A BRIEF SURVEY OF FREE-LIVING AMEBAE IN THAILAND AND HAMAMATSU DISTRICT, JAPAN

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Abstract. The aim of this study was to determine the presence of free-living amebae in aquatic habitats of human environments in Thailand and Hamamatsu district, Japan. Genus identification was based on the morphology of cyst and trophozoite forms and a flagellation test for genus *Naegleria*. The pathogenic potential was tested in mice by nasal instillation for genus *Naegleria* and *Acanthameba*. In 14 provinces of Thailand, amebae were isolated in 43 from 95 water samples and 67 from 120 soil swabs. Amebae of 49 isolates from waters were identified as *Acanthameba* (36.7%), *Naegleria* (28.6%), *Hartmannella* (20.4%), *Vahlkampfia* (12.2%) and *Vannella* (2%). Soil samples have significantly higher levels of *Acanthameba* and *Hartmannella* ($p<0.05$) but lower for *Naegleria* ($p<0.05$) and 7 unidentified amebae were found. In Hamamatsu district, Japan, 62 amebae of the same genera were isolated from 47 of 95 water samples. There were significantly higher levels of *Acanthameba* (22.6%) ($p<0.05$) but lower for *Naegleria* (4.8%) ($p<0.05$) than those of Thailand which each of them caused death in mice. Three unidentified amebae were isolated. This finding serves as additional evidence for the presence of free-living amebae under natural and the difference in distribution between tropic and subtropic areas.

INTRODUCTION

The free-living amebae are found worldwide in watery environments as diverse as sea water, tap water, bottled mineral water, chlorinated swimming pools, and sewage treatment plants. They are also found in soil and colonize a wide variety of plants and animals (De Jonckheere, 1991; John and Howard, 1995; Martinez and Vivesana, 1997). *Naegleria fowleri*, *Acanthameba* spp and *Balamuthia mandrillaris* can cause human infections and have been recognized as opportunistic pathogens of infections in immuno-compromised patients including those with AIDS (Szenasi *et al,* 1998; Viriyavejakul *et al,* 1997; Denny *et al,* 1997; Bonilla *et al,* 1999). Additionally, there are several reports of these free-living amebae that are able to support survival and growth of pathogenic legionellae in the ecosystems which found to be associated with sporadic episodes of respiratory illness in humans (Abu 1996; Addiss *et al,* 1989; Adeleke *et al,* 1996; Newsome *et al,* 1998).

Primary amebic meningoencephalitis (PAM) due to *N. fowleri* and *Acanthameba* keratitis have been reported from both Thailand (Viriyavejakul *et al,* 1997; Jongwutiwes *et al,* 2000) and Japan (Sugita *et al,* 1999; Ishibashi *et al,* 1987). *N. fowleri* was isolated from geothermal and industrial warm waters (De Jonckheere *et al,* 1991; Jariya *et al,* 1997). *Acanthameba* could be isolated in Japan from sandboxes, house dust, cooling tower water, soil and hot spring spas (Kuroki *et al,* 1998;Yagita *et al,* 1994; Yamaura *et al,* 1993; 1995) but only from contact len cases in Thailand (Roongruangchai and Supadirekkul, 1997). Co-occurrence of free-living amebae with bacteria and legionellae also found (Roongruangchai and Supadirekkul, 1997; Yagita *et al,* 1995). Legionellae occur widely in aquatic habitats and some human cases have been reported (Anonymous, 1997; Phatinawin *et al,* 2000; Srisawai, 2000; Tishyadhigama *et al*, 1995). The aim of our investigation was to detect the distribution of free-living amebae in natural soil and aquatic environments in tropic and subtropic areas.

MATERIALS AND METHODS

Sampling sites

The sampling sites were natural or man-made lakes, rivers, small streams, water receiving wells of water supply center, the public garden ponds and surrounding soils. Samples were collected from 14 provinces of Thailand in summer and Hamamatsu district, Japan in autumn.

Sample collection and ameba isolation

Using sterile 50-ml screw-cap bottles, 50-ml

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surface water samples were collected at various sites around the collecting places. Water temperature and pH were measured at sampling sites. Soil swab samples were collected by using sterile cotton-tipped applicators near the water's edge. All samples were processed in the laboratory within 2 hours of collection and followed as previously described (John and Howard, 1995). Briefly, the cultured plates after inoculation were observed daily under an inverted microscope. Amebae presented on the agar plate were subcultured and after growth on subsequence plates, isolated amebae were transferred to Page's saline with *Escherichia coli* for cultivation. Following 48-72 hours of growth in liquid media, amebae were harvested by centrifugation at 1,200*g*, 20˚C for 15 minutes.

Ameba identification and pathogenicity test

Amebae were identified on a morphological basis of cysts and trophozoites following the criteria as previouly described (Page, 1988). Enflagellation was induced by washing and suspending in sterile distilled water and incubating them with agitation at room temperature for 1 hour. All organisms were examined by phase-contrast inverted microscope. The virulence of pathogenic isolates of *Naegleria* and *Acanthameba* were determined by inoculating 5 mice/ isolate with 10^4-10^5 amebae. Ameba isolates were introduced into a single naris of the 21-day old male mice using Eppendorf pipet under anesthesia. Deaths were recorded daily and the cumulative percentage of mortality was calculated at 28 days after inoculation.

RESULTS

In Thailand, 95 water and 120 soil swab samples were collected from 5 areas in 14 provinces in March and April 1999. Amebae of 49 isolates were found in 46 water and 83 from 67 soil swab samples (Table 1). Ameba isolated were identified as *Acanthameba* (58), *Naegleria* (18), *Hartmannella* (30), *Vahlkampfia* (16) and *Vannella* (3). Seven unidentified amebae similar to leptomyxid ameba (John and Howard, 1995) were recovered. *Acanthameba* and other free-living amebae, except *Naegleria,* were recovered from all pH ranges of 5.0 to 7.0 and temperature ranges of 28˚ to 39˚C. *Naegleria* were found in water temperature ranges of 28˚ to 33˚C and at pH ranges of 6.5 to 7.0.

In Japan, 95 water samples were collected during September and November around Hamamatsu district. They were from Hamana Lake (21), Sanaru Lake (13), rivers (7), small streams (34), receiving wells of two water supply centers (14) and public garden ponds (6). Sixty-two ameba isolates were recovered from 47 (49.5%) positive samples. They were identified as *Acanthameba* spp (22.6%), *Naegleria* spp (4.8%), *Hartmannella* spp (41.9%), *Vahlkampfia* spp (19.4%), *Vannella* spp (6.5%) and 3 unidentified amebae (4.8%) as shown in Table 2. There were 18 thermotolerant amebae found in all genera; three *Acanthamebae* and two *Naegleria* except *Vannella*. Two thermotolerant isolates of genera *Naegleria* and *Acanthameba* caused

Table 1
Number of ameba isolates from 95 water and 120 soil swab samples collected during summer from 14 provinces of Thailand.

Ameba isolates (genus)	Sample		Total
	Water	Soil	
Acanthameba	18	40	58
Naegleria	14	4	18
Hartmannella	10	20	30
Vahlkampfia	6	10	16
Vannella	1	2	3
Unidentified amebae	0	7	7
Total	49	83	132

Table 2
Number of ameba isolates from water samples during autumn in Hamamatsu district, Japan.

Sources	Samples		Ameba Isolates					
	Total	Positive	*Acanthameba*	*Naegleria*	*Hartmannella*	*Vahlkampfia*	*Vannella*	Unidentified
Hamana Lake	21	5	3	0	3	2	0	1
Sanaru Lake	13	7	0	1	7	1	0	1
Rivers	7	0	0	0	0	0	0	0
Small streams	34	33	10	2	15	6	4	1
Ponds	6	1	1	0	0	1	0	0
Receiving wells	14	1	0	0	1	1	0	0
Total	95	47	14	3	26	12	4	3

Table 3
Comparison of number and percentage of ameba isolates from 95 water samples between Thailand and Japan.

Ameba isolates	Thailand (%)	Japan (%)
Acanthameba	18 (36.7)	14 (22.6)
Naegleria	14 (28.6)	3 (4.8)
Hartmannella	10 (20.4)	26 (41.9)
Vahlkampfia	6 (12.2)	12 (19.4)
Vannella	1 (2)	4 (6.5)
Unidentified amebae	0	3 (4.8)
Total	49 (100)	62 (100)

death in mice. Comparison of number and percentage of ameba isolated from 95 water samples between Thailand and Japan are presented (Table 3).

DISCUSSION

The environmental isolations of pathogenic free-living amebae, *Naegleria* spp and *Acanthameba* spp, are from air, soil and water, with water being the most numerous (Szenasi *et al,* 1998). In our investigation, the most common amebae distributed in moisted soil surface around water resources were *Acanthameba* spp. Although our sample size was small, the great difference between water and soil swab samples were noted with *Acanthameba* and *Naegleria*. *Naegleria* were found in lower numbers than *Acanthameba* isolates. In contrast to *Acanthameba, Naegleria* prefer stagnant and man-made thermal, clean water to warm, organically rich water in the natural resources (De Jonckheere *et al,* 1991; Jariya *et al,* 1997). Only two ameba isolates, *Naegleria* spp and *Acanthameba* spp, were thermophilic and pathogenic to mice. This showed that the thermophilic character is not evidence for pathogenic potential as the majority of the thermotolerant amebae in our investigation were not pathogen similar to that found in other places (Rohr *et al,* 1998; Breiman *et al,* 1990; Sanden *et al,* 1992). However we recovered some unidentified amebae which looked like leptomyxid amebae as reported found in a previous study (John and Howard, 1995).

Hartmannella spp was the dominant ameba distributed in natural water resources in both Thailand and Japan similar to that found in other countries from previous studies(Brown *et al,* 1983; Rohr *et al,* 1998; Breiman *et al,* 1990; Sanden *et al,* 1992). *Vahlkampfia* spp, and *Vannella* spp were other small amebae that we could investigated but in a small number which up to now their pathogenic potential has not been described (Newsome *et al*, 1998). These small amebae have also been reported as the host for *Legionella.* They have been investigated both *in vitro* and found naturally harboring *Legionella* in environmental sources (Breiman *et al,* 1990; Sanden *et al,* 1992; Newsome *et al*, 1998). This finding serves as additional evidence for the presence of either pathogenic or non pathogenic free-living amebae which can also be sources of infection and proliferation of human pathogenic bacteria under natural conditions.

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