Original Article

Molecular Identification of *Naegleria fowleri* and Pathogenic *Acanthamoeba* spp. in Chao Phraya River and Canals Around Siriraj Hospital, Thailand

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Background: Free-living *Naegleria fowleri* and *Acanthamoeba* spp. amoebae are causative agent of lethal brain infection in human. Due to high fatal rate of the patients, a survey of these pathogenic amoebae should be done for prevention and control in public health.

Objective: To investigate the presence and prevalence of *N. fowleri* and pathogenic *Acanthamoeba* spp. in the Chao Phraya River and canals located close to Siriraj Hospital (Bangkok, Thailand).

Materials and Methods: One hundred eighty-nine freshwater samples collected from 21 sites were investigated between January and April 2017. Sample sediments were incubated at 37°C (regular temperature) and 42°C (tolerant temperature) for five to seven days. All samples were identified by morphologic characteristics and polymerase chain reaction.

Results: The samples from eight sites (Bangkoknoi District Office, Taling Chan Floating Market, Wang Lang, Wat Amarinthararam, Wat Khruawan, Wat Pho Riang, Wat Rakhang, Wat Taling Chan) yielded 38% (72/189) *Naegleria* spp. at 37°C, and 19% (36/189) *Naegleria* spp. at 42°C by morphological identification. The thermotolerant *Naegleria* spp. samples (9.5%, 18/189) from Wang Lang and Wat Rakhang were identified as *N. fowleri*. Forty-five of 189 (23.8%) water samples taken from the Taling Chan Floating Market, Wang Lang, Wat Khruawan, Wat Pho Riang, Wat Taling Chan collection sites were positive for pathogenic *Acanthamoeba* spp.

Conclusion: Our findings suggested that fresh water from the Wang Lang, Wat Rakhang were contaminated with *N. fowleri*, whereas Taling Chan Floating Market, Wat Khruawan, Wat Pho Riang or Wat Taling Chan were with pathogenic *Acanthamoeba* spp. Interestingly, we found *N. fowleri* and pathogenic *Acanthamoeba* spp. from Wang Lang site.

Keywords: *Naegleria* spp., *Acanthamoeba* spp., Chao Phraya River, Thailand

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Free-living amoebae [FLA] are often referred to as amphizoic amoeba due to their ability to exist as free-living organisms in nature. FLA can independently survive and proliferate in nature, such as in soil, fresh water, and man-made aquatic environments[1]. Amoeba cysts are highly resistant and can persist in suboptimal conditions because of a process known as encystment[2]. Several FLA support the growth of and protect *Legionella pneumophila*, *Mycobacterium*, and *Chlamydia*-like bacteria from the harsh environments and ecosystems in which sporadic episodes of human respiratory illness occur[3]. Long-term support survival of *L. pneumophila* associated with *Acanthamoeba castellanii* vesicles should explain the spreading of *Legionella*[4].

Naegleria, *Acanthamoeba*, *Balamuthia*, and *Sappinia* are now known to cause brain infections in humans[5]. However, only *Naegleria fowleri* has been identified a causative agent in primary amoebic meningoencephalitis [PAM], which is a lethal brain infection in humans and animals[6,7]. In 1983, the first case of PAM was reported in Sisaket, Thailand[8]. Since that time, additional cases of PAM have been sporadically reported[9,10]. In 2014, two cases suffering from PAM were found in Udon Thani and Pattaya, Chonburi province[11,12]. The infection occurred when trophozoites penetrate the CNS via the olfactory nerve and causes fulminant meningoencephalitis, which is generally fatal. The clinical presentation of PAM is similar to severe bacterial meningitis. The standard investigation from CSF is important to achieve a conclusive diagnosis[12]. Between 2001 and 2003, a

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survey of the distribution of pathogenic *Naegleria* spp. in water reservoirs in provinces from the central (Bangkok, Nakhon Nayok, Nakhon Sawan, Saraburi, and Sukhothai), southern (Chumphon and Surat Thani) and western regions (Prachuap Khiri Khan) of Thailand was conducted. The results revealed pathogenic strains of *Naegleria* belonging to the species *fowleri* in Saraburi and Surat Thani provinces[13].

Acanthamoeba spp. commonly infects older and/or immunocompromised hosts[5,10]. *Acanthamoeba* are free-living protists that can cause fatal granulomatous amoebic encephalitis [GAE] and amoebic keratitis. *Acanthamoeba* spp. has worldwide distribution and inhabits a wide range of environments[2]. Between 1996 and 2006, from the publications reviewed, clinical features of *Acanthamoeba* keratitis were reported in contact lens wearers and non-wearers in 22 Thai patients with ocular trauma, use of contact lenses, associated eye diseases, systemic disease, and visual acuity issues attending the Faculty of Medicine Siriraj Hospital. Data revealed that *Acanthamoeba* infection as a cause of keratitis in any patients, not just contact lens wearer. Physicians should be aware and observe for early diagnosis[14].

A survey of *Naegleria* and *Acanthamoeba* was conducted in some natural water sources in Chiang Mai Province, Thailand between March and September 2007[15]. Sixteen thermo-tolerant positive samples were identified as 37.5% (6/16) thermophilic *Naegleria* spp., 18.8% (3/16) *Acanthamoeba* spp., and a 31.3% (5/16) mix of both species. The distribution of 38.2% (26/68) *Acanthamoeba* spp., and 35.3% (24/68) *Naegleria* spp., found in natural hot springs in Thailand were carried out from 13 provinces from central (Kamphaeng Phet, Lopburi, Phetchabun, Utai Thani) and southern (Chumphon, Krabi, Nakon Si Thammarat, Phang Nga, Phattalung, Ranong, Satun, Surat Thani, Trang) Thailand[16].

The emergence of pathogenic FLAs is causing heightened concern across Thailand, given the adverse and sometimes fatal health implications of these pathogens. Studies of the presence and prevalence of pathogenic FLAs that live in fresh water system located near Siriraj Hospital, Thailand's largest national tertiary referral center are scary knowing that these pathogenic FLAs can be involved PAM, GAE, and amoebic keratitis in the population who uses the freshwater. The aim of the present study was to investigate and survey the presence and prevalence of *N. fowleri* and pathogenic *Acanthamoeba* spp. in the Chao Phraya River and adjacent canals that are near Siriraj Hospital (Bangkok, Thailand).

Materials and Methods

Sampling sites

Sample collection and isolation: The number of water samples was described by Lek-Uthai, 2009[17]. Water samples were collected from four groups: I, the Chao Phraya River; II, Bangkoknoi Canal; III, Chak Pra Canal; and IV, Mon Canal at sites located near Siriraj Hospital, between January and May 2017.

One hundred eighty-nine water samples were collected from 21 freshwater system locations (piers), with nine samples taken from each collection site. The collection sites comprised of 12 piers on the Chao Phraya River (Phra Pinkloa, Rama VIII, Siriraj Hospital, Tha Chang, Tha Phra Arthit, Tha Prachan, Tha Tien, Thonburi Railway, Wang Lang, Wat Arun, Wat Kanlaya, and Wat Rakhang) and nine piers in three adjacent canals, Bangkoknoi Canal (District office Bangkoknoi, Wat Amarinthararam, and Wat Srisudaram), Chak Pra Canal (Talingchan floating Market, and Wat Talingchan), and Mon Canal (Wat Bang Sao Thong, Wat Chinorasaram, Wat Khruawan, and Wat Pho Riang) (Figure 1). These water sources are in residential areas and in public places. Study for *Naegleria* spp. and *Acanthamoeba* spp. were performed

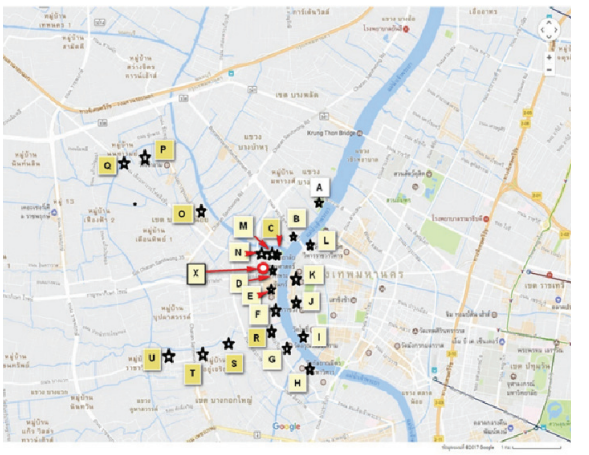

Figure 1. Map illustration of the 21 water sampling sites on the Chao Phraya River and adjacent canals located in close proximity to Siriraj Hospital (X): (A) Rama VIII; (B) Phra Pinkloa; (C) Thonburi Railway; (D) Siriraj; (E) Wang Lang; (F) Wat Rakhang; (G) Wat Arun; (H) Wat Kanlaya; (I) Tha Train; (J) Tha Chang; (K) Tha Prachan; (L) Tha Phra Arthit; (M) Wat Amarinthararam; (N) Bangkoknoi District Office; (O) Wat Srisudaram; (P) Wat Taling Chan; (Q) Taling Chan Floating Market; (R) Wat Khruawan; (S) Wat Chinorasaram; (T) Wat Pho Riang; and (U) Wat Bang Sao Thong.

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Using sterile 50 ml screw-cap tubes, 50 ml of surface water was collected in each of the nine bottles at various spots around each collection site pier. Water temperature and pH level were measured at each sampling site. All water samples were transported to the laboratory in ice-box and processed within four hours after sampling. All samples were processed in the laboratory following a previously described protocol[18]. The sediment from each sample was cultured on 1.5% non-nutrient agar plates seeded with a thin layer of non-viable *Escherichia coli* [NNA-*E. coli*] and duplicated onto plates. The plates were sealed with parafilm and incubated at 37°C and 42°C to identify thermophilic amoeba. Plates were observed daily for amoebic growth (appearance of clearing zones) for seven days using an Olympus IX70 inverted microscope (Olympus Corporation, Tokyo, Japan). Amoebae were harvested by centrifugation at 1,200 × g for 15 minutes at room temperature. Amoebae were identified by cyst and trophozoite morphology following the criteria previously described protocol[19]. Amoebic trophozoite and cyst stages were flushed from the culture plates and subjected to direct screening using Trichrome staining for each microscopic observation. After staining, the amoebae were examined using an Olympus BX51 light microscope (Olympus Corporation, Tokyo, Japan). Amoebae identified on the agar plate were sub-cultured and after growth on subsequence plates[7].

DNA extraction

Generally, the expression of trophozoite genes was observed during proliferation growth. The trophozoite stage of each amoebic isolate was harvested from plates with 5 mL of cold Page's Amoeba Saline [PAS] and transferred into 1.5 mL tubes. DNA was further extracted using a commercial QIAamp DNA Blood Mini Kit (Qiagen, Hilden, France) following the manufacturer's procedures and stored at -20°C until further analysis.

Polymerase chain reaction [PCR] amplification

Amplification of DNA templates of *Naegleria* spp. and *Acanthamoeba* spp. were performed in 25 µl per reaction. For *Naegleria* spp. amplification, the PCR mixture containing 10x DNA polymerase buffer 0.2 mM dNTP, 0.2 µM of specific primers (Table 1)[20-23], 2.5 µmol of *Taq* polymerase, and 2 µl of DNA. The reaction was performed at an initial incubation temperature of 94°C for three minutes, followed by 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds in a Gene Amp PCR 2400 thermal cycler (PerkinElmer, Inc., Waltham, MA, USA)[24]. The PCR product was incubated at 72°C for five minutes to ensure complete extension of all amplified molecules. Finally, PCR products were subjected to 1.8% agarose Tris-borate-EDTA gel electrophoresis at 100 V for 30 minutes

Results

One hundred eighty-nine water samples were collected from 21 freshwater system locations (piers), with nine samples taken from each collection site. The collection sites comprised of 12 piers on the Chao Phraya River and nine piers in three adjacent canals, Bangkoknoi canal, Chak Pra canal, and Mon canal. The samples from eight sites (Bangkoknoi District Office, Taling Chan Floating Market, Wat Amarinthararam, Wat Khruawan, Wang Lang, Wat Pho Riang, Wat Rakhang, and Wat Taling Chand) yielded 38% (72/189) *Naegleria* spp. at 37°C, and 19% (36/189) *Naegleria* spp. at 42°C by morphological identification (Table 2). The thermotolerant *Naegleria* spp. samples (9.5%, 18/189) from Wang Lang and Wat Rakhang were identified as *N. fowleri* (Table 3). These water sources were identified in samples by morphological characteristics. The morphologic characteristics are shown in Figure 2. Unstained and stained morphology of *Naegleria* trophozoite under

Table 1. Primers used for amplification of *Naegleria fowleri* and pathogenic *Acanthamoeba* spp.

Amoeba name	Primer	Sequence (5'→ 3')	Primer length	Reference
Naegleria fowleri	ITS (F) ITS (R)	5'GAACCTGCGTAGGGATCATTT3' 5'TTTCTTTTCCTCCCCTTATTA3'	450 bp	Tung et al., 2013[20]
	nfa1 (F) nfa1 (R)	5'ATGGCACTACTATTCCATCACCA3' 5'ATTCTATTCACTCCACAATCC3'	360 bp	Jeong et al., 2004[21]
Pathogenic *Acanthamoeba* spp.	JDP (F) JDP (R)	5'GGCCCAGATCGTTTACCGTGAA3' 5'TCTCACAAGCTGCTAGGGGAGTCA3'	450 bp	Howe et al., 1997[22]
	Ac6 (F) Ac6 (R)	5'GGCGAAGAACCTGCATCAGC3' 5'CAAACCAACTCCCGAGCCA3'	195 bp	Rahdar et al., 2012[23]

F = forward; R = reverse; bp = base pairs

Table 2. Occurrence of free-living amoebae in the Chao Phraya River around Siriraj Hospital grown in screening incubation at 37°C and 42°C was identified by morphological characteristics

No.	Location (n)	Temp., mean ± SD	pH, means ± SD	*Naegleria* spp.		*Acanthamoeba* spp.	
				37°C	42°C	37°C	42°C
1	Chao Phraya River (12)	28±0.5	6.5±0.5	2	2	1	0
2	Bangkoknoi canal (3)	29±0.5	7.5±0.5	2	2	0	0
3	Chack Pra canal (2)	28±0.5	6.5±0.5	2	0	2	0
4	Morn canal (4)	29±0.5	7.2±0.5	2	0	2	0
	Total (21)			8/21 (38%)	4/21 (19%)	5/21 (23.8%)	0 (0%)

Temp. = temperature; C = Celsius

Table 3. Positive locations of *Naegleria fowleri*, pathogenic *Acanthamoeba* spp. isolated in the Chao Phraya River around Siriraj Hospital and adjacent canals were identified by PCR technique

No.	Positive locations	*Naegleria fowleri*	Pathogenic *Acanthamoeba* spp.
1	Wang Lang	+	+
2	Wat Rakhang	+	-
3	Wat Takingchan	-	+
4	Takingchan Floating Market	-	+
5	Wat Khruawan,	-	+
6	Wat Pho Riang	-	+
Total		2/21 (9.5%)	5/21 (23.8%)

+ = positive for free-living amoebae [FLA]; - = negative for FLA

light microscope showed active progressive and directional movement (Figure 2A). The rounded cyst stage had a double smooth wall (Figure 2B). Trichrome stained of trophozoite showed typical semispherical eruptive pseudopodia and vesicular nucleus (Figure 2C), and cyst showing prominent vesicular nucleus (Figure 2D).

Forty-five of 189 (23.8%) water samples taken from the Taling Chan Floating Market, Wang Lang, Wat Khruawan, Wat Pho Riang, or Wat Taling Chan collection sites were positive for pathogenic *Acanthamoeba* spp. The unstained and stained morphology of *Acanthamoeba* stage were determined under light microscope. In fresh smear, *Acanthamoeba* trophozoites showed acanthopodia and nucleus (Figure 3A) and cysts showed multiple shapes, double wall cyst (Figure 3B). Trichrome stained trophozoites showed acanthopodia and prominent contractile vacuoles (Figure 3C). Additionally, morphological cysts showed multiple shapes, triangular, and square protrusion with a thicker double wall (Figure 3D) (Table 2).

Since amoebae were identified in water samples based on microscopic examination, the ITS (450 bp) and nfa1 (360 bp) genes of each isolate of thermo-tolerant amoebae were identified. Only Wang Lang and Wat Rakhang samples water (9.5%) were positive for *N. fowleri* (Figure 4). Six of twenty-one sites (28.5%) from the Chao Phraya River around Siriraj Hospital (Wang Lang and Wat Rakhang), Chak Phra Canal (Taling Chan Floating Market and Wat Taling Chan), and Mon Canal (Wat Khruawan and Wat Pho Riang) were obtained from different sites (Figure 1). Trophozoite and cyst stage of *Acanthamoeba* spp. (23.8%) isolated from the Chao Phraya River around Siriraj Hospital (Wang Lang), two canals near Siriraj Hospital Chak Phra Canal (Taling Chan Floating Market and Wat Taling Chan), and Mon Canal (Wat Khruawan and Wat Pho Riang) were identified by morphologic characteristics. The JDP and Ac6 genes of each isolate were amplified 450 bp and 195 bp

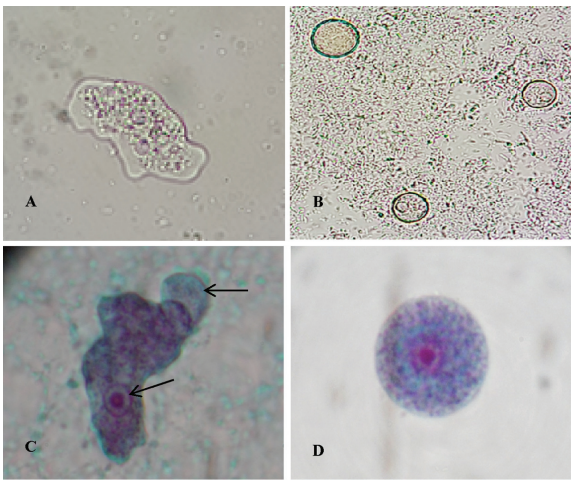

Figure 2. Unstained and stained morphology of *Naegleria* trophozoite and cyst under light microscope: A) x400 unstained *Naegleria* trophozoite showing active progressive and directional movement; B) x400 cyst showing rounded form with double wall cyst; C) x1,000 trichrome stained trophozoites showing typical semispherical eruptive pseudopodia (arrow) and vesicular nucleus (arrow); and D) x1,000 cyst showing prominent vesicular nucleus.

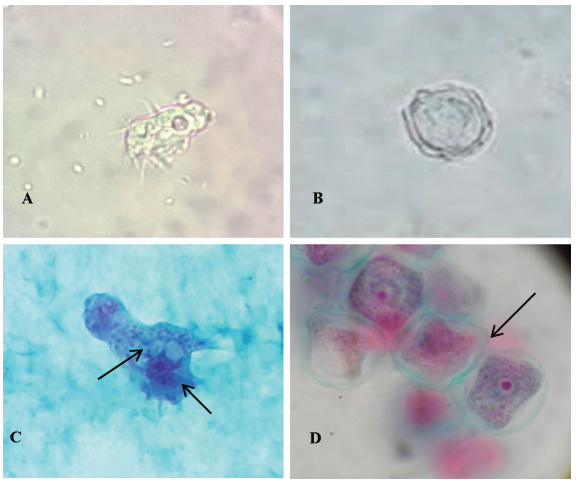

Figure 3. Unstained and stained morphology of *Acanthamoeba* trophozoite and cyst under light microscope: A) x400 unstained *Acanthamoeba* trophozoites showing acanthopodia and nucleus; B) x400 cysts with multiple shapes, and round, triangular, and square protrusions; C) x1,000 trichrome stained trophozoites, acanthopodia, (arrow) and prominent contractile vacuoles (arrow); and, D) x1,000 cyst with multiple shapes with thicker double wall (arrow).

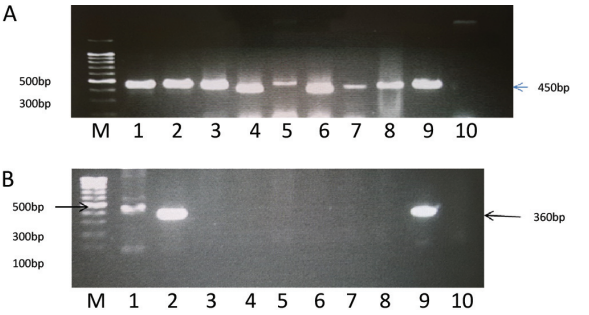

Figure 4. A) Gel electrophoresis of amplicon from *Naegleria* spp. PCR conditions showed specific band ITS 450 bp. Lane 1: *N. fowleri*, positive control showing specific band; Lanes 2 to 9: eight major thermophilic *Naegleria* spp. isolated from Wang Lang, Wat Rakhang, Wat Amarinthararam, Bangkoknoi District Office, Wat Taling Chan, Taling Chan Floating Market, Wat Khruawan, and Wat Pho Riang showing positive band; and, Lane 10: pathogenic *Acanthamoeba* spp. negative control. B) Gel electrophoresis of amplicon from *N. fowleri*. PCR conditions showed pathogenic specific band nfa1 at 360 bp. Lane 1: *N. fowleri*, positive control showing specific band; Lanes 2 and 9: *N. fowleri* isolated from Wang Lang and Wat Rakhang showing positive band; and Lane 10: pathogenic *Acanthamoeba* spp. negative control.

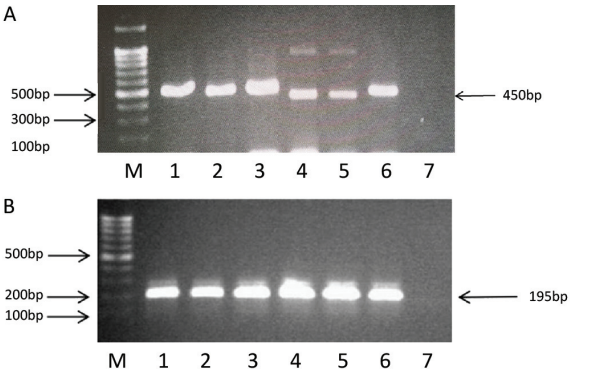

Figure 5. A) Gel electrophoresis of amplicon from *Acanthamoeba* spp. PCR conditions showed JDP band at 450 bp. Lane 1: pathogenic *Acanthamoeba* spp. positive control showing specific band; Lanes 2 to 6: five positive samples isolated from Wang Lang, Wat Taling Chan, Taling Chan Floating Market, Wat Khruawan, and Wat Pho Riang showing positive band at 450 bp; and Lane 7: pathogenic *N. fowleri* negative control. B) Gel electrophoresis of amplicon from *Acanthamoeba* spp. PCR conditions showed Ac6 band at 195 bp. Lane 1: pathogenic *Acanthamoeba* spp. positive control showing specific band at Lanes 2 to 6: Five isolated from positive samples Wat Taling Chan, Taling Chan Floating Market, Wang Lang, Wat Khruawan, and Wat Pho Riang showing positive band at 195 bp; and Lane 7: pathogenic *N. fowleri* negative control.

products. All of them were positive for pathogenic *Acanthamoeba* spp. (Figure 5) (Table 3).

Discussion

In the present study, the authors identified *N. fowleri* and pathogenic *Acanthamoeba* spp. in water samples taken from the Chao Phraya River and adjacent canals located near Siriraj Hospital. The thermotolerant *Naegleria* spp. samples (9.5%) from Wang Lang and Wat Rakhang were identified as *N. fowleri*. The water samples (23.8%) taken from the Taling Chan Floating Market Wang Lang, Wat Khruawan, Wat Pho Riang, and Wat Taling Chan, collection sites were positive for pathogenic *Acanthamoeba* spp.

In the present study, the occurrence of pathogenic *Acanthamoeba* spp. (23.8%) was higher than *N. fowleri* (9.5%). Nacapunchai et al, 2001[10] reported the presence of FLA in aquatic habitats of human environments in Thailand. In the Hamamatsu District of Japan, significantly higher levels of *Acanthamoeba* (22.6%) and *Naegleria* (4.8%) were found and caused death in mice. Previous studies reported that *N. fowleri* (10.49%) was isolated from fresh water (162 samples) from Lopburi province by histopathological studies in Rattus rattus. Furthermore, pathogenic *Naegleria* isolated from Pathum Thani (11.46%) and Samut Prakan (12%) were distinguished by characteristics of growth properties since the pathogenic form can grow in the in SCGYEM media[8]. In the present study,

the distribution of *N. fowleri* (9.5%) from Wang Lang and Wat Rakhang were similar to Lopburi, Pathum Thani and Samut Prakan provinces. From previous publications reported from Chiang Mai, Thailand, thermotolerant positive samples of *Naegleria* and *Acanthamoeba* were found in some natural water sources and flood waters[3,16]. However, the present study reported that *Acanthamoeba* spp. could not grow at 42°C. In Southeast Asia, FLA were investigated for presence of pathogenic FLA in Laos, Myanmar, and Singapore. The results of the study revealed the pathogenic species of *A. lenticulata* from Laos, and *A. triangularis* and *A. polyphaga* from Myanmar[24]. The present study found pathogenic *Acanthamoeba* spp. in five locations from Chao Phraya River and adjacent canals located near Siriraj Hospital (Table 3), which was similar to Nacapunchai et al, 2001[10]. The isolation of FLA in the present study provided us with a better understanding of the distribution and prevalence of *N. fowleri* and pathogenic *Acanthamoeba* spp. in Thailand. Moreover, this finding supplies additional evidence regarding the presence of pathogenic FLA, which can be a source of infection and proliferation of human pathogenic organism under natural conditions.

Conclusion

The present study results suggested that fresh water from Piers Wang Lang, Wat Khruawan, Wat Pho Riang, and Wat Rakhang may be areas where these pathogens are transmitted to the general public. Public health notices should be developed and posted, so the people who are living in these locations are aware of these and take proper precautions, thus, preventing or reducing the infections caused by these parasites.

What is already known on this topic?

N. fowleri and pathogenic *Acanthamoeba* spp. are causative agent of lethal brain infection in human. Due to the high mortality rate of these patients, a survey of these pathogenic amoebae has been carried out in Thailand between 1997 and 2017. However, there is no data of the distribution of *N. fowleri* and pathogenic *Acanthamoeba* spp. in Chao Praya River and canals around Siriraj Hospital. The surveillance of *N. fowleri* and pathogenic *Acanthamoeba* spp. in Bangkok should be followed by the health professionals.

What this study adds?

N. fowleri and pathogenic *Acanthamoeba* spp. were isolated and identified by morphological characteristics and PCR amplification. The results revealed that *N. fowleri* (18/189, 9.5%) was found in fresh water from the Wang Lang and Wat Rakhang sites. Pathogenic *Acanthamoeba* spp. (45/189, 23.8%) was also found in Taling Chan Floating Market, Wat Khruawan, Wat Pho Riang, and Wat Taling Chan sites. Interestingly, we found both pathogenic free-living (9/189, 4.7%) at the Wang Lang site.

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Potential conflicts of interest

The authors declare no conflict of interest.

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