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Occurrence of *Acanthamoeba* genotypes in Central West Malaysian environments

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ABSTRACT

Acanthamoeba species are ubiquitous free-living protozoa that can be found worldwide. Occasionally, it can become parasitic and the causative agent of acanthamoebic keratitis (AK) and Granulomatous Amoebic Encephalitis (GAE) in man. A total of 160 environmental samples and 225 naturally-infected animal corneal swabs were collected for *Acanthamoeba* cultivation. *Acanthamoeba* was found to be high in samples collected from environments (85%, 136/160) compared to infected animal corneas (24.89%, 56/225) by microscopic examination. Analysis of nucleotide sequence of 18S rRNA gene of all the 192 cultivable *Acanthamoeba* isolates revealed 4 genotypes (T3, T4. T5 and T15) with T4 as the most prevalent (69.27%, 133/192) followed by T5 (20.31%), T15 (9.90%) and T3 (0.52%). Genotype T4 was from the strain of *A. castellanii* U07401 (44.27%), *A. castellanii* U07409 (20.83%) and *A. polyphaga*AY026243 (4.17%), but interestingly, only *A. castellanii* U07401 was detected in naturally infected corneal samples. In environmental samples, T4 was commonly detected in all samples including dry soil, dust, wet debris, wet soil and water. Among the T4, *A. castellanii* (U07409) strains were detected high occurrence in dry (45%) followed by aquatic (32.50%) and moist (22.50%) samples but however *A. castellanii* (U07401) strains were dominant in dry samples of soil and dust (93.10%). Subsequently, genotype T5 of *A. lenticulata* (U94741) strains were dominant in samples collected from aquatic environments (58.97%). In summary, *A. castellanii* (U07401) strains were found dominant in both environmental and corneal swab samples. Therefore, these strains are possibly the most virulent and dry soil or dusts are the most possible source of *Acanthamoeba* infection in cats and dogs corneas.

1. Introduction

Acanthamoeba is a free-living amoeba. It was isolated from a variety of environments throughout nature and man-made habitats. It possesses a very high tolerance level of temperature, osmolarity, oxygen, water chlorination and pH (Martinez and Janitschke, 1985; Shoff et al., 2008; Woese et al., 1990; Init et al., 2010). The changes in surrounding circumstances such as limited food source, less oxygen and increase or decrease temperature enhance *Acanthamoeba* feeding trophozoite to the resistant cyst stage. The double-walled cysts of *Acanthamoeba* were noted to be viable for more than 20 years in laboratory conditions (Mazur et al., 1995).

It was first detected in 1913 from debris, known as *Amoeba polyphagus* (Puschkarew, 1913) but later renamed as *Acanthamoeba polyphaga.* Subsequently, the *Acanthamoeba* genus was established in accordance with reclassification of *A. castellanii* from *Hartmanella castellanii* (Page, 1967). Currently, more than 20 species of *Acanthamoeba* were identified based on morphological criteria, particularly of the cyst stage (Visvesvara et al., 2007). However, only few species such as *A. castellanii, A. culbertsoni, A. hatchetti, A. healyi, A. polyphaga, A. rhysodes, A. astronyxis, A. divionensis, A. griffini* and *A. palestinensis* were identified to be pathogenic and potentially cause infections in humans and animals (Martinez and Janitschke, 1985; Ofori-Kwakye et al., 1986; Ledee et al., 1995; Visvesvara et al., 2007).

Acanthamoeba is gaining attention and recognized as pathogen after the first clearly identified *Acanthamoeba* infections of the CNS which

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were reported in the early 1970s (Robert and Rorke, 1973). *Acanthamoeba* invasion of the CNS is secondary to local infection elsewhere in the body (e.g. skin, mucosa or lung) and reaches the brain by way of the bloodstream, causes granulomatous amebic encephalitis (GAE) (Martinez, 1980) that was documented especially in immunocompromised individuals. In addition, *Acanthamoeba* can also cause *Acanthamoeba* keratitis when it invade the eye produced a chronic progressive ulceration of the cornea led to severe ocular pain and loss of vision (Nagington et al., 1974). Eye infection is always due to direct contact of the cornea with amoeba, which may be introduced through minor corneal trauma or by exposure to contaminated water or contact lenses. To date, *Acanthamoeba* keratitis cases are increasing dramatically due to wearing of contact lenses, especially soft contact lenses (Stehr-Green et al., 1989). Other possible risk factors include foreign objects (stone, sand, soil, etc.) lodge in the eye causing irritation and rubbing, provides minor injuries that may end up with *Acanthamoeba* keratitis.

The increase of *Acanthamoeba* infection has been so alarming that molecular study is needed to further recognizes the species complexity and phylogeny. Sequencing of 18S rRNA genes has established several genotypes including T1-T12 (Stothard et al., 1998), T13 (Horn et al., 1999), T14 (Gast, 2001), T15 (Hewett et al., 2003), T16 (Corsaro and Venditti, 2010), T17 (Nupraset et al., 2010), T18 (Qvarnstrom et al., 2013) and T19 (Magnet et al., 2014). Among these genotypes, T4 was determined to be the most predominant in environmental and clinical samples and the causative agent of different diseases (Booton et al., 2005). Other genotypes such as T2, T3, T5, T6, T10, T11, T12, T15 and T18 were also described as pathogenic and occasionally reported in clinical samples (Booton et al., 2005; Qvarnstrom et al., 2013; Stothard et al., 1998; Walochnik et al., 2008). Thus, the distribution of *Acanthamoeba* genotypes in the environments should be made known to the public especially the high-risk groups in order for them to take necessary precaution against the infection.

Animals are potentially at high risk of *Acanthamoeba* natural infections though rarely reported. Several CNS and multisystemic infections by this amoeba were detected in autopsy specimens of dogs (Ayers et al., 1972; Pearce et al., 1985; Bauer et al., 1993; Brofman et al., 2003; Dubey et al., 2005; Kent et al., 2011), Indian buffalo (Dwivedi and Singh, 1965), sheep (Fuentealba et al., 1992) and kangaroo (Norton and Harvey, 1993). *Acanthamoeba* naturally infected the corneas of stray cats cannot be excluded from a possibility of causing *Acanthamoeba* keratitis (Ithoi et al., 2013), although keratitis cases in cats were more often due to virus, bacteria and fungi infections (Doyle, 2009). Infected animals can potentially contribute in spreading the pathogenic *Acanthamoeba* by contaminating the domestic environment, enhancing the opportunity to infect humans. In this study, we reported the occurrence and distribution of *Acanthamoeba* genotypes obtained from cultivable *Acanthamoeba* isolates from environmental and naturally infected animal (cats and dogs) corneal swabs samples. The investigation is to increase awareness of the occurrence of potential pathogenic *Acanthamoeba*, especially among Malaysian clinicians, public health authorities and general communities.

2. Materials and methods

2.1. Sampling site and sample

Most of the selected sampling sites were located at Selangor State and Kuala Lumpur Federal Territory, representing the most developed region of Malaysia (Tables 1A and 1B). Sampling sites were selected based on convenience and potential implications to public health. Environmental samples were collected from PAWS (Performing Animal Welfare Society) animal shelter (dust and debris), 15 recreational natural-rivers (water, debris and wet soil), 15 children playgrounds (dry soil) and at University Malaya Medical Faculty (dust from both outdoor and indoor environments).

Table 1A
Sampling sites for environmental samples.

Latitude (°N)	Longitude (°E)	Sampling site
3.13044	101.55183	PAWS animal shelter
		Recreational river (n = 15)
3.11164	101.81316	1. Sungai Pangsun
3.02607	101.48040	2. Sungai Congkak
3.21842	101.86485	3. Sungai Lopo
3.29973	101.61925	4. Sungai Kancing
3.43173	101.65667	5. Sungai Sendat
3.24277	101.61101	6. Sungai Rumput
3.05847	101.87191	7. Sungai Tekala
3.36603	101.636.78	8. Sungai Serendah
3.45140	101.64208	9. Sungai Kedondong
3.59907	101.73736	10. Sungai Chiling
3.33183	101.70248	11. Sungai Tua
3.15528	101.89915	12. Sungai Gabai
3.21842	101.76676	13. Sungai Kemensah
3.29875	101.62025	14. Sungai Templer
3.23694	101.63777	15. Sungai FRIM
		Playground (n = 15)
3.11814	101.63216	1. Section 12, PJ
3.11614	101.63216	2. Section 4, PJ
3.08500	101.64405	3. SJKC Chen Moh, PJ
3.11814	101.63216	4. Section 5, PJ
3.31770	101.27431	5. TMN MawarPutih
3.09141	101.72390	6. SK Seri Bahagia
3.74769	101.05555	7. SK Bagan Terap
3.74463	101.05549	8. SRA Bagan Terap
3.72561	101.08083	9. KG Bagan Terap
3.74769	101.05555	10. SMK Bagan Terap
3.72846	101.07302	11. SK Tok Kharifah
3.09337	101.68302	12. SMK Sri Sentosa
3.11078	101.61733	13. SMK Petaling
3.14775	101.77386	14. SK Taman Kosas
3.14795	101.77224	15. SMK Taman Kosas
3.11363	101.65314	Medical Faculty, UM

Table 1B
Sampling sites for corneal swab samples.

Latitude (°N)	Longitude (°E)	Sampling site
3.13044	101.55183	1. PAWS animal shelter
3.12473	101.65340	2. University Malaya
3.16046	101.69488	3. Chow Kit
3.16361	101.70967	4. KG Baru
3.18767	01.70469	5. Setapak
3.19897	01.73957	6. Wangsa Maju
3.22376	01.72533	7. Taman Melati
3.19834	101.68690	8. Sentul
3.15880	101.75745	9. Ampang
3.10709	101.60825	10. Petaling Jaya
3.09060	101.52958	11. Shah Alam
3.04390	101.58065	12. Subang Jaya
2.71861	101.94055	13. Ampangan
2.53642	101.80681	14. Port Dickson
2.73892	101.14114	15. Ulu Bendul
2.54354	102.16913	16.Gunung Datuk
2.86441	102.09411	17. Jeram Toi
2.84752	101.82610	18. Taman Mutiara
2.69029	101.90771	19. Seremban-2

The sampling sites at the PAWS were at the animal bedding areas (dust), the surface of animals' food and drinking containers (moist debris of these containers). While for the rivers, there were at the river side (wet soil), the wet surface of stone/rock that scattered along the river (wet debris was collected from the water margins of rock-surface) and the middle area of the river (running surface water). As for the children playground (dry soil), the selected site was at the area with many children were seen playing. At the University Malaya Medical Faculty, the sampling site for dust collection was at the wall surface of the buildings adjacent to the car parking areas (outdoor dust). While

the indoor dust samples were collected from the wall surface inside the lecture halls, seminar rooms and undergraduate laboratories.

No permits were required to collect water, debris, soil or dust samples from all of these public sampling sites. However, representatives from our research group showed a letter entitled 'request for permission to collect samples' from the Principle Investigator of this project to the authorities or the security personnel concerned, followed with the explanation of the study objectives.

While animal samples (corneal swab) were collected from infected stray cats and dogs with ocular signs. Both of these stray animals were from PAWS Animal Welfare. However, we also extended the collection of the infected stray cat samples from various areas as stated in Table 1B. This is due to the tame characteristic of the cat as compare to dog. Concerning PAWS Animal Welfare, it is located in Subang, approximately 15 km from Kuala Lumpur city. PAWS is a social house for unwanted, abandoned, injured and stray cats/dogs in the vicinity of Klang Valley (Kuala Lumpur and its adjoining areas in the state of Selangor). Most of these stray cats and dogs were brought in by the workers (dog catchers) of the Kuala Lumpur City Council to the PAWS. They were brought in twice a week, at the morning (~8.00–9.00 a.m.) and approximately 10–15 animals each time. Most of these newly sent animals were with several signs of skin diseases as well as ocular infections. However, they were then treated accordingly and taken care by the volunteer personals including the veterinarian and animal lovers. Therefore, most of the eye swab samples were collected in newly sent stray animals (had not been treated) as compare to long time PAWS pets.

The protocol of this study was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Malaya (UM IACUC), Kuala Lumpur [Ethics Reference number: PAR/29/06/2012/II (R)]. Written permission was also obtained from the management authorities of PAWS Animal Welfare Society. The objectives and protocols of the research were thoroughly discussed with the authorities in charge or animals owners.

2.2. Collection and cultivation of sample

A total of 500 mL of surface water samples from the middle of rivers were collected in sterile Schott glass-bottle from each of the 15 recreational natural-rivers and were first filtered through a nitro-cellulose membrane (1.2 μm pore size, Millipore). The trapped debris was flushed *in situ* with 3.0 mL of sterile distilled water into a sterile test tube. The debris suspension was pipetted with the disposable pipette and dropped 1 mL onto each of 3 non-nutrient agar (NNA) plates lawned with live *Escherichia coli,* (JM109, Promega, USA). Meanwhile, the debris, soil and dust samples were collected by swabbing using a sterile cotton-bud (3 cotton-buds from each sampled site) and placed onto 3 NNA-*E. coli* plates as above. Prior to the swab collection, the packaging of the cotton-bud was first prepared in a 15 mL test tube (Ithoi et al., 2013) that consists of cotton-bed, cotton-bud, 1.0 mL of normal saline and sterilized by autoclaved at 15 lbs pressure, 121 °C for 15 min. The sterile packaging was kept in the refrigerator until used.

The cold packaging tubes were placed on ice cubes in a covered polystyrene box (~10 ± 2 °C) and transported to the sampling site at PAWS as early as 7.00 a.m. after received appointment from PAWS operator. The infected animals with ocular signs of exudation or pus discharge, swollen eye lids, excessive tearing and white membrane on the eye surface were selected. This respective eye signs can be unilateral or bilateral but in this study all were seen unilateral. No anesthesia was use but the animals were physically restrained by a skilled operator from Kuala Lumpur City Council. Subsequently, the inferior eyelid was everted to prevent from blinking and the specimen was collected.

A cold wet cotton-bud was carefully withdrawn from packaging by holding the cotton-bud handle followed by swabbing the infected cornea as documented by Lamagna et al. (2015) with modification. The swabbing was carried out on the cornea in a single direction for at least five times and then lightly rolled up to the cotton bud that was then carefully placed back to its original packaging test tube. [The cold wet cotton-bud may induce retraction of protruding acanthopodia. This will cause trophozoite to become rounded, less adherent to the corneal surface and easier to be swab out. Therefore, using cold wet cotton-bud may increase the possibility in getting positive *Acanthamoeba* after cultivation of corneal swab samples (Ithoi et al., 2013)].

Samples were then transported to the Laboratory at the Department of Parasitology, University of Malaya. A swabbed cotton-bud and a cotton-bed in the packaging tube were carefully withdrawn and placed onto the surface of NNA-*E. coli.* The culture plates were sealed with parafilm and incubated at room temperature (26 ± 2 °C) for up to 14 days. Sets of negative controls were included in each experiment by inoculating with a non-swabbed cotton-bud and cotton bed from packaging onto NNA-*E. coli* plates and incubated as for the test samples.

2.3. Detection of Acanthamoeba by morphological and molecular techniques

All culture plates were observed daily for up to 14 days using light inverted microscope (Olympus BX51) under 200 followed by 400 times magnification before being discarded. *Acanthamoeba* trophozoites were identified by the presence of spike-like pseudopodia called acanthopodia and cysts with the present of wrinkle double walled. Both trophozoite and cyst characteristics were examined by using light microscope after a thin smear preparation of suspected *Acanthamoeba*-positive agar. The surface agar with positive *Acanthamoeba* by inverted microscope was marked circle on the bottom of the plate with a marker pen. The lid of the plate was then opened and the surface agar at marking area was gently scrapped with cover slip. It was then placed upside down and pressed the gel on the glass slide evenly, then followed by examined under a light microscopic with 1000 times magnification. Positive culture plate was then sub-cultured at least ten times. The subculture was carried out by placing a colony of 4–6 cysts onto a newly prepared NNA-*E. coli*, to achieve homogeneous cells culture. The trophozoites from individual isolate were then cultured in at least 5 plates to yield at least 5×10^3 cells and were harvested for total DNA extraction using the QIAamp DNA mini kit (Qiagen).

Acanthamoeba genus-specific primers, forward JDP1 (5′- GGG CCC AGA TCG TTT ACC GTG AA −3′) and reverse JDP2 (5′- TCTC ACA AGC TGC TAG GGG AGT CA −3′) were used in the amplification reactions as described by Schroeder et al. (2001). PCR amplicons with expected size ranges from 423 to 551 bp (known as ASA.S1) in the 18S rRNA gene were electrophorese on a 1.5% agarose gel and TBE (0.5 x Tris-borate EDTA) buffer containing 0.5 μg/ml ethidium bromide. The gel was then visualized under UV illumination chamber BioDoc-It™ Imaging System (UVP, United Kingdom). Amplicon sizes were estimated by comparing with the GeneRuler 100 bp DNA ladder Plus (Fermentas).

The ASA.S1 amplicons from representative isolates (192) were gel-purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany), then cloned using InsT/Aclone™ PCR product cloning kit (Fermentas). The ASA.S1 or diagnostic fragment 3 (DF3) was inserted into vector (plasmid pTZ57R/T) to form a DNA recombinant. The recombinant molecules were transformed into *Escherichia coli* (strain JM109) followed by selection of the white colonies carrying recombinant plasmids with a disrupted β-galactosidase gene.

The plasmid DNA in selected recombinant white colonies were confirmed by PCR amplification and were gel-purified using QIAprep® Miniprep kit (Qiagen, Hilden, Germany), followed by sequencing at both strands using the amplification primers in an ABI PRISM™Bigdye ™ terminator cycle sequencing ready reaction kit V.3.1 (Korea). The obtained sequences were aligned using the ClustalW2 software (Labarga et al., 2007). Each consensus sequence was blasted against all eukaryotic nucleotide sequences retrieved in the GenBank database (Altschul et al., 1990) to detect the nucleotide similarities. Phylogenetic analyses were performed based on the DF3 sequences of both our

Table 2A
Number and percentage of cultivable *Acanthamoeba* in environmental samples.

Location	Samples	Cultivable *Acanthamoeba* (%)
PAWS animal shelter		
i. Bedding	20	14 (70.0)
ii. Food containers	20	17 (85.0)
iii. Drink containers	20	9 (45.0)
15 Recreational rivers		
i.Water samples	15	15 (100)
ii.Swabs (rocks & stones)	15	11 (73.33)
iii.Wet soil	15	15 (100)
15 Children playgrounds		
Dry soil	15	15 (100)
Medical Faculty, UM		
i. dust from indoor wall surface	20	20 (100)
ii. dust from outdoor wall surface	20	20 (100)
Total	160	**136 (85.0)**

isolates and references published genotypes using neighbour-joining of MEGA version 4 software (Tamura et al., 2007). This was followed by Kimura 2-parameter algorithm and constructed tree by bootstrap analysis of 1000 replicates. The data on DNA sequencing of all these isolates (192) were grouped (11) according to their homology as GD1, GD2, GD3, GD4, GD5, GD6, GD7, GD8, GD9, GD10 and GD11; and have been deposited in GenBank with the accession numbers MF100899, MF100900, MF100901, MF100902, MF100903, MF100904, MF100905, MF100906, MF100907, MF100908 and MF100909, respectively.

3. Results

3.1. Cultivable Acanthamoeba as detected by microscopy

A total of 160 environmental samples and 225 corneal swabs (200 from cats and 25 from dogs) were collected as stated in Tables 2A and 2B. Of these total samples, 192 stable *Acanthamoeba* isolates were successfully isolated and designated as in Table 3. *Acanthamoeba* was highly detected in all the environmental samples with 100% positivity in surface water, wet soil, dry soil, dust samples and a bit lower in debris from the surface of river stones (73.33%), drinking containers (45.0%), food containers (85.0%) and bedding areas (70.0%). Meanwhile, for the infected corneal samples, there were 24.89% positive with *Acanthamoeba* of which both young and adult animals were included.

3.2. Molecular analysis of Acanthamoeba isolates base on DF3 sequence of 18S rRNA gene

All 192 *Acanthamoeba* isolates were subjected for PCR amplification using JDP primer set showed positive results by producing amplicons

Table 2B
Number and percentage of cultivable *Acanthamoeba* from corneal swab.

Animal corneal sample	Gender	Positive *Acanthamoeba* (%)		
		Adult	Kitten	Total
Cats (n = 200)	Male	5 (2.5)	10 (5.0)	15 (7.50)
	Female	14 (7.0)	19 (9.50)	33 (16.50)
Total		19 (9.50)	29 (14.50)	48 (24.0)
			Puppy	
Dogs (n = 25)	Male	1 (4.0)	2 (8.0)	3 (12.0)
	Female	1 (4.0)	4 (16.0)	5 (20.0)
Total		2 (8.0)	6 (75.0)	8 (32.0)
Grand total (N = 225)		21 (9.33)	35 (15.56)	56 (24.89)

Table 3
Groups and isolates of cultivable *Acanthamoeba* according to their source and DNA sequence similarity

Sub-group.	Sources and *Acanthamoeba* isolates
	PAWS (40 isolates):
P1	B1, B2, B3, B10, B11, B15, DC1, DC9, DC10, DC13, DC16, DC18, DC19, FC2, FC3, FC11 = 16
P2	B4, B8, B13, B17, B18, B19, DC15, DC16, FC6, FC7, FC8, FC9, FC14, FC17, FC18 = 15
P3	B12, B14, FC4, FC5, FC10, FC12, FC15, FC16, FC19 = 9 [dust from bedding (B); debris from food container (FC) and drinking container (DC)]
	15 Recreational rivers (41 isolates):
RW1	Water: w1, w2, w12, w14 = 4
RW2	: w3, w4, w5, w6, w7, w8, w9, w10,w11, w13, w15 = 11
RD1	Swab (Debris): d1, d2, d6, d7, d8, d9, d14, d15 = 8
RD2	: d3 = 1
RD3	: d4 = 1
RD4	: d5 = 1
RS1	Wet soil: s1, s2, s3, s4, s7, s8, s9, s10, s11, s12, s13, s14, s15 = 13
RS2	: s5, s6 = 2 [water (w); debris from rock surface (d); wet soil from the side of river 1–15 (s)]
	15 Children playground (15 isolates):
PS1	Dry soil: S1, S2, S4, S7, S8, S10, S12, S13, S14 = 9
PS2	: S3, S5, S6, S9, S11, S15 = 6
	Medical Faculty, University Malaya (40 isolates):
UID1	ID1, ID2, ID3, ID5, ID6, ID12, ID14, ID15, ID20 = 9
UID2	ID4, ID7, ID10, ID13, ID16 = 5
UID3	ID8, ID9, ID11, ID17, ID18, ID19 = 6
UOD1	OD1, OD2, OD3, OD4, OD15, OD18, OD20 = 7
UOD2	OD5, OD7, OD8, OD 9, OD10, OD12, OD19 = 7
UOD3	OD6, OD11, OD14, OD13, OD16, OD17 = 6 [dust from indoor wall (ID) and outdoor wall (OD)]
	Animal cornea (56 isolates):
AC1	CAM3-12, CAM11-35, CAF9-29, CAF6-22, CAF10-31, CKM3-15, CKM10-33, CKM11-38, CKM14-43, CKF1-9, CKF3-16, CKF6-23, CKF7-26, CKF11-40, CKF14-44, DAF1-2, DPM1-3, DPF1-6 = 18
AC2	CAM1-1, CAM3-13, CAM7-24, CAF1-2, CAF1-3, CAF1-4, CAF1-5, CAF1-6, CAF10-32, CAF11-36, CAF3-14, CAF11-37, CAF13-42, CAF15-46, CKM1-7, CKM1-8, CKM5-21, CKM9-30, CKM8-27, CKM11-39, CKF1-10, CKF2-11, CKF3-17, CKF3-18, CKF4-19, CKF4-20, CKF7-25, CKF10-34, CKF8-28, CKF12-41, CKF14-45, CKF19-47, CKF19-48, DAM1-1, DPM1-4, DPF1-5, DPF1-7, DPF1-8, = 38 [cat (C); adult (A); male (M); female (F); kitten (K); dog (D); puppy (P)]

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

with expected size ranges approximately between 400 and 500 bp known as ASA1 [or Diagnostic Fragment 3 (DF3) by Schroeder et al., 2001]. The obtained sequences from 192 isolates were aligned using the ClustalW2 software (Labarga et al., 2007) and were accumulated into 21 sub-groups (P1, P2, P3, RW1, RW2, RD1, RD2, RD3, RD4, RS1, RS2, PS1, PS2, UID1, UID2, UID3, UOD1, UOD2, UOD3, AC1 and AC2) according to their original source (site and sample) and DNA sequence homology. The designation and number of isolates in all these subgroups were summarized in Table 3.

Concurrently, the sequence from representative subgroups showed high homology (98–100%) with their closest matched reference of *Acanthamoeba* strains from GenBank, which were assemblage under genotype T1 to T19. The DNA sequences of isolate clones under subgroup RD2 showed 100% homology with their closest match reference, *A. griffini* (KJ446979) under genotype T3. A 100% homology with genotype T4 such as *A. castellanii* (U07409), *A. polyphaga* (AY026243), *A. castellanii* (U07401) were also observed in other groups such as GD2 (P2, RS1, UID3, UOD3), GD3 (RD4, UOD1) and GD4 (PS1, UID2, UOD2, AC1), respectively. Subsequently, 99% homology with genotype T4, *A. castellanii* (U07401), was observed in groups GD5 due to one base

Table 4
Homo- and hetero-geneity of cultivable isolates against reference *Acanthamoeba* species retrieved from GenBank.

Group (%)	Sub-group (n isolates)	homology with reference, bp number, Genotype, (total isolates detected)	Genotype (%)	BI	BS	No. of BI/BS/BD
GD1 (0.52)	RD2 (1)	100%, *A. griffini* (KJ446979), 466 bp, (1)	T3 (0.52)			
GD2 (20.83)	P2 (15) RS1 (13) UID3 (6) UOD3 (6)	100%, *A. castellanii*, (U07409), 463 bp, (40)	T4 (69.27)			
GD3 (4.17)	RD4 (1) UOD1 (7)	100%, *A. polyphaga*, (AY026243), 455 bp, (8)				
GD4 (20.31)	PS1 (9) UID2 (5) UOD2 (7) AC1 (18)	100%, *A. castellanii*, (U07401), 459 bp, (39)				
GD5 (23.95)	RS2 (2) PS2 (6) AC2 (38)	99%, *A. castellanii* (U07401), 459 bp, (46)			$A^{354} \rightarrow G$	1BS
GD6 (8.33)	P1 (16)	99%, *A. lenticulata* (U94741), 424 bp, (16)	T5 (20.31)	$-^{30} \rightarrow C$ $-^{55} \rightarrow C$ $-^{120} \rightarrow C$		3B1
GD7 (6.25)	RW1 (4) RD1 (8)	99%, *A. lenticulata* (U94741), 420 bp, (12)			$G^{12} \rightarrow T$ $G^{54} \rightarrow A$	2BS
GD8 (5.73)	RW2 (11)	98%, *A. lenticulata* (U94741), 420 bp, (11)			$A^{9\rightarrow}C$ $G^{10\rightarrow}A$ $C^{15\rightarrow}T$ $A^{16\rightarrow}C$ $G^{54\rightarrow}A$ $G^{346\rightarrow}A$ $A^{368\rightarrow}G$	7BS
GD9 (4.69)	P3 (9)	99%, *A. jacobsi*, (KC164249), 459 bp, (9)	T15 (9.90)		$C^{372} \rightarrow G$ $T^{393} \rightarrow C$	2BS
GD10 (0.52)	RD3 (1)	99%, *A. jacobsi*, (KC164249), 459 bp, (1)			$C^{381} \rightarrow T$ $T^{393} \rightarrow C$	2BS
GD11	UID1 (9)	99%, *A. jacobsi*, (KC164249), 459 bp, (9)			$G^{367} \rightarrow C$	1BS

Designation: GD (grand group), BI (base insertion), BS (base substitution), G (guanine), C (cytosine), A (adenine), T(thiamine), base deletion (BD) is excluded due to no finding in this study. *Acanthamoeba* isolates and their sub-groups were designated as in Table 3.

Table 5
Occurrence of *Acanthamoeba* genotypes and strains from environmental samples.

Group	Sub-group (n)	homology with reference, base pair number, Genotype, (n), genotype	Environmental samples, n (%)		
			Aquatic (water, watery soil/ debris)	Moist (moist debris)	Dry (dust, soil, debris)
GD1 n = 1	RD2 (1)	100%, *A.griffini* (KJ446979), 466 bp, (1), T3	1 [100%]		
GD2	RS1 (13)		13		
	P2 (15)	100%, *A. castellanii*, (U07409),		9	6
	UID3 (6)	463 bp, (40), T4			6
	UOD3 (6)	[40/77, 51.95%]	[13/40, 32.50%]	[9/40, 22.50%]	6 [18/40, 45.0%]
GD3	RD4 (1)	100%, *A. polyphaga*, (AY026243),	1		
	UOD1 (7)	455 bp, (8), T4 [8/77, 10.39%]	[1/8, 12.50%]		7 [7/8, 87.50%]
GD4	PS1 (9)				9
	UID2 (5)	100%, *A. castellanii*, (U07401),			5
	UOD2 (7)	459 bp, (21), T4			7
GD5	RS2 (2)	99%, *A. castellanii* (U07401),	2		
n = 77	PS2 (6)	459 bp, (8), T4 [29/77, 37.66%]	[2/29, 6.90%]		6 [27/29, 93.10%]
GD6	P1 (16)	99%, *A. lenticulata* (U94741), 424 bp, (16), T5		10	6
GD7	RW1 (4)	99%, *A. lenticulata* (U94741),	12		
	RD1 (8)	420 bp, (12), T5			
GD8	RW2 (11)	98%, *A. lenticulata* (U94741),	11		
n = 39		420 bp, (11), T5	[23/39, 58.97%]	[10/39, 25.64%]	[6/39, 15.38%]
GD9	P3 (9)	99%, *A. jacobsi*, (KC164249), 459 bp, (9), T15		7	2
GD10	RD3 (1)	99%, *A. jacobsi*, (KC164249), 459 bp, (1), T15	1		
GD11	UID1 (9)	99%, *A. jacobsi*, (KC164249),			9
n = 19		459 bp, (9), T15	[1/19, 5.26%]	[7/19, 36.84%]	[11/19, 57.89%]

n = total number of *Acanthamoeba* regarding to their strain and genotype

substitution ($A^{354} \rightarrow G$). Two of the groups (GD6 and GD7) showed 99% homology with *A. lenticulata* (U94741), genotype T5, due to insertion 3 bases ($-^{30} \rightarrow C$, $-^{55} \rightarrow C$ and $-^{120} \rightarrow C$) and substitution of 2 bases ($G^{12} \rightarrow T$ and $G^{54} \rightarrow A$), respectively. While group GD8 showed 98% homology with *A. lenticulata* (U94741), genotype T5, due to 7 bases substitution. Similarly, groups GD9, GD10 and GD11 respectively showed 99% homology with *A. jacobsi*, (KC164249), genotype T15, due to the differences in base substitutions. Subsequently, genotype T4 was detected in both environmental and corneal swab samples with the highest prevalent of 69.27%. The other three genotypes which were T5 (20.31%), T15 (9.90%) and T3 (0.52%) were only detected in environmental samples (Table 4). Among genotype T4, *A. castellanii* (U07401) strains were detected the highest percentage in dry samples of dust and soil (93.10%), and apparently the only strains found in corneal swab samples. While for T5 *A. lenticulata* (U94741) strains were detected high in samples collected from aquatic (58.97%) followed by moist (25.64%) and dry (15.38%) environments (Table 5). Genotype T3 of *A.griffini* (KJ446979) strain was the least detected which only in one debris sample from the stone surface of Sungai Lopo, a natural recreational river in Selangor state, Malaysia.

3.3. Phylogenetic analyses

The DF3 region of 18S rDNA gene sequences from *Acanthamoeba* isolates were summarized into 11 groups (GD1, GD2, GD3, GD4, GD5, GD6, GD7, GD8, GD9, GD10 and, GD11). They were used to construct the phylogenetic tree to illustrate the relationships between the test and reference sequences of *Acanthamoeba* genotypes T1-T19 (Table 6) retrieved from GenBank. The relationships among these isolates were examined by using neighbour-joining (NJ) analysis as showed in Fig. 1. Each genotype is seen strongly supported by moderate to high bootstrap values (bootstrap values from 56 to 100%). The tree showed multiple species of genotypes T3/T4/T11, T2/T6, T10/T12 and T9/T17 clumped together in same clade while genotypes T1, T5, T7, T8, T13, T14, T15, T16, T18 and T19 were placed in their own clade.

Concurrently, test isolate(s) under GD1 (*A. griffin,* T3), GD2 (*A. castellanii* U07409, T4), GD3 (*A. polyphaga,* T4), GD4 and GD5 (*A. castellanii* U07401, T4) were seen as polyphyletic as they were grouped in the same clade clustered with each other with high bootstrap value. Similarly, *A. castellanii* and *A. polyphaga* groups were assemblage under genotype T4 and have a very close relationship with the bootstrap value of 93%. Furthermore, *A. castellanii* strains were divided into two groups with similar bootstrap value of 100%. Subsequently, GD6, GD7 and GD8 were assemblage under genotype T5 (*A. lenctiulata*) and GD9, GD10 and GD11 were under genotype T15 (*A. jacobsi*) in which each of these genotypes clustered independently in their own clade (monophyletic group).

4. Discussion

In this study, sampling sites for environmental samples (water, soil, debris and dust) were selected based on places that were highly engaged with human activities and also accessible to stray animals especially cats and dogs. However, for stray dogs, corneal swab samples were only collected from PAWS animal shelter. The dogs were aggressive and needed experience assistant (dog catcher) to restrain on this stray during sampling. Therefore, dog samples were limited and could not be carried out without cooperation from experienced assistant. As for stray cats, their tame character made sampling easier thus was carried out in many other areas (Table 1B), whenever found with any eye sign such as exudation or pus discharge, swollen eye lids, excessive tearing and white membrane on the corneas. In this study, these signs were respectively seen only in unilateral eye of cats and dogs. Many workers documented that these signs were usually caused by several microorganisms such as herpes simplex virus (Martinez and Visvesvara, 1997), bacterial (*Pseudomonas aeruginosa* (Clarke and Niederkorn, 2006) and *Staphylococcus* (Giese and Weissman, 2002)), fungi [*Aspergillus flavus*, *Aspergillus fumigatus*, *Fusarium* spp., *Alternaria* spp.] and yeast [*Candida*] (Halde, 1986)]. However, all of these organisms were not detected as they were not part of this study objective.

To date, there is no conclusive report on the *Acanthamoeba* keratitis (AK) case in animal. Similarly, the warning sign due to *Acanthamoeba* infection in animal eye have not been establish although it was stated that the presence of *Acanthamoeba* could be associated with the above mentioned eye signs in stray cats (Ithoi et al., 2013). However, *Acanthamoeba* was detected in brain autopsy specimens of several animals that died due to granulomatous amoebic encephalitis (GAE), including gorillas, monkeys, dogs, ovine, bovine, horses, kangaroos as well as birds, reptiles, amphibians, fishes and even invertebrates (Visvesvara and Stehr-Green, 1990; Dykova et al., 1999; Visvesvara et al., 2007).

In environmental samples, cultivable *Acanthamoeba* was found highly prevalence (45.0% − 100%) in all locations and specimens including water, wet soil, wet debris, moist debris, dry soil and dust. High prevalence (24.89%) was also found in infected corneal swabs of stray

Table 6
Acanthamoeba GenBank reference sequences used in construction of phylogenetic tree.

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

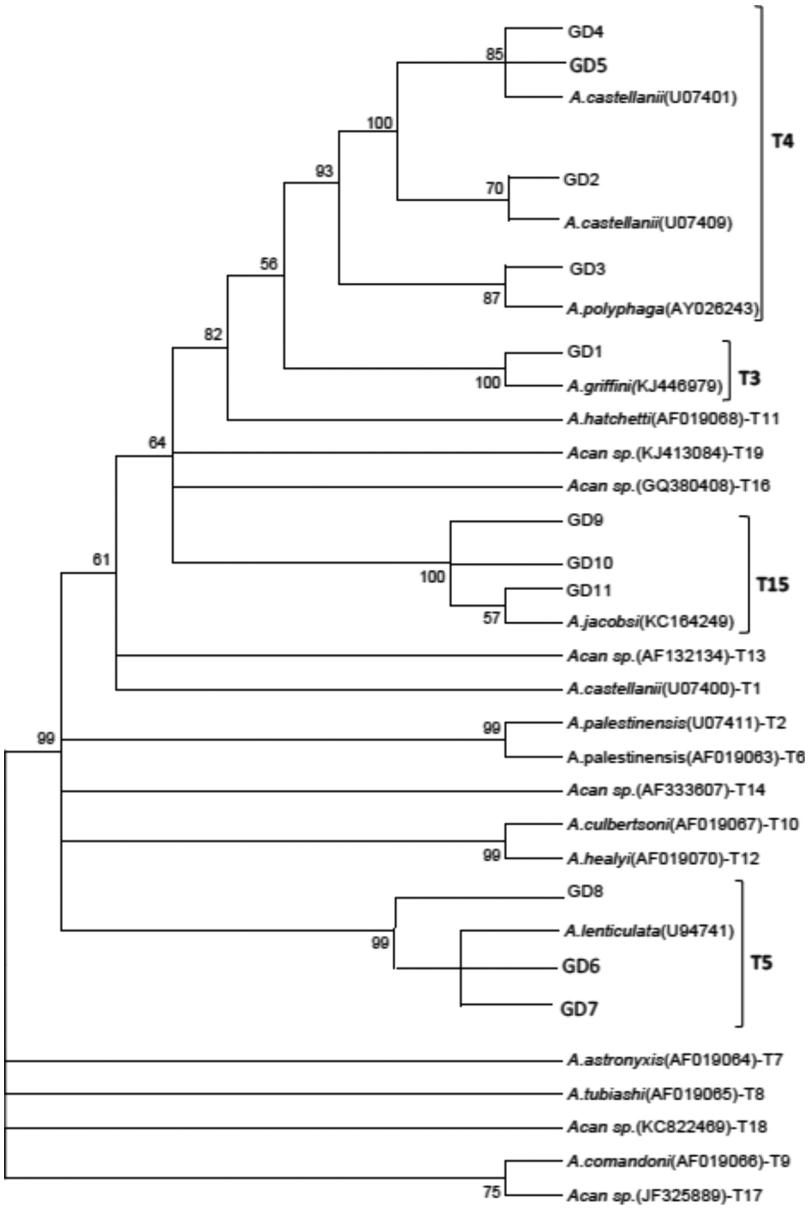

Fig. 1. Neighbor-joining tree depicting the relationships between test isolates and reference strains representing genotypes of *Acanthamoeba*. Numbers at the nodes are percentage-bootstrapping value on 1000 replicates. GenBank accession numbers and genotypes for reference sequences are indicated at the ends of the *Acanthamoeba* isolates designations.

animals of which 24.0% in cats and 32.0% in dogs. However, the prevalence of *Acanthamoeba* in dogs may not represent the correct value due to small sample size (only 25 samples) and this was our limitation in getting infected corneal swab especially from dogs during this study. Meanwhile, from a molecular method, all the genotypes detected in this study (T3, T4, T5 and T15) were based on the entire dataset of diagnostic fragment 3 (DF3) region of *Acanthamoeba* 18S rRNA gene. The DF3 is one out of the three highly variable portions of the 18S rDNA (Gast, 2001; Stothard et al., 1998) and was noted to be the best to use in constructing phylogenetic tree that shows the genetic variability among isolates. Its topology showed a good target to locate intra-genotype differential variation rather than a tool for genotype assignment (Gast, 2001; Stothard et al., 1998; Cave et al., 2009). Consequently, the genotyping data based on DF3 sequences revealed clades that were associated with AK (T2, T3, T4, T5, T6 and T11) and GAE (T1, T4, T10, T12 and T18) (Ledee et al., 2003; Qvarnstrom et al., 2013).

Acanthamoeba genotype T3 (isolates in group GD1) has 100% homology with *A. griffini* KJ446979 was found in a single debris sample (RD2-d3) from recreational river, Sungai Lopo. *A. griffini* was reported to be commonly found in tap-water sources (Edagawa et al., 2009) and freshwater environment (Lorenzo-Morales et al., 2006). Furthermore, it was detected in many human amoebic keratitis worldwide (Maghsood et al., 2005). Several studies have determined that genotype T3 contributed to human AK by contaminating contact lens/cases (Booton et al., 2002, 2005; Yera et al., 2008).

Genotype T4 has the highest percentage (69.27%) followed by T5 (20.31%) and T15 (9.90%). Genotype T4 comprises of several species and strains which respectively were 100% (isolates in group GD4) and 99% (GD5) homology with *A. castellanii* U07401; isolates under group GD2 were 100% homologous with *A. castellanii* U07409 and GD3 had 100% homology with *A. polyphaga* AY026243. The isolates particularly under groups GD4 and GD5 (that were homologous with *A. castellanii* U07401) showed the highest prevalence (44.27%) and they were detected in dry soil, dust at the indoor and outdoor walls as well as infected animal corneal swabs. Group GD2 showed 20.83% prevalence and was detected in the dust from animal bedding, moist-debris from food-containers in PAWS shelter, wet-soil from riversides and dust from indoor and outdoor walls., *A. polyphaga* showed less prevalence (4.17%) and was detected in wet debris from stone surface and outdoor wall's dust. On the other hand, we also detected genotype T5 (GD6, GD7 and GD8) that showed 99% homology with *A. lenticulata* U94741 from rivers water, wet debris from stone surfaces, dust from animal

bedding and moist debris from drinking/food containers. Lastly, GD9, GD10 and GD11 of genotype T15 that showed 99% homology with *A. jacobsi* KC164249 were found in dust from animal bedding, moist debris from animal food containers, wet debris from stone surface and dust from indoor walls.

The above results fully dependent on cultivable *Acanthamoeba* and there is a high possibility that not all *Acanthamoeba* have good growth in *in-vitro* cultivation. Therefore, the actual existence of *Acanthamoeba* might be more in terms of prevalence and genotypes if sample collection was repeated and a more sensitive diagnostic method such as RT-PCR was used., However, this result gave us information of the predominant genotypes T4 in wet and dry samples while T5 was in aquatic and moist debris.

Concurrently, T4 was also reported to be the most commonly detected genotype in wet and dry environments worldwide (Fuerst et al., 2003; Lorenzo-Morales et al., 2006; Maghsood et al., 2005; Niyyati et al., 2009). In addition, it was also the most frequently reported genotype in clinical cases such as in patients with AK and GAE (Booton et al., 2005). Evidence of *Acanthamoeba* genotype T4 contributing to humans AK has been reported worldwide (Spanakos et al., 2006; Booton et al., 2002; Yera et al., 2007, 2008; Maghsood et al., 2005). Booton et al. (2005) reported that 72% (179/249) of AK cases was due to genotype T4 and it was detected in 53% of environment samples. Genotype T4 was also predominantly detected in central nervous systems (CNS) of immunocompromised patients, causing GAE (Martinez and Janitschke, 1985).

Among all the isolate groups and genotypes detected, only GD4 and GD5 (homologous with *A. castellanii* U07401, T4) occurred in both environmental and animal corneal samples. Therefore, these groups of isolates of genotype T4 are capable of living on animal corneal surface as well as in the soil ecosystem (viable in dry heat of the sun). They can accumulate together with dust particles and subsequently transferred by blowing wind as an outdoor and indoor dust. As for the corneal infections acquired by stray animals (cats/dogs), it could be due to the behavior of the animals that caused the soil/dust particles to enter their eyes. Their paws that were used for cleaning their face would probably be contaminated with *Acanthamoeba* cysts/trophozoites that can be transferred onto their eyes. Other cause is likely to be when the animals fight with each other leading to injury in the eyes thus encouraging *Acanthamoeba* infection. Eye infection due to *Acanthamoeba* usually occurs in one eye but can occur bilaterally (Lee and Gotay, 2010).

Subsequently, the strains of *A. castellanii*,U07401 (GD4-AC1 and GD5-AC2) were detected in all characteristics of young, adult, male and female of both animals. Transmission of *Acanthamoeba* is always correlated with low immunity and corneal injury/trauma as compared to poor hygiene or dirty environment (Ithoi and Ahmad, 2013). Younger animals (kittens and puppies) having less immune protection, coupled with active movement (playing), may also be a frequent cause in getting contaminated foreign particles (soil/dust-*Acanthamoeba* cyst) to enter their eye. In addition, these strains of *Acanthamoeba* may have been involved with contaminating contact lens and caused keratitis in Malaysian cases. Further prospective controlled study on human corneal sample is needed to confirm of this statement.

Genotype T5 (isolates from GD6, GD7 and GD8 groups) that showed 99% homology with *A. lenticulata* U94741 were seen dominant in aquatic environments (58.97%, in water from all 15 rivers and debris on stones surfaces) followed by moist samples (25.64%, in debris from drinking/food containers) and less in dry area (15.38%, in dust from animal bedding). None of the T5 was detected in dry soil from children playground and dust samples from indoor/outdoor walls surfaces, suggesting that this genotype is less resistant to dryness or heat (from sun light). Genotype T5 preferred habitat that is moist/wet conditions including nasal mucosa cavity (Cabello-Vílchez et al., 2013; Khan, 2006) and those sites that have high moisture content including central air-conditioners (Chan et al., 2011). It was also reported to cause human keratitis (Spanakos et al., 2006; Ledee et al., 2009) and in immunosuppressed, as such documented in a fatal disseminated of heart transplant patient (Barete et al., 2007). Therefore, high occurrence of genotype T5 in aquatic environments may possibly play an important role as a potential source of *Acanthamoeba* infection to those involved with water related activities, especially immunosuppressed individuals.

Regarding T15 *A. jacobsi*, their occurrences in domestic environments are still lacking except in water samples (Hewett et al., 2003; Evyapan et al., 2014; Corsaro et al., 2017). However, in this study we found T15 [the strains of *A. jacobsi* (KC164249), isolates under groups GD9, GD10 and GD11] was dominant in dry samples (57.89%, dust from indoor and animal bedding) followed by moist samples (36.84%, debris from animal food containers) and aquatic (5.26%, one debris samples from stone surface). It was not detected in the playground's dry soil and outdoor dry dust samples. This result may due to the resistant of T15 cyst to the dryness but not under the continuous sun heat. Probably, the T15 cyst-dust particles that accumulated in indoor environments can easily transform to trophozoite stage when its growth requirements are met in the moist condition (such as food/drinking containers of cats/dogs that is rich with their food source). *A. jacobsi,* T15 which was previously reported as non-keratitis causing strain (Flint et al., 2003), until recently the clinical isolate of T15 was reported in Italy and this finding represents the first association of T15 with AK in human (Cave et al., 2009).

The occurrences of AK cases in humans were rare and usually due to accidental findings (Christopher and Stuart, 1998; Ledbetter, 2011). In the recent years, however, cases of AK have risen worldwide, parallel with the increasing use of contact lens wear. In this study, all four detected *Acanthamoeba* genotypes (T3, T4, T5 and T15) have been reported as having pathogenic characteristics that eventually cause AK and GAE in humans. Therefore, the presence of these genotypes in our environment could be considered as a public health issue and should be made known especially to high-risk group or populations, such as contact lens wearers and immunocompromised patients. Awareness on the pathogenicity of *Acanthamoeba* infections is important in health control program in both humans and animals.

Health education on environmental (water, soil and dust) and physical (corneal trauma and immunosuppressed) risk factors of *Acanthamoeba* infection should be promoted to the public especially to those who choose to wear contact lens. As for pet cats or dogs, veterinarians should consider *Acanthamoeba* along with virus, bacteria and fungi in the diagnosis of eye or brain infection. The owner of these pets must always keep their pets in a good and clean environment in their homes, with appropriate cages to minimize the exposure with infected stray animals and contaminated environments.

In conclusion, only one strain (*A. castellanii*, U07401) was detected from infected corneal samples of stray cats and dogs. Therefore, this strain is believed to be the most virulent than many other pathogenic strains of *Acanthamoeba* found in Malaysian environment. Dry soil and dust are the most possible source of *Acanthamoeba* infection in cats and dogs.

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