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# Molecular identification of free-living amoebae of the Vahlkampfiidae isolated in Mexico

Johan F. De Jonckheere [1], Eunice Guzmán Fierros [2] and Fernando Lares Villa[2]

[1] *Scientific Institute of Public Health, B-1050 Brussels, Belgium*
[2] *Technological Institute of Sonora, Cd. Obregon, Sonora, 85000, Mexico*

## Summary

We performed a search for the presence of the vahlkampfiid amoebae in six different states of Mexico. We obtained five *Naegleria* isolates belonging to four described species. Two of them, *N. indonesiensis* and *N. byersi*, were previously though to have restricted geographic distribution. The four *Tetramitus* isolates belong to two known species, one of which has recently been described from Arizona.

**Key words**: 5.8S rDNA, internal transcribed spacer, *Naegleria*, *Tetramitus*

## Introduction

There have been several cases of primary amoebic meningoencephalitis (PAM) caused by the free-living amoeba *Naegleria fowleri* in Mexico (Valenzuela et al., 1984, Lopez-Corella et al., 1989, Lares-Villa et al., 1993, Vargas-Zepeda et al., 2005). As a result, many investigations on the presence of free-living amoebae in the environment were performed in Mexico. Identifications of the Vahlkampfiidae were conducted mainly on morphological basis (Rivera et al., 1993), but sometimes isoenzymes were used (De Jonckheere and Rivera, 1984, Rivera et al., 1989). While morphology does not allow one to identify *Naegleria* species, isoenzymes identify species which have been described previously and of which control protein extracts are run on the same gel. Two high temperature-tolerant *Naegleria* species, *N. australiensis* and *N. lovaniensis*, could be identified by this method amongst the isolates from Mexico. However, several *Naegleria* isolates in these studies could not be identified, and the only other member of the Vahlkampfiidae identified is *Willaertia magna*. Strains of *N. fowleri* have also been isolated from the environment and PAM cases in Mexico (Lares-Villa et al., 1993).

By sequencing parts of the rDNA it is now possible to identify species and describe new species amongst isolates of the genus *Naegleria* and the other genera of the Vahlkampfiidae (De Jonckheere, 1998; De Jonckheere and Brown, 2005). With the use of this DNA sequencing method, one of the earlier untyped *Naegleria* isolates from Mexico was described as *N. mexicana* (De Jonckheere, 2004).

Using these more reliable molecular methods we performed a new survey on the presence of the Vahlkampfiidae in Mexico.

## Material and Methods

Samples of freshwater sediments were taken at different sites in Mexico in December 2005. The sampling area covered the states of Sonora, Sinaloa, Jalisco, Michoacán, Estado de Mexico and Distrito Federal (Table 1). Water bodies sampled were ponds, rivers, lakes and a water reservoir. The samples were kept for a week at room temperature and then inoculated in triplicate onto plates with non-nutrient (NN) agar coated with *Escherichia coli* (Page, 1988). The sealed plates were incubated at 20 °C, 37 °C and 42 °C, respectively.

Only isolates that showed the typical vahlkampfiid morphology were further investigated. Flagellate formation was tested in distilled water at room temperature. For this, a piece of agar with the migrating ring of amoebae was cut from the agar plate and suspended in 0.2 ml of distilled water. The tubes were inspected for up to 24 h with an inverted microscope.

DNA was isolated from pelleted trophozoites using the UNSET method (Hugo et al. 1992). The ITS1, 5.8S and ITS2 rDNA were PCR-amplified using an ITS forward primer and an ITS reverse primer, corresponding to the 3' end of the SSU rDNA and the 5' end of the large subunit (LSU) rDNA, respectively (De Jonckheere, 2004). Two pairs of ITS primers were employed, one pair designed for amplifying from *Naegleria* spp. specifically (De Jonckheere, 1998) and a second less specific primer pair for amplifying from vahlkampfiid species in general (De Jonckheere and Brown, 2005). The PCR product was sequenced (both strands) with the amplification primers without cloning with a Beckman CEQ2000 sequencer using the CEQ Dye Terminator Cycle Sequencing kit (Beckman Coulter Inc., Fullerton, CA, USA).

Total ITS1, 5.8S and ITS2 sequences of the vahlkampfiid isolates were aligned with those of published sequences of different genera (De Jonckheere, 2004; De Jonckheere and Brown, 2005). The sequences differing from those published before have been submitted to EMBL (accession numbers AM418424 to AM418426).

## Results

Except one sample from Michoacán, all samples yielded amoebae (Table 1). The sample from the state of Jalisco, a water reservoir along the road, and one out of two samples from a lake in the Districto Federal, only yielded isolates at 20°C incubation. The majority of the vahlkampfiids were isolated at 42°C incubation, while at the two lower incubation temperatures strains of *Acanthamoeba* predominated in the samples. While most *Acanthamoeba* spp. will not grow at 42°C, strains

**Table 1.** Isolation and morphological identification of free-living amoebae from samples in Mexico

| Sample N° | Place (State) | Water Temperature (°C) | 20 °C | 37 °C | 42 °C |
|---|---|---|---|---|---|
| 1 | Xochimilco Lake (Distrito Federal) | 18 | N | - | - |
| 2 | Xochimilco Lake (Distrito Federal) | 15.5 | + (Ac) | N | - |
| 3 | Pond in La Marquesa (Estado de Mexico) | 6 | + (Ac) | + (Ac) | + (Ac) |
| 4 | Pond in Atlacomulco (Estado de Mexico) | 10.5 | N/+(Ac) | + (Ac) | - |
| 5 | Cuitzeo Lake (Michoacán) | 18 | + (Ac) | V | V |
| 6 | River 30 km from Jalisco border (Michoacán) | 15.5 | - | - | - |
| 7 | Water reservoir in Tequila (Jalisco) | 19 | N | - | - |
| 8 | River 40 km from Mazatlan (Sinaloa) | 17 | + (Ac) | + (Ac) | N? |
| 9 | Lake Guamuchil (Sinaloa) | 20 | + (Ac) | + (Ac) | + |
| 10 | Brook in El Carizo (Sinaloa) | 19.5 | + (Ac) | + (Ac) | V |
| 11 | Rio Mayo (Sonora) | 20 | + (Ac) | + (Ac) | V |
| 12 | Laguna Obregon (Sonora) | 23 | + (Ac) | + (Ac) | N |

+ : positive for amoebae

- : negative for amoebae

(Ac) : is added to + if the amoeba is morphologically identified as *Acanthamoeba*

N : *Naegleria* identified by its typical cysts and eruptive pseudopod formation of amoebae

V : vahlkampfiid, with eruptive pseudopod formation of amoebae, but the cysts different from those of *Naegleria*

? : species of this *Naegleria* could not be identified by molecular methods

of the latter genus probably overgrew the vahlkampfiids at lower temperatures. Only in sample 4 could a *Naegleria* sp. be isolated, while an *Acanthamoeba* strain was also present. The *Acanthamoeba* isolates were not typed by molecular methods.

Four *Naegleria* spp. were identified, *N. clarki, N. pagei*, *N. indonesiensis* and *N. byersi* (Table 2). The strain of *N. indonesiensis* differs by one substitution in the ITS2 sequence from that of the type strain (EMBL AJ243444), while the sequences of the *N. clarki* isolate is totally identical to those of the type strain (EMBL X96575). One of the two isolates of *N. pagei* differs a bit in the ITS2 sequence from that of the type strain (EMBL AJ566633). In strains of *N. byersi* two ITS2 sequence lengths are known, 115 bp and 116 bp, respectively (De Jonckheere, 2004). The isolate from Sonora has the shorter ITS2 sequence (EMBL Y10194). One strain, isolated at 42°C incubation from a river in Sinaloa and identified as *Naegleria* by the typical cyst morphology, could not be identified as the PCR failed to produce a good product for sequencing.

**Table 2.** Molecular identification of the vahlkampfiid isolates and their ability to transform into flagellates

| Isolate N° | Flagellates | Species (sequence difference with type strain) |
|---|---|---|
| 1/20 | + | *Naegleria clarki* |
| 2/37 | - | *Naegleria indonesienis* (ITS2: 1 subst.) |
| 4/20 | + | *Naegleria pagei* (ITS2: 1 indel; 4 subst.) |
| 5/37 | - | *Tetramitus entericus* (ITS1: 1 insert; 5.8S: 1 subst.) |
| 5/42 | - | *Tetramitus entericus* (ITS1: 1 insert; 5.8S: 1 subst.) |
| 7/20 | + | *Naegleria pagei* |
| 10/42 | - | *Tetramitus hohokami* |
| 11/42 | - | *Tetramitus hohokami* |
| 12/42 | + | *Naegleria byersi* |

Two *Tetramitus* spp. were identified, *T. entericus* (EMBL AJ698841) and *T. hohokami* (EMBL AM294981). The latter is a new species recently described and isolated from samples in Arizona (De Jonckheere, 2007). The two *T. hohokami* strains were isolated at 42°C incubation from a brook in Sinaloa and a river in Sonora. The sequence of the two *T. hohokami* isolates is totally identical to that of the type strains from Arizona. The two strains of *T. entericus*, isolated at different incubation temperatures from the same sample from Cuitzeo Lake in Michoacán, have the same sequence but differ by one G insert in a four G repeat in the ITS1 from the type strain (EMBL AJ98841), and the 5.8S is identical to that of strain C101 (EMBL AJ698856), which has a one bp substitution compared to the type strain (De Jonckheere and Brown, 2005).

## Discussion

While previous researchers of free-living amoebae in Mexico have tried to isolate pathogenic *N. fowleri*, we have attempted to find as much different Vahlkampfiidae strains as possible. Instead of isolating at high temperature (44-45°C) for the isolation of *N. fowleri*, we have, therefore, used three different incubation temperatures. By using 42°C instead of 44-45°C we have actually tried to avoid isolating *N. fowleri*, and the related *N. lovaniensis*. We seem to have succeeded, though this might also have been due to the low water temperatures in winter season. The place La Marquesa is situated at high altitude and was actually chosen for its cold climate, but we did not isolate any vahlkampfiid amoeba from this place, only strains of *Acanthamoeba*.

While only *N. fowleri*, *N. lovaniensis*, *N. australiensis* and *W. magna* had been identified in Mexico with certainty before, recently a strain each of *N. tihangensis* and *N. americana* has also been isolated (Guzmán Fierros et al., in preparation). We are adding four other *Naegleria* spp. to this list and two *Tetramitus* spp. Especially the isolation of *N. indonesiensis* and *N. byersi* from Mexico is very interesting. Indeed, only one strain of *N. indonesiensis* is known and we confirm that this species does not transform into flagellates under the usual conditions (De Jonckheere, 2002). Only three strains of *N. byersi* are known, from Australia, Papua New Guinea and Bangladesh, and this is the only species known to have a group I intron, while other *Naegleria* spp. have a group I twintron (De Jonckheere, 2004). It will be interesting to investigate whether the Mexican isolate of *N. byersi* also has this group I intron.

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**Address for correspondence:** Johan F. De Jonckheere, Research unit for Tropical Diseases, Christian de Duve Institute for Cellular Pathology, Avenue Hippocrate, 74-75, B1200 Brussels, Belgium. Fax: 32-2-762.68.53. E-mail: jdjonckh@ben.vub.ac.be

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