and neck stiffness occur in about half of the cases. Nausea and vomiting may also be noted (Martinez, 1987). In contrast to *Naegleria* (other free living species) infection, which is characterized by a diffuse meningoencephalitis, *Acanthamoeba* CNS disease is focal granulomatous encephalitis.

Martinez (1980) gave a summary of the neuropathological features for 15 patients with GAE. At the affected areas, the leptomeninges contain a moderate amount of purulent exudates. The cerebral hemispheres show moderate or severe edema with foci of softened tissue and associated hemorrhagic necrosis. Lesions are usually multifocal and more posterior, including the upper portion of the spinal cord. The olfactory bulbs generally are not involved.

Lesions of CNS in GAE are characterized by necrosis with hemorrhagic foci and localized leptomeningitis. The chronic inflammatory exudates over the cortex comprise mostly mononuclear cells with a few polymorph nuclear leukocytes. The brain substance may have a prominent granulomatous reaction with foreign body giant cells; these multinucleated giant cells may not be present in immunosuppressed patients (Carter *et al*., 1981 and Martinez, 1987). Amoebae reach the brain via the bloodstream; therefore, invasion of the CNS is centrifugal, from the deeper tissues toward the brain surface. Trophozoites and cysts occur in most infected tissues and around blood vessels (Martinez, 1987).

Acanthamoeba reaches the CNS by hemaogenous spread from a primary focus of infection elsewhere in the body, most probably of the skin, mucosa, or lungs. Table 3 is an example of *Acanthamoeba* species infecting humans and the tissues involved. Within the infected primary tissues, there occurs a chronic granulomatous reaction like that seen in the brain, with multinucleated giant cells, trophozoites, and cysts. Similar lesions have been described from other tissues as well, including prostate, thyroid, uterus, and pancreas. These lesions are probably the result of hematogenous dissemination of amoebae from the primary focus in the skin or lungs, or possibly even from a secondary CNS lesion (Martinez, 1987).

Another important disease, *Acanthamoeba* keratitis, is a chronic infection of the cornea caused by several species of *Acanthamoeba* e.g.:*A. castellani, A. culbertsoni, A. hatchitti, A. polyphaga,* and *A. rhyosodes.* Infection is by direct contact of the cornea with amoebae, which may be introduced through minor corneal trauma or by exposure to contaminated water or contact lenses. The wearing of contact lenses and the use of homemade saline solutions are important risk factors associated with the

disease. Saline solutions contaminated with protein residues from contact lenses promote the growth of bacteria and yeast that, in turn, are a source of food for the amoebae. Amoebae attach to the contact lenses stored in contaminated solutions, and then are transferred to the eye when lenses are placed over the cornea. It become established as part of the conjuctival flora and may invade the corneal stroma through a break in the epithelium or through the intact epithelium (John *et al*., 1989), producing an infection that progresses to *Acanthamoeba* keratitis.

Acanthamoeba keratitis usually develops over a period of weeks to months and is characterized by severe ocular pain, often out of proportion to the degree of inflammation (Mannis *et al*., 1986), affected vision, and a stromal infiltrate that is frequently ring shaped and composed predominantly of neutrophils (Ma *et al*., 1981). *Acanthamoeba* keratitis is a serious ocular infection and, if not properly managed, can lead to loss of vision and even loss of the eye (Key *et al*., 1980).

In advanced cases of *Acanthamoeba* keratitis there may be a marked stromal infiltrate and necrosis. The whitish inflammatory infiltrate, often appear ring shaped around the corneal ulcer,consists mainly of polymorph nuclear leukocytes and macrophages, with a few lymphocytes (Mathers *et al*., 1987) in most of the reports, neutrophils, and not lymphocytes, are the predominant infiltaring cells. Corneal ulceration may progress to perforation (Lindquist *et al*., 1988). *Acanthamoeba* eye infections also have been described as conjunctivitis (Nagington *et al*., 1974), iritis (Bos *et al*, 1981 and Lund *et al*., 1978), scleritis (Mannis *et al*, 1986), and uveitis (Jones *et al*, 1975 and Nagington, 1974). *Acanthamoeba* tropozoites and cysts occur within infected corneal tissue.

GAE tends to occur in persons who are debilitated, chronically ill, or immunocompromised. In contrast, *Acanthamoeba* keratitis usually occurs in healthy individuals, and infection is by direct invasion of the cornea through trauma to the eye or the wearing of contaminated contact lenses. Trophozoites are the invading stage in *Acanthamoeba* infections. However, in theirinfections, whether of the eye or the CNS, cysts and trophozoites form could occur the tissue.

Table 3. Human Infection Caused by Species of *Acanthamoeba*

Species of *Acanthamoeba*	CNS infection	Eye infection	Other tissues	Reference
A. astronyxis	X		Adrenal, lymph, node, sinus, skin thyroid	Gullett *et al*. (1979)
A. castellanii	X	x	Lung, prostate, bone,muscle,sinus, skin	Martinez (1982) Martinez *et al*.(1977) Moore *et al.* (1985) Borochovitz *et al*. (1981) Gonzalez *et al*. (1986)
A. castellanii	X	x	Liver, spleen, uterus, skin	Martinez *et al*. (1977) Wiley *et al.* (1987) Mannis *et al.* (1986) May *et al.* (1992)
A. castellanii		x		Cohen *et al*. (1987)
A. castellanii	X			Ofori *et al.* (1986)
A. polyphaga		x		Lindquist *I et al.* (1988)
A. castellanii	X	x		Cleland *et al.* (1982) Wilhelmus *et al*. (1986)

1.8 Cutaneous Acanthamebiasis

Cutaneous infections caused by *Acanthamoeba* are most commonin patients with AIDS, with or without CNS involvement (Casper*et al.,* 1999; Duluol *et al.,* 1996; Helton *et al.,* 1993 and Murakawa *et al.,* 1995). Cutaneous disease has also been documented fornon-HIV-infected patients with amebic encephalitis, for patientsundergoing immunosuppressive therapy for organ transplantation(Slater *et al.,* 1994 and Van Hamme *et al.,* 2001), or for individuals with immunological diseases (Gordon *et al.,* 1992;Martinez, 1982; Oliva *et al.,* 1999; Slater *et al.,* 1994 and Visvesvara *et al.,* 1983). The cutaneous form of the disease is characterizedby the presence of hard erythematous nodules or skin ulcers(Bonilla*et al.,* 1999; Chandrasekar *et al.,* 1997; Friedland *et al.,* 1992; Gonzalez*et al.,* 1986; Helton *et al.,* 1993; Levine*et al.,* 2001; May *et al.,* 1992;Murakawa *et al.,* 1995; Rosenberg & Morgan2001; Tan *et al.,* 1993 and Torno *et al.,* 2000). Earlymanifestations of the cutaneous form of acanthamebiasis includethe presence of firm papulonodules that drain purulent materialand then develop into nonhealing indurated ulcerations (May*et al.,* 1992 andRosenberg *et al.,* 2001). Occurrence of disseminated skin lesions may be the presentingmanifestation of *Acanthamoeba* infection (Murakawa*et al.,* 1995 and Tan *et al.,* 1993). Whetherskin lesions represent a primary focus of infection or are theresult of hematogenous dissemination from other sites such asthe respiratory tract, sinuses, or the CNS is not known (Friedland*et al.,* 1992).The reported mortality rate from cutaneous infection for individualswithout CNS involvement is approximately 73%, while that fromcutaneous infection accompanied by CNS disease is 100% (Torno*et al.,* 2000).

Histologic examination of cutaneous lesions generally showsfoci of necrosis surrounded by inflammatory cells, vasculitis,trophozoite and cyst forms (Friedland *et al.,* 1992). However, the histologic appearanceof skin lesions may mimic that of fungi, viruses, mycobacteria,or inflammation due to a foreign body (Chandrasekar *et al.,* 1997; Gordon *et al.,* 1992 and Singhal *et al.,* 2001). Organismsin tissue sections have been mistaken for yeast forms of *Blastomycesdermatiditis* (Tan *et al.,* 1993), sporangia of *Rhinosporidium seeberi*, *Cryptococcusneoformans*, or *Prototheca*

wickerhamii (Sison *et al.,* 1995). Also, cases ofcutaneous acanthamebiasis have been misdiagnosed as bacillaryangiomatosis (Khalife *et al.,* 1994), cat scratch fever (Sison *et al.,* 1995), *Penicillium marneffei*infection (Casper*et al.,* 1999), Kaposi's sarcoma (Chandrasekar *et al.,* 1997) or cells with cytomegalovirusinclusions (Teknos *et al.,* 2000). Therefore, when a single biopsy specimen does notreveal trophozoites when *Acanthamoeba* is suspected, the examinationshould be repeated with a different specimen (Migueles& Kumar, 1998 and Oliva *et al.,* 1999).

1.9 Signs and symptoms

Acanthamoeba keratitis can occur in patients of any age, sex or race, but mostly manifests in young, healthy adults. The list of risk factors included: corneal foreign body, contact with non-sterile water, bullous keratopathy, neurotrophic keratopathy, herpes simplex keratitis, radial keratotomy, swimming and scuba diving, basement membrane dystrophy, contact lens wear and bacterial keratitis. Cases sometimes arise with no identifiable risk factors. Essentially, any event that disrupts the corneal epithelium is a potential risk factor for *Acanthamoeba* keratitis.

Patients with *Acanthamoeba* keratitis typically present with a unilateral, red, painful eye. Initially, there is typically a non-specific epitheliopathy which can progress to ulceration with infiltration. Limbititis occurs as the initial finding in 94% of early stage cases and in 84% of late-stage cases (Bacon *et al.,* 1993 and Bernauer *et al.,* 1996). Another common finding is radial keratoneuritis, or perineuritis; this involves irregularly thickened corneal nerves in the anterior to mid-stroma with shaggy borders. Other clinical signs of *Acanthamoeba* keratitis include irregular epithelial defect, corneal microcysts, punctate keratopathy, bullous keratopathy, disciform stromal keratitis, pseudodendritic keratitis, anterior uveitis and a granulomatous stromal reaction. While *Acanthamoeba* keratitis has historically been associated with stromal ring infiltrate formation, only 6% of early cases and 16% of late cases actually present in this manner clinically (Bacon *et al.,* 1993 and Bernauer *et al.,* 1996).

About half of patients report significant pain, the rest experience only mild irritation and foreign-body sensation (Tabin *et al.,* 2001 and Roters *et al.,* 2001). Those who do report pain often present a degree much worse than the clinical appearance suggests.

In GAE, a person may suffer with headaches, stiff neck, nausea and vomiting, tiredness, confusion, lack of attention to people and surroundings, loss of balance and bodily control, seizures, and hallucinations . Signs and symptoms progresses over several weeks, and death generally occurs. . (http://www.wrongdiagnosis.com/a/acanthamoeba/complic.htm).

1.10 Immunology

The immune defense mechanisms that operate against *Acanthamoeba*have not been well characterized. Exposure to *Acanthamoeba* appearsto be common since the presence of antibodies to the ameba havebeen demonstrated in serum samples from most asymptomatichealthy individuals (Cerva, 1989; Chappell *et al*., 2001; Cursons*et al*.,1980; Marciano-Cabral*et al.,* 2000; Newsome *et al.,*1992 and Powell *et al.,*1994). The prevalence of *Acanthamoeba* cysts and trophozoitesis high in the environment, the incidence of fatal infectionappears to be low. Indeed, *Acanthamoebae* have been isolated from thenasopharynx of apparently healthy individuals (Badenoch *et al.,* 1988; Cerva*et al.,* 1973; Michel *et al.,* 1994; Newsome *et al.,* 1992; Rivera *et al.,* 1991, 1984). Whether *Acanthamoeba* causes transient infectionsin these individuals and stimulates host defense responses as well which result in the elimination of the organismis not known. Chappell *et al*.(2001) noted that seriousocular disease and CNS infections are rare but mucosal infectionsmay contribute significantly to large numbers of undiagnosedsinus or pulmonary infections. The presence of a higher titersof *A. polyphaga*-specific immunoglobulin M (IgM) and a lowertiters of IgG antibodies in serum from rheumatoid arthritis(RA) patients than from matched controls were thought to be due to persistentor repeated antigenic stimulation by *A. polyphaga* (Jeansson and Kvien, 2001). However, it is not known whether an immune reaction to *A. polyphaga* antigensresults in symptoms in RA patients.

Protection from lethal infection may involve both innate andacquired immunity (Cursons*et al.*, 1980). In experimental animal infections, the age of the animal, the mouse strain, the immune status ofthe host, as well as the infecting dose, temperature tolerance, and thevirulence of the ameba strain appear to be important factorsin the outcome of a murine infection (Marciano-Cabral*et al.*, 2001). An innateresistance factor which provides the first line of defense against invading organisms, is activated by *Acanthamoeba* infection (Ferrante and Rowan-Kelly, 1983 andToney and Marciano-Cabral, 1998). However, the consequences of such activation in responseto infection with *Acanthamoeba* are not known. Whether complementactivation results in increased pathogenesis by generating C3aand C5a components, which act as mediators of inflammation andtissue damage, or aids in eliminating amebae remains to be defined.Activation of complement may result in generation of opsonicfactors such as C3b, which plays a role in recognition of amebaeby phagocytic cells (Ferrante and Rowan-Kelly, 1983). Alternatively, complement may protectthe host by lysing amebae, although *in vitro*, highly pathogenicspecies of *Acanthamoeba* such as *A. culbertsoni* are more resistantto complement lysis than are nonpathogenic *Acanthamoeba* spp.(Toney and Marciano-Cabral,1998). It has also been shown that pathogenic strains of *Acanthamoeba* interact and bind more efficiently withhuman C1q than nonpathogenic strains. Binding of C1q byamebae cause blocked binding sites for C1 (the first component ofthe classical complement cascade), which serves to inhibit theclassical pathway (Walochnik *et al.*, 2001). Thus, resistance to complement lysismay constitute a mode of immune evasion which contributes tothe establishment of infection and dissemination of *Acanthamoebae*within the host (Toney & Marciano-Cabral, 1998 and Walochnik *et al.*, 2001).

The role of antibodies in *Acanthamoeba* infection also remainsunresolved. Antibodies may prevent attachment to host cells,inhibit the motility of amebae, or neutralize ameba cytotoxicfactors (Cursons *et al.*, 1980; Ferrante & Abell, 1986; Marciano-Cabral & Toney,1998 and Stewart *et al.*,1994). However, it has been reportedthat *Acanthamoeba* can degrade human IgG and IgA antibodies byserine

proteases (Kong *et al.*, 2000). In mice, immunization with *A. culbertsoni*antigens using intranasal, intraperitoneal, intravenous, ororal routes of administration purportedly provided protectionagainst a lethal challenge (Rowan-Kelly & Ferrante, 1984). However, multiple immunizationswere required to impart protection. Several workers reported that, of the immunogens tested(e.g., culture fluid, amebic sonicate, freeze-thawed extract,or live amebae), amebic sonicate was the best protectionagainst intranasal challenge with *A. culbertsoni.* This Protectionwas very specific, where immunization with immunogens from other *Acanthamoeba* speciesdid not protect mice challenge with *A. culbertsoni* (Bhaduri *et al.*, 1987;Culbertson, 1971 and Ferrante, 1991).

There have been few studies to assess the interaction of *Acanthamoeba*with specified cells of the immune system. The earliest response of the host against amebae consists ofan influx of neutrophils to the site of infection. However, human neutrophils fail to kill *Acanthamoeba* unless the neutrophilsare treated with Tumor Necrosis Factor. *In vitro,* killing of *Acanthamoeba* by lymphokine-treatedneutrophils requires the presence of both antibodies and complement (Ferrante & Abell, 1986). Macrophages may play a more importantrole than neutrophils in killing *Acanthamoeba*.These cells are capable of injuring amebae and comprise themajor cellular component of granulomas frequently encounteredin tissues containing *Acanthamoeba* cysts (Marciano-Cabral & Toney, 1998). Masihi*et al.* (1986) studied the effect of the mycobacterium-derived immunopotentiating agents muramyl dipeptide and trehalose dimycolateagainst intranasal *Acanthamoeba* infections in mice. Treatmentof mice with these macrophage-activating agents prior to infectionprotected 40 and 30% of the animals, respectively, against alethal infection with *A. culbertsoni*.*In vitro* studies withmurine macrophages activated *in vivo* with *Bacillus* Calmette - Guérindemonstrated that activated macrophages were more efficientin injuring *Acanthamoeba* than were unstimulated macrophages. The activated macrophages also were more efficient ininjuring *Acanthamoeba* than were unstimulated macrophage-likecells maintained as continuous cell lines (Marciano-Cabral & Toney,1998). In addition, Stewart *et al.* (1992) reportedthat rat macrophages, similar

to murine macrophages, undergochemotaxis to amebae and kill trophozoites *in vitro*. Thus, although the full range of specific macrophage factors responsible forinjuring *Acanthamoeba* has yet to be defined, it is apparentthat macrophages activated with immunomodulators are capableof phagocytizing and destroying amebae (Marciano-Cabral & Toney,1998).

Recent reports indicate that microglial cells, resident macrophagesof the brain, also exert amebicidal activity (Marciano-Cabral *et al.*, 2000). Microglialcells obtained from newborn rat pups and cocultured with *A.castellanii* were shown to destroy amebae by both phagocyticand lytic processes. These observations that microglia undergoinducible expression of proinflammatory cytokine genes suggesta mode by which *Acanthamoeba* effects neuropathology (Marciano-Cabral *et al.*, 2000). Studiesalso have been performed on *Acanthamoeba*-microglia interactionsby using highly pathogenic *A. culbertsoni* and weakly pathogenic*A. royreba* amebae. Shin *et al*. (2000) reported that microglial cellscocultured with virulent *A. culbertsoni* exhibited cytopathicchanges consistent with those described for cells undergoingapoptosis while microglial cells cocultured with weakly pathogenic*A. royreba* did not. In view of these observations, ithas been postulated that virulent *Acanthamoeba* strains escapethe amebicidal activity of macrophages and macrophage-like cellswhile, in contrast, weakly pathogenic species are targeted bymacrophages and are lysed, ingested, and destroyed (Marciano-Cabral *et al.*, 2000; Marciano-Cabral and Toney,1998 andShin *et al.*, 2000).

Except in the case of amebic keratitis, the defenses of a healthy host seem sufficient to prevent *Acanthamoeba* infection. Patients who contract granulomatous amebic encephalitis usually have impaired humoral and/or cell-mediated immunity. However, there are reports of patients with no demonstrable underlying disease or predisposing factor (www.gsbs.utmb.edu/microbook/ch081.htm).

1.11 Diagnosis

When *Acanthamoeba* infection is suspected, fresh cerebrospinal fluid or tissue (*eg*, corneal biopsy, corneal scrapings, and CNS tissue) should be collected aseptically and examined immediately. Specimens intended for culture must never be

frozen or refrigerated and should be maintained at 20ºC to 30ºC during transport to the laboratory or prior to processing within the laboratory. If immediate examination is not possible, amoebae could survive in sterile fluid or otherwise sterile tissue for several days at room temperature prior to processing in the laboratory. Suitable transport medium for transport of tissue biopsy sample or corneal scrapings is the minimal essential medium (MEM). *Acanthamoebae* could be cultured on nonnutrient agar spread with washed *E. coli* or *E. aerogenes.*

The laboratory diagnosis of GAE is made by identifying amebic forms of *Acanthamoeba* in the CSF or amebae and cysts in brain tissue. Only in a few cases has *Acanthamoeba* been isolated from patients with GAE. *A. culbertsoni* (Gogate *et al*., 1984 and Lalitha *et al,* 1985) and *A.rhysodes* (Cleland *et al*., 1982 and Gogate *et al*., 1984) have been cultured from CSF, an *Acanthamoeba* sp. (Harwood *et al*., 1988) and *A. palestinensis* (Ofori-Kwakye *et al*., 1986) have been cultured from aspirated and biopsied brain material, respectively.

The diagnosis of *Acanthamoeba* keratitis is made by identifying amebae cultured from corneal scrapings or by histologic examination of infected corneal tissue. As in GAE, *Acanthamoeba* could be cultured on nonnutrient agar spread with gram-negative bacteria followed by incubation at 30ºC or room temperature rather than 37ºC, then identifying the trophozoite after staining with Giemsa stain.

Species identification is based on indirect immunofluorescent antibody staining. The two species most frequently identified in *Acanthamoeba* keratitis were *A. castellanii* and *A. polyphaga*. *A. castellanii* is the species that has most often been identified in cases of GAE and ocular infection. Other techniques which could be used are the calcofluor procedure by Wilhelmus *et al*. (1986) and Indirect Fluorescent Antibody Technique (Epstein *et al.,* 1986) which both require the fluorescence microscopy.

Histopathologic preparations of infected tissue could be stained using the conventional hematoxylin and eosin procedure or by the more specialized staining procedures of Heidenhain's hematoxylin, Gomori's chromium hematoxylin, periodic acid-Schiff, Bauer chronic acid-Schiff, and silver methenamine (McClellan *et al*., 1988). The special staining techniques are useful for demonstrating the presence of

cysts in corneal tissue. Indirect fluorescent antibody staining and calcofluor white staining (Silvany *et al*., 1987) also may be used.

Herpes simplex keratitis is the disease most commonly mistaken for *Acanthamoeba* keratitis (Johns *et al.*, 1987; Mannis *et al.*, 1986; Moore and McCulley, 1989). The single most consistent clinical symptom of *Acanthamoeba* keratitis is severe ocular pain, which is not characteristic of an infection limited to the cornea and generally not present in herpes simplex keratitis. Additional distinguishing features of *Acanthamoeba* keratitis include a history of direct exposure to soil or water, wearing contact lenses, scleritis, and failure of cultures from the inflamed eye to reveal bacteria, fungi, or viruses (Mannis *et al*., 1986).

1.12 Molecular methods

The molecular methods have been used to detect the species, strains (subgeneric) and pathogenicity of *Acanthamoeba* (John, 1993; Martinez and Visvesvara, 1997; Pussard and Pons, 1977 and Visvesvara**,** 1991). These methods are suitablefor both clinical and environmental applications. Severalresearch groups demonstrated the usefulnessof PCR methods for detection of *Acanthamoeba* (Dykova *et al*., 1999; Howe *et al.*, 1997; Kong and Chung, 1996; Lehmann *et al.*, 1998; Mathers *et al* ., 2000 and Vodkin *et al* ., 1992). As few as 1 to 10 trophozoites can be detected.It is also possible to enhance detection of individual amoebain very dilute liquid clinical samples with fluorescent in situ hybridization (FISH) (Stothard *et al.*, 1999). Several molecular approaches increasethe reliability of specimen identification, but the use of DNAsequence variation appears to be the most promising. The variationis observed in restriction fragment length polymorphisms (RFLP) of completeor partial nuclear 18S rRNA genes (Chung *et al* ., 1998;Kim *et al* .,1996 andKong & Chung, 1996 and Kong *et al.*, 1995),complete mitochondrial 16S rRNA genes (Chung *et al* ., 1996and Yu *et al* ., 1999), and of thecomplete mitochondrial genome (Byers *et al* ., 1990;Chung *et al* ., 1996;Gautom *et al* ., 1994;Kilvington *et al* ., 1991;Kong *et al* ., 1995 andYagita & Endo, 1990).It is also observed in the DNA sequences of complete or partial18S rRNA genes (Dykova *et al* ., 1999;Mathers *et al* ., 2000;Stothard *et al* .,

1998 andWalochnik *et al* ., 2000a) and in randomlyamplified polymorphic DNA (RAPD) analysis of whole-cell DNA (Alves , 2000).

1.13 Cultivation

The simplest way to cultivate *Acanthamoeba* is on the surface of nonnutrien agar (1.5%) spread with *Escherichia coli* or *Enterobacter aerogenes.* Other gram-negative bacteria could also be used. The agar is prepared with either Page's ameba saline (Page, 1988) or distilled water.

The amebae feed on the bacteria and growth enters stationary phase to form cyst, the cysts will remain viable for months, possibly years. A plaque is formed behind the advancing amebae front where amebae have cleared the bacteria. The amoebae front appears much like a line of precipitate on the agar surface.

Although amebae are easy to grow on agar with bacteria, the presence of bacteria often makes it difficult to study the amebae. Consequently, a number of liquid media have been developed for the axenic cultivation of *Acanthamoeba*. Amoebae from the agar surface could be transferred with a bacterial inoculating loop and placed in liquid medium with antibiotics. Streptomycin (100 µg/ml) was needed if contaminated bacterial strains are sensitive, and only sensitive strains should be used as their food. However, in environmental samples, either gentamicin (50 µg/ml) or a combination of penicillin (100 units per ml) and streptomycin (100 µg per ml) could be used. Although the pathogenic free-living amebae will grow in axenic culture, not all strains of free-living amebae will grow axenically. In general, the complex bacteria-free media that have been developed for axenic cultivation of free-living amebae contain a phosphate buffer and either liver extract, yeast extract, peptone, or casein and are supplemented with serum. Chemically defined media have been described for the cultivation of *Acanthamoeba* species (Adam, 1959), *A. culbertsoni* (Shukla *et al*., 1990) and *A. polyphaga* (Ingalls and Brent, 1983).

1.14 Treatment

There is no complete treatment recorded for GAE because most cases were diagnosed after death. However, There are three reports of persons having recovered from *Acanthamoeba* CNS infection. A 7-year-old girl with a single *Acanthamoeba*–

induced granulomatous brain tumor recovered following total excision of the mass and treatment with ketoconazole. *A. palestinensis* was cultured from her brain biopsy material (Ofori-Kwakye *et al*., 1986). The second report involves a 40-year-old man with *Acanthamoeba* meningitis who recovered following treatment with penicillin and chloramphenicol. *A. culbertsoni* was repeatedly cultured from the patient's CSF (Lalitha *et al*., 1985). The third case, for whom complete recovery cannot be claimed because the patient returned home and follow-up was not done, was a 30-year-old man with chronic meningoencephalitis who was treated with sulfamethazine and from whose CSF *A. rhysodes* was cultured (Cleland *et al*., 1982).

Sulfadiazine initially was shown to protect mice from CNS infection by *Acanthamoeba* (Culbertson *et al*., 1965). However, a later report (Rowan-Kelly *et al*., 1982) showed it to be of no chemotherapeutic value once the amebae had become established in the CNS. Rifampicin, given prophylactically, has showed full protection in mice infected by *A. culbertsoni* and to cure mice when treatment started 1 day after intranasal inoculation (Das *et al*., 1991).

Studies *in vitro* have shown *A. culbertsoni* to be highly sensitive to polymyxin E (0.02 μg/ml), sulfisoxazole (0.78 μg/ml), sulfadiazine (1.56 μg/ml), polymyxin B (1.56 μg/ml) (Ferrante & Mocatta, 1984), and the antipsychotic phenothiazine compounds trifluoperazine dihydrochloride and chlorpromazine hydrochloride (Schuster & Mandel, 1984). The phenothiazine compounds were also shown to be active against *A. polyphaga.* Because of the cysts formed in tissues, it is important for a potentially effective drug for GAE to be one that is able to destroy cysts and trophozoites. Otherwise, a possible relapse could occur after the course of treatment has ended.

Many *Acanthamoeba* keratitis patients required corneal transplants in order to manage the disease. Even so, there were reported instances of surgical enucleation. However, with present therapies, *Acanthamoeba* keratitis can be managed by medical treatment alone if infection is identified soon enough (Moore & McCulley, 1989). However the first successful medical cure of *Acanthamoeba* keratitis was reported by Wright *et al*. (1985) and involved the use of a combination of dibromopropamidine

and propamidine isethionate ointment and drops and neomycin drops. The success of this treatment regimen has been confirmed by others (Cohen *et al*., 1987; Lindquist *et al.*, 1988; Moore & MCCulley, 1989). Signs of toxicity of propamidine and dibromopropamidine have been reported in one patient (Yeoh *et al.*, 1987); however, when treatment was discontinued, there seemed to be a recurrence of the *Acanthamoeba* keratitis.

Other successful treatment regimens have used, in addition to topical propamidine, topical miconazole and systemic ketoconazole (Wilhelmus *et al.*, 1986), topical miconazole and neosprin with epithelial debridement (Lindquist *et al*, 1988), and topical clotrimazole (Driebe *et al.*, 1988). A recent report describes the successful treatment of three *Acanthamoeba* keratitis patients with oral itraconazole, a new antifungal agent, topical miconazole, and surgical debridement of the lesions (Ishibashi *et al*, 1990). There is no good animal model for *Acanthamoeba* keratitis, eventhough

Acanthamoeba CNS infections can be studied in mice and other laboratory animals.

Therapeutic options for treatment of cutaneous acanthamebiasisare not clearly established. Patients given combination treatmentshave shown improvement, but the majority have died (Dunand *et al.,* 1997; Hunt*et al.*, 1995 andSison *et al.,* 1995). Therapy is less successful when CNS was involved.However, successful treatments of cutaneous acanthamebiasisusing itraconazole, pentamidine, 5-fluocytosine, and topicalchlorhexidine gluconate and ketoconazole cream have been reported(Helton *et al.*, 1993 and Slater *et al.,* 1994).Six Sudanese patients who had chronic keratites were documented by laboratory methods to be AK cases, good response to oral ketoconazole therapy was observed. At 18 months follow up period, four patients remain symptoms and signs free (Abdel Mageed et al 2006).

1.15 Prevention and control

Associated factors with *Acanthamoeba* keratitis in contact lens wearers were (1) using nonsterile homemade saline, (2) disinfecting lenses less frequently than recommended, and (3) wearing lenses while swimming (Stehr-Green *et al*, 1987).

Acanthamoeba cysts remain viable in commercially prepared ophthalmic saline solutions from 14 to 90 days, in ophthalmic cleaning solutions from 1 to 90 days, and in ophthalmic disinfectants from 6 hours to 14 days (Brandt *et al*, 1989). More than 18 different solutions tested for killing *Acanthamoeba* but none of them were able to kill *Acanthamoeba* cysts in less than 6 hours exposure. Contact lens disinfection using heat has been shown to be more effective in killing *Acanthamoeba* trophozoites and cysts than cold disinfection (Ludwig *et al*, 1986). Contact lenses wearer should closely follow the manufacturer's recommendations for wear, care and disinfection of the lenses. Homemade saline solutions remain an important risk factor associated with *Acanthamoeba* keratitis. Contamination of the contact lens care system with bacteria or fungi encourages the survival and growth of *Acanthamoeba* (Donzis *et al.*, 1989).

A. polyphaga and *A. hatchetti* have been isolated from laboratory eyewash stations, especially stations containing reservoirs (Bier & Sawyer, 1990 and Tyndall *et al.*, 1987). Eyewash stations having reservoirs should be flushed weekly, otherwise they present a potential health hazard to users, particularly to those wearing contact lenses. Alternatives to conventional chlorination that have been tested with free-living amebae include addition to the water of Baquacil (Dawson *et al*, 1983), chlorinated cyanurates (Engel *et al*, 1983) and chlorine dioxide (Dawson & Brown, 1987). Chlorination remains the single most effective disinfectant system for controlling pathogenic free-living amoebae in public waters, with emphasis being placed on the level of residual free chlorine which must be 0.5 mg/liter.

It has been proposed that *Acanthamoebae* may be natural hosts for *legionella* and that human infection is acquired not by inhaling free bacteria but rather by inhaling living or dead amebae full of *legionellae* (from 50 to 1000 or more bacteria per amoeba) (Rowbotham, 1980). Although virulent strains of *L. pneumophila* will kill amebae, bacterial virulence does not appear to be affected by growth in the amoebae (Tyndall & Domingue, 1982).

Rationale

Recently, *Acanthamoeba* have become known to cause eye infection in people using contact lenses with very grave consequences, and as no enough information exists as to the situation in Malaysia, this study has been designed to evaluate the epidemiological situation in Malaysia as a number of patients effected with corneal ulcers have been discovered who were infected with *Acanthamoeba* type of organisms.

Objectives

General objectives:

To detect the presence and the Molecular Characterization of *Acanthamoeba* from Malaysian Environment.

Specific objectives:

1. To culture and isolate the amoeba from environmental samples.
2. To observe the morphology of amoeba using Field, Iron Haematoxylin and Trichrome Stain.
3. To use the PCR technique for the detection of genus and pathogenicity of *Acanthamoeba* isolates.
4. To use the restriction enzymes for the detection of *Acanthamoeba* species.

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2.0 MATERIALS AND METHODS

2.1 Study design

It's describted cross-sectional horizontical study.

2.2 Study area

This study was carried out in Malaysian environment.

2.3 Preparation of *Acanthamoeba* media

2.3.1 Nutrient agar and *E. coli* culture

Ten gms of nutrient agar powder were mixed with 500 ml PAS (appendix 1.1). After being autoclaved at 15 ibs pressure, at 121°C at 15 minutes, the warm agar (45°C - 60°C) were transferred into 10 ml aliquots of the sterile universal bottles. The bottles were then slanted at room temperature to solidify and kept at 4°C until use.

Escherichea coli colonies were retrieved from stock nutrient agar plates with cultured *E. coli* by using a sterile bacteriological loop and inoculated into the nutrient agar slant. The inoculums were then incubated at 37°C for at least 48 hours before harvesting. This inoculation could be kept in 4°C as stock after sealing with parafilm.

2.3.2 Non-nutrient agar

Fifteen gmsof non-nutrient agar powder (Sigma) was added to 1000 ml of Page′s Amoeba Saline (PAS) solution (appendix 1.1). Themixture was autoclaved at 15 Ibs pressure, at 121°C for 15 minutes, while the nutrient agar was still warm (45 - 60°C), approximately 10 ml were poured into each of the sterile disposable petri dishes and left at room temperature to solidfty. A 1000 ml volume of non-nutrient agar can usually make up to 90 agar plates. After polymerization, the Petri dishes were sealed and were stored at 4°C until used.

2.3.3 Preparation of non-nutrient agar with *E. coli* lawn

One (1) ml of sterile Phosphate Buffer Saline (PBS) solution (appendix 1.2) was addedto the *E. coli* culture slant. The solution was pipetted to resuspend the *E. coli* from slant nutrient agar surface. A loopful or one drop of a heavy suspension of *E. coli* was placed in the center of a non-nutrient agar plate followed by 1 ml of PBS.

The diluted suspension was then spread evenly on the agar surface. The plates were then left at room temperature for 30 minutes and they were ready to use or sealed with parafilm and kept at 4°C until use.

2.4 Collection of samples

2.4.1 Air conditioner exposure

38 non–nutrient agar with *Escherichia coli* lawn (NNA—*E. coli*) plates were exposed right in front of the air conditioner for 10 minutes. The exposed plates were then covered and transported to the laboratory.

2.4.2 Dusty air exposure

14 non–nutrient agar with *Escherichia coli* lawn (NNA—*E. coli*) plates were left uncovered at different areas of University of Malaya compound. These areas were at the side of the main road where many cars are passing by. The plates were placed at 30, 60 and 120 minutes then covered with their cover, sealed with parafilm and immediately transported to the laboratory for cultivation.

2.4.3 Water and sewage

Water from the surface of the lakes and sewage were collected in 500 ml sterile blue-caped bottles (14 bottles). The filled bottles were then transported to the laboratory for further processing.

2.5 Preparation of samples and cultivation

Samples from steps 2.4.1 and 2.4.2 were incubated at room temperature, at 37°C and 44°C respectively. The plates were examined under the inverted microscope to identify the presence of amoeba during the 10 days incubation. The positive plates were sub-cultured and incubated at the respective temperatures. Centrifugation technique was used to concentrate lakes and sewages water samples while air conditioner and dust samples, after incubation at room temperature, 37°C and 44°C for up to 10 days, were subjected to direct subculture and reincubated at the same degree of temperature.

On the other hand, the water sample was strained through two layers of muslin. For each water sample, an aliquot of 50 ml was used for centrifugation at 3000 rpm for 15 minutes at room temperature. The supernatant was then discarded leaving 1 ml

which was then used to resuspend the sediment. The resuspended solution was then filtered through 0.45 µm pore size, 47 mm diameter cellulose ester membrane (inoculum) using a vacuum/pressure pump.

The inoculum was placed up-side down at the center of *NNA-E.coli* plate followed by sealing with parafilm. Duplicated plates were incubated at room temperature, 37°C and 44°C respectively. They were then examined daily for 10 days to detect the presence of amoeba.

2.6 Detection of amoebae

The presence of amoebae (*Acanthamoeba* sppand *Naegleria* spp) in the culture plates was observed under the inverted light microscope (20 and 40 X magnification). After 10 days observation, the negative plates were discarded, while the positive plates were used for further investigation. In the positive plates, the amoeba was seen as a plug at the surface of the agar. This surface was then scrapped and placed on the glass-slide, covered with glass- slip and examined for the presence of trophozoites or cysts and their morphology whether they are *Acanthamoeba* or *Naegleria* under light and phase contrast microscopes.

2.7 Sub cultivation of amoeba

Sub culture of amoeba was carried out on the cysts stage of amoeba to isolate the *Acanthamoeba* and *Naegleria*. The surface of NNA-*E.coli* containing cysts was cut approximately 1×1 cm by using sterile scalped. This agar was then then inverted up-side down and placed on top of the new NNA-*E.coli* plate. The plate were then incubated atrespectives temperatures for 7 days, where most of the trophozoites have been accumulated and become cysts. Only the surface area containing *Acanthamoeba* cysts was collected for further subculture for at least ten times to obtain enough colonies *Acanthamoeba* spp. Several subcultures of NNA-*E. coli* with *Acanthamoeba* plates were were prepared for further study.

2.8 Collection of *Acanthamoeba*

For each of NNA-*E.coli* with *Acanthamoeba*plate, 10 ml of PBS were added, pipetted with Pasteur pipette to remove the *Acanthamoeba* trophozoites from the NNA-*E.coli* surface. The mixture was then transferred into centrifuge tube,

centrifuged at 1,000 rpm for 20 minutes at room temperature. The supernatant was then discarded and the sediment was then washed by adding sterile PBS solution, resuspended and centrifuged at 3000 rpm, at room temperature for 5 minutes. The supernatant was discarded and the washing steps were carried out at least three times to minimize the presence of *E. coli*. The clean *Acanthamoeba* sediment was used in direct examination, staining or DNA extraction.

2.9 Staining

2.9.1 Unstained preparation

The 7-10 days *NNA-E. coli* with *Acanthamoeba* plates were processed as in step 2.8. A clean *Acanthamoeba* sediment was transferred to a slide diluted with 1 ml PAS (appendix 1.1). Approximately 50 microlitres of this mixture was droped on a glass-slide, covered with cover slip and photographed for permanent record.

2.9.2 Trypan Blue stain

The 7-10 days *NNA-E. coli* with *Acanthamoeba* plates were processed as in step 2.8. The *Acanthamoeba* sediment was mixed with approximately 100 ul of 0.4% Typan Blue Stain (appendix 1.3) followed by resuspension. This suspension was allowed to stand for 5 min at room temperature and then transferred to a slide, covered with cover slip and photographed for permanent record. The Trypan Blue Stain was used to detect the viability of the amoeba before DNA extraction.

2.9.3 Modified Field`s stain

A sediment from step 2.8 was used to produce a smear on the glass-slides. This sediment was resuspended in 1 ml PBS and approximately 100 ul of this suspension was droped on glass-slide. The suspension was then spread with new glass-slide or pasture pipette to form a thin smear. These slides were then dried in semi-moist container at 37º C and fixed with methanol for 5 minutes. The slides were then dipped in 1% methanolic eosin for 2 minutes and several drops of 3% acid alcohol were then added, enough to discolor the eosin. Then the slides were gently rinsed with tap water for 10 seconds. Two drops of 1% Field A per slide was added, spread around the smears and immediately dipped the slides five times in clean

distilled water (less than 5 seconds for each time). The slides were then air dried and mounted with DPX and photographed for permanent record.

Preparation and protocol of Modified Field`s stain as in appendix 1.4.

2.9.4 Trichrome stain (for *Acanthamoeba* cyst):

A thin smear of *Acanthamoeba* suspension was prepared as in step 2.9.3. The slides with *Acanthamoeba* smear were dried and placed in Shaudin fixative for 1 hour at room temperature. They were then dipped in 70% alcohol plus iodine for 1 minutes, then placed successively in clean 70% alcohol for two times, followed by 50% ethyl alcohol solutions for 3-5 minutes in each solution. The slides were then placed in trichrome stain for 6-8 minutes, followed by decolorized in 90% acidified ethyl alcohol (1 drop glacial acetic acid in 10 ml alcohol) for 10-20 seconds or until stain barely runs from smear. The slides were then placed in 95% ethyl alcohol for 5 minutes, carbolxylene for 10 minutes and placed xylene for 10 minutes, followed by mount in DPX. After 24 hours, the slides were examined under light microscopy and photographed for permanent record.

The trichrome stain used was as in appendix 1.5.

2.10 *Acanthamoeba*spp. genomic DNA Extraction

The DNeasy Tissue kit (QIAGEN) was used to isolate genomic DNA from *Acanthamoeba* trophozoites. In a 2 ml micro centrifuge tube, 1 ml suspended *Acanthamoeba* trophozoites from step 2.7 were centrifuged for 5 minutes at 400 rpm. The supernatant was discarded and the pellet was resuspened in 200 ulPBS solution. Twenty (20) µl of proteinase K (QIAGEN) and 200 µl of Buffer AL were added to the sample and pulse-vortexed for 10 seconds. This mixture was then incubated at 70 ^{o}C for 10 minutes. Then 200 µl of absolute ethanol (96-100%) was added to the sample and pulse-vortexed for 10 seconds. The mixture was then was carefully applied to the DNeasy Mini spin column (in a 2 ml collection tube) and centrifuged at 8000 rpm for 1 min. Bound DNA was washed with 500 µl Buffer AW1 by centrifugation at 8000 rpm for 1 minute, followed by 500 µl buffer AW2 at 24,000 rpm for 3 minutes. The above washing was achieved by packaging the DNeasy mini spit column according to the manufacturer's. After washing, the column was then

placed in a clean 1.5 ml microcentrifuge tube and 200 µl of buffer AE was added. The column was incubated at room temperature for 5 minutes. Purified DNA was released from the column by centrifugation at 8000 rpm for 1 minute. The eluted which was containing purified DNA was aliguoted and store at 4°C until used.

2.11 Primers

In this study, *Acanthamoeba* specific primers (Table 4) were used to detect the genus, species and pathogenicity of *Acanthamoeba* . These primers were designed by Michael *et al* (1992). The primers which were designed to detect genus of *Acanthamoeba* were forward primer ACARNA. For 1383 (TCCCCTAGCAGCTTGTG) and reverse primer ACARNA. rev1655 (GTTAAGGTCTCGTTCGTTA) annealed to the 18S rDNA gene of *Acanthamoeba* genus to yield a 272 fragment . The other primers were designed to detect the pathogenicity of *Acanthamoeba*, which were ACARNA . for1345 and ACARNA . rev1830. which were also annealed to the 18S rDNA gene of pathogenic *Acanthamoeba* to yield 485 bpprominent band at 450 bp alone or with other minor multiple bands. The third primers were forward primer ACA18.for 2209 (CGGGCTTGTGAGGTCTC) and reverse primer ACA58.rev 92 (GATGATTCACTGATCCCTG) which were designed to detect the species of *Acanthamoeba* to yield a prominent band of about 600 bp that varied within 100 bp.

Table 4. *Acanthamoeba* specific primers

Template	Sequence	Identifies	References
18S rDNA			
ACARNA.for1383 ACARNA.rev1655	TCCCCTAGCAGCTTGTG GTTAAGGTCTCGTTCGTTA	Genus	Michahel *et al* ., 1992
18S rDNA			
ACARNA.for1345 ACARNA.rev1830	CGCGAGGGCGGTTTA GCTGGCTAGGCGCGCAG	Pathogen	Michahel *et al* ., 1992
18S-5.8S ITS			
ACA18.for 2209 ACA58.rev 92	CGGGCTTGTGAGGTCTC GATGATTCACTGATCCCTG	Species	

Key;

for = forward. rev = reverse.

2.12 Positive control

Acanthamoeba castellani was cultured in 5 %Peptone yeast extract glucose (PYG) medium (appendix 4.1) the sample was obtained from the National University of Singapore and was used as a positive control.

2.13 Polymerase Chain Reaction (PCR) amplification

Amplification was carried out in a 50 ul PCR reaction mix and the concentration of the reaction component was based on that recommended by the manufacturer. In preparing the PCR reaction mixtures, a master mix containing all the reaction component except the DNA template was first prepared and equally dispensed into each reaction tube. Appropriate volumes of DNA template were added to the respective reaction mix to give a final volumes of 50 ul each.The solution was then

mixed well and transferred immediately into an Eppendorf PCR thermocycler for PCR amplification. The reaction mixture to a total volume of 50 ul comprised of :

DNA template	2.0-4.0 µl of *Acanthamoeba* genomic DNA
10 X PCR buffer	*0.1 X*
dNTP (10 mM) (Fermentas)	0.2 mM
Forward primer (10 pmole/µl)	*0.5 pmole*
Reverse primer (10 pmole/µl)	*0.5 pmole*
$MgCl_2$ (25 mM)	1.5 mM
Taq DNA polymerase (1 U/µl)	0.05 U
Sterile water	as appropriate, topping up to 50 µl

The DNA samples from the 14 isolates of *Acanthamoeba* were amplified with standard PCR amplification condition of thirty cycles of denaturation 94 ^{o}C for 1 minute, annealing 50 ^{o}C for 1 min and extention, at 72 ^{o}C for 2 min.At the end of the last cycle,the reaction was held at extension temperature (72 ^{o}C) for an extra 5 min. For every run of the PCR amplification, reaction mixtures without the DNA template were also included as negative controls.

2.13.1 Agarose gel

The amplified band from PCR fragment and plasmids can be visualized by separating molecules of different sizes using agarose gel electroporesis. 1080 mg of agarose (Research Biolabs) was melted in 60 ml 0.5 X Tris-borate EDTA Buffer (appendix 4.2), 4 ul of mg/ml ethidium bromide EtBr was added to the melted gel and the resultant solution was poured into a casting tray with comb in place. It was left to solidify at room temperature before it was placed in an electrophoresis tank containing 0.5 X TBE (appendix 1.6)buffer. Then 2 ul of loading dye was mixed with 10 ul of amplified DNA mixture and loaded into the wells. 100 bp ladder (Research Biolabs)was run alongside the samples to serve as indicator of the size of the bands. After electrophoresis at 90 volts for 60 min, the fluorescent EtBr–intercalated DNA bands were viewed over an UV transilluminator.

2.14 Restriction Fragment Length Polymorphism

To confirm and exploit the sequence variation of the products, aliquots of 14 *Acanthamoeba* isolates (with primers ACA18.for 2209 and ACA58.rev 92), were directly digested with *Taq1* (Research Biolabs). Eleven out of these 14 PCR product of *Acanthamoeba* were digested with *Hae111*(Research Biolabs), *Mbo 1* (Research Biolabs) and *Rsa 1* (Research Biolabs). Twenty ul of each PCR product was mixed with 3-5 ul of 10 x restriction buffer that was provided by manufacturer, 25 ul of dH2O and 10 U of each restriction enzymein a microcentrifuge tube. Each mixture was then immediately incubated at 37^{o}C in a water bath for 2 - 4 hours. The reaction was then inactivated by incubated at 65 ^{o}C – 80 ^{o}Cfor 20 min.

2.14.1 Electrophoresis and analysis

The digested PCR products were electrophoresis on a 3% agarose gel (appendix 4.3) incorporated with EtBr (0.5 ug/ml) at 70 volts for 1.5 hours alaongside a 100 bp DNA ladder (Research Biolabs)as the molecular weight marker. The gel was then visualized under UV transillumination and photographed.

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3.0 Results

3.1 Microscopical examination

From a total of 80 samples (air conditioner, 38 samples; dust,14 samples; lake, 14 samples and sewage 14 samples) collected from the University of Malaya environment and by using inverted microscope, only fourteen isolates (17.5%) (Acl1, Acl2, Acs3, Acd4, Acs5, Acs6, Acs7, Acd8, Acs9, Acd10, Acd11, Acd12, Acd13 and Acl14) were showed morphological characteristics similar to *Acanthamoeba* morphology.

3.2 Air conditioner samples

All the 38 samples collected were negative for *Acanthamoeba*.

3.3 Dust samples

From a total of 14 samples collected from dust, 6 samples were positive for *Acanthamoeba* while the other 8 samples were negative for *Acanthamoeba* (table 3.2).

(Table 5) isolation of *Acanthamoeba* from dust samples

No	Date of collection	Isolation at r. temp.	Isolation at 37°C	Isolation at 44°C	Total
4	20/11/2002	1	-	-	1
5	19/12/2002	1	2	-	3
5	5/1/2003	1	1	-	2
Total		3	3	0	6

Exposing the non-nutrient agar with *E. coli* lawn plates to the dust was performed for 30 mins, 1 hour and 2 hours (table 3.3)

(Table 6) Exposing the agar to the dust

No	Exposing for 30mins/positive for *Acanth.*	Exposing for 1 hour/positive for *Acanth.*	Exposing for 2 hour/positive for *Acanth.*	Total
4	2 / 0	1 / 1	1 / 0	1
5	2 / 0	1 / 1	2 / 2	3
5	2 / 0	1 / 0	2 / 2	2
Total	0	2	4	6

3.4 Lakes samples

A total of 14 samples were collected from lakes. Three isolates out of these 14 were positive for *Acanthamoeba*, 9 were negative (table 3.4)

(Table 7) isolation of *Acanthamoeba* from lakes samples

No	Date of collection	Isolation at r. temp.	Isolation at 37°C	Isolation at 44°C	Total
4	20/11/2002	-	1	-	1
3	19/12/2002	-	-	-	-
3	5/1/2003	-	2	-	2
4	6/1/2005	-	-	-	-
Total		0	3	0	3

3.5 Sewages samples

From a total of 14 samples collected from sewages, 5 samples were positive for *Acanthamoeba* while the other 11 samples were negative for *Acanthamoeba* (table 3.5)

(Table 8) isolation of *Acanthamoeba* from sewages samples

No	Date of collection	Isolation at r. temp.	Isolation at 37°C	Isolation at 44°C	Total
2	20/11/2002	-	1	-	1
3	19/12/2002	-	-	-	-
3	5/01/2003	-	2	-	2
3	6/01/2003	-	2	-	2
3	15/01/2003	-	-	-	
Total		0	5	0	5

3.6 Staining and Morphology

Unstained preparation, Trypan Blue stain, Modified Field`s stain and Trichrome stain were used to study the morphology of *Acanthamoeba*cysts and trophozoites (figures 3.1- 3.10). Trophozoites of all isolates were irregular in shape, measuring 25 to 40µm in length with highly vacular cytoplasm and some with acanthopodia. The 14 positive isolates for *Acanthamoeba* showed many different shapes of cysts (obtained by prolonged incubation on NNA-*E. coli*).

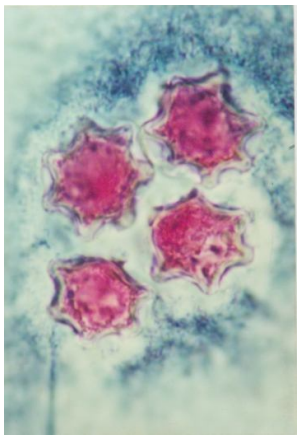


Figure 1. *Acanthamoeba* cyst under Trichrome stain(400X)
Note star shaped cyst and unseen nucleus

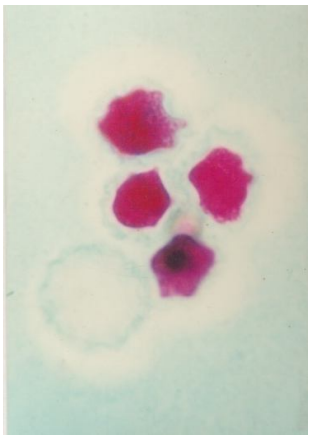


Figure 2. *Acanthamoeba* spp. Cyst under Trichrome stain (400X) Note wrinkled wall cyst and unseen nucleus

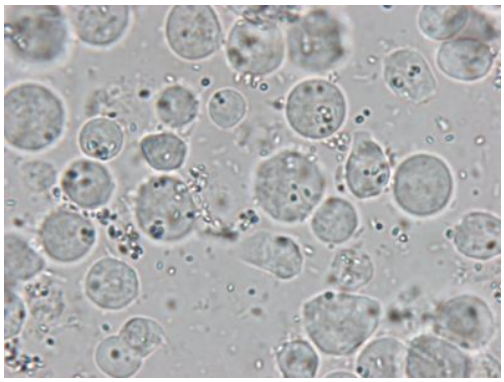


Figure 3. wet preparation
(400X) note the highly vacuole

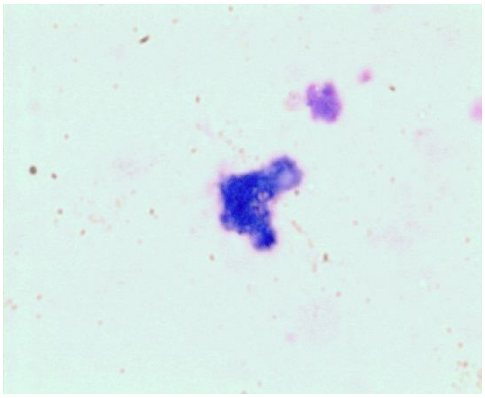


Figure 4 Modified Field`s
(400X) note the highly vacuole Stain, note the acanthopodia

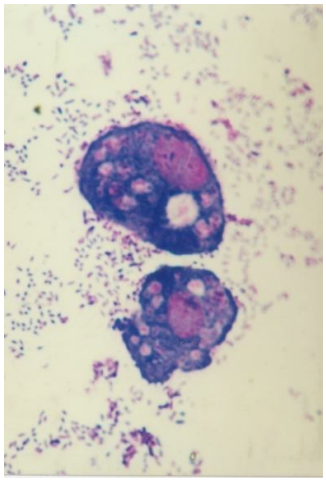


Figure 5. *Acanthamoeba* spp.
Trophozoite under modified field's stain (400X)
Note large pink nucleus and acanthopodia

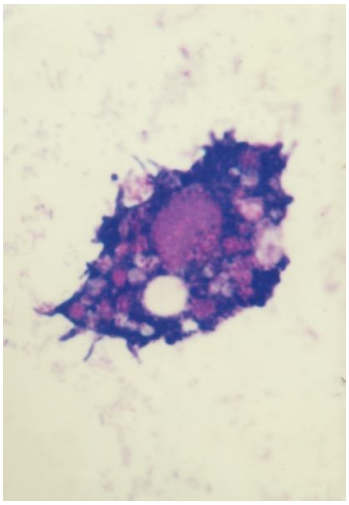


Figure 6. *Acanthamoeba* trophozite under modified fild's stain
Note large pnk nucleus and acanthopodia

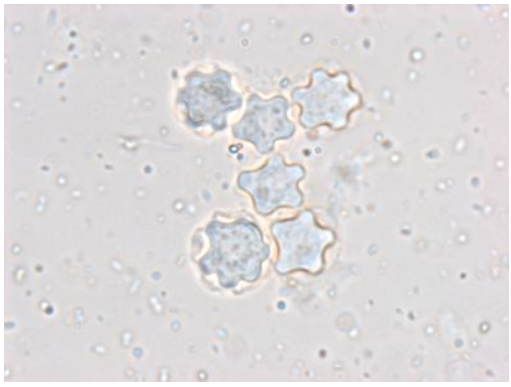
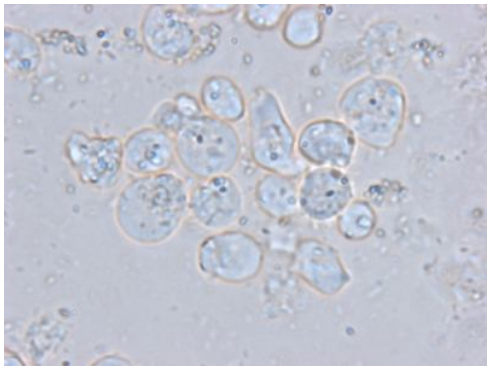


Figure 7, wet preparation (400X), note the star shape cyst



Figur 8. wet preparation (400X) Cytoplasm trophozoite

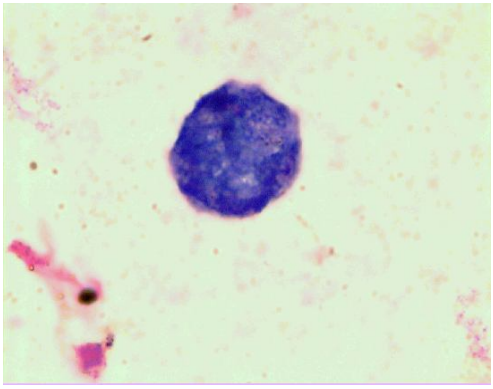


Figure 9. Modified Field`s stain (400X) note the acanthopodia trophozoite

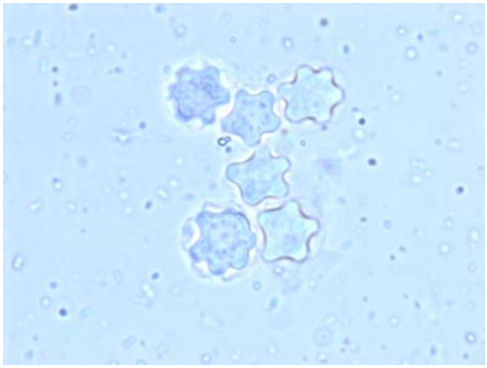


Figure 10. wet preparation (400X) note the star shape cyst

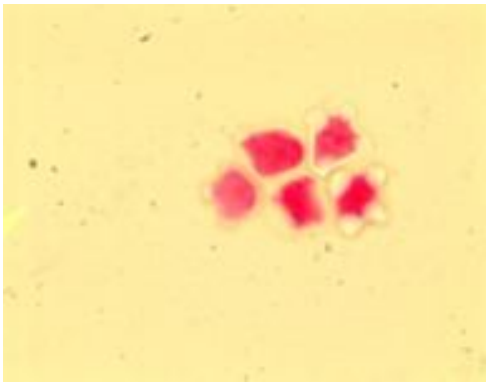


Figure 11. Tichrome Stain(400X) some are retangular, some are star shape cyst

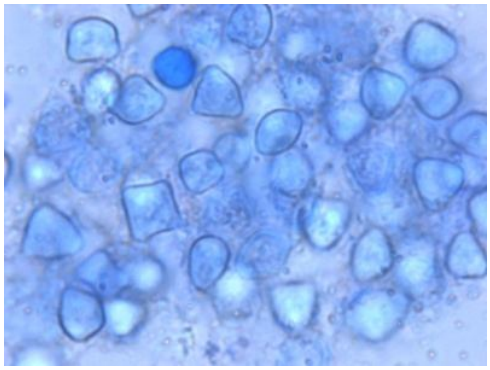


Figure 12. Typan Bluestain (400X)Some are retangular and some are star shape and some are roundcysts

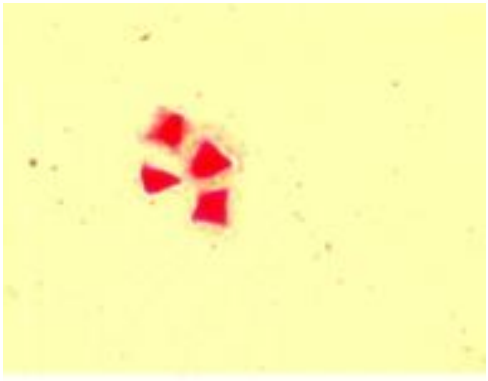


Figure 13. Tichrome Stain (400X) retangular and triangle cysts shape

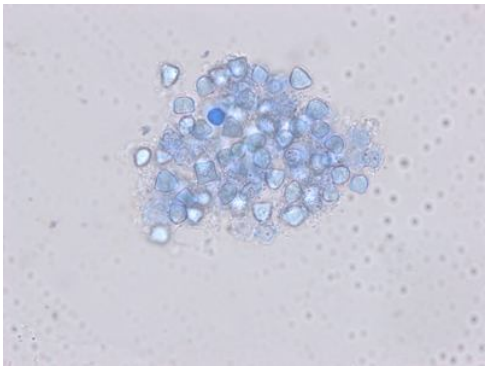


Figure 14. Typan Blue Stain(400X) some are retangular, some are traingle shape some are round cysts shape

3.7 Polymerase Chain Reaction (PCR)

3.7.1 *Acanthamoeba* genus

To detect *Acanthamoeba* genus, PCR was performed on genomic DNA of the 14 positive isolates for *Acanthamoeba* using ACARNA.for1383 and ACARNA.rev1655 (Table 4) primers. The PCR product of the 14 *Acanthamoeba* isolates and the known positive control (*A. castellani*) were electrophoresed on agarose gels containing ethidium bromide. Bands of 272 bp size were observed (figures 1 and 2). These PCR products seemed to be similar with the result reported by Michael H. *et al*., (1992) which showed *Acanthamoeba* genus.

3.7.2 Pathogenicity of *Acanthamoeba*

ACARNA.for1345 and ACARNA.rev1830 (Table 4) primers were used to detect the pathogenicity of the 14 *Acanthamoeba* isolates. After amplification with the genomic DNA of the 14 positive isolates for *Acanthamoeba*and agarose gels electrophoresis (figure 17), two distinct patterns were produced. Six isolates (Acl1 , Acd4, Acs5, Acs8, Acd13and the positive control) had a prominent band (485 bp) which indicated that these were non pathogenic isolates, while the other 9 isolates (Acl2, Acs3, Acs6, Acs7, Acs9, Acd10, Acd11, Acd12 and Acl14) showed multiple bands without 485 bp., which was indicative of pathogenic isolates (Michael H. *et al*.,1992)

3.7.3*Acanthamoeba* species and RFLP

To verify that *Acanthamoeba* had been isolated, ACA18.for 2209 and ACA58.rev 92 (Table 1 page 5) primers were used in the amplification of genomic DNA for the 14 positive isolates. After amplification, the PCR products

were electrophoresed on agarose gels containing ethidium bromide and then visualised under UV illumination . Aliquots of the reaction were digested with Taq 1 restriction enzyme and electrophoresed on the same gel (figures 4 and 5). The 14 positive isolates for *Acanthamoeba* were divided into three species. Eight isolates (Acl1, Acs3, Acd4, Acs5, Acs6, Acs7, Acd8 and Acd10) were categorised as *A. castellani* because these 8 isolates showed sizes similar to that of the positive control (known *A. castellani*). Three isolates were categorised as *Acanthamoeba* spp Lb., (Acl2, Acd11 and Acl14) because the three isolates showed identical multibe band sizes. Three isolates were categorised as *Acanthamoeba* spp. Gc., (Acs9, Acd12 and Acd13) because they also showed identical multiple band sizes which were nonethless different than *Acanthamoeba* spp. Lb. Figures 6, 7, 8, 9, 10, and 11 present RFLP patterns obtained by using three kinds of restriction enzymes; *Hae111*, *Mbo 1* and *Rsa 1* to digest aliquots from the same reaction for the 11 non star shaped *Acanthamoeba* isolates (*A. castellani* and *Acanthamoeba* spp. Gc). With every restriction enzyme, each species (*A. castellani* and *Acanthamoeba* spp. Gc) showed identical multiple sizes which differed from the other species.

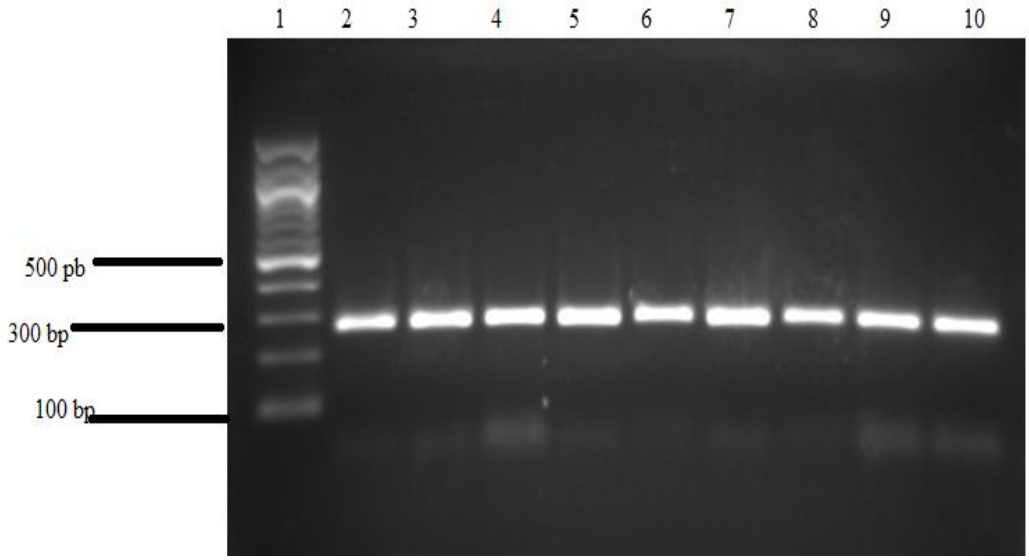


Figure 15. Amplification of 1383-1655 18s rDNA target from *Acanthamoeba* isolates. Amplified with an annealing temperature of 50º C.

Lane 1, 100 bp DNA ladder. Lane 2, positive control (*A. castellani*). Lane 3, Acl1. Lane 4, Acl2.

Lane 5, Acs3. Lane 6, Acd4. Lane 7, Acs5. Lane 8, Acs6. Lane 9, Acs7. Lane 10, Acs8.

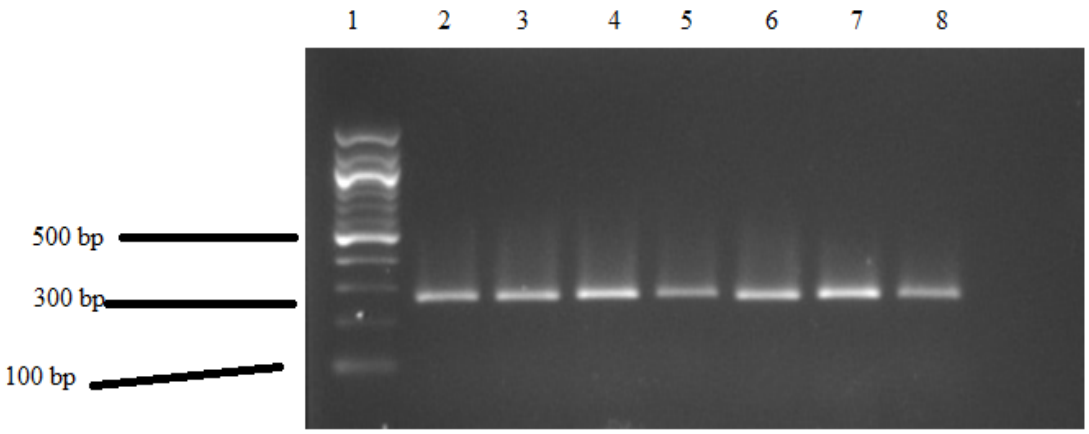


Figure 16. Amplification of 1383-1655 18s rDNA target from *Acanthamoeba* isolates. Amplified with an annealing temperature of 50º C.

Lane 1, 100bp DNA ladder. Lane 2, positive control (*A. castellani*). Lane 3, Acs9. Lane 4, Acd10. Lane 5, Acd11. Lane 6, Acd12. Lane 7, Acd13. Lane 8, Acl14.

All the14 *Acanthamoeba* isolates produced 272 bp. These PCR products was unique for the genus of *Acanthamoeba*(Michael*et al*., 1992).

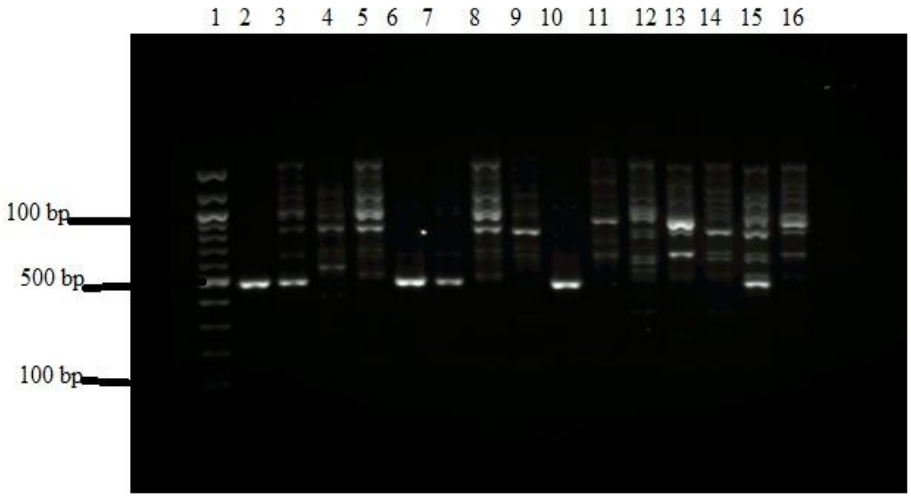


Figure 17. Amplification of 18S rDNA 1345-1830 at restrictive conditions (50º C annealing temperature). Lane 1, 100 bp DNA ladder. Lane 2, positive control (known *A. castellanii*) lane 3, Acl1; lane 4, Acl2; lane 5, Acs3; lane 6, Acd4; lane 7, Acs5; lane 8, Acs6; lane 9, Acs7; lane 10, Acd8; lane 11, Acs9; lane 12, Acd10; lane 13, Acd11; lane 14, Acd12; lane 15, Acd13; lane 16, Acl14.

485 bp discrete band indicated non pathogenic isolates. Non or faint 485 bp but with other multiple bands indicated pathogenic isolates.

The 5 Non Pathogenic isolates are; *Acanthamoeba castellanii* Lebb strains (Acl1 , Acd4, Acs5, Acs8) lanes 3,6,7,and 10 respictively. and*Acanthamoeba* sp. Gc1 strain (Acd13) lane 15 while all the other 9 isolates are pathogenic.

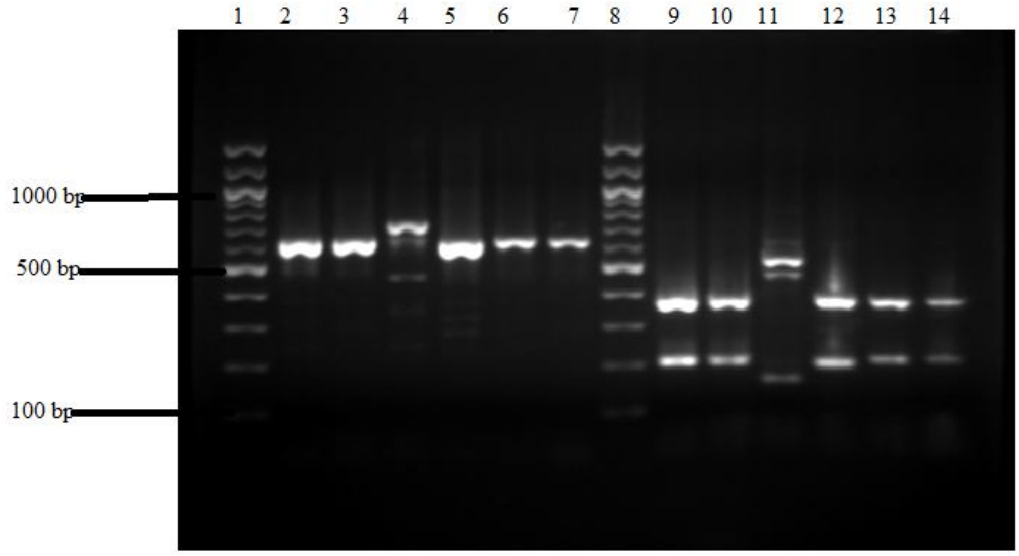


Figure 18. Amplification of Acanthamoeba 18S rDNA 2209-5.8S rDNA 92 and restriction analysis of the PCR product. After Amplification, one aliquot of the reaction mix was analyzed by gel electrophoresis. Another aliquot was digested with Taq I and analyzed on the same gel.

Lane 2, positive control (known *A. castellanii*). Lane3, *A. castellanii*. Lane 4, *A.* sp.Lb. Lane 5, *A. castellani.* Lane 6, *A. castellanii*. Lane7, *A. castellani*. Lanes 9-14, respectively, represent the Taq I digested in the same order as lanes 2-7. Lanes 1 and 8, 100 bp DNA ladder

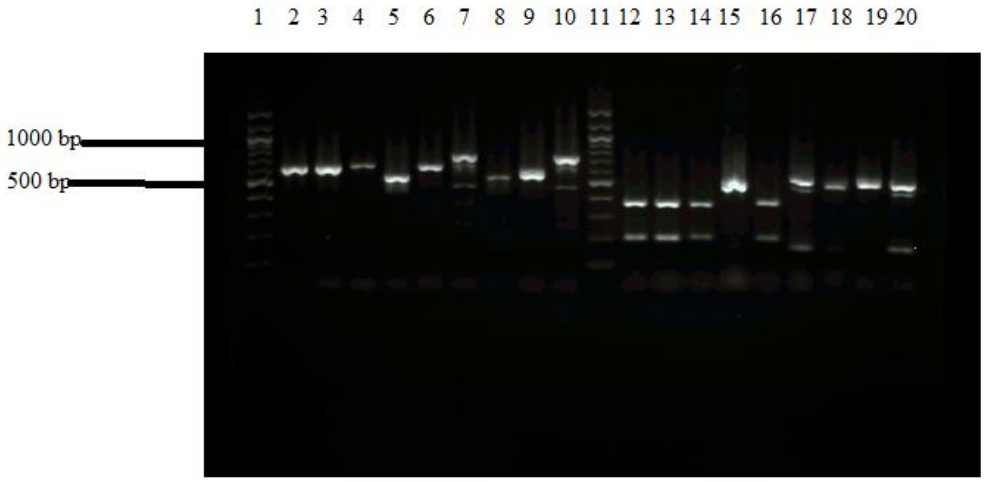


Figure 19. Amplification of Acanthamoeba 18S rDNA 2209-5.8S rDNA 92 and restriction analysis of the PCR product. After Amplification, one aliquot of the reaction mix was analyzed by gel electrophoresis. Another aliquot was digested with Taq I and analyzed on the same gel.

Lane 2, *A. castellanii*. Lane3, *A. castellanii.* Lane 4, *A. castellanii.* Lane 5, *A.* sp. Gc. Lane 6, *A. castellanii*. Lane7, *A.* sp. Lb. Lane8, *A.* sp. Gc. Lane9, *A.* sp. Gc. Lane10, *A.* sp. Lb. Lanes 12-20, respectively, represent the Taq I digested in the same order as lanes 2-10 Lanes 1 and 11, 100 bp DNA ladder.

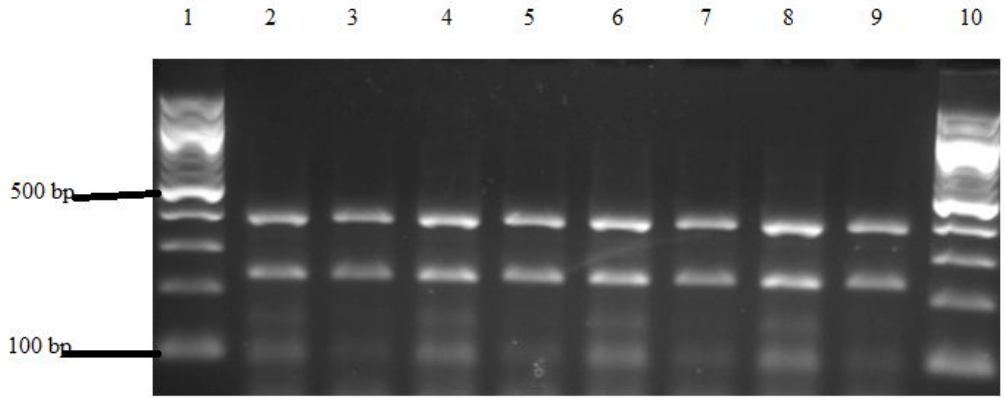


Figure 20. Amplification of the 8 *A. castellani* isolate18S rDNA 2209-5.8S rDNA 92, digeted with HaIII restriction enzyme.
Lane1; 100 bp DNA ladder. Lane2; Acl1. Lane3; Acs3. Lane4; Acd4. Lane5; Acs5. Lane6; Acs6. Lane7; Acs7. Lane8; Acd8. Lane9; Acd10. Lane10; 100 bp DNA ladder. All isolates showed same identical bp size

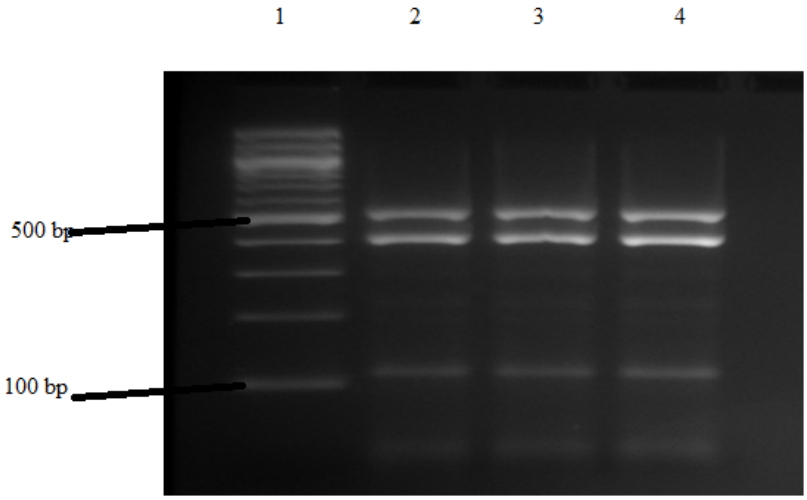


Figure 21. Amplification of the 3 *A.* isolate18S rDNA 2209-5.8S rDNA 92, digeted with HaIII restriction enzyme.
Lane1; 100 bp DNA ladder. Lane2; Acs 9. Lane3; Acd 12. lane4; Acd 13.
All isolates showed same identical bp size

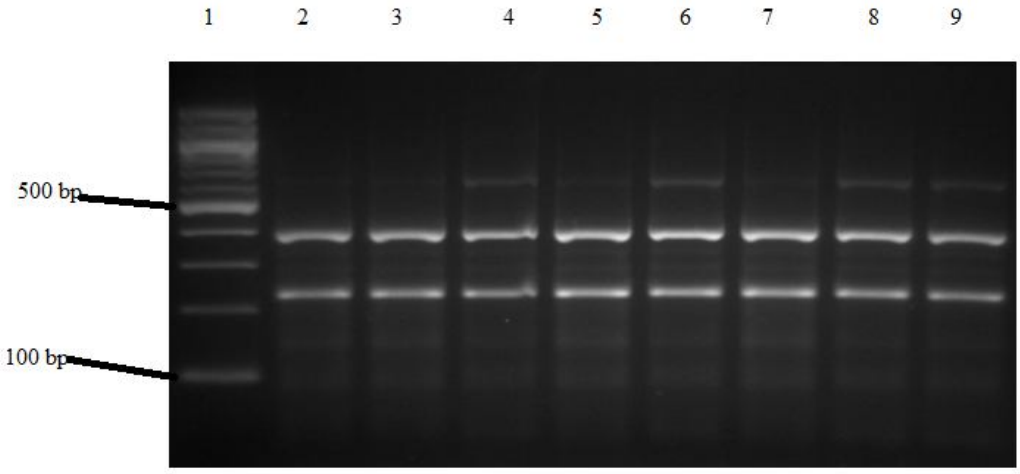


Figure 22. Amplification of the 8 *A.castellani* isolate18S rDNA 2209-5.8S rDNA 92, digeted with RsaI restriction enzyme
Lane1; 100 bp DNA ladder. Lane2; Acl1. Lane3; Acs3. lane4; Acd4. lane5; Acs5. lane6; Acs6. lane7; Acs7. lane8; Acd8. lane9; Acd10. lane10;100 bp DNA ladder. All isolates showed same size

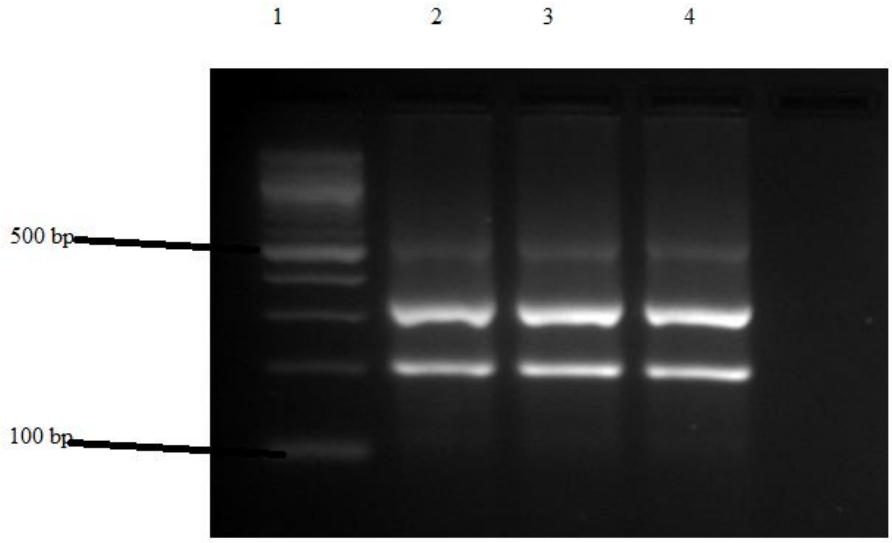


Figure 23. Amplification of the 3*A.* isolates18S rDNA 2209-5.8S rDNA 92, digeted with RsaI restriction enzyme
Lane1; 100 bp DNA ladder. Lane2; Acs 9. Lane3; Acd 12. lane4; Acd 13.
All isolates showed same identical bp size

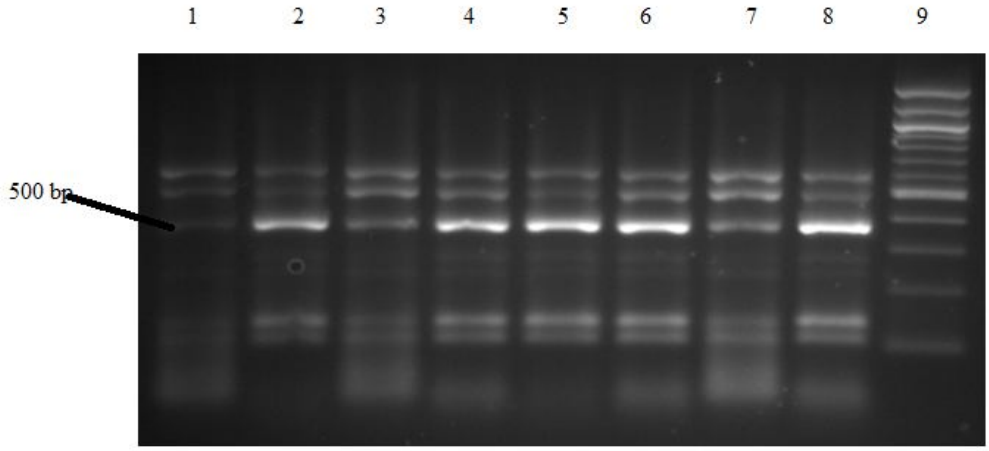


Figure 24. Amplification of the 8 *Acanthamoeba castellani* isolate18S rDNA 2209-5.8S rDNA 92, digeted with MboI restriction enzyme.
Lane1; 100 bp DNA ladder. Lane2; Acl1. Lane3; Acs3. lane4; Acd4. lane5; Acs5. lane6; Acs6. lane7; Acs7. lane8; Acd8. lane9; Acd10. lane10;100 bp DNA ladder
All isolates showed same size

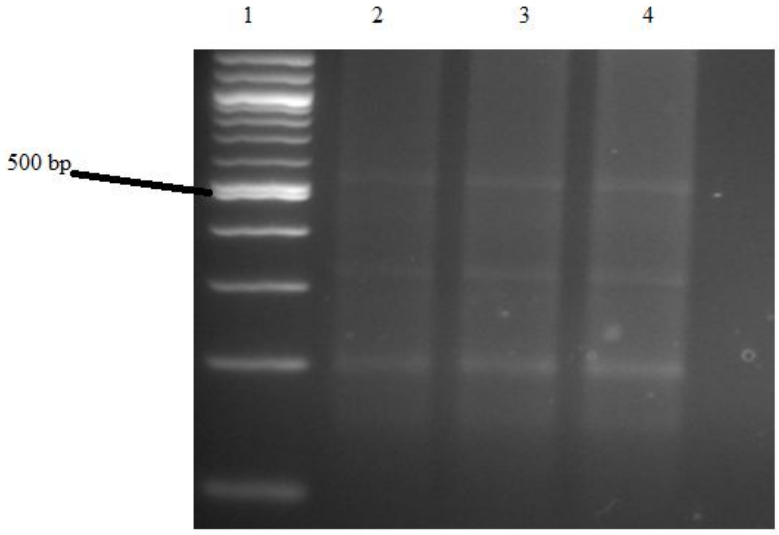


Figure 25. Amplification of the 3*A.* isolates18S rDNA 2209-5.8S rDNA 92, digeted with MboI restriction enzyme
Lane1; 100 bp DNA ladder. Lane2; Acs 9. Lane3; Acd 12. lane4; Acd 13.
All isolates showed identical size

4. **Discussion**

Many studies have reported the presence of *Acanthamoeba* in drinking water, swimming pools, and rivers. These water sources have an obvious role in prevalence *Acanthamoeba* keratitis among people. Since *Acanthamoeba* has an extensive dispersion, it is expected that many individuals will be exposed to the protozoan hazard (Niyyat *et al* 2009, Rezaeian *et al* 2008, De Jonckheere *et al* 1979 and Rivera F *et al* 1993). For this reason, it has been claimed that more than 80% of healthy people have antibodies against *Acanthamoeba* (Chappell Cl *et al* 2001).

In this study, from a total of eighty samples (air conditioner, thirty eight samples; dust, fourteen samples; lake, fourteen samples and sewage fourteen samples) collected from the University of Malaya environment and by using inverted microscope, only fourteen isolates (17.5%) (Acl1, Acl2, Acs3, Acd4, Acs5, Acs6, Acs7, Acd8, Acs9, Acd10, Acd11, Acd12, Acd13 and Acl14) showed morphological characteristics similar to *Acanthamoeba* morphology.

The presence of *Acanthamoeba* genus was most frequent in dust (42.8%; 6 out of 14 samples) followed by sewages (35.7%; 5 out of 14 isolates) and finally lakes (21.4%; 3 out of 14 isolates).

The result of the PCR product at 272 bp concurred with the results reported by Michael H. *et al.*, (1992) which identified them as *Acanthamoeba* genus. The results of the PCR product at 485 bp and multiple bands are agreement with the results reported by Michael H. *et al.*, (1992) which identified as non pathogenic and pathogenic *Acanthamoeba* respectively. In the same study area (Kuala Lumpur) Chan

Lili *et al* 2011, reported that by Utilizing microscopy and PCR approaches, *Acanthamoeba* species were detected in 20 out of the 87 dust samples collected (23%).

In sudan, A total of four hundred water samples were examined by PCR to detect *Vibrio cholerae* toxin gene (toxA) and *Acanthamoeba* 18 S RNA gene. The result showed that eight water samples contained both *Vibrio cholerae* and *Acanthamoeba*. Furthermore, it was found that only one water sample contained *Vibrio cholerae* compared to thirteen samples which contained *Acanthamoeba* only. The study showed that both *Vibrio cholerae* and *Acanthamoeba* species can be detected in the same natural water samples collected from different cholera endemic areas in Sudan. Eighty nine percent of detected *Vibrio cholerae* was found with *Acanthamoeba* and eleven percent was found alone (Salah Shanan *et al* 2011).

In Egypt, species of the genus *Acanthamoeba* were isolated from 33.3% of inlet water samples of the Damanhour Drinking Water Treatment Plant, Acanthamoeba species were detected at higher percentages (66.7%, 40%, and 43.1%, respectively) in the examined Nile water of Egypt by Al-Herrawy, Hamadto *et al*, and Hilali *et al*. Also, during the same work, *Acanthamoeba* spp. were isolated from 16.7% of the examined finished water samples of the Damanhour Drinking Water Treatment Plant. In other studies in Egypt, Al-Herrawy *et al* recorded a higher occurrence (58.6%), 33 while Hamadto *et al* recorded a lower occurrence (4%).

In a study, that was conducted for the first time in Iran, the results showed that about half samples (30 samples) were infected with *Acanthamoeba*. This result demonstrates the considerable spreading of *Acanthamoeba* in different regions

among the country. This can be due to high resistance of *Acanthamoeba* against water chlorination as well as tolerance of it to the relatively high temperature (HR Bagheri *et al* 2010).

In Spain, *Acanthamoeba* contamination was found in 48 out of 88 (59.5%) tap water samples (Lorenzo-Morales J *et al* 2005). In another study in Thailand, 56 samples were collected from 28 natural water resources that six samples were *Naegleria* spp., 3 samples were *Acanthamoeba*, 5 were mixed, and 2 samples were unidentified (Anchalee W *et al* 2009).

In African regions, the majority of authors were interested to detect and identify *Acanthamoeba* species in clinical samples. Recently, some cases of *Acanthamoeba* keratitis were reported in South Africa (Dini *et al*., 2000), in Mali (Quinio *et al*., 2000) and in Ghana (Leck *et al*., 2002). A case of meningoencephalitis caused by *Acanthamoeba* spp. was diagnosed in Dakar (Ndiaye *et al*., 2005).

In the United Kingdom, *Acanthamoeba* spp. were isolated from 26.9% of tap water samples by Kilvington *et al* 2004. The difference in detection rates of free-living amoebae in different countries and localities may be influenced by geographical conditions and raw water sources.

Based on the results presented in this study, the occurrence of *Acanthamoeba* in Malaysian environment was confirmed. Potentionally pathogenic isolates of *Acanthamoeba* were isolated and maintained in our laboratory. Future research needs to be carried out for the speciation and determination of the pathogenicity of other strains of these environmental isolates. Furthermore efforts need to be put forward to develop a method that could examine the concentration of these free-living amoebae

in the aquatic environments. This will be of great help for the safety of recreational activities.

The prevalence of *Acanthamoeba* was moderately high.

Conclusion;

The use of molecular methods to identify free-living amoebae of genus *Acanthamoeba* could provide a more rapid means to diagnose infections caused by those amoebae. The culture method is more reliable, easier than direct DNA extraction and analysis for the detection of *Acanthamoeba* species.

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APPENDICES

1.0 Solution preparation

1.1 Page's Amoeba saline (PAS) solution

NaC1	0.120 g
$MgSO_4.7H_2O_4$	0.004 g
$CaCl_2 2H_2O$	0.004 g
$Na_2 HPO_4$	0.142 g
KH_2PO_4	0.136 g
D H_2O	1000 ml

All the above chemicals were mixed with 1000 ml of distilled water and aliquoted in to 2 bottles of 500 ml. They were then autoclaved at 121ºC, 15 Ibs pressure for 15 minutes and kept in room temperature until used.

1.2 Phosphate Buffer Saline (PBS) Solution

a) Stock I

Na_2 H PO_4 .H_2O	13.8 g
d H_2O	500 ml

b) Stock II

$Na_2 HPO_4$	14.195 g
d H_2O	500 ml

Working Solution:

Stock I	28 ml
Stock II	72 ml
Na Cl	1.7 g

D H_2O 100 ml

The above solutions were mixed thoroughly, aliquoted and autoclaved. They were then kept at room temperature until used.

1.3 Typan blue stain

0.20% trypan blue (prepared from 0.4% trypan blue, Sigma T 8154 diluted 1:1 with 0.9% NaCl). This working solution is stable for several months at room temperature.

1.4 Modified field stain

To prepare 100 ml of 1% field stain A:

Field′s stain A powder 1 gm.

Distilled water (hot) 100ml

1- Weigh the powder on a piece of clean paper (preweighed) and transfer it to Pyrex beaker.

2- Measure the water and heat to boiling.

3- Add the hot water to the stain and mix to dissolve the powder.

4- When cool, filter the stain in to a clean bottle.

5- Label the bottle and store it at room temperature.

The stain stable idefinitely.

To prepare 100 ml of 1% methanolic eosin:

Eosin powder 1gm.

Methyl alcohol 100ml.

1- Weigh the eosin powder on a piece of clean paper and transfer it to a clean, leak-proof, brown bottle of 100 ml capacity.

2- Add 100 ml of water and mix to dissolve the stain.

3- Label the bottle and store it at room temperature.

The stain stable idefinitely.

* For use: transfer a small amount of the stain to a bottle with a cap in to which a dropper can be inserted.

Protocol of Modified field stain:

- Air dry the slide of *Acanthamoeba* smear in semi-moist container at 30°C.

- fix the slide with methanol for 5 minutes.

- dip in 1% methanolic eosin for 2 minutes.

- add several drops of 3% acid alcohol to discolour the eosin.

- rinse gently in tap water for 10 seconds.

- add 2 drop of field stain A.

- immediately dip the slide five times in clean distilled water.

- air dry and mount with DPX.

1.5 Trichrome stain

1- Schaudinn fixative

2- Iodine-alcohol.

3- Trichrome stain.

4- Carbolxylene.

Schaudinn fixative:

Mercuric chloride, saturated aqueous	200 ml
Ethyl alcohol, 95%	100ml
Glacial acetic acid	12ml

Protocol:

1- place in shaudin fixative for 5 minutes at 50°C or 1 hour at room tempreture.

2- Place in 70% alcohol plus iodine for 1 minutes (10 minutes for PVA)

3- Place successively in 70% , 70% and 50% ethyl alcohol solutions for 3-5 minutes in each solution.

4- Place in trichrome stain for 6-8 minutes.

5- Decolorized in 90% acidified ethyl alcohol (1 drop glacial acetic acid in 10 ml alcohol) for 10-20 sec or until stain barely runs from smear.

6- Place in 95% ethyl alcohol for 5 minutes

7- Place in carbolxylene for 10 minutes.

8- Place in xylene for 10 minutes.

9- Mount in Permount.

1.6 TBE (Tris Borate EDTA) buffer, 5 X stock solution

Tris base	54g
Boric acid	27.5g
EDTA (pH8.0)	0.5 M, 20 ml
dH_2O	to 1 L

For preparing 1 X working solution, the stock solution is diluted with sterile dH_2O (100 ml 5 X TBE buffer mixed with 400ml dH_2O.

2.0 Chemicals and reagents

2.1 Research Bio labs (Singapore)

- Oligonucleotides primers (Primer residues are listed in Tables 1 and 2 pg 45 and 53)

- 100 bp DNA ladder

- Taq 1, supplement with:

1X NEBuffer Taq 1

100 mM Nacl

10 mM Tris-Hcl

PH 7.9 @25°C

- Hae111 supplemented with:

1X NEBuffer 2

50 mM Nacl

10 mM Tris-Hcl

10 mM $Mgcl_2$

1 mM DTT

PH 7.9 @ 25°C

- Mbo I, Research biolabs (Singapore), supplement with:

1X NEBuffer 3

100 mM Nacl

50 mM Tris-Hcl

10 mM $Mgcl_2$

1 mM dithiothreitol

PH 7.9 @ 25°C

- RSA1, supplement with:

1X NEBuffer 2

50 mM Nacl

10 mM Tris-Hcl

10 mM $Mgcl_2$

2.2 Scharlau Chemie S.A. (Spain)

- Ethanol, absolute (C_2H_5OH)

2.3 Sigma Chemical Company (U. S. A.)

- EDTA (Ethylenediaminetetraacetic acid; $C_{10}H_{14}N_2O_8Na_2.2H_2O$)

- Trizma Base (Tris[hydroxymethyl]aminomethane; $C_4H_{11}NO_3$)

- Agarose Type 1-A

- Boric Acid (H_3BO_3)

- Sodium phosphate monobase; monohydrate ($NaH_2PO_4.H_2O$)

3.0 Commercial kits

3.1 MBI Fermentas (U.S.A.)

- *Taq* DNA polymerase (recombinant) Kit (supplement with 10 X PCR buffer and $MgCl_2$)

- 1 kb DNA ladder

3.2 Qiagen (Germany)

- QIAamp DNA Mini Kit

(Buffer AE, Buffer AL, Buffer AW1, Buffer AW2, QIAamp spin column and collection tube, and QIAGEN proteinase K)

- QIAquick Gel Extraction Kit

(Buffer EB, Buffer PE, Buffer QG, and QIAquick spin column and collection tube)

- QIAprep Spin Miniprep Kit

(Buffer EB, Buffer N3, Buffer P1, Buffer P2, Buffer PE, and QIAprep spin column and collection tube, RNase A)

4.0 Instruments

Centrifuge : KUBOTA 2010 (Japan)

Centrifuge : refrigerated (Hettich 16R, Hettich zentrifugen)

Centrifuge : Micro, refrigerated (EBI 12R, Hettich zentrifugen)

Centrifuge :mini, tabletop (C-1200, NATIONAL LABNET)

Gel documentation device (DOC-088.XD, UVItech)

Gel electrophoresis equipment-horizontal (EASY-CAST™ Electrophoresis Syste, B1, Owl Scientific)

Gel viewing monitor (VISTA, Norbain)

Incubator : orbital shaker (NOVOTRON®; INFORS AG)

Fluorecent microscope, Olympus (Japan)

Microscope, inverted microscope, Nikon TMS (Japan)

Oven: microwave (N-904, NEC)

PCR thermal cycler (Mastercycler® personal; eppendorf)

Power pack (EPS-250 Series II, C.B.S.)

Refrigerator/-70 ^{o}C (ULTRA LOW, MDF-U5086W, Sanyo)

Refrigerator/ freezer (GR562, GoldStar)

Video copy processor (P91, Mitsubishi)

Vortex (ZX^{3}, VELP Scientifica)

UV transilluminator (3UV™ Transluminator, LMS-20E, UVP)

Water bath (WBC-1510W, JEIO TECH)

Weighing machine (VI-3mg, ACCULAB)