ZYMOGRAM PATTERNS OF *NAEGLERIA* SPP ISOLATED FROM NATURAL WATER SOURCES IN TALING CHAN DISTRICT, BANGKOK

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Abstract. A genetic approach was cited for species detection of the ameba genus *Naegleria* using allozyme electrophoresis to characterize the trophozoite stage of three strains of *Naegleria fowleri* isolated from patients with primary amebic meningoencephalitis, five thermophilic (45ºC) *Naegleria* spp isolated from natural water sources in the Taling Chan district, and a reference control strain, *Naegleria fowleri* CDC VO 3081. Isoenzymes of ameba whole-cell extracts were analyzed by vertical polyacrylamide slab gel electrophoresis to determine whether there was any correlation between different strains of the ameba. The results showed that five out of fifteen enzymes; aldehyde oxidase (ALDOX), aldolase (ALD), a-glycerophosphate dehydrogenase (a-GPDH), xanthine dehydrogenase (XDH), and glutamate oxaloacetate transaminase (GOT), were undetectable in the pathogenic strains, while the other enzymes; esterase (EST), fumerase (FUM), glucose-6-phosphate dehydrogenase (G-6-PDH), glucose phosphate isomerase (GPI), isocitate dehydrogenase (IDH), lactate dehydrogenase (LDH), leucine aminopeptidase (LAP), malic enzyme (ME), glucose phosphomutase (GPM), and malate dehydrogenase (MDH), were detected. *Naegleria fowleri* strains were biochemically the most homogeneous. They showed intraspecific isoenzyme variation that allowed them to be grouped. In contrast, the allozyme patterns (EST 1-7, IDH) of *Naegleria* spp isolated from the environment showed interspecific isoenzyme variations from the pathogenic *Naegleria* strain. In conclusion, this study recognized the zymograms of the *Naegleria fowleri* strains were heterogenically different from the thermophilic 45ºC *Naegleria* spp isolated from the environment.

INTRODUCTION

Naegleria fowleri is a small, asexual, free-living amebo-flagellate commonly found in fresh water. It is a causative agent of fatal human primary amebic meningoencephalitis (PAM). Although PAM is rare, this central nervous system disease is lethal within one week in most cases (Cerval, 1969; Carter, 1970; Laemmli, 1970; De Jonckheere *et al*, 1977; Jariya *et al*, 1983; Charoenlarp *et al*, 1988; Sirinavin *et al*, 1989; Poungvarin and Jariya, 1991; Wang *et al*, 1993). The majority of fatal infections due to *N. fowleri* occur in young people exposed to warm water in ponds, swimming pools, and natural water sources. *N. fowleri* is thermophilic and generally found in natural and artificially-heated water, in which this species can proliferate intensely (Singh and Das, 1972; Kasprzak and Mazur, 1974; De Jonckheere *et al*, 1975; Griffin, 1983; Jariya *et al*, 1988; 1990; Mangkalanond *et al*, 1998; Tiecharoen *et al*, 2001). The identification of *N. fowleri* depends on its morphology and physiological characteristics (Beaver *et al*, 1984; Jariya *et al*, 1997; Permin and Grelauel, 1989). However, a genetic approach to species differentiation of amebae of the genus *Naegleria* using allozyme electrophoresis is used to detect genetic differences in *Naegleria*. In this study, we applied a simplified isoenzyme pattern procedure to specimens of *Naegleria* isolated from the environment, in order to 1) conduct a preliminary biochemical study of *N. fowleri* Thai strains, and 2) to identify other thermophilic *Naegleria* isolated from natural water sources.

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MATERIAL AND METHODS

Ameba isolation

Three hundred and fifty water samples were

taken over one year, during the years 2001-2002, in five tambons in Taling Chan district, Bangkok, Thailand. Sterile 500 ml glass containers were filled at the surface of the water. Water plants and detritus taken from the water were placed in sterile plastic bags. The temperature of the water was taken at the sampling sites, while the pH was measured in the laboratory with a glass electrode. All samples were taken in the afternoon and processed the same day. For isolation of *Naegleria* spp, 250 ml water samples were filtered with slight suction through sterile 5.0 μm cellulose acetate membranes (Gelman, Michigan), 47 nm in diameter. The inverted membranes and solids were incubated at 45ºC on non-nutrient agar 1.5% Difco Bacto-Agar in ameba saline: NaCl, 0.12 g; $MgSO_4$ .7 H_2O, 0.004 g; $CaCl_2$ .2 H_2O, 0.004 g; Na_2HPO_4, 0.142 g; KH_2PO_4 0.136 g; distilled water 1,000 ml, then overlain with a thin pellicle of living *Escherichia coli* as a food source.

In contrast to the *N. fowleri* isolation technique used previously, the temperature was increased from 42ºC to 45ºC to suppress the growth of other free-living amebae. A temperature of 45ºC selectively favors the pathogenic *N. fowleri* over the known non-pathogenic variants isolated from the same water sources. However, a highly pathogenic strain was identified when the plates were examined daily for growth over 3 to 4 days. An inverted microscope (x200) was used to identify the *Naegleria* species by their morphological characteristics. Two agar squares were cut from the corresponding plate for species identification using biochemical methods. For the biochemical procedure, this culture was carried out to prevent fungal overgrowth, and to obtain the isolate in sufficient quantity. By polyacrylamide gel electrophoresis, 5 out of 16 samples, having similar molecular weights to *N. fowleri,* were isolated.

Cultivation of amebae

N. fowleri 3081 (CDC) and three *N. fowleri* Thai strains were maintained in Nelson media to which 10% heat inactivated fetal calf serum had been added, in the Department of Parasitology, Faculty of Medicine at Siriraj Hospital, Mahidol University. Initial isolates of *N. fowleri* and *Naegleria* spp isolated from the environment, were made in 1986 from a Thai patient who died at Siriraj Hospital (Si). Additional isolates were from patients who died at Ramathibodi Hospital (Ra) and Chachoengsao Hospital (Cha), in 1987. Isolated *Naegleria* spp from natural water sources were cultured in SCGYEM (Chang, 1997), then inoculated into Nelson media (Nelson and Jones, 1970) to give cell populations of around 10^4 cells/ml, and were then incubated at 37ºC.

Sample preparation

To study the zymograms of these amebae, and to make a comparison with the four reference strains of *N. fowleri,* samples of the four reference strains of *N. fowleri* were prepared. Flasks of early stationary phase cultures were placed in an ice bath for five minutes and agitated to detach adhering cells. The trophozoite form was produced by adding 0.01% p-aminobenzoic acid. The trophozoites were washed three times with distilled water, then the suspension was ultrasonicated for 5 minutes in an ice bath and centrifuged at 10,000*g* at 4ºC for 30 minutes. The supernatant was then collected for further isoenzyme study.

Isoenzyme identification

The environmental *Naegleria* strains were identified by detecting the fifteen enzymes, as in

Table 1
List of thermophilic *Naegleria* strains collected from natural water sources.

Places (tambon)	Samples (No)	Temperature (ºC)	pH	NNE		PAGE-positive strains
				37ºC	45ºC	
Taling Chan	60	28-34	6-6.8	29	2	[a]
Chim Plee	75	30-35	6-6.8	32	2	2
Bang Prom	70	30-36	6-6.8	38	6	2
Bang Ramard	75	30-36	6-6.8	37	4	1
Bang Cherknang	60	30-36	6-6.8	26	*2*	[a]
Total	340			162	16	5

[a]Different molecular weight from control group.

Table 2. Allozyme electrophoresis was carried out using vertical polyacrylamide slap gel following the Takai method (Takai, 1984). The gel was composed of a 5% gel stacking layer and a 7.5% gel separating layer. The gel buffer for the stacking gel was 0.5 M Tris-HCl pH 6.8, for the separating gel 1.5 M Tris-HCl pH 8.9, and for the electrode buffer 0.005 M Tris-0.038 M glycine pH 8.3. Loci were designated numerically according to increasing electrophoretic mobility. Approximately 30 µl of sample supernatant were loaded in a separate pocket of stacking gel. The proteins contained in the supernatant were subjected to electrophoresis for 3 hours at a constant voltage of 300 volts. Electrophoresis was done at 5ºC and stopped when the indicator dye (bromophenol blue) had migrated about 10 cm into the separating gel. The methods used for enzyme visualization were those of Steiner and Joslyn (1979), and Tsukamoto (1984), with slight modification. A reference sample of homogenous *Escherichia coli* was included in all gels to ensure that no isoenzyme of bacterial origin was scored as a *Naegleria* allozyme. In contrast, the four reference strains of *N. fowleri* were the positive controls in this research.

Table 2
Enzymes used for the isoenzyme study of *Naegleria* species.

Enzyme classification no.	Enzyme
1.2.3.1	Aldehyde oxidase (ALDOX)
4.1.2.13	Aldolase (ALD)
3.1.1.1	Esterase (EST)
4.2.1.2	Fumerase (FUM)
1.1.1.49	Glucose-6-phosphate dehydrogenase (G-6-PDH)
5.3.1.2	Glucosephosphate isomerase (GPI)
2.6.1.1	Glutamate oxaloacetate transaminase (GOT)
1.1.1.42	Isocitate dehydrogenase (IDH)
1.1.1.27	Lactate dehydrogenase (LDH)
1.1.1.40	Malic enzyme (ME)
1.1.1.8	a-Glycerophosphate dehydrogenase (a-GPDH)
2.7.5.1	Glucosephosphomutase (GPM)
1.2.1.37	Xanthine dehydrogenase (XDH)
1.1.1.37	Malate dehydrogenase (MDH)
1.1.1.3	Leucine aminopeptidase (LAP)

RESULTS

The results showed the zymograms of control species of *N. fowleri* and *Negleria* spp isolated from natural water sources in Taling Chan district (Table 1). Fifteen isoenzymes were sought in this experiment, of which five (ALDOX, ALD, ADH, a-GPDH, GOT) (Table 2) were undetectable. Ten distinct isozyme patterns (ME-1,2, LDH-1, MDH-2, GPI-1,3,4, GPM, EST-2, IDH)(Table 3, Figs 1,2,3) were observed with a high density homogenic band in the reference control. Among the four strains of *N. fowleri*, only *N. fowleri* 3081 showed different zymograms for FUM-1, G-6-PDH-1,2,3,4, LDH-2, MDH-1, EST-1, LAP-1,3, while the other *N. fowleri* strains showed a dense band for GPI-2,5, EST-3,5, LAP-2. However, *N. fowleri* 3081, Ra and Cha showed GPM faint band of allele b, while the band of allele a was in the Si strain. The five isolates of

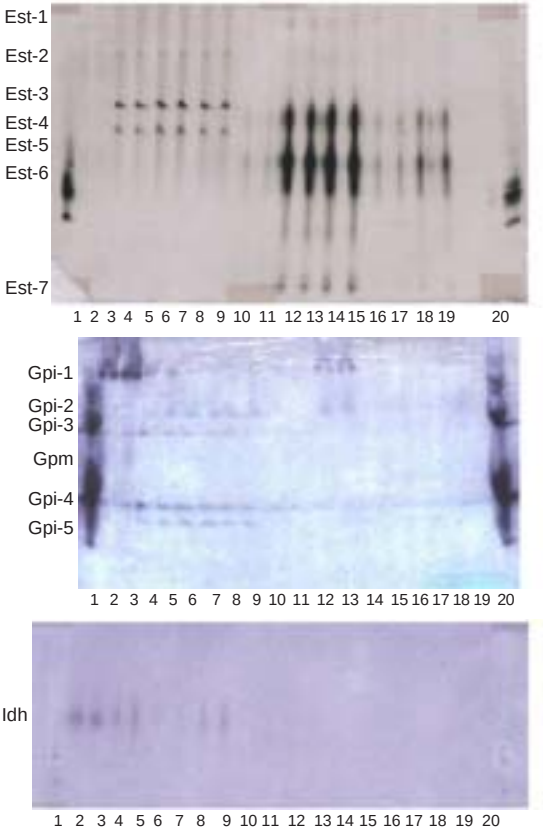

Fig 1–Est, Gpi, Gpm and Idh banding patterns of *Naegleria* spp. 1,20=*E. coli*; 2,3=CDC; 4,5=Si; 6,7=Ra; 8,9=Cha; 10,11=isolate No.1; 12,13=No.2; 14,15=No.3; 16,17=No.4; 18,19=No.5.

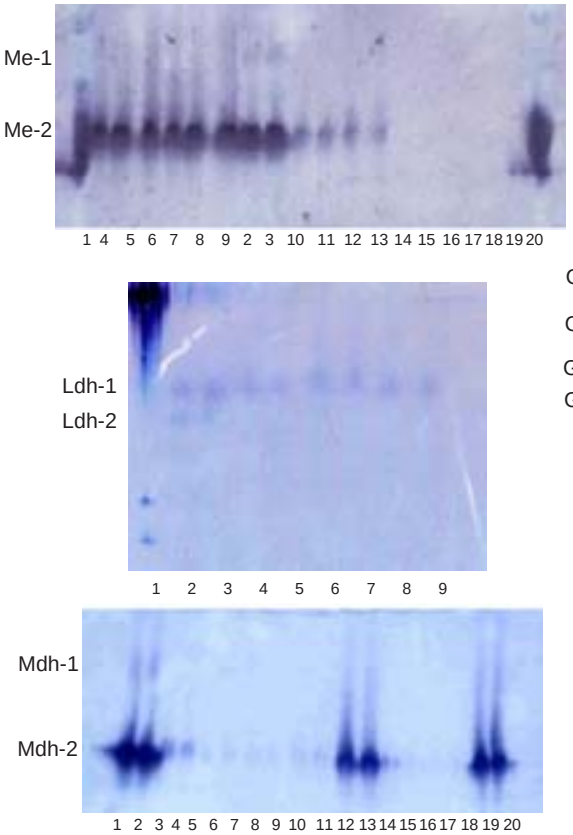

Fig 2–Me, Ldh, and Mdh banding patterns of *Naegleria* spp. 1,20=*E. coli*; 2,3=CDC; 4,5=Si; 6,7=Ra; 8,9=Cha; 10,11=isolate No.1; 12,13= No.2; 14,15=No.3; 16,17=No.4; 18,19=No.5.

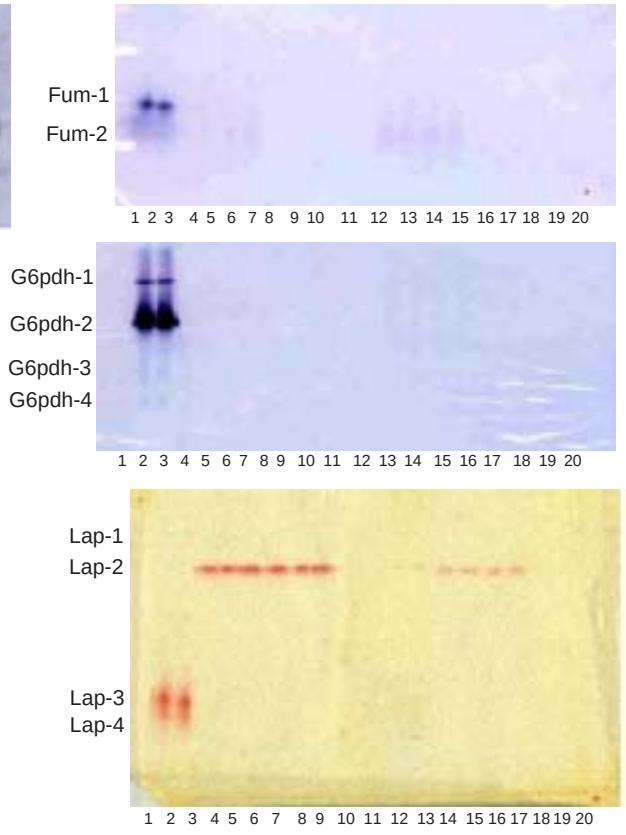

Fig 3–Fum, G-6-pdh, and Lap banding patterns of *Naegleria* spp. 1,20=*E. coli*; 2,3=CDC; 4,5=Si; 6,7=Ra; 8,9=Cha; 10,11=isolate No.1; 12,13= No.2; 14,15=No.3; 16,17=No.4; 18,19=No.5.

the 45ºC-tolerant (Nos 1,2,3,4,5) (Figs 1,2,3 and Table 3) were closely related, characterized by a dense band in MDH-2, GPI-4. However, most isoenzymes were undetectable in five natural isolates, while they were present in the referent pathogenic strains. Some interspecific variation occurred among the isolates. Nos 1,2 had the ME-2 band, while Nos 2,3 had allele EST-1, FUM-2 and Nos 3,4 had LAP-2. *Escherichia coli* was used as a negative control, because the characteristics are biochemically dissimilar from *Naegleria* isolates to be useful as out-groups in this type of isoenzyme study.

DISCUSSION

Isoenzyme electrophoresis by zymogram pattern has become a convenient and reliable method of distinguishing the named species of *Naegleria*, since these characteristics demonstrate the extraordinary diversity of the strain, which contrasts with the apparent homogeneity of the cytomorphologic structure (Permin *et al*, 1985; Adam *et al*, 1989). In this study, allelic profiles (10 enzymes; 29 loci) of various strains of *Naegleria* species demonstrate the genetic interpretation of four strains of *N. fowleri* and five samples of thermophilic species isolated from the environment. Most of the reference control *N. fowleri* strains showed homogeneous zymograms (9/10 enzymes) and a few of them showed slightly heterogeneous differences in this group. There were some intraspecific variations in isozyme patterns in the control group, since diversity also exists to a lesser degree in *N. fowleri* strains from a very homogeneous group, for most enzymatic systems tested (Szenasi *et al*, 1998). These results may explain the diversification seen in pre-

Table 3
Allelic profiles of various isolations of *Naegleria* species.

Loci	Isolations								
	CDC	Si	Ra	Cha	No.1	No.2	No.3	No.4	No.5
ME-1	a	a	a	a	0	0	0	0	0
ME-2	a	a	a	a	a	a	0	0	0
LDH-1	a	a	a	a	0	0	0	0	0
LDH-2	a	0	0	0	0	0	0	0	0
MDH-1	a	0	0	0	0	0	0	0	0
MDH-2	a	a	a	a	a	a	a	a	a
GPI-1	a	a	a	a	0	a	0	0	0
GPI-2	0	a	a	a	0	a	0	0	0
GPI-3	a	a	a	a	0	0	0	0	0
GPI-4	a	a	a	a	a	a	a	a	a
GPI-5	0	a	a	a	0	0	0	0	0
GPM	b	a	b	b	N D	N D	N D	N D	N D
EST-1	a	0	0	0	0	a	a	0	0
EST-2	a	a	a	a	0	0	0	0	0
EST-3	0	a	a	a	0	0	0	0	0
EST-4	0	0	0	0	a	a	a	a	a
EST-5	0	a	a	a	0	0	0	0	0
EST-6	0	0	0	0	a	a	a	a	a
EST-7	0	0	0	0	a	a	a	a	a
FUM-1	a	0	0	0	0	0	0	0	0
FUM-2	a	a	a	0	0	a	a	0	0
G-6-PDH-1	a	0	0	0	0	0	0	0	0
G-6-PDH-2	a	0	0	0	0	0	0	0	0
G-6-PDH-3	a	0	0	0	0	0	0	0	0
G-6-PDH-4	a	0	0	0	0	0	0	0	0
LAP-1	a	0	0	0	0	0	0	0	0
LAP-2	0	a	a	a	0	0	a	a	0
LAP-3	a	0	0	0	0	0	0	0	0
IDH	a	a	a	a	0	0	0	0	0

Loci are designated numerically according to increasing electrophoretic mobility.
The allele is designated alphabetically according to increasing electrophoretic mobility.
ND = not done

vious studies (Pernin, 1984). From these results, obtained for only five thermo-tolerant strains, it is apparent that these zymograms patterns possibly represent important differences in the reference control group of *N. fowleri*. Among the thermo-tolerant strains it is suggested that the zymograms are distinguishable because of the corresponding heterogeneity of the alleles. Some of the isoenzymes (MDH-2, GPI-4) were similar to the reference control, due to the fact that these strains constitute a particular group, defined by the common biological properties in the *Naegleria* group (Visvervara and Healy, 1988; Robinson *et al*, 1992). This study was concerned with a limited sample of various particular strains, selected for their biological properties (thermo-tolerance at 45ºC), but gives an indication of the extraordinary diversity of free-living *Naegleria*. The present work is a first step in studying the zymograms of *N. fowleri* Thai strains and thermophilic *Naegleria* strains isolated from the environment.

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