

Morphological and Physiological Characterization of Philippine *Naegleria* Isolates

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Naegleria is a free-living amoeba with a transient flagellate stage and a resistant cyst in its life cycle. One species, *Naegleria fowleri*, causes primary amoebic meningoencephalitis (PAM) in humans. Eight environmental isolates of *Naegleria* were obtained from the University of the Philippines in Diliman, Mt. Arayat, Marikina, Taal Island, and Calamba, Laguna. They were subsequently maintained as clonal cultures derived from single cells. Together with the local clinical isolate that was previously designated as *N. philippinensis* (RITM-1) and a Japanese clinical isolate of *N. fowleri* (IT9511) for comparison purposes, all isolates were characterized using morphological and physiological parameters. Differences in cyst morphology, rate of encystment, rate of enflagellation, and thermal tolerance at 45°C indicate that the Philippine isolates may actually belong to other species that have not yet been described.

Keywords: cyst morphology, encystment, enflagellation, thermal tolerance

Naegleria is a free-living amoeboflagellate whose life cycle consists of three states: the actively dividing and feeding amoeba or trophozoite stage, the transient, non-feeding bi-flagellate stage, and the resistant, pore-plugged cyst stage (Marciano-Cabral 1988). Environmental factors can cause the transformation of the trophozoite to either the flagellate or the cyst stage. For instance, trophozoites that are placed in nutrient-deprived buffers such as distilled water or amoeba saline will exhibit enflagellation, although they can quickly revert back to their amoeboid form (Woodworth et al. 1982, Cable & John 1986). The characteristics of *Naegleria* makes it a model organism for cellular transformation (Fulton 1967). Food deprivation for extended period of time or desiccation will lead to the encystment of the trophozoite (Marciano-Cabral 1988; Ma et al. 1990). Some species of *Naegleria* are thermotolerant, enabling them to survive as cysts up to 45°C. In fact,

a few even proliferate as trophozoites at 43-46°C (Aufy et al. 1986; Dobson et al. 1997).

One species, *N. fowleri*, is an opportunistic pathogen that causes primary amoebic meningoencephalitis (PAM), an extremely rare and fatal human disease acquired by people who come into contact with contaminated waters, soil, or vegetation. The amoebae enter the nasal passages, disrupt the olfactory mucosa, pass through the submucosal nervous plexus and the cribriform plate, and finally infect the olfactory bulbs through the olfactory nerves (Martinez et al. 1973, Parija & Jayakeertee 1999, Jardim et al. 2000). Two other species, *N. australiensis* and *N. italica*, are also pathogenic to mice but have not yet been known to infect humans (De Jongheere 1981, Scaglia et al. 1982). A fourth species, designated *N. philippinensis*, was isolated from the brain aspirate of a patient who manifested clinical symptoms of PAM. When subjected to mouse lethality test, enflagellation kinetics, immunological reactions, and isozyme analyses, the

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isolate was found to be distinct from the pathogenic *N. fowleri* and *N. australiensis* (Simeon et al. 1990, Matias et al. 1991, Perez-Orozco 1991, Silva-Tahat & Enriquez 1991).

Once *Naegleria* has been obtained from the environment, certain methods are immediately employed to characterize the isolate, mostly with the aim of determining if it is potentially pathogenic. These common methods include cyst morphology, rate of encystation, and temperature tolerance. Cyst morphology can distinguish, up to a certain extent, the pathogen from non-pathogenic *Naegleria* species, particularly if the presence or absence of a lip or rim on the cyst pore is to be considered. Physiological characteristics such as rate of encystation and thermal tolerance at 45°C can also help differentiate certain isolates (Shuster 1975, De Jonckheere & Van de Voorde 1977). However, there is yet no known study that examines whether or not the various species of *Naegleria* exhibit different rates of encystation. Recently, molecular methods have also been used; one example is the sequence analysis of the 5.8 S rDNA gene and internal transcribed spacers which was employed for phylogenetic evaluation and species confirmation of the *Naegleria* isolates (Pelandakis et al. 2000, Fontanilla et al. 2001).

This paper characterizes eight Philippine environmental isolates of *Naegleria* and compares them with the clinical isolates *N. philippinensis* and *N. fowleri*. Characterizations are based on (1) selected morphological characters such as cyst size, the number of cyst pores and presence or absence of lip or rim on the pores; and (2) physiological characters, particularly on rate of encystation, rate of encystation, and temperature tolerance at 45°C.

Materials and Methods

Isolation and Screening of *Naegleria*

Soil and water samples were obtained from different sites of the Philippines. Both water and soil samples suspended in distilled water were filtered. The filter paper used for each site was placed at room temperature in bacterized 1.5% non-nutrient agar (NNA) plate containing *Klebsiella oxytoca*. The plates were observed everyday for three to five days for the presence of trophozoites or cysts. The agar plates were then flooded with distilled water and scraped gently using a hockey stick. The suspension was subsequently washed three times by centrifugation at 200 to 2500 rpm for five minutes. The presence of flagellates near the surface of the cell suspension was determined from 1 to 5 hours by microscopic inspection. One to two

hundred microliters were obtained near the surface of the cell suspension positive for flagellates and transferred to new bacterized agar plates. The plates were then set aside for 3 to five days until trophozoites and/or cysts appeared. A piece of agar containing relatively few and closely arranged cells were cut and placed in new bacterized agar plates and maintained at room temperature. The screening for encystation was repeated two more times.

Establishment of Cloned *Naegleria* isolates

Five hundred microliters of 1.5% NNA was poured over a microscope slide to form a thin layer that was allowed to solidify. Cysts or trophozoites were harvested from the agar plates using sterile distilled water, and then a small volume (25-50 µl) of the cell suspension was added on top of the solidified NNA on the microscope slide. The cell suspension was spread by moving the slide from side to side before observing it under an inverted microscope. A piece of the NNA that contained only one cyst or trophozoite was cut using a very sharp and thin point of a Pasteur pipette. The cut NNA was transferred to a new agar plate containing heat-killed *E. coli*. The agar plate was finally observed for the growth of amoebae after two days.

Eight clonal cultures from five different sites were established. These were the Calamba, Laguna isolates (PNCA-1 and PNCA-1), Mt. Arayat isolate (PNMA-1), University of the Philippines, Diliman isolates (PNML-1 and PNML-2), Marikina isolate (PNMR-1), and Taal isolates (PNTL-1 and PNTL-2). Two previously cloned clinical isolates of *Naegleria* were also included in this study. These were *N. philippinensis* (RITM-1) obtained by the Research Institute for Tropical Medicine and characterized by Enriquez and co-workers at the Natural Science Research Institute, UP Diliman, and *N. fowleri* (IT9611) provided by the Department of Parasitology, Kurume University School of Medicine, Japan. (See table 1 for a brief description of the sources of all the isolates). The ten isolates were then subjected to morphological and physiological characterization.

Morphological Characterization

Cysts were harvested by flooding the agar plates with sterile distilled water and by scraping the surface. The cell suspension was washed three times by spinning at 2500 rpm for 5 minutes. The cysts were resuspended in 2.0-ml sterile distilled water and fixed with 1.0-ml Lugol's solution. Thirty cells were measured for the cyst diameter using a calibrated eyepiece micrometer, after which the measurements were checked for significant differences among the clones using Duncan's Multiple Range Test (DMRT). The number of pores per cyst and the presence or absence of a rim or lip for each pore were also determined.

Table 1. Cyst morphology of the *Naegleria* isolates used in this study.

Isolate	Source	Cyst diameter			Number of pores		Lip or rim on pores
		mean (μm)	range (μm)	mode (μm)	range	mode	
PNCA-1	Water sample from a hot spring, Calamba, Laguna	8.83 ^c	7-10	9	3-6	5	+
PNCB-1	Water sample from a hot spring, Calamba, Laguna	11.20 ^a	8-16	11	4-7	5	+
PNMA-1	Soil sample from Mt. Arayat, Pampanga	10.66 ^b	9-12	11	3-8	5-6	+
PNML-1	Soil sample from the University of the Philippines-Diliman Campus, Quezon City, Metro Manila	10.66 ^b	9-13	10	5-7	5	+
PNML-2	Soil sample from the University of the Philippines-Diliman Campus, Quezon City, Metro Manila	10.44 ^b	9-13	10	5-7	5	+
PNMR-1	Soil sample from Nerikins City, Metro Manila	10.10 ^b	9-11	10	4-6	5	+
PNTL-1	Soil sample from Tail Island, Batangas	12.20 ^b	9-15	12	5-8	6	+
PNTL-2	Soil sample from Tail Island, Batangas	12.63 ^b	10-15	12	5-8	6	+
<i>N. fowleri</i> (IT9611)	Brain aspirate from a 25-year old female Japanese from Tsu City, Saga Prefecture, Japan	11.03 ^b	8-15	10	2-5	5	-
<i>N. philippinensis</i> (RITM-1)	Brain aspirate from a 12-year old male Caucasian who swam in a pool in Matbella, Cavite	10.30 ^a	8-13	10	4-7	5	+

a - not significantly different among each other based on DMRT

b - not significantly different among each other based on DMRT

c - values obtained are significantly different from all of the isolates based on DMRT

Physiological Characterization

A. Rate of Encystment

Two-day old clonal cultures in NNA plates with heat-killed *E. coli* were harvested and resuspended in 1.0-ml sterile distilled water. One hundred microliters of the cell suspension was transferred to a small disposable Petri dish containing a thin layer of solidified 1.5% NNA (1.0-ml). Individual trophozoites were observed for encystment every hour starting at 0 hour under a microscope. Encystment was noted if the cytoplasmic contents of the amoeba were fully enclosed in a double-walled structure. The time it took for the cells for each clone to encyst was recorded. The experiment was undertaken three times with three trials for each.

B. Rate of Enflagellation

Cells were harvested in the same manner as described above. Three aliquots of 100-ml each for every clone were placed into wells in a multi-well titer

plate. Individual cells were observed for enflagellation every 15 minutes under an inverted microscope, and the time it took for cells to enflagellate was recorded. Enflagellation was observed when the cells began to round up and eventually assume the pear shape. These cells also swam instead of crawled in an amoeboid movement at the bottom of the plate. Three replicates were conducted, with each replicate having three trials.

C. Thermal Tolerance

Cells were subcultured in 1.5-% NNA plates containing heat-killed *E. coli* in small disposable Petri dishes. These cultures were maintained at room temperature (24°C) for two days or when there was noticeable increase in number of trophozoites before they were incubated at 45°C for 2 days. They were then observed under an inverted microscope for the presence of trophozoites, cysts, or both. These cells were subcultured in new bacterized 1.5% NNA and

maintained for three days at room temperature (24°C). Should the cells survive the thermal tolerance test, noticeable growth would have been observed at room temperature if inspected under an inverted microscope. The experiment was done twice.

Results

Morphological Characterization (Table 1)

PNCA-1 from Calamba had the smallest cyst diameter with a mode of 9- μ m while the Taal isolates (PNTL-1 and PNTL-2) had the largest with 12- μ m. Duncan's Multiple Range Test showed that measurements for PNCA-1, PNMA-1, PNML-1 and 2, PMMR-1, *N. fowleri*, and *N. philippinensis* were not significantly different from each other. DMRT also showed that measurements for PNTL-1 and PNTL-2 were not significantly different.

The number of cyst pores varied from 2 to 8 among the different isolates and even within each isolate, with mode ranging from 5 to 6. *N. fowleri* showed the least number of pores with as little as 2 whereas PNMA-1, PNTL-1 and 2 exhibited as much as 8 pores.

All the environmental isolates and the clinical isolate *N. philippinensis* revealed a distinct wall or rim on their pores, which was clearly absent in *N. fowleri* (Fig. 1).

Physiological Characterization (Table 2)

Encystment rate of individual trophozoites showed that the Calamba isolates (PNCA-1 and PNCA-1), Mt. Arayat isolate (PNMA-1), *N. fowleri* (IT9699), and *N. philippinensis* (RITM-1) took longer to encyst with 4 hours on average with PNMA-1 encysting as late as 6 hours in one replicate. The rest encysted in 2 to 3 hours.

Enflagellation of individual trophozoites also revealed that the clinical isolates *N. fowleri* and *N. philippinensis* enflagellated from 3.0 to 4.0 hours and 4 hours, respectively, compared to 0.5 to 1.25 hours in the environmental isolates. Although *N. philippinensis* began enflagellating at 4 hours, their movement was limited to cells rotating or wriggling in place.

At 45°C, only *N. fowleri* and the Mt. Arayat (PNMA-1) and UP Diliman (PNML-1 and 2) isolates showed thermotolerance. *Naegleria fowleri* persisted as trophozoites at 45°C while the other three isolates encysted.

Discussion

Morphological Characterization

The differences in cyst diameter of seven out of the ten isolates were statistically insignificant. This agrees with the results of other studies which show that even isolates of the same species or even cells within an isolate may have varying cyst diameters. For instance, the diameter may range from 6.5 to 12.5- μ m in *N. fowleri* and as much as 14.5- μ m in *N. lovaniensis*. The cyst of the pathogenic *N. australiensis* can sometimes measure up to 19.5- μ m. *N. gruberi*, which forms a species complex that may consist of several species, can have cysts as large as 20- μ m (De Jonckheere & Van de Voorde 1977, Stevens et al. 1980, De Jonckheere 1981, Robinson et al. 1992). Clearly, species identification of the isolates based on cyst diameter is not possible.

The number of cyst pores likewise cannot distinguish the different isolates, although the Taal isolates had the most number of pores based on the mode and the range. In *N. fowleri*, the number of pores ranges from 0 to 5 per cyst while in other species, most notably *N. lovaniensis* and *N. australiensis*, the number can reach up to 8 pores (Stevens et al. 1980, De Jonckheere 1981). On the other hand, only *N. fowleri* so far has not been found to have a volcano-like rim or lip on its pore. In fact, even *N. lovaniensis*, which is closely related to *N. fowleri* morphologically, has a slightly thickened rim on its pores (De Jonckheere 1981). The observations indicate that all

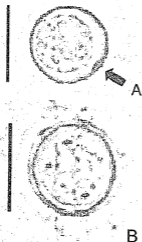


Figure 1. Cysts of two *Naegleria* isolates used in this study. (A) *N. philippinensis* with a rim or lip on the cyst pore (arrow). Note the absence of the rim or lip on *N. fowleri* (B). Bar = 10- μ m.

Table 2. Physiological characterization of the *Naegleria* isolates used in this study.

Isolate	Source	Rate of Encystment* (hours)	Rate of Enflagellation* (hours)	Thermal Tolerance at 45°C**	Dominant Stage at 45°C**
PNCA-1	Water sample from a hot spring, Calamba, Laguna	3-4	0.5-0.75	-	NA
PNCB-1	Water sample from a hot spring, Calamba, Laguna	4	1.25	-	NA
PNMA-1	Soil sample from Mt. Arayat, Pampanga	3-6	0.5-0.75	+	Cyst
PNML-1	Soil sample from UP-Diliman Campus, Quezon City, Metro Manila	2-3	0.75	+	Cyst
PNML-2	Soil sample from UP-Diliman Campus, Quezon City, Metro Manila	2-3	0.75	+	Cyst
PNMR-1	Soil sample from Marikina City, Metro Manila	2-3	0.5-0.75	-	NA
PNTL-1	Soil sample from Taal Island, Batangas	2-3	0.5-0.75	-	NA
PNTL-2	Soil sample from Taal Island, Batangas	2-3	0.5-0.75	-	NA
<i>N. fowleri</i> (IT9611)	Brain aspirate from a 25-year old female from Tosu City, Saga Prefecture, Japan	4	3-3.75	+	Troph
<i>N. philippinensis</i> (RITM-1)	Brain aspirate from a 12-year old male Caucasian who swam in a pool in Marbella, Cavite	4-5	4	-	NA

NA - not applicable

Troph - trophozoite

* two replicates with three trials each

** two replicates

the isolates in the study other than IT9611 are clearly not *N. fowleri*. In addition, correlation of pathogenicity and absence of the rim cannot be assumed since pathogenic *N. australensis* and *N. philippinensis* have rims on their pores.

Physiological Characterization

A. Rate of Encystment

Several factors that affect encystment include accumulation of metabolic waste products from bacteria, food deprivation, lack of oxygen, desiccation, and crowding (Marciano-Cabral 1988). In observing individual cells for encystment in a thin NNA with no *E. coli*, the factors of crowding and waste product accumulation of bacteria were eliminated. In spite of this, different rates of encystment were still noted in this study. Such variations could be genetic in nature. However, distinction among the different species based on encystment rate may be limited. Since *N. fowleri* takes 4 hours to encyst in nutrient-deprived

conditions, then it is safe to conclude that isolates that encyst in 2 hours or less are probably not *N. fowleri*. For this same reason, the local isolates that do encyst at 4 hours or longer (e.g. *N. philippinensis*, PNCA-1, PNCB-1 and PNMA-1) may also form one or more species that are set apart from those that encyst early.

B. Enflagellation Test

Even if enflagellation is not considered a parameter in identifying *Naegleria* species, it can be used as additional information in comparing different species (Fulton & Dingle 1967). Most literature on transformation studies dwell on cell populations being induced to enflagellate. However, even if time for maximum enflagellation differed between *N. fowleri* and *N. gruberi* (Fulton & Dingle 1967, Patterson et al. 1981), such studies are problematic in that cells do not enflagellate synchronously, which Cable & John (1986) observed. A more accurate parameter for measuring rate of enflagellation would be at the level

of individual cells. This study demonstrated that the clinical isolates took considerably longer to enflagellate compared to the environmental isolates. In fact, only very few flagellated *N. philippinensis* cells, whose movements were limited to wiggling or rotating in place, were noted. Such observation could be attributed to the different stages of enflagellation. Preflagellated cells do not normally swim about like normal flagellates; instead, they tremble, then spin, before they suddenly swim (Fulton 1977). The cells noted in *N. philippinensis* were still probably in their preflagellated stage. This study showed that even if most of the local environmental isolates could not be distinguished among each other based on rate of enflagellation, they were certainly distinct from the pathogenic *N. fowleri* and the other local isolate, *N. philippinensis*.

Thermal Tolerance Test at 45°C

Among the local isolates, only the PNML-1, PNML-2, and PNMA-1 survived at 45°C, forming cysts at that temperature. This would further confirm that the three isolates were not *N. fowleri* because the latter predominates as trophozoites at 43°C (Auly 1986). There are five other known species of *Naegleria* that are thermophilic: *N. cartei*, *N. lovaniensis*, *N. morganensis*, *N. niuginiensis*, and *N. sturti* (Dobson et al. 1997). The cyst diameters of *N. cartei*, *N. morganensis*, *N. niuginiensis*, and *N. sturti* are generally larger (mean of 11.8 – 18.2-µm) than the thermotolerant isolates in this study, but the number of cyst pores are just as variable. It is possible that the three local isolates could be any of these thermophilic species, or they could be new species (Dobson et al. 1997).

Correlation of Morphological and Physiological Characteristics with Molecular Data

Previous work done by Fontanilla and co-workers (2001) on the sequence of the 5.8 S rDNA gene and internal transcribed spacers (ITS) of the same *Naegleria* isolates used in this study. Results revealed that the 5.8 S rDNA gene was highly conserved for the Philippine isolates while the ITS regions, particularly ITS 2, were extremely polymorphic. High degree of variation in the ITS was attributed to base substitutions, insertions (either of single nucleotides or entire repeat motifs), and deletions, thus leading to differences in length. Cluster analysis of the sequences together with those of other known *Naegleria* isolates using UPGMA algorithm and Euclidean measure generated a dendrogram showing the local isolates as distinct from each other and from the known isolates. This conclusion explains why the data obtained from morphological and physiological characterizations in the current study are varied.

However, PNML-1 and PNML-2 from UP Diliman had identical sequences for the 5.8 S rDNA gene and ITS, and the same thing was observed for PNTL-1 and PNTL-2 from Taal. It is highly likely that, based also on the morphological and physiological data, PNML-1 and PNML-2 belong to one species while PNTL-1 and PNTL-2 belong to another species.

Summary

Results based on morphological and physiological parameters clearly show that the Philippine *Naegleria* isolates do not form a homogenous group and may actually belong to different species. This conclusion was further strengthened by molecular data from the authors' previous work. The results also confirm the species designation of the clinical isolate *N. philippinensis* as distinct from the pathogenic *N. fowleri*.

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