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Detection of free living amoebae, *Acanthamoeba* and *Naegleria*, in swimming pools, Malaysia

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Abstract. This study reports the detection of *Acanthamoeba* and *Naegleria* species in 14 swimming pools around Petaling Jaya and Kuala Lumpur, Malaysia. Sampling was carried out at 4 sites (the platforms (P), wall (W), 1 meter from the wall (1) and middle (2)) of each swimming pool. These free living amoebae (FLA) were detected under light and inverted microscopes after being cultured on the surface of non-nutrient agar lawned with *Escherichia coli*. *Acanthamoeba* species were detected in higher number of culture plates from all sampling sites of all the swimming pools. While *Naegleria*, were detected in fewer culture plates at 3 sampling sites (absent at site P) of 8 swimming pools. This suggested that the thick double-walled cysts of *Acanthamoeba* were more resistant, thus remaining viable in the dry-hot areas of the platforms and in chlorinated water of the swimming pools whereas *Naegleria* cysts, that are fragile and susceptible to desiccation, preferred watery or moist areas for growth and proliferation. The prevalence of both FLA was highest at site W (76.2%), followed by site 1 (64.7%), lowest at site 2 (19.4%), and could be detected at all 3 sampling levels (top, middle and bottom) of these 3 sites. The surface of site W might act as a bio-film that accumulated all kinds of microbes providing sufficient requirement for the FLA to develop and undergo many rounds of life cycles as well as moving from top to bottom in order to graze food. Other factors such as human activities, the circulating system which was fixed at all swimming pools, blowing wind which might carry the cysts from surroundings and the swimming flagellate stage of *Naegleria* could also contribute to the distribution of the FLA at these sampling sites. Both FLA showed highest growth (80.4%) at room temperature (25-28ºC) and lesser (70.0%) at 37ºC which might be due to the overgrowth of other microbes (*E. coli*, fungi, algae, etc). While at 44ºC, only *Acanthamoeba* species could survive thus showing that our swimming pools are free from potentially pathogenic *Naegleria* species. However, further study is needed in order to confirm the virulence levels of these amoebae isolates.

INTRODUCTION

Free-living amoebae of the genera *Acanthamoeba* and *Naegleria* are known to cause disease in human. Infections by these amoebae are still unfamiliar to many physicians, pathologists and laboratory workers. However these amoebae have recently received more attention as a result of an increase in cases of *Acanthamoeba* keratitis (AK), granulomatous amoebic encephalitis (GAE), primary amoebic meningoencephalitis (PAM) and problems with effective treatment (Seidel *et al.*, 1982; Moore *et al.*, 1986; Schuster & Visvesvara, 1998; Kamel *et al.*, 2003). Swimming was reported to be associated with corneal ulcer or AK due to *Acanthamoeba* (Mathers *et al.*, 1996) and PAM due to *Naegleria* (Wiwanitkit, 2004) in healthy people. While GAE is usually found in immuno-suppressive patients due to *Acanthamoeba* infections of the central nervous system and brain (Martinez, 1980). Both of these amoebae are ubiquitous in nature and can be found in almost all environments

worldwide. In Malaysia, the government's call to citizens to spend more quality time with family has resulted in the increase of water-related activities in swimming pools and recreational areas in the country. This may contribute to the incidence of FLA and waterborne infections. The popularity of wearing contact lens especially among teenagers may enhance the chances of contracting AK, and the risk is increased when wearing it while swimming (Stehr-Green *et al.*, 1987). Pathogenic FLA have the ability to multiply and grow well in tropical climate and in water body with high temperatures of 40-45°C (De Jonckheere, 1991). Besides that, it was reported that FLA isolates from warm swimming pools or hot spring were more pathogenic than those from natural fresh water (De Jonckheere, 1987). This study was carried out to detect these two FLA in commonly used swimming pools in Malaysia in order to provide public health information.

MATERIALS AND METHODS

Preparation of nutrient agar (NA) plates for maintenance of *Escherichia coli* strain JM109

A 23g of NA (Difco) was dissolved in 1000 ml of distilled water. This mixture was then autoclaved at 15 lbs pressure, 121°C for 15 minutes and allowed to cool until 50°C. Twenty (20) ml of this warm agar was distributed aseptically to each disposable petri dish (90 X 15 mm, Greiner) and left to solidify at room temperature. These NA plates were used in maintaining *Escherichia coli* or kept inside clean plastic bags and stored at 4°C until use. The maintaining procedure was carried out by pipetting 20 µl of *E. coli* (JM109 strain) from glycerol stock (Promega) and streaked onto a NA plate, followed by incubation at 37°C for 24 hours. The next day, the *E. coli* colonies growing on the agar surface were scrapped off using disposable loop and further streaked onto several NA plates and incubated for another 48 hours prior to harvest. Alternatively, these plates were stored at 4°C and subcultured every 2 weeks.

Preparation of non-nutrient agar (NNA) plates with *E. coli* lawn

A 15g of NNA (American bacteriological agar, Pronadisa) was dissolved in 1000 ml of PAS solution (0.12g NaCl, 0.004g MgSO4.7H2O, 0.004g CaCl2.2H2O, 0.142g Na2HPO4 and 0.136g KH2PO4; all these chemicals from Sigma were diluted in 1000 ml distilled water). The mixture was autoclaved and solidified in Petri dish as the above procedure used in the preparation of NA plates. These NNA plates were then lawned with *E. coli* (as stated below) or alternatively kept at 4°C until use.

Growth of *E. coli* in NA plate was harvested by adding 2 ml of PAS solution followed by pipetting for several times to detach the *E. coli* colonies from the NA surface. The bacterial suspension was then pooled into a bijou bottle and diluted with 2-3 ml of PAS solution. Subsequently, 10 drops of the bacterial suspension was transferred onto each NNA plate and spread evenly on the agar surface using a sterile spreader. At this stage, plates could be directly used for cultivation (of free-living protozoa) purpose or kept at 4°C for up to 3 weeks before use.

Sampling sites

Fourteen (14) swimming pools (SP) located in Petaling Jaya and Kuala Lumpur, Malaysia, as listed in Table 1 were selected. Four (4) sampling stations – site P, site W, site 1 and site 2 – of each SP were selected, which were the platform floor (Figure 1A), SP wall (Figure 1B), within 1 meter from the wall (Figure 1C) and middle of the pool (Figure 1D) respectively. All sampling sites, except P were further divided into 3 sub-sampling sites: WA, WB, WC; 1A, 1B, 1C; 2A, 2B and 2C respectively.

Collection of samples

Six (6) sterile cotton wools were used to scrape the dust or debris from each of the sites P, WA, WB, WC and subsequently

Table 1. List of the 14 swimming pools at Petaling Jaya and Kuala Lumpur, Malaysia

No:	Designation	Location of swimming pool
1	UM	Sport Centre, University of Malaya, Kuala Lumpur
2	VA	Vista Angkasa, Kuala Lumpur
3	PH	Pantai Hill Park, Kuala Lumpur
4	UT	University Tower, Petaling Jaya
5	VS	Vista Serdang, Serdang
6	ER	Ehsan Ria Condominium, Petaling Jaya
7	1P	Pantai Panorama I, Kuala Lumpur
8	2P	Pantai Panorama II, Kuala Lumpur
9	SL	Sri Langit, Seputeh, Kuala Lumpur
10	SS	Sri Seputeh, Seputeh, Kuala Lumpur
11	CD	CASA Damansara, Petaling Jaya
12	PR	Prima 16, Section 17, Petaling Jaya
13	LV	Li Villas, Section 17, Petaling Jaya
14	TI	Taman Indah Persona, Section 17, Petaling Jaya

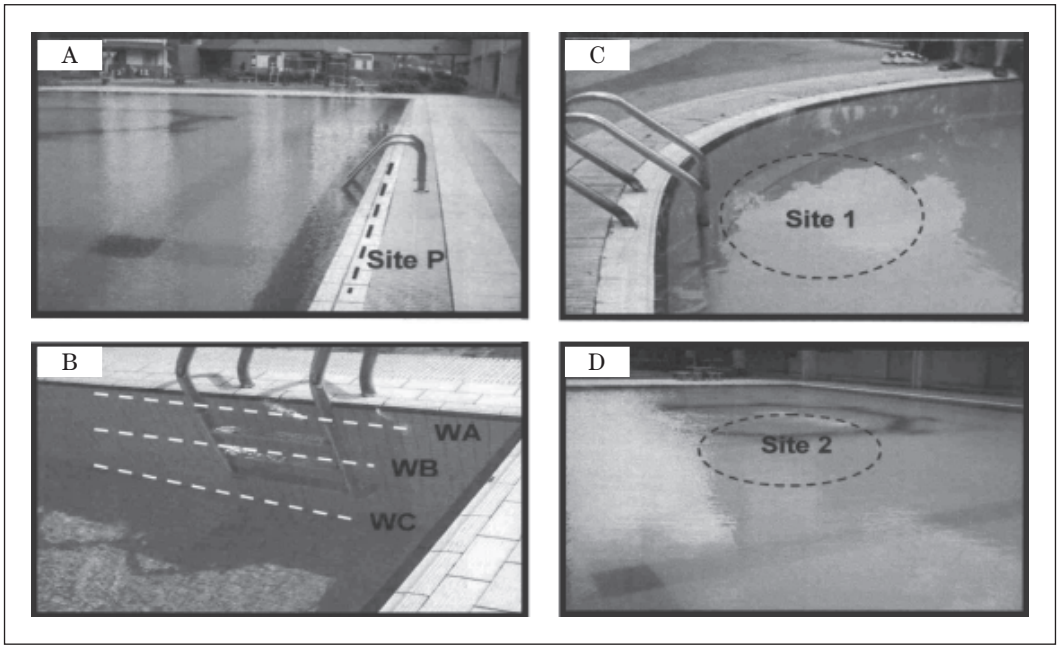

Figure 1. Sampling sites of the swimming pool

(A) The platform (Site P) where the wet visitors used to walk in and out of the swimming pool (SP) by using staircase, (B) The SP wall (Site W) which was divided into the top (WA), middle (WB) and bottom (WC) areas, (C) The area within one meter from SP wall (Site 1) where visitor used to jump and swim, (D) The middle of the SP (Site 2) which was less disturbed by swimmers

placed individually in sterile plastic bags and labeled accordingly. Besides that, 6 water samples (1500 ml each) were collected from each of the sites 1A, 1B, 1C, 2A, 2B and 2C using 1.5 litre clean mineral bottles. All the samples were then transported to the laboratory for further processing. Note: Since the mineral bottles could not be steriled by autoclave, therefore all of these bottles were first cleaned with 10% NaOCl (sodium hypochlorite or clorox), followed by sterile autoclaved water and 70% alcohol. The bottles were then dried in 37ºC incubator which was regularly cleaned by swabbing with 70% alcohol.

Preparation of samples for cultivation

Cotton wool scrapped samples were directly streaked in the middle of the NNA-*E. coli* plates and labeled accordingly. A group of 2 plates from each of the sampling and sub-sampling sites were then incubated at room temperature (25-28ºC), 37ºC and 44ºC respectively. For the water samples, they were processed prior to cultivation. The bottle containing the water sample was shaken vigorously to mix the contents and 1000 ml was filtered through a 1.2 µm pore size cellulose nitrate membrane (Millipore) by mild suction using a vacuum or pressure pump (Millipore). A new sterile membrane was used for each water sample. Filtration was stopped when 3-5 ml of the water sample was left above the membrane. The trapped debris (including FLA) on the membrane surface was carefully washed *in situ* with 60 ml of sterile distilled water and subsequently distributed into 6 portions of 10 ml each inside a 15-ml centrifuge tube (Falcon). The tubes were then centrifuged at 2000 rpm for 15 minutes. The supernatant was discarded and the pellet from each tube was transferred and spread evenly onto NNA-*E. coli* plate. A group of 2 plates were incubated as mentioned above. Similarly, the negative control plates were also carried out by inoculation with sterile cotton wool or sterile distilled water and the plates were incubated at the respective temperatures.

Detection of FLA

All cultured plates were examined daily for up to 14 days by inverted and light microscopes before being discarded. The presence of FLA could be seen by the clear tracks on the *E. coli* lawn, produced by the feeding trophozoites of *Acanthamoeba and Naegleria*, which were readily apparent after 48-72 hours of incubation. The specific morphological appearances of the trophozoites, cysts and flagellates were identified accordingly, based on the reports by several workers (Carter, 1970; Pussard & Pons, 1977; Page, 1988). The images of the selected organisms were photographed using light (Olympus BX51) and inverted (Olympus CKX41) microscopes which were attached to a photo adapter and a computer installed with imaging software.

Enflagellation test for *Naegleria* species

Culture plates that were seen to contain *Naegleria* trophozoites were added with 3 ml of PAS solution or sterile distilled water and placed onto a shaker at 50 rpm (Heto). Observations for the highly motile flagellates were carried out every 30 minutes for up to 6 hours with inverted and light microscopes followed by photography.

Subcultivation and isolation of FLA

Subculture was carried out for all the positive plates with growth of *Naegleria* or *Acanthamoeba* or both. The plate was observed under the inverted microscope to identify the best spot of FLA growth, followed by a circle mark at the bottom of the Petri dish with a marker pen. The cover of the plate was opened and 1 cm^2 of the NNA-*E. coli* was cut with a sterile scalpel blade. The cut agar was lifted and placed side down on a new NNA-*E. coli* plate. The plate was then sealed with parafilm (Parafilm 'M'®) and incubated at the respective temperatures. This subculture was carried out after 2-3 weeks of cultivation. Performing subculturing several times facilitated the isolation of *Acanthamoeba* species. While for *Naegleria* species, isolation was achieved by adding 3 ml of PAS and left for 1 hour or

overnight (to allow for the transformation of *Naegleria* trophozoites into the flagellate stage). The plate was then placed under the inverted microscope to observe the flagellate stage swimming in PAS solution. It was then pipetted using sterile disposable pipette (without disturbing the agar surface) and placed onto a new NNA-*E. coli* plate. This step was carried out several times until monogenous culture was obtained.

RESULTS

Presence of FLA in NNA-*E.coli* plates from sampling sites of the swimming pools

The data of the presence of *Acanthamoeba* (A), *Naegleria* (N) and both *Acanthamoeba* and *Naegleria* (AN) species, are summarized in Table 2, and the growth capability of these FLA at room temperature, 37ºC and 44ºC are recorded in Table 3. Plates that did not show any FLA growth by day 14 were discarded and recorded as negative.

Morphological observation of FLA

The trophozoite stage of *Acanthamoeba and Naegleria* were first observed after the second and third days of culture. The cyst stage of these FLA could be seen after at least 5 days of cultivation. The flagellate stage of *Naegleria* was seen in the watery surface of the agar or could be induced by enflagellation test. All the three stages are illustrated in Figure 2(A-M). The sizes of trophozoites and cysts of *Naegleria* and *Acanthamoeba* isolates are summarized in Table 4.

DISCUSSION

In this study, culturing of samples were carried out as soon as possible in order to prevent contamination by excessive overgrowth of environmental bacteria and fungi. Water samples that could not be processed immediately were kept at 2–10ºC for no more than 24 hours. High ratio of bacteria to amoebae (i.e., >10 to 1) was reported to inhibit the growth of *Acanthamoeba* (Wang & Ahearn, 1997). Both *Acanthamoeba* and *Naegleria* showed an excellent growth in a non-nutrient agar seeded with nonmucoid bacteria (*E. coli*). *Escherichia coli* was reported to be a good food source for these FLA, while the mucoid bacteria is believed to impede phagocytosis by amoebae and lead to bacterial overgrowth (Schuster, 2002). After cultivation, the positive plates may consist of only *Acanthamoeba* or *Naegleria*, or both (Table 2). The detection of FLA in the cotton wool scrapped samples from the platform (site P) and wall (site W), indicated that these sites might trap the cysts or trophozoites of either *Acanthamoeba* or *Naegleria*, or the mixture of both FLA, and accumulated (by proliferation) at the respective scrapped areas. The water samples collected from sampling sites 1 and 2 which involved the surface, middle and bottom of the swimming pools with the water circulation system, were actually not adequate to detect the presence of FLA. Moreover, the 6 culture samples used for each of the surface, middle and bottom areas were actually divided portions of water samples originally from a bottle of water collected at the respective areas of the swimming pools. The positive plates of these FLA were then subcultured for the purpose of colonisation, stabilisation and maintenance for further subsequent studies. Additionally, subcultivation could also remove flocs which were commonly found to obstruct the detection and the growth of FLA, especially from water samples. Flocs are actually conglomerates of living and dead bacterial cells, often including filamentous strains, precipitated salts, trapped inorganic particles (sand) and organic fibers (Eikelboom, 2000).

Out of the 14 sampled swimming pools, *Acanthamoeba* was detected in all and *Naegleria* in 8 of them. Furthermore, *Acanthamoeba* was detected in higher number of positive culture plates at all of the four sampling sites, including the dry and hottest areas (site P). This suggests

Table 2. Presence of *Acanthamoeba* and *Naegleria* species at the sampled sites of the 14 swimming pools

Sampled Site (No and type of sample at each SP)	FLA	swimming pool (refer to Table 1) 1	2	3	4	5	6	7	8	9	10	11	12	13	14	Total
		Number of positive plate with FLA out of each 6 cultivation plates respectively														
Site P (Platform)																
(6 SCB)	A	2	2	5	6	1	6	4	6	6	5	2	2	2	4	53
	N	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	AN	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Site W (wall)																53
WA (TOP)	A	3	3	1	3	2	5	2	6	6	5	4	6	1	2	49
(6 SCB)	N	2	2	1	2	–	–	–	–	–	–	–	–	–	–	7
	AN	1	1	2	1	1	–	–	–	–	–	–	–	3	1	10
WB (MIDDLE)	A	2	3	2	3	2	5	3	4	5	5	2	6	3	1	46
(6 SCB)	N	1	–	1	–	1	–	–	–	–	–	–	–	–	–	3
	AN	1	1	2	1	2	–	–	1	–	–	–	–	2	–	10
WC (BOTTOM)	A	3	5	2	5	1	6	4	5	6	5	2	4	4	5	57
(6 SCB)	N	–	–	–	–	1	–	–	–	–	–	–	–	–	–	1
	AN	1	1	1	1	2	–	–	1	–	–	–	–	2	–	9
Site 1 (within 1 meter from wall)																192
1A (TOP)	A	2	1	1	2	2	4	2	5	5	4	3	5	2	4	42
(6 PWS)	N	2	1	1	2	–	–	–	–	–	–	–	–	–	–	6
	AN	2	2	2	1	2	–	–	–	–	–	–	–	1	–	10
1B (MIDDLE)	A	2	1	1	3	2	4	3	5	5	5	2	6	3	3	45
(6 PWS)	N	1	1	–	–	–	–	–	–	–	–	–	–	–	–	2
	AN	1	2	1	–	–	–	–	–	–	–	–	–	–	–	4
1C (BOTTOM)	A	4	2	3	4	1	4	4	4	5	5	2	4	4	3	49
(6 PWS)	N	–	–	–	–	–	–	–	–	–	–	–	–	1	–	1
	AN	2	1	1	–	–	–	–	–	–	–	–	–	–	–	4
Site 2 (middle area)																163
2A (TOP)	A	–	–	1	1	1	1	2	2	2	1	1	1	1	1	15
(6 PWS)	N	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	AN	–	–	–	–	–	–	–	–	–	–	–	–	–	1	1
2B (MIDDLE)	A	–	–	–	1	1	1	3	3	2	1	1	1	–	1	15
(6 PWS)	N	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	AN	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2C (BOTTOM)	A	3	–	2	1	–	2	2	2	1	–	1	1	–	2	17
(6 PWS)	N	–	1	–	–	–	–	–	–	–	–	–	–	–	–	1
	AN	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Total number of plates with and without FLA															49
	A	21	17	18	29	13	38	29	42	43	36	20	36	20	26	388
	N	6	5	3	4	2	–	–	–	–	–	–	–	1	–	21
	AN	8	8	9	4	7	–	–	2	–	–	–	–	8	2	48
Plate with FLA	35	30	30	37	22	38	29	44	43	36	20	36	29	28	457	
Plate without FLA		25	30	30	23	38	22	31	16	17	24	40	24	31	32	383
Total samples used		60	60	60	60	60	60	60	60	60	60	60	60	60	60	840

Designation: FLA (free living amoeba), A (*Acanthamoeba* only), N (*Naegleria* only), AN (both *Acanthamoeba* and *Naegleria*), Site P, W, 1 and 2 (sampled sites refer to figure 1), - (non growth plate), SP (swimming pool), SCB (scrapped cotton buds), PWS (portion of water samples)

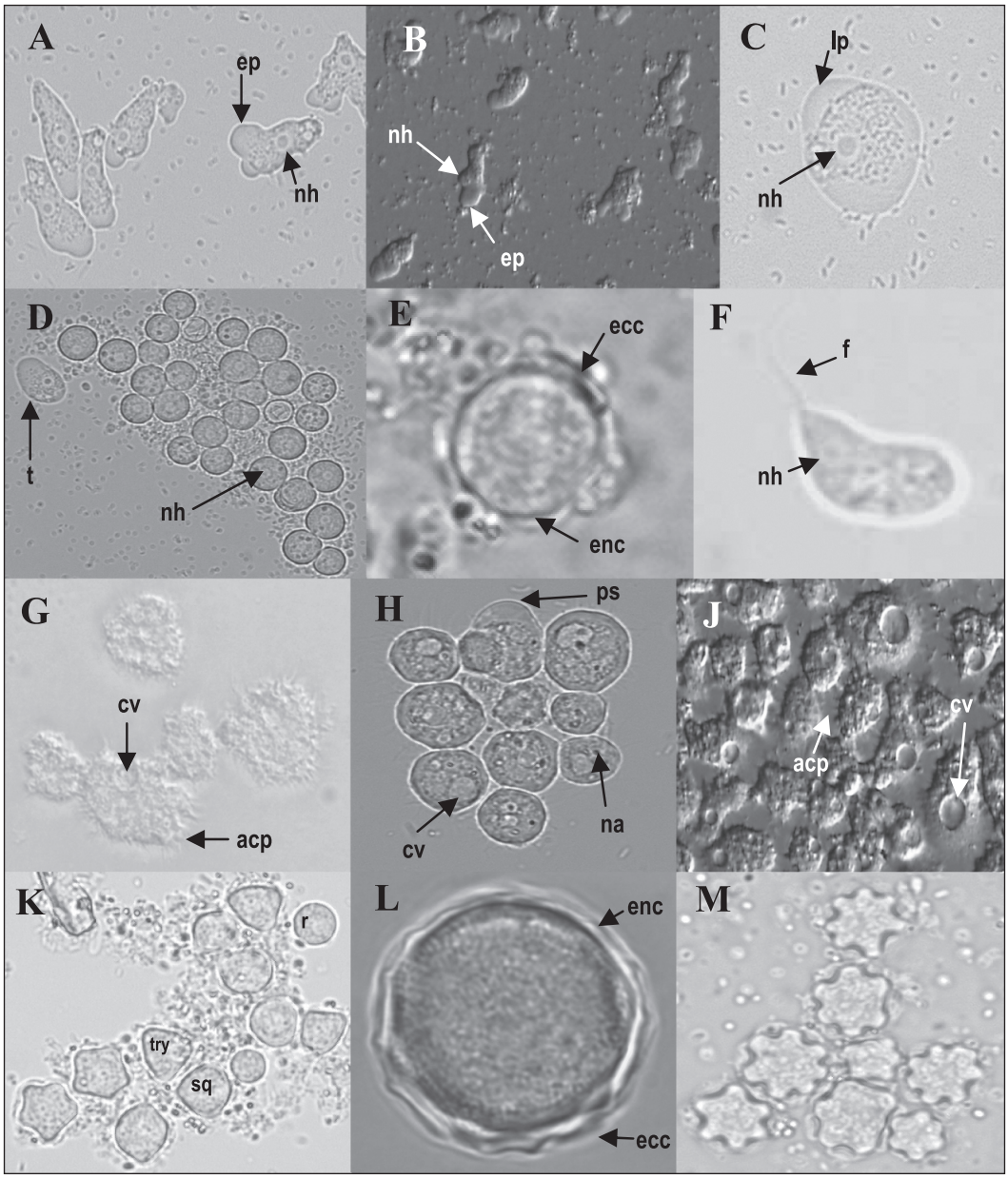

Figure 2. Morphological observation of *Neagleria* (**A, B, C, D, E, F**) and *Acanthamoeba* (**G, H, J, K, L, M**) species by using light (LiM) and inverted (InM) microscopes

(A, LiM X 400; **B**, InM X 200) Trophozoites showing typical eruptive pseudopodia / lobopodia,
(**C**, LiM X 1000; **H**, LiM X 400**)** Rounded form of trophozoite/s in cold buffer after detached from agar surface,
(**D**, LiM X 400) Newly formed cysts in colonial with a trophozoite,
(**E,** LiM X1000) A single cyst showing smooth ectocyst and endocyst,
(**F,** LiM X 1000**)** Flagellate stage showing one visible flagella,
(**G**, LiM X 400) Various shapes of trophozoites showing acanthopodia after attached at the surface of glass slide,
(**J**, InM X 200) Trophozoites with prominent contractile vacuole,
(**K**, LiM X 400) Cysts with multiple shape, round, triangle and square,
(**L**, LiM X 1000) A rounded cyst exhibiting wrinkle ectocyst and endocyst,
(**M**, LiM X 400) Cysts with 5-7 angles found in 14 days agar culture.

nh (nucleus with clear halo), ep (eruptive pseudopodia), lp (lobopodia), t (trophozoite), enc (endocyst), ecc (ectocyst), f (flagella), acp (acanthopodia), cv (contractile vacuole), ps (pseudopodia), na (nucleus without halo), r (round), try (triangle), sq (square)

Table 3. Growth capability of *Acanthamoeba* and *Naegleria* species at room temperature (25-28ºC), 37ºC and 44ºC in non-nutrient agar lawned with *E. coli*

FLA	Swimming pool (refer to Table 1) 1	2	3	4	5	6	7	8	9	10	11	12	13	14	Total plate
	Number of cultivation plate at room temperature (20 – 28ºC)														
A	9	9	10	9	8	15	12	18	20	15	12	18	9	15	179
N	3	1	3	2	2	–	–	–	–	–	–	–	1	–	12
AN	5	8	5	4	4	–	–	2	–	–	–	–	5	1	34
-ve FLA	3	2	2	5	6	5	8	–	–	5	8	2	5	4	55
Total	20	20	20	20	20	20	20	20	20	20	20	20	20	20	280
	Number of cultivation plate at 37ºC														
A	9	6	6	17	5	19	14	20	20	20	5	15	8	9	173
N	3	4	–	2	–	–	–	–	–	–	–	–	–	–	9
AN	3	–	4	–	3	–	–	–	–	–	–	–	3	1	14
-ve FLA	5	10	10	1	12	1	6	–	–	–	15	5	9	10	84
Total	20	20	20	20	20	20	20	20	20	20	20	20	20	20	280
	Number of cultivation plate at 44ºC														
A	3	2	2	3	–	4	3	4	3	1	3	3	3	2	36
N	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AN	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
-ve FLA	17	18	18	17	20	16	17	16	17	19	17	17	17	18	244
Total	20	20	20	20	20	20	20	20	20	20	20	20	20	20	280

Designation:
Culture plates contain A (*Acanthamoeba* only), N (*Naegleria* only), AN (both *Acanthamoeba* and *Naegleria*), -ve FLA (without any free living amoebae), – (non growth plate)

Table 4. Size of trophozoite and cyst of *Naegleria* and *Acanthamoeba* isolates

Naegleria (total 24 isolates)	Range of trophozoites diameter (µm)	Range of cysts diameter (µm)
UM2n, UM4n, VA6n, VA7n, PH8n, UT14n, VS25n, 2P28n, SS34n, SL37n, SL39n, CD43n, CD45n, LV47n, 1P50n, 1P52n, PR55n, PR56n (18 isolates)	15–20	
PH10n, UT15n, ER16n, ER18n, 2P26n, SS35n (6 isolates)	15–25	
UM2n, UM4n, VA6n, VA7n, PH8n, UT14n, VS25n, 2P28n, SS34n, SL37n, SL39n, CD43n, CD45n, LV47n, 1P50n, 1P52n, PR55n, PR56n, PH10n, UT15n, ER16n, ER18n, 2P26n, SS35n (24 isolates)		8–10
Acanthamoeba (total 23 isolates)		
UM4a, VA6a, UT14a, UT15a, ER18a, VS25a, 2P28a, SS34a, SS35a, SL37a, SL39a, CD43a, CD45a, LV47a, 1P50a, 1P52a (16 isolates)	20–25	
UM2a ,VA7a, PH8a, PH10a, 2P26a, PR55a, PR56a (7 isolates)	25–30	
UM2a, UM4a, VA6a, PH10a, VA7a, UT14a, UT15a, ER18a, VS25a, 2P28a, SS34a, SS35a, SL37a, SL39a, 1P52a, PR55a, PR56a (17 isolates)		8–10
2P26a (1 isolate)		8–13
PH8a, LV47a (2 isolates)		10–13
CD45a, 1P50a (2 isolates)		10–15
CD43a (1 isolate)		10–20

that the thick double-walled cysts of *Acanthamoeba* are more resistant to most environmental conditions and thus are viable in dry areas as well as in highly chlorinated water. *Acanthamoeba* cysts had been reported to be viable for more than 20 years under laboratory conditions (Mazur *et al.*, 1995). It is the only amoeba that could survive in high saline environments such as seawater and ocean sediment (De Jonckheere, 1991, 1987). In a comparison test, the cysts of pathogenic strain of *Acanthamoeba culbertsoni* were still viable after 3 hours of exposure to 40 µg/ml of free chlorine, whereas cysts of *Naegleria fowleri* were destroyed after an hour exposure to 0.5 µg/ml of free chlorine (De Jonckheere & De Voorde, 1976). *Naegleria* cysts are believed to be less resistant and need moist environment for survival. This is supported by our study, in that it was detected in less number of culture plates, and only from moist samples (wet scrapped cotton wools and debris from filtered water) collected from three sampling sites (W, 1 and 2) of the respective swimming pools. It was not detected in dry areas (platform) of all the 14 swimming pools. In moist and watery conditions, *Naegleria* cysts are believed to rapidly excyst to the asexual trophozoite stage.

Among the chosen sampling sites, both FLA showed highest distribution at site W, followed by site 1 and the lowest at site 2. The surface of site W was always covered with algae which might enhance the growth of other organisms such as bacteria, other microbes, worm-like and flea-like organisms, etc, thus providing sufficient requirement for the FLA to attach and obtain food. The presence of the detritus, filamentous cyanobacteria and eubacteria may also provide food sources for the FLA (Kyle & Noblet, 1985). The cysts from the environment may first attach to the surface at the top area, excyst to trophozoites with sluggish downward movement while grazing food, then multiply, colonize and form cysts. Therefore, the FLA was distributed at site W from the top to the bottom areas of the swimming pools SP1, SP3, SP4, SP5 and SP13. As for site 1of SP1 and SP3, the presence and distribution of FLA at all levels might be due to the dispersion of FLA from site W caused by human activities. In this observation, site W was a favourite resting place for people (especially children and poor swimmers) where they held on to the top of the wall and moving their legs, thus helping to disperse the FLA to site 1. Additionally, site 1 was always having people moving in and out (from/to the platform), swimming and jumping from the platform. These activities might carry dust with FLA (from platform) as well as contributing to water circulation in these areas. It was also possible that the transient flagellate stage of *Naegleria* trophozoites from the wall, swim to this area. While for site 2, *Naegleria* was only present in SP2 and SP14, and *Acanthamoeba* was seen in all swimming pools except SP2. This area was less disturbed because it was only visited by good swimmer. However, as all the swimming pools were equipped with water circulator, thus aiding in the dispersion of FLA from the top to the bottom of the pools and vice versa, especially at sites 1 and 2. The presence of FLA at site 2 most possibly was due to the wind which blow and carried the cysts from the nearby environment. The cysts of FLA from the surrounding may easily be blown into the uncovered swimming pools due to the their locations at the ground level surrounded by dusty trees, buildings, construction sites, walking lanes and some were without shower facilities (SP2, SP3, SP5 and SP13). There was a possibility that cyst colonies adhered to the wall and was only removed when the SP wall was scrapped or cleaned, as for SP1. The highest detection of *Acanthamoeba* in SP8 and SP9 might be due to the higher number of visitors, in addition to the dusty environment nearby. Results also showed that *Acanthamoeba* was more frequently detected at the bottom while *Naegleria* was at the top surface (Table 2), thus showing that the *Acanthamoeba* cysts being more compact, bigger and was probably heavier than the *Naegleria* cysts.

For the temperature tolerance test, both of the FLA were found to be suitable and best grown at room temperature (25-28°C), followed by 37°C incubation. The growth rate was slower at 37°C which might be due to the overgrowth of *E. coli*, which sometimes made the detection of FLA difficult. At 44°C incubation, none of the *Naegleria* was able to grow. We believe that all of the *Naegleria* detected were from the non-pathogenic species due to its inability to survive at high temperature (44°C). On the contrary, 36 (12.8%) of the culture plates showed positive growth of *Acanthamoeba* at 44°C and the trophozoites were able to undergo binary fission. The movement of the *Acanthamoeba* trophozoite at this temperature was very slow (less active), not exceeding 3000 µm in distance from the original area where the sample was placed on the agar surface. The trophozoites and cysts on the agar surface were less in number and found apart from each other and were smaller in size. A previous report of the temperature tolerance test noted that most of the amoebae that survived at high temperature were considered to be potentially pathogenic (De Jonckheere, 1991), but further molecular study is needed in order to confirm the virulence levels of these *Acanthamoeba* isolates.

For the morphological observation, the detection of flagellate (transient form trophozoite) stage was one of the important indicators for the rapid screening to differentiate the *Naegleria* from *Acanthamoeba* species. Flagellate stage could easily be detected, when the surface of the agar culture is watery due to the settling down of the moisture during cultivation. All those culture plates without accumulation of moisture at the surface could enhance the appearance of flagellate stage by adding distilled water or PAS solution. In this study, all the culture plates which were suspected to contain *Naegleria* showed the transient flagellate stage (Figure 2F). Furthermore, *Naegleria* species were observed to disappear abruptly when the cultures were not subcultured frequently or when there was increased accumulation of waste product. In cases with both FLA in the same culture plates, the *Acanthamoeba* would suppress the growth of *Naegleria*. Therefore both of these amoebae were isolated and maintained in separate culture plates for further study. Several selected *Naegleria* and *Acanthamoeba* species (Table 4) were able to be maintained in the laboratory. The trophozoites and cysts from all *Naegleria* isolates showed similarity in shape with the size ranging from 15-25 µm and 8-10 µm respectively. The cyst shape was round in with double thin and smooth wall (Figures 2D, 2E) while the trophozoites showed eruptive pseudopodia (lobopodia) (Figures 2A, 2B, 2C).

On the contrary, the *Acanthamoeba* isolates had trophozoites ranging from 20-30 µm in size. The cysts were variable in size and could be categorised into 5 groups: 8-10 µm, 8-13 µm,10-13 µm, 10-15 µm and 10-20 µm (Table 4). They were variable in shape, with isolates consisting of only one-shape, such as star-shaped, round-shaped (Figure 2L), or mixtures of 2 to 5 shapes (Figures 2K, 2M) such as oval, triangular, rectangular, pentagonal, hexagonal and heptagonal. Almost all of the cysts had double walls with diameter of less than 18 µm. The above observation concurred with previous reports of the cysts characteristics from Group II and Group III species. According to Pussard & Pons (1977), species in Group 1 are designated on the basis of having a large cyst (18 µm or more) in comparison to that of species in the other groups. Species in Group II are less than 18 µm in diameter and characterised as having a wrinkled ectocyst and an endocyst which could be stellate, polygonal, triangular or oval. Group III species also exhibited cysts with less than 18 µm in diameter but the ectocyst is thin and smooth and the endocyst is usually round but may have 3-5 gentle corners. Detection of *Acanthamoeba* cysts with diameter ranging from 10-20 µm from culture plate of isolate CD43a suggests that this culture plate consisted of a mixture of

Group I and Group II species. Likewise, although the species of *Acanthamoeba* can be classified by the morphology of the cysts, studies have shown that the cysts morphology can change in response to the culture conditions (Visvesvara *et al.*, 1975; Stratford & Griffith, 1978; Armstrong, 2000). Therefore, the identification at species level needs further study by using other approaches such as polymerase chain reaction (PCR) and deoxyribonucleic acid (DNA) sequencing. The trophozoite stage showed a specific feature of acanthophodia (Figures 2G, 2J) especially when they adhered on the surface of agar culture. Some of these trophozoites also showed sluggish movement with the aid of non-active pseudopodia.

In summary, observation of both *Naegleria* and *Acanthamoeba* by microscopy has permitted the differentiation of both FLA at the genus level. There were striking differences between the trophozoites of *Naegleria* and *Acanthamoeba*. The trophozoites of *Naegleria* were smaller in size compared to those of *Acanthamoeba*. The lobopodia and acanthopodia structures were only exhibited by the trophozoites of *Naegleria* and *Acanthamoeba* respectively. In addition, *Naegleria* trophozoites moved in a unidirectional manner due to the activities of the pseudopodia, compared to those of *Acanthamoeba* which showed slow movement without any direction. The *Naegleria* cysts were also more uniform in shape (Figure 2D) and usually found accumulated in compact colonies compared to those of *Acanthamoeba* which had multiple shapes and in dispersed colonies (Figure 2K).

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