Tropical Biomedicine 30(1): 131–140 (2013)

Acanthamoeba genotype T4 detected in naturally-infected feline corneas found to be in homology with those causing human keratitis

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Received 2nd October 2012; received in revised form 20 January 2013; accepted 24 January 2013

Abstract. A total of 10 out of 65 cornea swab samples from cats with eye symptoms showed *Acanthamoeba*-like morphology after cultivation. By PCR and DNA sequencing of *Acanthamoeba* diagnostic fragment 3 (DF3), all 10 isolates from the positive samples were categorized into two homologous groups of AfC1 (PM1, PM2, PM3, PF6, KM7, KF8, KMK9) and AfC2 (PM4, PM5, KFK10) due to the presence of bases A^{354} and G^{354}, respectively. Furthermore, DF3 of AfC1 and AfC2 showed 100% similarity with Genbank reference isolates with the accession numbers DQ087314, EU146073 and U07401, GU808323, which were *Acanthamoeba castellanii* strains genotype T4 originating from human keratitis. This finding suggests that *A. castellani strains* have the capability to infect cats and human under favorable conditions.

INTRODUCTION

Acanthamoeba is one of the free-living bacterivores in nature and it has been isolated from diverse habitats worldwide including soil, sand, water, dust, air, etc. ranging from the tropics to the arctic regions. Acanthamoebiasis in animals was noted in wild squirrels (Lorenzo-Morales *et al.*, 2007), dogs, monkeys, a bull, a kangaroo and an Indian buffalo (Schuster & Visvesvara, 2004). In addition, most of the reported cases in dogs were acute to chronic infections of the central nervous system (CNS) (Ayers *et al.*, 1972; Pearce *et al.*, 1985; Bauer *et al.*, 1993; Brofman *et al.*, 2003) and multisystemic infections (Dubey *et al.*, 2005; Kent *et al.*, 2011). Eye infections are rarely reported, although fairly common especially in pets. Eye infections usually occur after the surface of the cornea is accidentally scratched or when hard particles lodge in the eyes. Common warning signs for eye infection in pets are eye discharge, squinting, redness, cloudiness, difficulty in keeping the eyelids open, and attempts to rub and scratch the eye. In felines, eye infections are frequently encountered in veterinary clinics and can be caused by a variety of pathogens including virus, bacteria and fungi (Doyle, 2009). However, other pathogens such as free living amoebae, especially the *Acanthamoeba* species, are rarely reported. To the best of our knowledge, *Acanthamoeba* has not been naturally isolated from animal eyes, including felines (*Felis domesticus*) except a patent note (Ledbetter, 2011).

In humans, *Acanthamoeba* is known as the causative agent of acanthamoebic keratitis (AK) when it directly infects the corneas of the eyes following a trauma through exogenous sources (Jones *et al.*, 1975) and occurs mostly in wearers of all types of contact lens (Stehr-Green *et al.*, 1989). Microtrauma to the corneal epithelium is assumed to be an important factor in facilitating the invasion of *Acanthamoeba* (Kinnear, 2004). Others are opportunistic

systemic infections, including fatal acanthamoebic granulomatous encephalitis (AGE), nasopharyngeal and cutaneous infections (Marciano-Cabral & Cabral, 2003). *Acanthamoeba* infections in humans are increasing and occurring worldwide. Infected pets could potentially contribute to the increase of pathogenic *Acanthamoeba* by contaminating the domestic environment and opens an opportunity to infect humans. Therefore, information on the occurrence of this amoeba in domestic animals is needed for the improvement of healthcare structures in both humans and animals.

MATERIAL AND METHODS

Location of the sampling sites

All selected sampling sites are located around Kuala Lumpur (latitude 3.13900°N and longitude 101.68686°E), the capital city of Malaysia. They comprise of four places, namely Society for The Prevention of Cruelty to Animals (SPCA) of Ampang Jaya, National Zoo of Hulu Kelang, PAWS Animal Welfare Society of Subang and Kampung Orang Asli (KgOA) of Kuala Pangsun at Hulu Langat, between latitude 3.13044°N–3.21046°N and longitude 101.55183°E–101.87990°E (Table 1). These sampling sites were selected based on the benefits, convenience and potential implications to public health. All cats from the National Zoo live individually in cages that are cleaned regularly. The SPCA and PAWS shelter the unwanted, abandoned, injured and stray cats around the city of Kuala Lumpur. Cats at the KgOA of Kuala Pangsun live freely as pets, in the houses of the Malaysian Aboriginal community.

Ethics statement

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 the SPCA and National Zoo. Authorities of the PAWS Animal Welfare Society and the owners of cats at KgOA agreed verbally without any written permission. The objectives and protocols of the research were thoroughly discussed with the authorities in charge or the cat owners.

Collection and cultivation of feline corneal swab samples

A small piece of facial cotton (1.0 cm^2) and a cotton bud were packed in a 15-mL test tube and wetted with 1.0 mL of normal saline. The tube was then autoclaved at 15 lbs pressure, 121°C for 15 minutes. The sterile packaging tubes were kept in the refrigerator at 4°C until used. For sampling, the cold packaging tubes were placed on ice cubes (or ice pack) in a covered polystyrene box and transported to the sampling site. Cornea swabs were carried out among cats (male, female, adult, and kittens) with symptoms of watery eyes with gray discharge and redness. A cold wet cotton-bud was carefully withdrawn by holding the cotton bud handle followed by swabbing of the infected cornea. It was then placed back to its original test tube. Precaution was always taken to maintain the packaging in a cold (10±2°C) condition and to avoid any possible contamination. They 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 placed onto the surface of a non-nutrient agar plate overlaid with a thin layer of live *Escherichia coli*, JM109 (Promega), sealed with a parafilm, placed in a clean container and incubated at room temperature (26±2°C).

Detection of *Acanthamoeba* by morphological and molecular techniques

All culture plates were examined daily for up to 14 days using a light inverted microscope (Olympus BX51) before being discarded. The morphology was observed under a 200 followed by a 400 times magnification for specific characteristics of *Acanthamoeba* trophozoites (acanthopodia and pseudopodia) and cysts (wrinkle double walled), and were photographed and recorded. Initially, culture plates with positive *Acanthamoeba*-like cells could exist as mixed isolates. The positive culture plate was sub-cultured for at least ten times and

each sub-culture was carried out by placing a colony of 4-6 cysts onto a new agar plate lawned with JM 109. The trophozoites were cultured and harvested for total DNA extraction using the QIAamp DNA mini kit (Qiagen, Hilden, Germany).

Amplification reactions using *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 carried out according to the protocol described by (Schroeder *et al*., 2001). PCR amplicons known as ASA.S1 (ranged 423 to 551 bp) were fractioned by 1.5% agarose electrophoresis stained with a TBE (0.5 x Tris-borate EDTA) buffer containing 0.5 µg/ml ethidium bromide and visualized under UV illumination in the chamber of a BioDoc-It™ Imaging System (UVP, Cambridge, United Kingdom). Amplicon sizes were estimated by comparison with the GeneRuler 100 bp DNA ladder Plus (Fermentas Life Science, Canada).

The ASA.S1 amplicons from 10 representative isolates were gel-purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany), followed by cloning using the InsT/Aclone™ PCR product cloning kit (Fermentas). The ASA.S1 or diagnostic fragment 3 (DF3) was inserted into vectors (plasmids, pTZ57R/T) to form a DNA recombinant. The recombinant molecules were transformed into *Escherichia coli* (strain JM109, Promega) followed by selection of the white colonies carrying recombinant plasmids with a disrupted β-galactosidase gene.

The plasmid DNA in selected recombinant 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 isolates and references published genotypes using the neighbour-joining of MEGA version 4 software (Tamura *et al*., 2007). This was followed by Kimura 2-parameter algorithm and constructed tree by a bootstrap analysis of 1000 replicates. The new data on DNA sequencing of all 10 selected isolates of PM1, PM2, PM3, PM4, PM5, PF6, KM7, KF8, KMK9 and KFK10 were deposited in Genbank with the accession numbers JX494391, JX494392, JX494393, JX494394, JX494395, JX494396, JX494397, JX494398, JX494399 and JX494400, respectively.

RESULTS

Microscopic observation of *Acanthamoeba* species

The trophozoite and cyst stages were observed as early as the second and five days of cultivation, respectively. Ten (10) isolates from the cornea of 65 cats were successfully grown in the laboratory. All positive isolates were from PAWS (6) and KgOA (4), which were isolated from 6 adult males, 2 adult females, and 1 each of male and female kitten (Table 1). All of these isolates showed good growth at 26±2°C. On the moist agar surface, the trophozoites of all isolates showed the characteristics of a spike-like acanthopodia and pseudopodia that passively protruded at surface extensions surrounding the cell. On continuous cultivation, the trophozoites became stagnant and slowly transformed to a cyst with wrinkled, thick double walls.

Molecular detection and characterization of *Acanthamoeba* species

After the PCR amplification, the *Acanthamoeba* genus-specific primer set produced amplicons approximately 460 bp against all 10 isolates (Figure 1), known as ASA.S1 (Schroeder *et al*., 2001). Analysis of the DF3 sequence using the ClustaIW programme revealed two homologous groups. *Acanthamoeba* isolated from the corneas of cats in group 1 (AfC1) consisted of 7 isolates (PM1, PM2, PM3, PF6, KM7, KF8,

Table 1. Location of the sampled sites and the presence of *Acanthamoeba* in feline corneal swab samples

Location		Site	Gender	No. of corneal sample cultured in agar plate						Grand total
				Positive (isolate)		Negative		Total		
latitude °N	longitude °E			Adult	Kitten	Adult	Kitten	Adult	Kitten	
3.15880	101.75745	SPCA	Male			2		2		12
			Female			10		10		
3.21046	101.75737	Zoo	Male			5		5		5
			Female							
3.13044	101.55183	PAWS	Male	5(PM1,PM2,PM3, PM4,PM5)		16		21		31
			Female	1(PF6)		9		10		
3.20655	101.87990	KgOA	Male	1(KM7)	1(KMK9)	1	7	2	8	17
			Female	1(KF8)	1(KFK10)	1	4	2	5	
								52	13	
		Grand total		8	2	44	11			65

Society for The Prevention of Cruelty to Animals (SPCA), National Zoo of Malaysia (Zoo), PAWS Animal Welfare Society (PAWS), Aborigines village of Kuala Pangsun (KgOA), adult male cat from PAWS (PM), adult female cat from PAWS (PF), adult male cat from KgOA (KM), adult female cat from KgOA (KF), male kitten from KgOA (KMK), female kitten from KgOA (KFK)

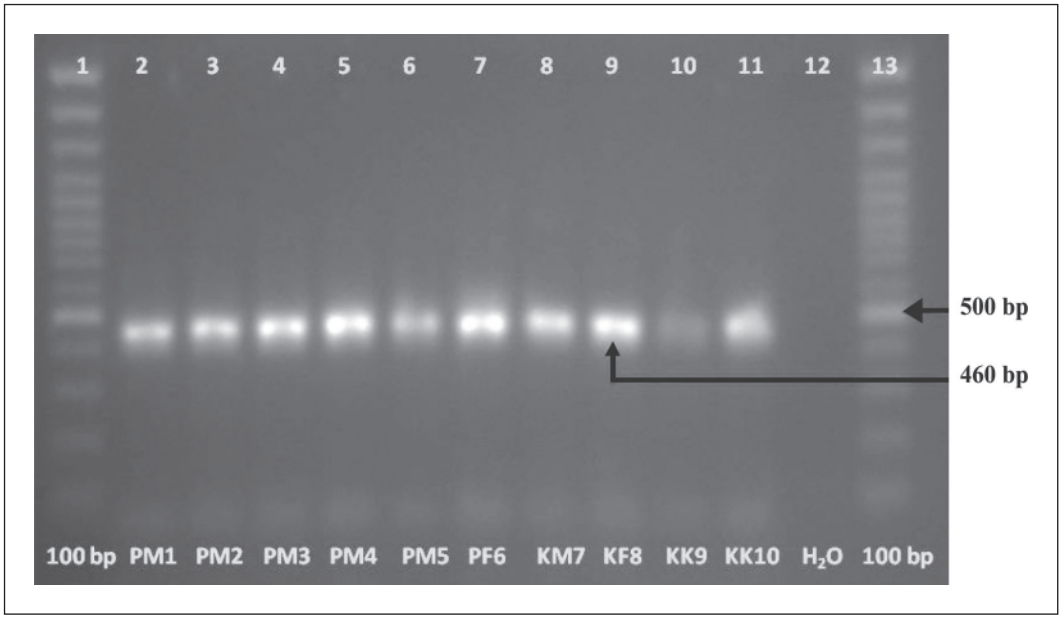
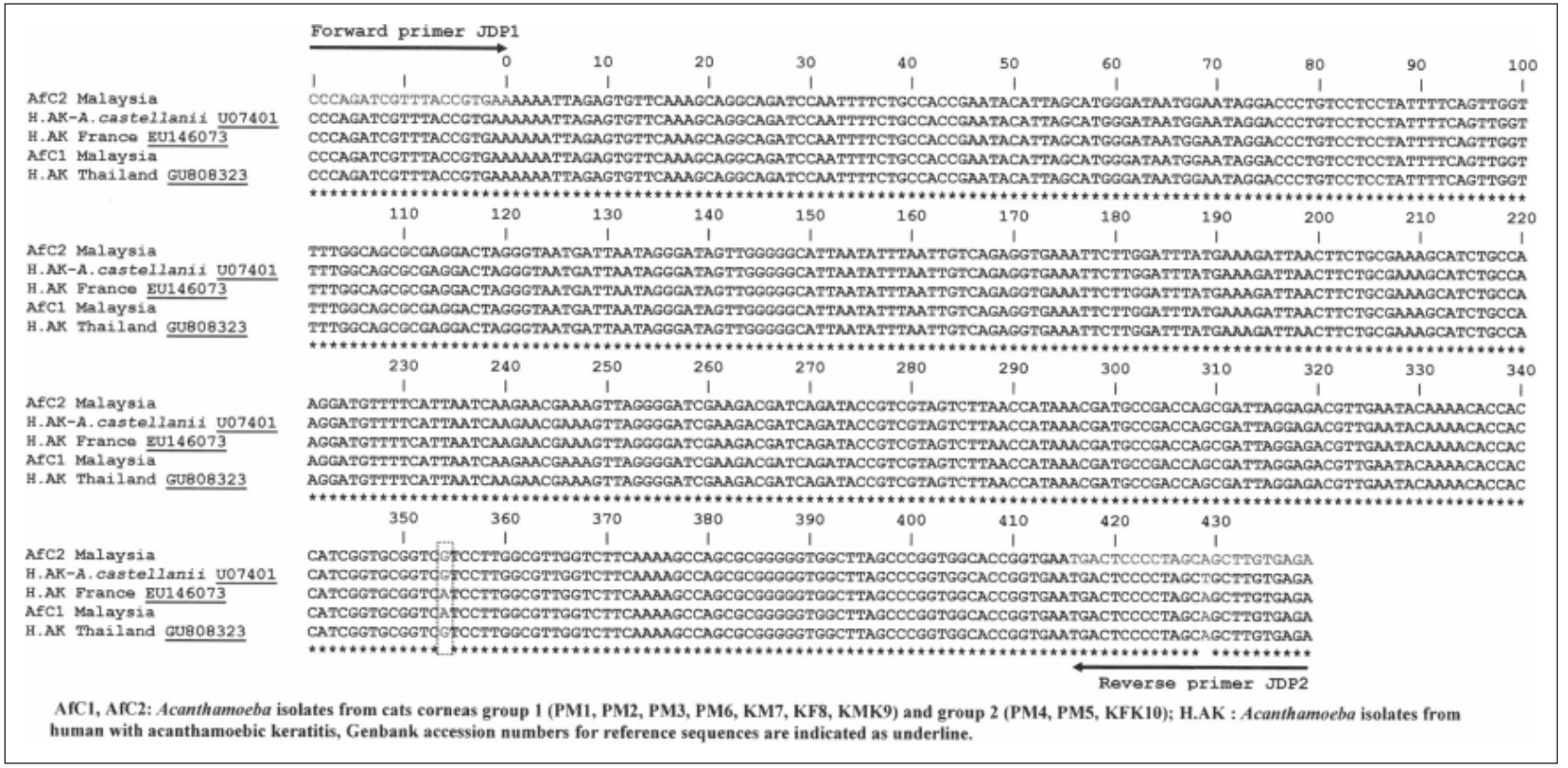


Figure 1. PCR amplicons ASA.S1 of *Acanthamoeba* isolates from cornea of cats. Lane 1 and 13 are standard DNA ladders 100 bp, lane 2 to 11 are representative of *Acanthamoeba* isolates, lane 12 is control sterile water

KMK9) and AfC2 consisted of 3 isolates (PM4, PM5, KFK10), due to the presence of bases A^{354} and G^{354}, respectively. After blasting with reference isolates from the Genbank data base, AfC1 and AfC2 respectively showed 99% and 100% homology with *Acanthamoeba castellanii* CDC:0184:V014 (U07401) genotype T4, originating from human keratitis (Figure 2). Subsequently, AfC1 showed 100% identity with human keratitis isolates (DQ087314 and EU146073) from France and AfC2 with human keratitis (GU808323) and CSF (GU808321), both isolated from Thailand. Phylogenetic analyses of our



AfC1, AfC2: ***Acanthamoeba*** **isolates from cats corneas group 1 (PM1, PM2, PM3, PM6, KM7, KF8, KMK9) and group 2 (PM4, PM5, KFK10); H.AK :** ***Acanthamoeba*** **isolates from human with acanthamoebic keratitis, Genbank accession numbers for reference sequences are indicated as underline.**

Figure 2. DNA sequence of *Acanthamoeba* diagnostic fragment 3 (DF3) of test and Genbank published isolates

representative and reference isolates assemblage genotype T1 to T17 showed that all of the representative isolates were assemblage *Acanthamoeba* genotype T4. The relationship between these isolates was summarized in the phylogenetic trees that consist of *Acanthamoeba* assemblage genotypes T1, T2, T3, T4, T10, T11, T12, T13, T14 and T15 (Figure 3). Genotypes T5, T6, T7, T8, T9, T16 and T17 were excluded as they are more distant from our representative isolates.

DISCUSSION

Despite being the causative agent of acanthameobic keratitis in humans worldwide, *Acanthamoeba* infection that causes feline eye disease is not known. To determine whether this amoeba could be considered as one of the possible pathogens infecting the eyes of cats, this study was carried out to detect *Acanthamoeba* in naturally-infected feline corneas with

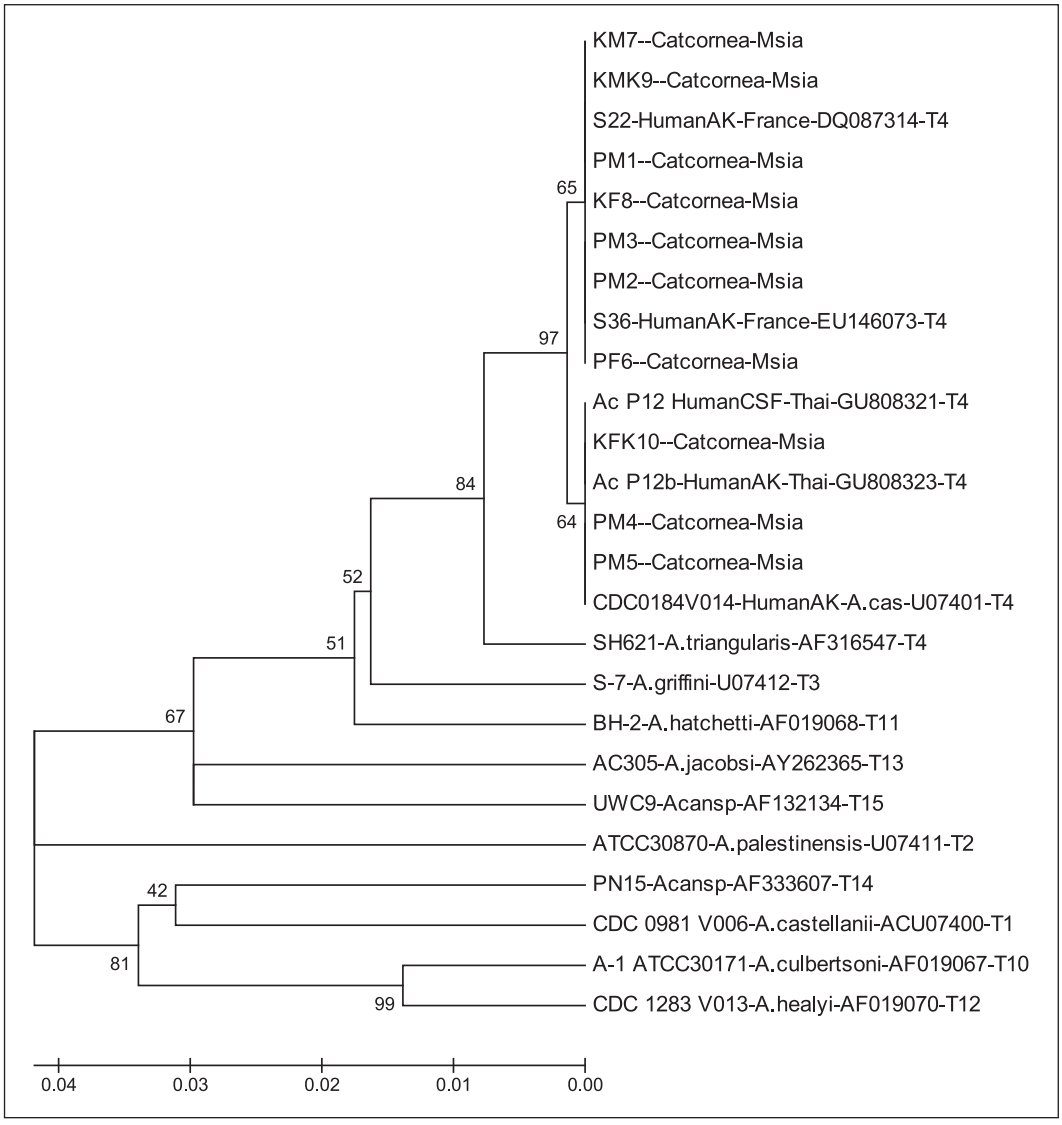


Figure 3. Neighbour-joining tree depicting the relationships between test isolates and reference strains representing respective genotypes of *Acanthamoeba*. Numbers at the nodes are percentage-bootstrapping values on 1000 replicates. Genbank accession numbers and genotypes for reference sequences are indicated at the ends of the *Acanthamoeba* isolates designations

symptoms of keratitis. In felines, eye discharge (often thick and yellow, gray or green in colour) is a valuable warning sign of pathogenic infection which could progress very quickly and cause permanent eye damage (Doyle, 2009). Cats with eye discharge, swelling and redness were selected and used in our current study. During cornea swabbing, the cotton bud used was wet and cold (10±2°C) in order to induce any *Acanthamoeba* trophozoites present to round up without protruding the acanthopodia. Rounded trophozoites may be less adherent to the corneal surface, making them easier to be swabbed out. Using this technical precaution, there was an increased chance of any *Acanthamoeba* trophozoites and cysts from the infected corneas to be cultured. After cultivation, 10 out of 65 corneal swab samples were identified positive for *Acanthamoeba*-like species, based on the morphological characteristics of the trophozoite and cyst stages. Each motile trophozoite showed the characteristics of both pseudopodia and acanthopodia, where both structures passively protruded from surface extensions surrounding the cell. These structures may act as fingers that attach to the cornea surface, thus needing a cold object (cotton bud) to release these 'fingers' from the corneal surface. Similarly, when the swabbed cotton bud was placed in the test tube, the cold condition could prevent the *Acanthamoeba* trophozoites from protruding acanthopodia and pseudopodia which would allow them to adhere to the test tube wall. This would improve the detection rate in samples with lower amoeba cells.

All of these isolates generated amplicons known as ASA.S1 by PCR using the genus-specific primer set, JDP1 and JDP2, designed from 18S rRNA gene of *Acanthamoeba* (Schroeder *et al*., 2001). DNA sequences of these amplicons (or DF3) from each representative isolate were matched to two homologous groups of AfC1 (PM1, PM2, PM3, PF6, KM7, KF8, KMK9) and AfC2 (PM4, PM5, KFK10 isolates) due to the presence of bases A^{354} and G^{354}, respectively. The DF3 region of 18S rRNA gene was recently used to identify the sequence variation, in which 17 different genotypes (T1-T17) have been established, T1-T12 (Stothard *et al*., 1998), T13 (Horn *et al*., 1999), T14 (Gast, 2001), T15 (Hewett *et al*., 2003), T16 (Corsaro & Venditti, 2010) and T17 (Nuprasert *et al*., 2010). Each genotype showed at least 6% sequence divergence among different genotypes. However, the pathogenicity of *Acanthamoeba* could be limited to some genotypes; genotype T4 occurs most commonly with human keratitis (Khan, 2006). Similarly, based on the DF3 sequence variation, the identification of all 10 isolates from the cornea of cats were blasted with *Acanthamoeba* DNA sequences retrieved from the GenBank database, and were all found to be assemblage genotype T4 with most matching 99%-100% with 5 isolates originally from human keratitis, of *Acanthamoeba castellanii* CDC:0184:V014 (U07401) from the USA (Gast *et al*., 1996), *Acanthamoeba* spp. (DQ087314, EU146073) from France (NCBI, unpublished), Korea (EF140625) (NCBI, unpublished) and Thailand (GU808323) (Nuprasert *et al*., 2010). Additionally, they matched with *Acanthamoeba* isolate (GU808321) from the CSF of a patient from Thailand (Nuprasert *et al*., 2010). Besides that, they have a 99%–100% match with many other reference isolates from environmental samples isolated from many regions in the world. This suggests that all *Acanthamoeba* isolates in this study (from cornea of cats) are the strains of *Acanthamoeba castellanii* genotype T4 that occur worldwide and have the capability to infect humans and animals under favorable conditions.

Most veterinarians believe that feline keratitis (inflammation of the cornea) results from foreign objects which lodge in the eye, causing irritation and rubbing. Feline eye disease associated with vision loss, such as keratitis is a dry eye syndrome leading to reduced production of tears and could be associated with mucoid discharge. Clinical signs include redness, discharge from the eye (discharge is initially watery and as the disease progresses it turns into thick puss), pain, sensitivity to light (photophobia), swelling, corneal edema, impaired vision or blindness. These symptoms were usually diagnosed as due to parasitic, viral or

fungal infections (Doyle, 2009) and free living amoeba was never in the list of considerations, except a recent patent note (Ledbetter, 2011). However, in this study, free living amoeba, *Acanthamoeba* genotype T4 was detected in 15.38% of the cats with related symptoms. Although we could not rule out other pathogens (virus, bacteria and fungus) as these were not looked for, we strongly believe that assemblage genotype T4 of *Acanthamoeba* has the capability to infect and may be possible pathogens infecting the eyes of cats. Moreover, the infections could have been higher in animals compared to humans due to the higher possibility of corneal injuries resulting from fighting or from injuries due to hard particles (stone, sand, soil, etc.) accidentally lodged in the eyes. Rubbing or scratching of the eyes with the front paws could worsen the infection in animals. In pets, a cone shaped instrument that covers the head will prevent the pet from rubbing their eyes and prevent further worsening of the condition.

In the current study, the cats with positive *Acanthamoeba* were from two sampling sites, the PAWS Animal Welfare Society and Malaysian Aborigines (Orang Asli) villages (KgOA) of Kuala Pangsun. All cats at the PAWS Animal Welfare Society were abandoned, injured, unwanted pets or neighborhood strays, originating from a variety of environment and brought in by the City Council dog-catchers. However, cats at Kuala Pangsun live as pets and move freely in dirty environments, in and out from houses and have close contact especially with children of the Aboriginal community. These cats help to control unwanted pests (rodents, lizards, snakes, etc.) in the home. Only *Acanthamoeba* strains of *A. castellanii* T4 were detected in all cats, although a variety of *Acanthamoeba* assemblage genotype T4 of *Acanthamoeba polyphaga*, *Acanthamoeba quina*, *Acanthamoeba hatchetti* and *Acanthamoeba triangularis* exist in Malaysian dust (Chan *et al*., 2011). This suggests that *A. castellanii* genotype T4 strain is most commonly associated with infections in cat eyes. Furthermore, this finding is in keeping with the increase in human acanthamoebic keratitis, especially in contact lens wearers in Malaysia (Kamel *et al*., 2003). Risk factors of acanthamoebic keratitis are use of home-made saline solutions, inappropriate cleaning of contact lenses, corneal abrasions or trauma due to injury by a foreign body, exposure to contaminated water, air or contact lens (Martinez & Visvesvara, 1997; Kilvington *et al*., 2004; Visvesvara *et al*., 2007). There is no report on the identification of the associated *Acanthamoeba* genotype in human acanthamoebic keratitis; however, we strongly believe that *Acanthamoeba* assemblage T4 of *A. castellanii* (U07401) strains could be one of the possible causative agents of human acanthamoebic keratitis in Malaysia. Additionally, *Acanthamoeba* infection in humans and animals could be more common than currently recognised since most Malaysians, especially cats lovers, live in close contact within the same environment.

Despite being a carrier of many other infectious diseases to man, the presence of pathogenic *Acanthamoeba* T4 in pet cats could be considered a public health concern among those who keep cats as pets in their homes. The presence of many unneutered stray cats at restaurants, food stalls and slum areas could contribute to the overpopulation of unwanted felines. Therefore, improvement of healthcare structures such as neutering pet animals, environmental hygiene and health education on pathogenic free-living amoebae infections should be considered in intervention programmes in order to control infection in both animals and humans.

Acknowledgements. This research was funded by the University of Malaya Research Grant, UMRG RG187/10HTM. The funding organization had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors wish to acknowledge the cooperation and technical support (i.e. holding cats) given by the staff of Society for The Prevention of Cruelty to Animals (SPCA), National Zoo, PAWS Animal Welfare Society and the entire Aboriginal community in Kampung Orang Asli Kuala Pangsun, in making this survey a success.

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