โครงการ การประเมินการตรวจหา IgG แอนติบอดีที่จำเพาะต่อเชื้อ *Acanthamoeba* ที่ทำให้เกิดโรคกระจกตาอักเสบเพื่อช่วยในการวินิจฉัยและศึกษาชนิดของแอนติบอดีโดยใช้วิธี two dimensional gel electrophoresis และ mass spectrometry

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เดือน กรกฎาคม ปี 2559

สัญญาเลขที่ **TRG5780034**

รายงานวิจัยฉบับสมบูรณ์

โครงการ การประเมินการตรวจหา IgG แอนติบอดีที่จำเพาะต่อเชื้อ *Acanthamoeba* ที่ทำให้เกิดโรคกระจกตาอักเสบเพื่อช่วยในการวินิจฉัย และศึกษาชนิดของแอนติบอดีโดยใช้วิธี two dimensional gel electrophoresis และ mass spectrometry

ผศ.ดร. พรทิพย์ เหลื่อมหมื่นไวย์
ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
และ มหาวิทยาลัยขอนแก่น

กิตติกรรมประกาศ

รายงานการวิจัยฉบับนี้ ได้มาจากการดำเนินงานโครงการวิจัยเรื่อง การประเมินการตรวจหา IgG แอนติบอดีที่จำเพาะต่อเชื้อ *Acanthamoeba* ที่ทำให้เกิดโรคกระจกตาอักเสบเพื่อช่วยในการวินิจฉัยและศึกษาชนิดของแอนติบอดีโดยใช้วิธี two dimensional gel electrophoresis และ mass spectrometry ความสำเร็จของงานเกิดขึ้นจากการได้รับการสนับสนุนจากหลายฝ่าย ซึ่งต้องขอขอบคุณ ร.ศ ธิดารัตน์ บุญมาศ ซึ่งเป็นอาจารย์ที่ปรึกษาที่ให้คำแนะนำที่มีประโยชน์ต่อการทำงาน และหัวหน้าภาควิชาปรสิตวิทยาที่สนับสนุนการทำวิจัย รวมทั้งฝ่ายวิจัยของคณะแพทย์ที่ให้ความอนุเคราะห์สถานที่ในการทำวิจัย และขอขอบคุณสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) และสถาบันวิจัยและพัฒนาของมหาวิทยาลัยขอนแก่นที่ได้ให้การสนับสนุนทุนวิจัยเป็นเวลา 2 ปี ซึ่งผลจากทำวิจัยที่ได้นี้นอกจากจะได้รับการตีพิมพ์ในวารสารนานาชาติแล้ว ตัวอย่างเชื้อ *Acanthamoeba* สายพันธุ์ต่างๆ ที่ได้จากการศึกษาในครั้งนี้ ยังสามารถนำมาใช้ในการศึกษาคุณสมบัติของเชื้อสายพันธุ์ที่แตกต่างกัน ทั้งในด้านของคุณสมบัติของการเป็นแอนติเจน ความทนทานต่อสภาวะแวดล้อมต่างๆ เช่น อุณหภูมิ สารเคมีที่ใช้ในการฆ่าเชื้อในห้องปฏิบัติการหรือโรงพยาบาล หรือความทนทานต่อน้ำยารักษาสภาพ contact lens เพื่อใช้เป็นข้อมูลในการพัฒนาหรือปรับปรุงน้ำยาฆ่าเชื้อให้มีประสิทธิภาพ หรือนำมาทดสอบกับยาเพื่อศึกษาประสิทธิภาพของยาที่จะสามารถพัฒนาผู้ป่วยที่ติดเชื้อ *Acanthamoeba* ให้มีประสิทธิภาพมากขึ้น

พรทิพย์ เหลื่อมหมื่นไวย์

หัวหน้าโครงการ

กรกฎาคม 2559

รูปแบบ บทคัดย่อ (ภาษาไทย)

รหัสโครงการ: TRG5780034

ชื่อโครงการ: การประเมินการตรวจหา IgG แอนติบอดีที่จำเพาะต่อเชื้อ *Acanthamoeba* ที่ทำให้เกิดโรคกระจกตาอักเสบเพื่อช่วยในการวินิจฉัยและศึกษาชนิดของแอนติบอดีโดยใช้วิธี two dimensional gel electrophoresis และ mass spectrometry

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เอกสารแนบหมายเลข 2

รูปแบบ **Abstract (ภาษาอังกฤษ)**

Project Code : TRG5780034

Project Title : Evaluation of human IgG antibodies to *Acanthamoeba* keratitis for serodiagnosis and identification of the antigen component by two dimensional gel electrophoresis and mass spectrometry

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Project Period : 2 years

Evaluation of human IgG antibodies to *Acanthamoeba* keratitis for serodiagnosis and identification of the antigen component by two dimensional gel electrophoresis and mass spectrometry

Abstract

In Thailand, there are reports of *Acanthamoeba* contaminated natural water sources in Central, Northern and Southern parts, while there have been no report in Northeastern parts. *Acanthamoeba* is a causative agent of cutaneous lesions and sinus infections, vision- threatening keratitis and a rare but fatal encephalitis, known as granulomatous amoebic encephalitis. *Acanthamoeba* can be diagnosed by culture, polymerase chain reaction and serodiagnosis. The aim of this study are to isolate and identify *Acanthamoeba* in natural water sources in the Northeastern part of Thailand and *Acanthamoeba* isolates collected by this study have been used to investigate the immune response of IgG antibody by immunoblotting. In this study, 63 natural water samples from 11 provinces in northeast Thailand were collected and cultured on non-nutrient agar plates. Positive culture were subsequently analyzed by PCR and sequencing. The results showed that 10 samples out of 63 were positive (15.9 %). Phylogenetic analysis revealed that seven samples wereT4, one sample was similar toT3, and the other two samples were similar to T5.This is the first report demonstrating the contamination of *Acanthamoeba* species in natural water sources in northeast Thailand. In addition, T4 isolate from this study was used for investigation of IgG immune response. the result revealed that protein bands at around 14 and 23 showed highly reaction with *Acanthamoeba* patients, whereas, there are no reactive in all sera of healthy control samples and other parasite infections used in this study.

Keywords: *Acanthamoeba*, Culture, Genotyping, Polymerase chain reaction, Sequencing

Introduction

Acanthamoeba is a genus of free-living amoebae that can be found in various environments, e.g., in the soil and in natural and artificial water sources (Marciano-Cabral et al. 2000; Maghsood et al. 2005; Mahmoudi et al.2015). There have been reports of serious diseases being caused by *Acanthamoeba*, including acanthamoebic keratitis (AK), corneal inflammation, and granulomatous amoebic encephalitis (GAE) (Siddiqui and Khan 2012; Maghsood et al.2005). In addition, they are also a causative agent of pneumonitis, sinusitis, dermatitis, chronic granulomatous lesions, etc. (Khan 2006). At present, *Acanthamoeba* consists of 20 genotypes, namely T1–T20, based on nuclear small subunit ribosomal RNA sequence analysis (Fuerst et al. 2015). The clinical isolates include T1, T2a, T3, T4, T5, T6, T10, T11, T12, T15, and T18; the T4 genotype is most frequently isolated from patients (Siddiqui and Khan 2012). In Thailand, several cases of *Acanthamoeba* infection have been reported: one patient with a peptic ulcer (Thamprasert et al.1993), four cases of GAE (Sangruchietal.1994), and 24 patients with acanthamoebic keratitis (Wanachiwanawin et al. 2012). There are many techniques have been used to diagnose Acanthamoeba infection such as culture, PCR and serodiagnosis. ELISA technique had been used to detection *Acanthamoeba* antibodies, the researchers found that anti-*Acanthamoeba* IgG antibody in patients had significantly higher than healthy sera. In addition, there have report of protein profile of *Acanthamoeba* spp. and immunoreactivity for IgG using immunoblotting and the results revealed the prominent band at 29 kDa. However, they did not study the potential of IgG antibody for serodiagnosis of *Acanthamoeba* infection. Previous studies have reported *Acanthamoeba* contamination of natural water sources in the central, northern, and southern parts of Thailand (Lekkla et al. 2005; Nuprasert et al. 2010). However, there have been no reports on the distribution of *Acanthamoeba* in the northeast, even though most people in this area are farmers and have traditionally engaged in activities that involve natural water sources. Therefore, the aim of this study was to isolate and identify the genotypes of *Acanthamoeba* in natural water samples collected from provinces located in the northeastern part of Thailand, using nuclear rRNA sequences. The objectives of this study are to isolate and identify *Acanthamoeba* in natural water sources in the Northeastern part of Thailand and *Acanthamoeba* isolates collected by this study have been used to investigate the immune response of IgG antibody by immunoblotting.

Objectives

1. To isolate and identify the genotypes of *Acanthamoeba* in natural water sources
2. To evaluate the immunogenicity of T4 isolate for serodiagnosis application by using immunoblotting

In this reports, there are two parts of study design

Study design part I: this study has been published.

1. Sample collection and isolation of *Acanthamoeba* spp.

In this study, 63 natural water samples from 11 provinces in the northeastern part of Thailand were collected and tested for the presence of *Acanthamoeba* spp. Water samples (500 ml) from each site were collected in sterile plastic bags. Samples were centrifuged at 377 ×g for 5min, after which 20 µl of each sediment was pipetted onto 1.5 % non-nutrient agar (NNA) plates upon which was spread 5 µl of heat-killed Escherichia coli. The edges of the culture plates were then sealed with parafilm. The culture plates were incubated at 30 °C for 10 days and were observed daily under a stereomicroscope to ensure that there was no *Acanthamoeba* growth. Positive plates were investigated by morphological identification under a light microscope. The positive samples were then sub-cultured by cutting off small pieces of the gels and transferring them to new NNA plates in order to purify them of other organisms, particularly fungi. *Acanthamoeba* spp. of each of the positive samples were then examined by polymerase chain reaction (PCR).

2. Polymerase chain reaction

Five to ten cells of Acanthamoeba from each positive sample were used for PCR analysis (Laummaunwai et al. 2012). The DNA amplification reactions were then performed in a Perkin-Elmer GeneAmp 9600 thermal cycler using genus-specific primers, as previously described (Schroeder etal.2001): DP1(5-GGCCCAGATCGTTTACCGTGAA-3) and JDP2 (5-TCTCACAAGCTGCTAGGGGAGTCA-3) in a 50 µl volume containing 50 mM KCl buffer, 10 mM Tris– HCl, 2.5 mM MgCl2, 200 mM dNTP, 0.5mM of each primer, and 0.08 units Taq DNA polymerase (Applied Biosystems, Waltham MA), pH 8.3. The cycling conditions consisted of an initial denaturing phase at 94 °C for 10 min and 35

repetitions at 94°C for 1 min, annealing temperature of each primer pair at 56 °C for 1 min and 72 °C for 1 min. The primer extension phase was prolonged to 10 min at 72 °C in the last cycle. The PCR products were visualized by electrophoresis on 2 % agarose gel and then subjected to sequencing (Ward Medic, Bangkok, Thailand).

3. Phylogenetic and genotyping analysis

Nucleotide sequences of each isolate were then compared with the reference sequences deposited in the GenBank data- base using the Basic Local Alignment Search Tool (BLAST) program. To identify the genotype level, phylogenetic analysis was performed by comparing the obtained sequences with the reference strains. The phylogenetic tree was constructed using MEGA (Molecular Evolutionary Genetics Analysis) software, version 6 (Tamura et al. 2013).

Results

Ten out of 63 (15.9 %) samples were positive based on mor- phological analysis (Fig. 1) and PCR results (Fig. 2). After sequencing of PCR products, each of the nucleotides equences was examined for similarity using the BLAST program (www.ncbi.nlm.nih.gov/BLAST); the results revealed that all sequences were 98–99 % identical to Acanthamoeba species located in GenBank. All 18S rDNA sequences obtained from this study have been deposited in GenBank (accessionnumber KT897265–KT897274) (Table 1). The genotype was identified by phylogenetic construction using MEGA 6 software. The evolutionary relationship showed seven isolates—UB1, UB2, SU2, MU1, KK1, KK2, and KK3—which were clustered into genotype T4, while the SUI isolate was similar to T3, and the other two (SUI, MSI) isolates were similar to T5 (Fig. 3). The locations of the 11 provinces and areas of those provinces in which *Acanthamoeba* were found are shown in Fig. 4.

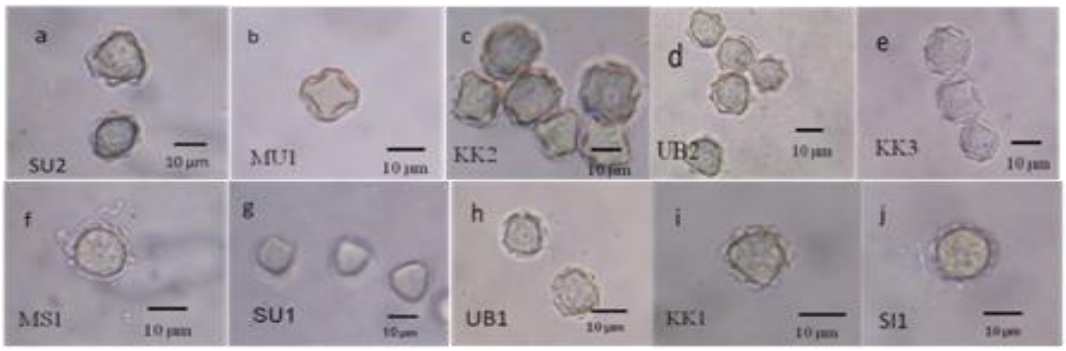

Fig.1 Double-walled cyst of Acanthamoeba spp. identified under light microscope from 10 isolates(a SU2,b MU1, c KK2,d UB2,e KK3,f MS1,g SU1, h UB1, i KK1, j SI1). Scale bar is 10 μm

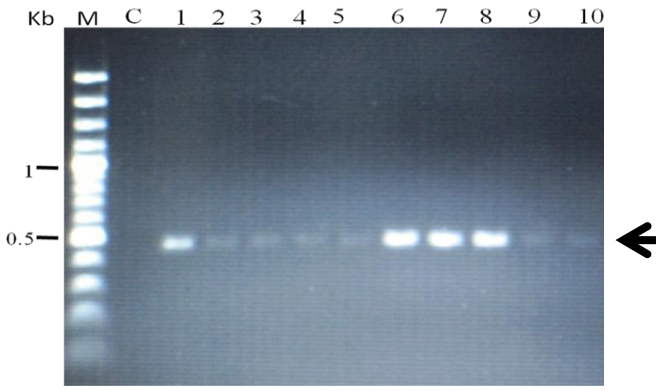

Fig. 2 Agarose gel electrophoresis of PCR products: lane M, 100 bp DNA ladder; lane C, negative control; lane 1, SU2 isolate; lane 2, MU1 isolate; lane 3, KK2 isolate; lane 4, UB2 isolate; lane 5, KK3 isolate; lane 6, MS1 isolate; lane 7, SU1 isolate; lane 8, UB1 isolate; lane 9, KK1 isolate; lane 10, SI1 isolate. The arrow indicates the specific band of approximately 500 bp

Table 1 *Acanthamoeba* strains isolated from natural water sources in this study

Isolate	Sampling area	Province	NNA	PCR	Genotype	GenBank
KK1	Marsh	Khon Kaen	+	+	T4	KT897265
KK2	Lagoon	Khon Kaen	+	+	T4	KT897266
KK3	Lagoon	Khon Kaen	+	+	T4	KT897267
UB1	Marsh	Ubon Ratchathani	+	+	T4	KT897273
UB2	Canal	Ubon Ratchathani	+	+	T4	KT897274
SU1	Rice field water	Surin	+	+	T3	KT897271
SU2	Marsh	Surin	+	+	T4	KT897272
MS1	Rice field water	Maha Sarakham	+	+	T5	KT897268
MU1	Marsh	Mukdahan	+	+	T4	KT897269
SI1	Canal	Sisaket	+	+	T5	KT897270

NNA, samples cultured on non-nutrient agar (NNA) plates

PCR, polymerase chain reaction

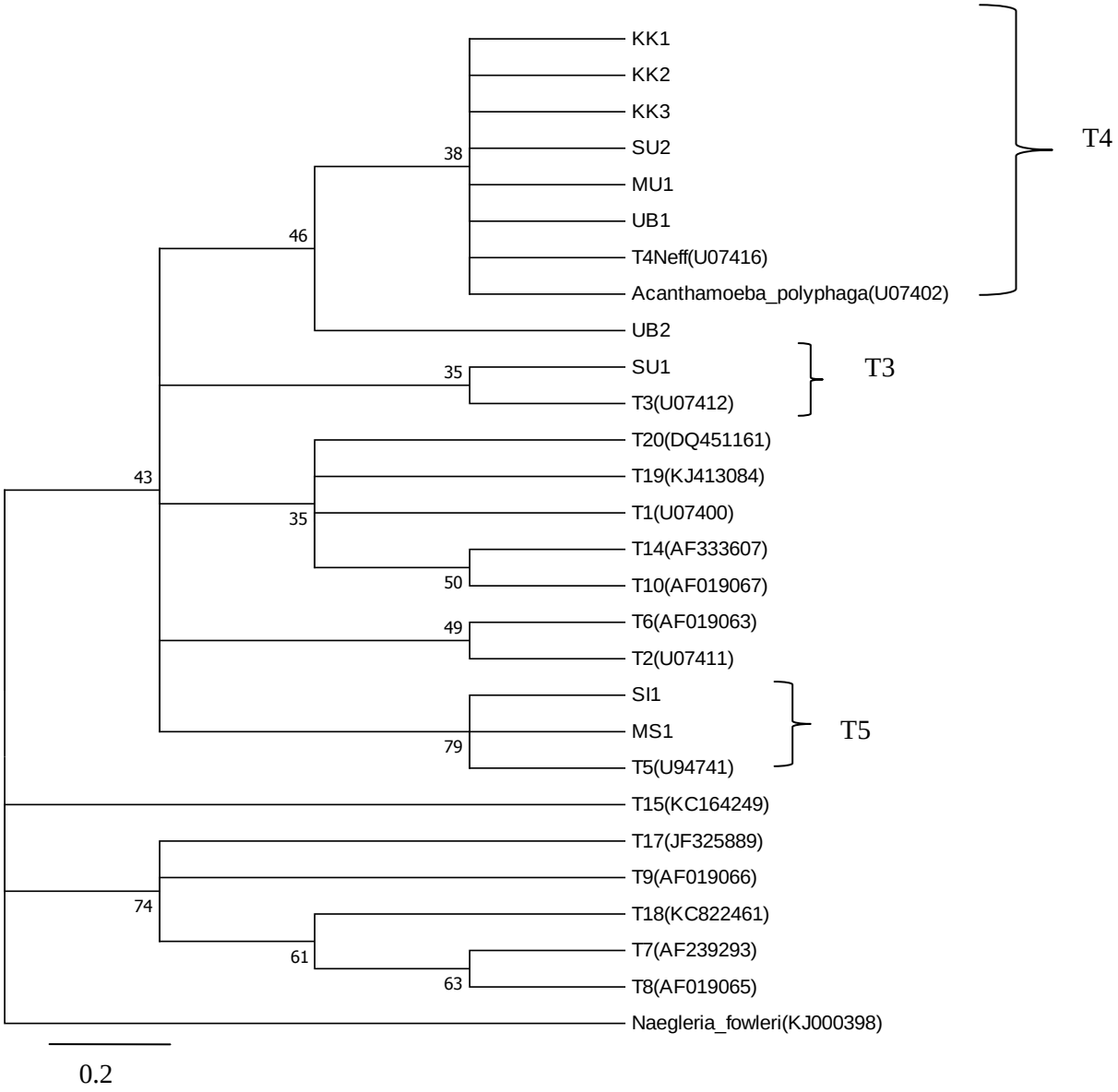

Fig. 3 The phylogenetic relationship of 10 *Acanthamoeba* isolates obtained in this study was analyzed by neighbor-joining based on 18S rRNA gene sequences using MEGA 6 software. Numbers on branches represent the percent of bootstrap values using 1,000 replicates. *Naegleria fowleri* was used as an outgroup

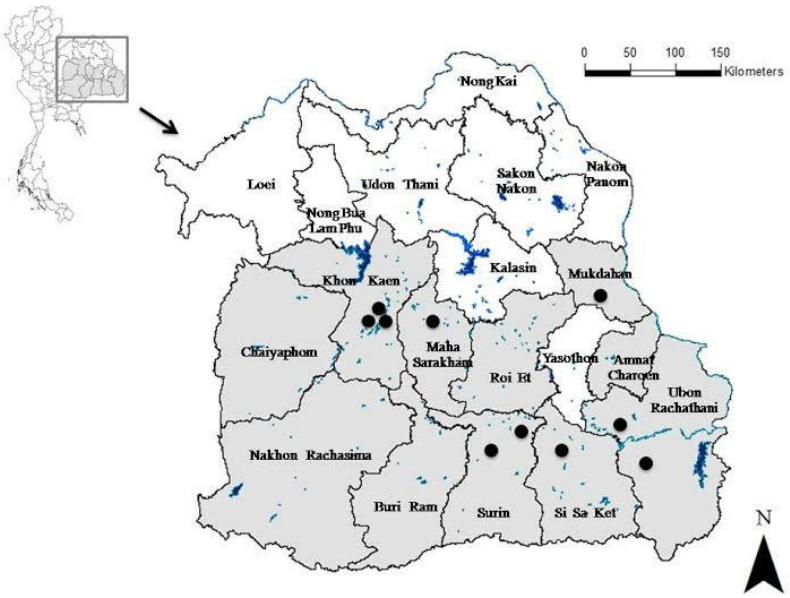

Fig. 4 Map showing the location of provinces in the northeastern part of Thailand. The gray color indicates the areas from which water was collected for investigation. Black dots indicate positive samples found in the region

Discussion

In Thailand, environmental studies have previously been conducted only on *Acanthamoeba* in the central, southern and northern regions (Nacapunchai et al. 2001; Lekkla et al. 2005; Nuprasert et al. 2010; Wannasan et al. 2013). This study is the first report of the occurrence of *Acanthamoeba* genotypes in natural water sources in the northeastern part of Thailand. The northeastern part of Thailand comprises nearly one third of the country's total area. Most of the residents are farmers whose lives are impacted by the conditions of natural water sources. It is important, therefore, that these natural water sources be investigated. Water samples in the study were collected from natural sources associated with human activities, including agriculture, fishing and traditional cultural practices, in order to investigate the risk of *Acanthamoeba* infection. Many cases of infection with *Acanthamoeba* in Thailand have been reported, including

patients with keratitis, GAE, peptic ulcers and sinusitis (Thamprasert et al. 1993; Sangruchi et al. 1994; Jongwutiwes et al. 2000; Sukthana et al. 2005). However, many cases of acanthamoebic keratitis in Thailand have also gone unreported.

In this study, the presence and identification of *Acanthamoeba* was based on morphology and molecular techniques using PCR and nucleotide sequencing. After phylogenetic analysis, the *Acanthamoeba* isolates obtained in this study were classified into T3, T4 and T5 genotypes; according to current knowledge, these *Acanthamoeba* genotypes are pathogenic and virulent strains (Siddiqui and Khan 2012). The T4 genotype has been identified as the major causative agent of keratitis and GAE (Booton et al. 2005; Maghsood et al. 2005). The T5 genotype also has been reported as being associated with keratitis and GAE, while the T3 genotype has been recognized as an agent of keratitis (Siddiqui and Khan 2012). The present study showed that six of ten isolates were closely related to the T4 genotype. This was in accordance with previous reports that the T4 genotype is the most abundant in the environment worldwide (Koltas et al. 2015; Mahmoudi et al. 2015; Todd et al. 2015). In a recent study, samples from freshwater ponds in Bangkok and the surrounding areas were collected and investigated for the distribution of *Acanthamoeba* genotypes, and the results revealed the presence of genotypes T17, T4, T3 and T9 (Nuprasert el al. 2010). It has been shown that T17 and T9 genotypes are not associated with disease (Siddiqui and Khan 2012). Therefore, this study is the first to reveal the pathogenic strain of the T5 genotype.

In this study, 15.9% of collected water samples were positive. However, the samples were randomly collected from each source. This is also the first study to focus exclusively on *Acanthamoeba* genotype levels in northeast Thailand.

In Thailand, *Acanthamoeba* diagnosis is available only in some hospitals in large cities and not in rural areas. Therefore, it is necessary to spread awareness of *Acanthamoeba* to clinicians and public healthcare providers in rural areas in Thailand. Nucleotide sequence data from this study are available in the GenBank database under accession numbers KT897265–KT897274.

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Study design part II (during working)

1. Human sera

Human sera were obtained from serum bank of Khon Kaen University (2514-2515). Each serum was aliquoted and stored at −80°C until used. Samples of human sera were categorized into three groups of subjects. Group 1 comprised of 2 patients with infected with *Acanthamoeba* whose diagnosis was confirmed by PCR with genus specific primer for *Acanthamoeba*. Group 3 comprised of 20 patients with other parasitologically confirmed parasitic infections (Hookworm, strongyloidiasis, opisthorchiasis) Group 4 was made up of ten healthy Thai adults. The examinations of their stool samples, collected at the same time as the serum samples, were performed using the formalin–ethyl acetate concentration method [10]. These gave no evidence of intestinal parasitic infections. The study protocol was approved by the Scientific–Ethical Committee of Khon Kaen University. Informed consent was obtained from the study subjects using a standard approved procedure.

2. *Acanthamoeba* strain, cultivation, and protein preparation

Acanthamoeba used in this study was obtained from natural water source (KT897268). This environmental isolate had its pathogenicity previously demonstrated. Trophozoites were maintained in axenic cultures in peptone-yeast-glucose (PYG) medium, as previously described, and samples for the protein profile analysis were directly taken from these cultures. Cells were harvested at 2000 × g for 10 min and washed twice in phosphate-buffered saline (PBS) buffer (pH 7.2), prior to resuspension in 1 ml of 25 mM Tris–HCl, pH 7.2. Cell suspensions were then lysed by sonication in an ice bath for pulse on 1 s and pulse off 9 with a 1-min interval between pulses. Lysates were centrifuged (12,000 × g, 15 min, 4 ˚C) to separate soluble and insoluble protein fractions. Soluble protein then kept at -20 ˚C until used.

3. SDS-PAGE and immunoblotting

The protein components of Acathamoeba antigens were separately resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on a 12% gel prepared using the method of Laemmli (1970) [11]. Antigen containing 300 μg protein per lane of 7-cm width was loaded onto the gel. After electrophoresis, the resolved polypeptides were electrophoretically transferred to a nitrocellulose membrane for immunoblotting [12]. The antigen-blotted nitrocellulose

membrane was immersed in a blocking solution [1% skimmed milk in 0.1-M phosphate-buffered saline, pH 7.5, containing 0.1% Tween 20® (Sigma, St. Louis, MO)] and cut vertically into strips of 0.5×5.5 cm. One strip was incubated with one serum sample (diluted 1:100 in the blocking solution) for overnight at 4 ˚C and then washed five times with blocking solution, and then incubated for 2 h at RT with peroxidase-conjugated monoclonal anti- human total IgG (Zymed Laboratories, South San Francisco, CA) in blocking solution at a dilution of 1:1,000 After washing, the strips were incubated in diaminobenzidine (Sigma) in 50-mM Tris– HCl, pH 7.6. The blot was developed at RT with agitation until dark brown bands appeared. The strips were then washed with distilled water and air dried.

Result

Acanthamoeba protein stained with coomassie brilliant blue reviewed rather complex protein as shown in figure 1. and multiple major bands could be observed at 14 to 125 kDa. These bands have resulted in immunoreactivity with sera of some healthy person but not reactivity with *Acanthamoeba* patients. The total of 10 sera were tested including two sera of *Acanthamoeba* keratitis and *Acanthamoeba* gastritis. Immunoreactivity of *Acanthamoeba* infection showed positive at around 14 and 23 kDa, particularly in the gastritis patient showed highly reaction more than keratitis patient. While the other sera, strongylodiasis and opisthorchiasis gave no reactivity at 23 and 14 kDa, and serum from Hookworm infective gave positive only band at 23 kDa but weak reaction when compared with gastritis patient. In all sera of healthy control sample have neither reactivity at 14 nor 23 kDa as shown in figure 2.

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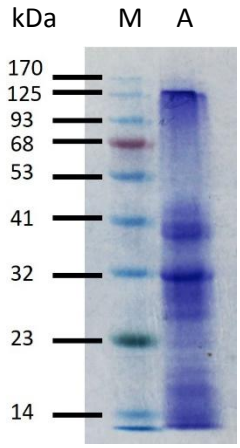

Fig 1. Protein profile of *Acanthamoeba* antigen in Coomassie blue staining, M is molecular weight marker and A is crude antigen prepared from *Acanthamoeba* trophozoites

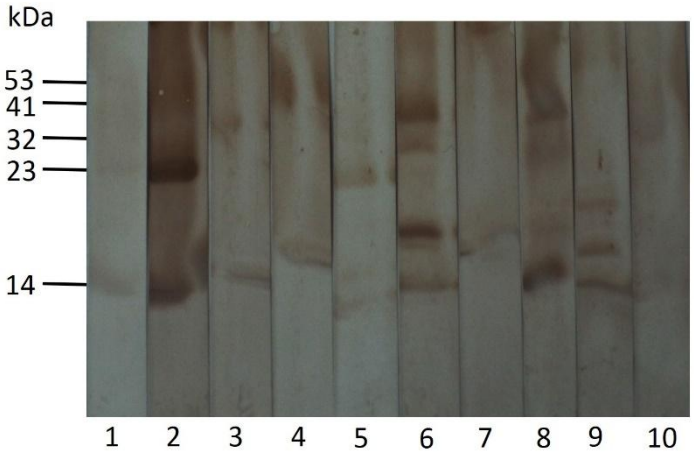

Fig2. IgG immunoreactivities of ten sera from *Acanthamoeba* infection, *Acanhtamoeba* keratitis patient (lane1); *Acanthamoeba* gastritis patient (lane 2); three sera from the patients with *Opisthorchis viverrini* (lane 3), *Strongyloides stercoralis* (lane4) and Hookworm (lane 5); five sera from healthy control (lane 6-10).