

POTENTIALLY PATHOGENIC FREE-LIVING AMOEBAE IN SOME FLOOD-AFFECTED AREAS DURING 2011 CHIANG MAI FLOOD

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SUMMARY

The survey was carried out to investigate the presence of potentially pathogenic free-living amoebae (FLA) during flood in Chiang Mai, Thailand in 2011. From different crisis flood areas, seven water samples were collected and tested for the presence of amoebae using culture and molecular methods. By monoxenic culture, FLA were detected from all samples at 37 °C incubation. The FLA growing at 37 °C were morphologically identified as *Acanthamoeba* spp., *Naegleria* spp. and some unidentified amoebae. Only three samples (42.8%), defined as thermotolerant FLA, continued to grow at 42 °C. By molecular methods, two non-thermotolerant FLA were shown to have 99% identity to *Acanthamoeba* sp. and 98% identity to *Hartmannella vermiformis* while the two thermotolerant FLA were identified as *Echinamoeba exundans* (100% identity) and *Hartmannella* sp. (99% identity). This first report of the occurrence of FLA in water during the flood disaster will provide information to the public to be aware of potentially pathogenic FLA.

KEYWORDS: Flood; Chiang Mai; Free-living amoebae.

INTRODUCTION

Free-living amoebae (FLA) are ubiquitous in nature, mainly in soil and water. Among them, *Naegleria*, *Acanthamoeba*, *Balamuthia* and *Sappinia* are now known to cause brain infections in humans^{7,8,27}. Moreover, *Acanthamoeba*, *Hartmannella* and *Vahlkampfia* can be causative agents of amoebic keratitis in humans^{1,17}. Several FLA are also known to play a role as vectors of several intracellular pathogenic microorganisms, such as *Legionella pneumophila*⁴, *Mycobacterium*¹⁸ and *Chlamydia*-like bacterium²⁰ as they can support the growth of those micropathogens and protect them from the harsh environment.

Surveys in Thailand showed the presence of FLA in the environment including water^{14,16,21}. Recently, FLA were detected in soil and water samples in Chiang Mai areas³⁴. As FLA are abundant in soil, they may be dispersed during flood and as a result human may have increased a risk of getting infected. It is therefore interesting to see if the pathogenic FLA are abundant in water during recent major floods in the country. The present study looked for the occurrence of FLA in the water during the 2011 flood disaster in Chiang Mai, Thailand. The results will provide more useful information to the public so as to have increased awareness of these FLA which can cause severe, life-threatening diseases.

MATERIALS AND METHODS

Sample collection: At end of September 2011, approximately 50 mL

of water samples were collected from each of seven flood crisis areas in Chiang Mai including Pracha Sampan Intersection (cmf1), Chang Klan Road (cmf2), Nawarat Bridge (cmf3), Chiang Mai-Lampun road (cmf4), Chiang Mai Land Village (cmf5), Nong Hoi Road (cmf6) and Charoen Pradet Road (cmf7) (Fig. 1). Samples were transported to the laboratory in the Department of Parasitology, Faculty of Medicine, Chiang Mai University, and processed on the same day.

Amoebae culture: To detect free-living amoebae, 10 µL of the sediment after centrifugation (1,200g, 10 min, RT) was dropped onto the middle of NNA-*E. coli* plates (1.5% non-nutrient agar pre-coated with heat-inactivated *Escherichia coli*). The plates were incubated at 37 °C for two weeks and daily observed for the growth of amoebae using an inverted microscope. If the amoebae existed, Page's amoeba saline solution (PAS) was applied to the culture and amoebae were harvested by scraping the agar surface with spatula. In case of fungal contamination, sub-culturing was performed by cutting the uncontaminated area of agar harboring amoebae and transferring to a new plate. Collected amoebae were subjected to trichrome staining for morphological identification and sub-culturing at 42 °C in order to examine the thermotolerance characteristics³⁴.

Morphological identification: The morphological criteria used to identify amoebae were based on the previous publication³⁰. *Acanthamoeba* cysts are characterized by a double-walled structure with an outer wrinkled wall, while its trophozoite represents fine,

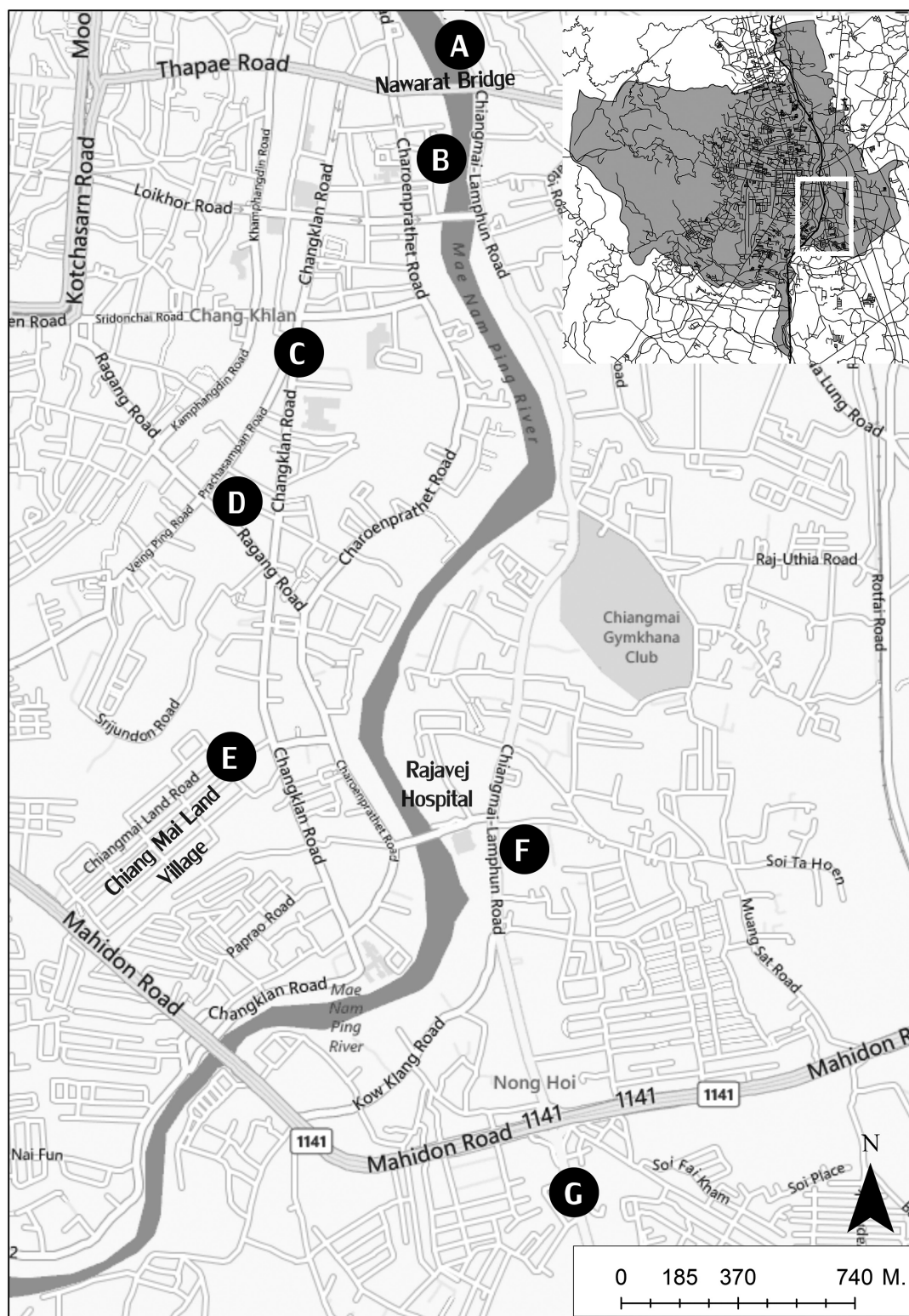


Fig. 1 - Map showing the sampling sites of flood crisis areas in Chiang Mai 2011. Whole picture of Mueang District, Chiang Mai is shown on the upper right. **A:** Nawarat Bridge (cmf3). **B:** Charoen Prate Road (cmf7). **C:** Chang Klan Road (cmf2). **D:** Pracha Sampan Intersection (cmf1). **E:** Chiang Mai Land Village (cmf5). **F:** Nong Hoi Road (cmf6). **G:** Chiang Mai-Lampun road (cmf4).

tapering, hyaline projections called acanthopodia, *Naegleria* cyst bears single layer and smooth cyst wall, where as its trophozoite possesses a large karyosome surrounded by a halo and typical blunt pseudopodia, lobopodia^{30,34}. Morphological examination and photography were done under a light microscope (Olympus CHA) with 1,000x magnification. *Acanthamoeba castellanii* originated from a keratitis patient of Siriraj Hospital was used as positive control. Enflagellation experiment was done to verify the presence of *Naegleria* spp³⁰. Briefly, the suspected trophozoites grown on a NNA-*E. coli* plate were suspended in sterile distilled water and left at room temperature for at least one hour. The presence of flagellate form containing two long flagella³⁴ can be periodically examined under the light microscope.

Molecular identification: DNA preparation was performed by boiling method²⁹. In brief, the amoebae were harvested from the culture plates, washed twice with PAS, and centrifuged at 5,000xg for five min at room temperature. After discarding the supernatant, the remaining cell sediment was suspended and directly heated at 95 °C for 10 min. Following brief centrifugation, 5 µL of the supernatant was freshly used for PCR. Whole sediments of the original water samples that showed no growth in culture were heated and used for PCR as described above. Two different sets of PCR employed in this study included FLA PCR designed for the detection of 18S rDNA of FLA²⁸ and *Acanthamoeba* spp.-specific PCR (ACA PCR) targeted to 18S rDNA of *Acanthamoeba*²⁴. All PCR reactions were done in 50 µL reactions containing 5 µL of 10x PCR buffer (Fermentas®), 1.25 units of *i*-Taq™ DNA polymerase (Fermentas®) and 0.2 mM of each dNTP (Applied Biosystems). The MgCl₂ concentrations were 3 and 4 mM and the primer concentrations were 0.8 µM and 0.5 µM for FLA PCR and ACA PCR, respectively. For FLA PCR, the reactions were performed by incubation for seven min at 94 °C, followed by 40 cycles of one min at 94 °C, one min at 63 °C and three min 30 s at 74 °C, with a final extension at 74 °C for 10 min²⁸. The reaction cycles for ACA PCR were pre-incubation step at seven min at 95 °C, followed by 20 cycles of one min at 95 °C, one min at 60 °C and two min at 72 °C. This was followed by 25 cycles of one min at 95 °C and two min at 72 °C²⁴. The PCR was carried out using DNA engine thermal cycler PTC-100 (MJ Research, USA). PCR products were analyzed by electrophoresis on 1.5% agarose gels and purified using QIAquick PCR purification kit (QIAGEN). The purified PCR products were sent to 1st Base DNA sequencing services (Singapore) for sequencing at both directions using the same primers used in the same PCR. The obtained

sequencing data were compared with all published sequences in GenBank using BLASTn at National Center for Biotechnology Information [http://blast.ncbi.nlm.nih.gov/] and submitted to the GenBank database.

RESULTS

Agar plate culture: At 37 °C culture, all seven water samples collected from the flood affected areas showed FLA growth on the second day of incubation. The growth areas of FLA, as shown by the presence of clear zones on 1.5% NNA-*E. coli* plates, were then cut and sub-cultured at 42 °C. Three out of seven samples (42.8%) were able to grow at 42 °C (Table 1).

Morphological identification: The amoebae growing at 37 °C were collected and subjected to trichrome staining. *Acanthamoeba* and *Naegleria* were concurrently found in most of the flood samples (5/7, 71%) stained with trichrome. *Acanthamoeba* sp. (Fig. 2A) were observed in five samples (except cmf6 and cmf7), while *Naegleria* sp. (Fig. 2B) were detected in six samples (except cmf6). The presence of *Naegleria* spp. in six samples was in correspondence with positive enflagellation test (Table 1). *Acanthamoeba*-like trophozoites showing fine short acanthopodia were also detected (Fig. 2C). Additionally, morphologically unidentified FLA including round double-walled cysts bearing one nucleus with central karyosome (Fig. 2D, E), small round cysts with unstained nucleus (Fig. 2F), small trophozoites presenting short spiny pseudopodia resembling *Echinamoeba* (Fig. 2G, H) and *Hartmannella*-like trophozoites with elongated shape (Fig. 2I) were often seen.

Molecular identification: The amoebae growing on agar culture plate at 42 °C or 37 °C were harvested and identified by PCR. Using the FLA PCR screening, cmf1 yielded a DNA fragment of approximately 1,000 bp (Fig. 3), whereas cmf3 to cmf6 yielded a distinctive band at 800 bp. However, cmf2 and cmf7 were negative by FLA PCR. When the ACA PCR was employed, the approximately 500-bp specific band for *Acanthamoeba* spp. was observed only in cmf1 (Fig. 4). Purified PCR products obtained from ACA PCR (cmf1) and FLA PCR (cmf3, cmf4, cmf5 and cmf6) were used for sequencing. Results from BLASTn revealed that sequence from cmf1 belonged to *Acanthamoeba* sp. (99% maximum identity with *Acanthamoeba* sp. UNB13 from Brazil Accession No. JQ268234, followed by 96% maximum identity with *Acanthamoeba castellanii* Accession No. GU001160). FLA isolated from cmf4 was

Table 1
FLA detected during 2011 Chiang Mai flood

Isolate	Growth at 37 °C	Growth at 42 °C	Enflagellation test	FLA-PCR	ACA-PCR	Sequencing data
cmf1	G ^a	NG ^b	+	Pos	Pos	<i>Acanthamoeba</i> sp.
cmf2	G	NG	+	Neg	Neg	ND ^c
cmf3	G	G	+	Pos	Neg	Invalid data
cmf4	G	G	+	Pos	Neg	<i>Echinamoeba</i> sp.
cmf5	G	G	+	Pos	Neg	<i>Hartmannella</i> sp.
cmf6	G	NG	-	Pos	Neg	<i>Hartmannella</i> sp.
cmf7	G	NG	+	Neg	Neg	ND

^a G: growth, ^b NG: no growth, ^c ND: not done.

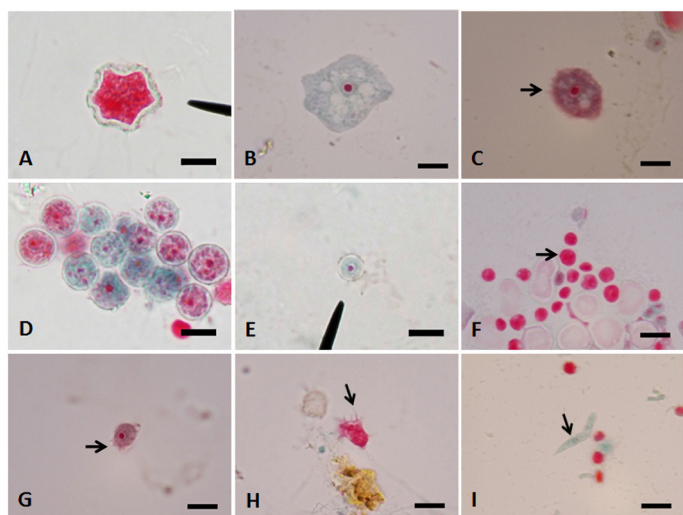


Fig. 2 A-I - Trichrome staining of free-living amoebae under light microscopy (1,000x), bar = 10 µm. **A:** A cyst of *Acanthamoeba* sp. **B:** A large trophozoite of *Naegleria* sp. **C:** *Acanthamoeba*-like trophozoites (arrow showing fine short acanthopodia). **D, E:** Different sizes of unidentified double-walled cysts with distinct nuclei. **F:** Unidentified amoebae with small round cysts (arrow). **G, H:** Small *Echinamoeba*-like trophozoites (arrow showing few spiny short pseudopodia). **I:** An elongated cylindrical trophozoite of *Hartmannella*-like amoeba (arrow).

Echinamoeba exundans (100% identity with *E. exundans* Accession No. AF293895), while those from cmf5 and cmf6 were most closely related to *Hartmannella* sp. (Accession No. HM363627) and *Hartmannella vermiformis* (Accession No. FJ940709) with 99 and 98% maximum identity, respectively. On the other hand, we failed to obtain unambiguous sequencing data from cmf3. It was also impossible to further analyze FLA isolated from cmf2, cmf3 and cmf7 since they were lost during culture. The sequences of FLA reported in this study were deposited in GenBank (Accession Number: JX507295-JX507297).

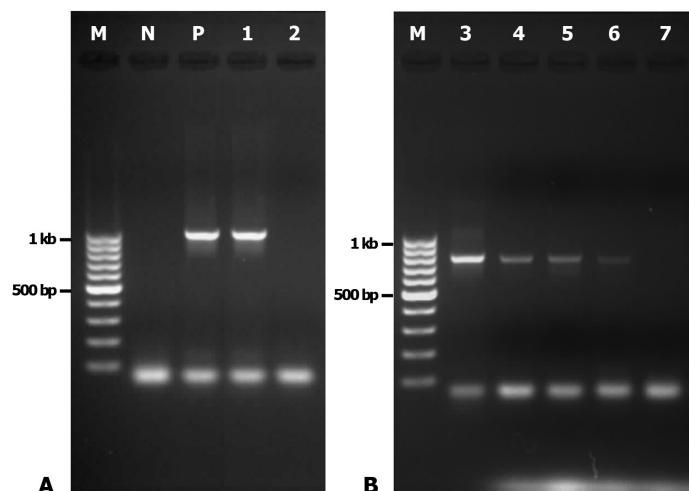


Fig. 3 - Gel electrophoresis of amplicons from FLA PCR conditions. **A:** N - negative control. Lane P (positive control) and lane 1 (cmf1) showing the specific bands (1 kb) for *Acanthamoeba*. Lane 2 (cmf2) showing negative result. **B:** Lanes 3, 4, 5 and 6 (cmf3, cmf4, cmf5 and cmf6, respectively) showing the bands (~800 bp) for other free-living amoebae. Lane 7 (cmf7) showing negative result. M - 100 bp Ladder DNA (Fermentas®).

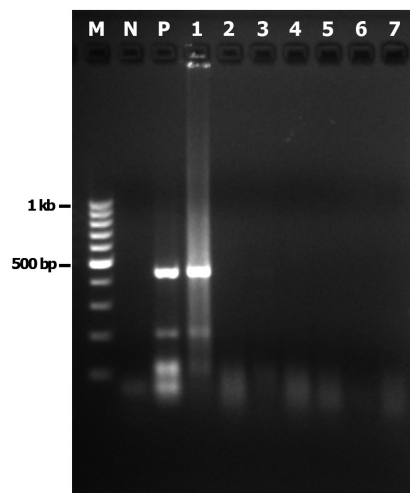


Fig. 4 - Gel electrophoresis of amplicons from ACA PCR conditions. N - negative control. Lane P (positive control) and lane 1 (cmf1) showing the approximately specific bands (500-bp) for *Acanthamoeba*. Lanes 2, 3, 4, 5, 6 and 7 (cmf2, cmf3, cmf4, cmf5, cmf6 and cmf7, respectively) showing negative results. M - 100 bp Ladder DNA (Fermentas®).

DISCUSSION

In Thailand, at least 17 cases of human brain infection caused by *Acanthamoeba*, *Naegleria* and *Balamuthia*^{13,25,35}, 29 cases of acanthamoebic keratitis^{6,15,33}, and one case of *Acanthamoeba* infection of gastric ulcer²⁶ have been reported from several provinces. Although there has never been any case of brain infection due to FLA reported from Chiang Mai, the importance of FLA cannot be neglected. In the previous survey of natural water sources in Chiang Mai in 2009, FLA frequently found were *Naegleria* spp. (37.5%) and *Acanthamoeba* spp. (18.8%). The number of positive thermotolerant FLA from water samples (62.5%) was higher than that from soil samples (37.5%)³⁴. As far as the flood conditions are concerned, such potentially pathogenic FLA in the environment may disperse and increase during the flood. Surprisingly, neither thermotolerant *Naegleria* nor *Acanthamoeba* was found in the present survey. Although the number of samples in the present study was low, detection of thermotolerant FLA were demonstrated in three out of seven water samples analyzed.

To our knowledge, this is the first report of the occurrence of FLA during flood disaster in Thailand. It was not surprising that several amoebae including *Acanthamoeba* and *Naegleria* were detected by microscopic examination after 37 °C incubation in all flood water samples. The presence of *Acanthamoeba* and *Naegleria* in this survey is due to their abundance in nature and ability to dwell in unsanitary conditions. However, both *Acanthamoeba* and *Naegleria* isolated from the flood samples failed to grow at 42 °C. This was unexpected as the previous FLA survey in Chiang Mai showed the occurrence of both thermotolerant *Acanthamoeba* and *Naegleria* in water and soil samples³⁴. Instead, thermotolerant *Hartmannella* and *Echinamoeba* were identified for the first time in Chiang Mai in this survey.

In the present study, five out of seven samples were successfully amplified by FLA PCR. The usefulness of FLA PCR, of which its primers are targeted at the conserved regions of *Acanthamoeba* 18S rDNA, was stated in some studies showing a detection range of several

FLA such as *Naegleria*, *Acanthamoeba*, *Hartmannella*, *Vahlkampfia*²⁸, *Echinamoeba*, *Vannella* and *Protacanthamoeba*⁹ and also ciliated freshwater protozoan *Tetrahymena*³. The negative results of FLA PCR in two of the flood samples might in part be due to the presence of unknown FLA which could not be detected by this PCR. The discrepancy between the sequencing data and microscopic examination and enflagellation test (Table 1) might be the result of sub-culturing procedures that lead to the overgrowth of predominant FLA. Moreover, some samples used for DNA preparation were harvested from continuous sub-culturing, not from the first inoculation as done for microscopic examination. Therefore, FLA detected by PCR could be only the subset of population of the entire samples. In contrast, FLA detected by microscopic examination represented the amoebae grown on the whole culture plates. Regarding cmf3, despite a distinct band obtained by FLA PCR, we could not get the valid sequencing data. It is possible that more than one species of amoebae were present in cmf3 resulting in heterogeneous PCR products and hence ambiguous sequence. In such a failure, axenic culture or cloning by limiting dilution should be considered in future surveys.

Among the non-thermotolerant FLA, only cmf1 and cmf6 were successfully sequenced showing the close relationship to *Acanthamoeba* sp. and *Hartmannella vermiformis*, respectively. The FLA isolated from cmf1 was most similar to *Acanthamoeba* sp. (JG268234, 99% identity). It also had 96% identity to *Acanthamoeba castellanii* (GU001160). It is widely accepted that temperature tolerance is a characteristic of potential pathogenicity, particularly for *Acanthamoeba*^{10,31}. It is therefore unlikely that *Acanthamoeba* identified in this study is virulent. As for *Hartmannella*, no evidence supporting correlation between thermotolerance and pathogenicity has been demonstrated but the health impact of non-thermotolerant *Hartmannella* could not be ignored.

Among the three thermotolerant FLA detected in this study, cmf4 and cmf5 were successfully sequenced and identified as *Echinamoeba exundans* and *Hartmannella* sp., respectively. Although *Echinamoeba* has been occasionally reported from aquatic sources, e.g. lake, leaf litter²², hot water systems of hospitals²³, water bodies⁹ and hot springs². To our knowledge this genus has never been described as a human pathogen. *Hartmannella* is ubiquitous in nature and has recently been associated with amoebic keratitis as it was found to co-infect with *Acanthamoeba* or even with *Vahlkampfia*^{1,12,17}. Even if *Echinamoeba* and *Hartmannella* of thermotolerant isolates investigated in this study are not considered to be as serious as *Acanthamoeba* or *Naegleria*, their important role as potential vectors of pathogens could not be overlooked. *Acanthamoeba*, *Echinamoeba* and *Hartmannella* have been reported to serve as vectors harboring several human pathogenic bacteria, such as, *Legionella pneumophila*^{4,9}, *Exophiala dermatitidis*⁵, *Pseudomonas aeruginosa*^{9,19}, *Comamonas acidovorans*, *Escherichia coli*, *Proteus mirabilis*, *Vibrio cholerae*³² and *Mycobacterium*¹¹. Thus the findings of such amoebae in this survey during flood in Chiang Mai should provide evidence for awareness of outbreaks of human infections caused by these FLA.

RESUMO

Amebas potencialmente patogênicas de vida livre em algumas áreas afetadas durante a inundação de 2011 em Chiang Mai

A pesquisa foi feita para investigar a presença de amebas de vida livre (FLA) durante a inundação em Chiang Mai, Tailândia, ano de

2011. A partir de diferentes áreas de inundação sete amostras de água foram coletadas e testadas para a presença de amebas usando métodos moleculares e de cultura. Através da cultura monoxênica, FLA foi detectada em todas as amostras após incubação a 37 °C. As FLA crescendo a 37 °C foram identificadas morfolologicamente como *Acanthamoeba* spp, *Naegleria* spp e algumas amebas não determinadas. Somente três amostras (42,8%) definidas como FLA termotolerantes continuaram a crescer a 42 °C. Por métodos moleculares duas FLA termotolerantes tiveram 99% de identidade com a *Acanthamoeba* sp e 98% de identidade com *Hartmannella vermiformis* enquanto as duas FLA termotolerantes foram identificadas como *Echinamoeba exundans* (100% de identidade) e *Hartmannella* sp (99% de identidade). Este primeiro relato da ocorrência de FLA em águas durante inundações informa ao público que ele deve estar atento de FLA potencialmente patogênica.

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