Acta Protozool. (2006) 45: 91 -96

ACTA
PROTOZOOLOGICA

Isolation and Molecular Identification of Vahlkampfiid Amoebae from an Island (Tenerife, Spain)

Johan F. DE JONCKHEERE

Scientific Institute of Public Health, Brussels, Belgium

Summary. Four vahlkampfiid amoebae were isolated from freshwater samples taken on the island of Tenerife. Ribosomal DNA sequence analysis identified two isolates to belong to the genus *Naegleria*, representing novel species with their closest relatives *N. gallica and N. philippinensis* respectively. The two other isolates belong to the genus *Vahlkampfia* and represent also novel species, with as their closest relatives *V. avara* and *V. inornata* respectively. The new *Vahlkampfia* spp. are more divergent from their nearest relatives than the new *Naegleria* spp. are to their closest relatives. The latter is probably due to the already high number of described *Naegleria* spp. in contrast to the few known *Vahlkampfia* spp. There are no morphological features in these new species of either genus that allow their identification. Only molecular typing can discriminate species within the vahlkampfiids.

Key words: 5.8S rDNA, cyst morphology, internal transcribed spacers, *Naegleria canariensis* n. sp., *N. tenerifensis* n. sp., *Vahlkampfia ciguana* n. sp., *V. orchilla* n. sp

INTRODUCTION

Since the discovery that *Naegleria fowleri* can cause fatal disease in man a lot of searches to find this free-living pathogen in the environment has led to the recognition of a high diversity of species in the genus *Naegleria* (for an overview see De Jonckheere 2002, 2004). Although large parts of the world have been sampled to look for the presence of *Naegleria* spp. still a lot of geographic areas have not been covered. In one of these areas not covered, Madagascar, a recent case of PAM indicates *N. fowleri* is also present on this island (Jaffar-Bandjee *et al*. 2005).

Also other vahlkampiids have acquired an interest because of their possible implication in keratitis. However, amoebic keratitis is due to *Acanthamoeba* spp. and the implication of other free-living amoebae in the disease remains controversial (De Jonckheere 2002).

Recently a report was published on the occurrence of *Acanthamoeba* strains in Tenerife, Canary Islands, Spain (Lorenzo-Morales *et al*. 2005). There is no information on the presence of *Naegleria* on this island, and no cases of PAM are known from this place. I have, therefore, tried to isolate vahlkampfiids from freshwater on this island. Such an investigation has become quite

Address for correspondence: Johan F. De Jonckheere, Research unit for Tropical Diseases, Christian de Duve Institute of Cellular Pathology, Avenue Hippocrate 74-75, B-1200 Brussels, Belgium; Fax: 32-2-7626853; E-mail: jdjonckh@ben.vub.ac.be

easy because of the quick and accurate identification of new isolates using ITS and 5.8S rDNA sequences for the identification of *Naegleria* (De Jonckheere 1998) and other vahlkampfiids (De Jonckheere and Brown 2005).

MATERIALS AND METHODS

Samples of freshwater sediments were taken at different sites on the island of Tenerife, Spain, beginning of March 2005. Water bodies sampled were three agricultural water reservoirs in concrete, one disused and almost empty barrage, a small river and a water display near the swimming pool of a hotel (Table 1). Kept for a week at room temperature, the samples were inoculated onto plates with non nutrient (NN) agar plates coated with *Escherichia coli* (Page 1988). The sealed plates were incubated at room temperature (RT), 37°C and 42°C.

Only isolates that showed the typical vahlkampfiid morphology were further investigated. To be sure to have pure cultures the isolates were subcultured several times by cutting a piece of agar with the migrating ring of amoebae and placing it upside down on a fresh plate. Isolates grown from 37°C plates were tested for growth at 42°C. Flagellate formation was tested in distilled water (DW) at RT.

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 the ITS forward primer and the 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 pair for amplifying from vahlkampfiid species in general (De Jonckheere and Brown 2005). The PCR product was sequenced (each strand in opposite direction) 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).

The 5.8S rDNA sequences of vahlkampfiid isolates other than *Naegleria* were aligned with those of published vahlkampfiid genera (De Jonckheere 2004, De Jonckheere and Brown 2005) using the programme ClustalX (Thompson *et al*. 1997). The total ITS1, 5.8S and ITS2 sequences of the *Naegleria* isolates were aligned with those of published sequences of other *Naegleria* which have identical 5.8S sequences. The alignment was manually adjusted. Phylogenetic trees were constructed using the DNAPARS (parsimony), DNADIST (distance matrix), NEIGHBOR (neighbor-joining and UPGMA), FITCH, KITSCH, DNAML and SEQBOOT (bootstrapping) programs of the PHYLIP (version 3.572c) package (Felsenstein 1989).

The complete ITS sequences have been deposited in EMBL with the accession numbers AJ973124 till AJ973127.

RESULTS

Isolation and morphological genus identification. There were so many amoeba isolates overgrowing each other on the plates at RT that they could not be cultured separately for identification. At 37°C two *Naegleria* isolates were recovered and one other vahlkampfiid genus (Table 1). At 42°C one isolate, belonging to another vahlkampfiid genus than *Naegleria*, was recovered. The vahlkampfiids were isolated from the samples of an agricultural water reservoir in concrete, the barrage and the small stream. Subculture at 42°C of the strains isolated at 37°C yielded growth for strain T3(37), not for strains TE1(37) and TE4(37). The two *Naegleria* isolates TE1(37) and TE3(37) transformed readily into flagellates when suspended in DW, the two other vahlkampfiid isolates did not.

Molecular identification. The two *Naegleria* isolates, TE1(37) and TE3(37), have identical 5.8S rDNA sequence which correspond to that of *N. gruberi* senso strictu, *N. pringsheimi*, *N. italica*, *N. clarki*, *N. australiensis*, *N. tihangensis*, *N. philippinensis*, *N. mexicana*, *N. gallica*, *N. endoi* and *N. laresi* (De Jonckheere 2004). But it is the ITS2 sequence that determines the species (De Jonckheere 2002, 2004). Strain TE1(37) has the same sequence length of 170 bp for the ITS2 (Table 2) as *N. gallica* (De Jonckheere 2004) and *N. dunnebackei* (Visvesvara *et al.* 2005). It differs in the ITS2 sequence by two bp substitutions from *N. gallica* (Table 3) and four bp substitutions from *N. dunnebackei*. I propose the name *N. canariensis*, denoting the name of the Canary Islands, Spain, from where the strain was isolated. The ITS2 sequence length of 110 bp (Table 2) in strain TE1(37) is not detected in any described *Naegleria* sp. Its ITS2 sequence differs by 5 bp from *N. philippinensis* (Table 4), 6 bp from *N. endoi* and *N. byersi*, 7 bp from *N. mexicana* and 13 from *N. gruberi* sensu stricto. I propose the name *N. tenerifensis*, denoting the name of the Isle of Tenerife, Spain, from where the strain was isolated.

Strain TE3(42) represents a new vahlkampfiid with unique ITS1, 5.8S and ITS2 sequences (Table 2). Its closest relative is *V. avara* (Table 5) with which it clusters with all methods used in trees based on the 5.8S rDNA sequences (Fig. 1), but there is a lot of sequence difference in all parts sequenced (Table 5). I propose the name *V. ciguana*, denoting the name of the place from where the strain was isolated, Embalso de Ciguana in Tenerife, Spain. Strain TE4(37) represents a new vahlkampfiid with unique ITS1, 5.8S and ITS2 sequences (Table 2). Its closest relative is *V. inornata* (Table 6), with which it clusters with all methods used in trees based on the 5.8S rDNA sequences (Fig. 1), but there is an enormous sequence difference in ITS1 and

Table 1. Amoeba isolation at 37°C, and morphological vahlkampfiid identification in samples from Tenerife.

Sample	Place	Amoeba growth	vahlkampfiid identification	Enflagellation
TE1	Buenavista del Norte (WR)	+	*Naegleria*	+
TE2	Buenavista del Norte (WR)	-	NA	NA
TE3	Embalso de Ciguana	+	*Naegleria**	+
TE4	Rio Orchilla	+	vahlkampfiid	-
TE5	San Miguel (WR)	+	-	NA
TE6	Adeje (hotel water display)	+	-	NA

WR - agricultural water reservoir in concrete.
* also a *vahlkampfiid* isolate at 42°C.

Table 2. Lengths (in bp) of ITS1, 5.8S and ITS2 rDNA sequences of strains and proposed new species names.

Isolate	ITS1	5.8S	ITS2	Total	Closest relative	Proposed new species
TE1(37)	33	175	170	378	*N. gallica*	*N. canariensis* n. sp.
TE3(37)	33	175	110	318	*N. philippinensis*	*N. tenerifensis* n. sp.
TE3(42)	31	155	161	347	*V. avara*	*V. ciguana* n. sp.
TE4(37)	141	153	368	662	*V. inornata*	*V. orchilla* n. sp.

Table 3. Sequence lengths (in bp) of *N. gallica* and strain TE1 (37) (differences from *N. gallica* in parenthesis).

	N. gallica Capd60	*N. canariensis* n. sp strain TE1 (37)
ITS1	33	33 (0)
5.8S	175	175 (0)
ITS2	170	170 (2 substitutions)

Table 4. Sequence lengths (in bp) of *N. philippinensis* and strain TE3 (37) (differences from *N. philippinensis* in parenthesis).

	N. philippinensis RJTM	*N. tenerifensis* n. sp. strain TE3 (37)
ITS1	33	33 (0)
5.8S	175	175 (0)
ITS2	113	110 (2 substitutions, 3 deletions)

Table 5. Sequence lengths (in bp) of *V. avara* and strain TE3 (42) (differences from *V. avara* in parenthesis).

	V. avara CCAP1588/1A	*V. ciguana* n. sp. strain TE3 (42)
ITS1	28	31 (5 substitutions, 3 insertions)
5.8S	155	155 (13 substitutions)
ITS2	152	161 (32 substitutions, 9 insertions)

Table 6. Sequence lengths (in bp) of *V. inornata* and strain TE4 (37) (differences from *V. inornata* in parenthesis).

	V. inornata CCAP1588/2	*V. orchilla* n. sp. strain TE4 (37)
ITS1	320	141 (almost no overlap)
5.8S	154	153 (36 substitutions, 6 indels)
ITS2	185	368 (almost no overlap)

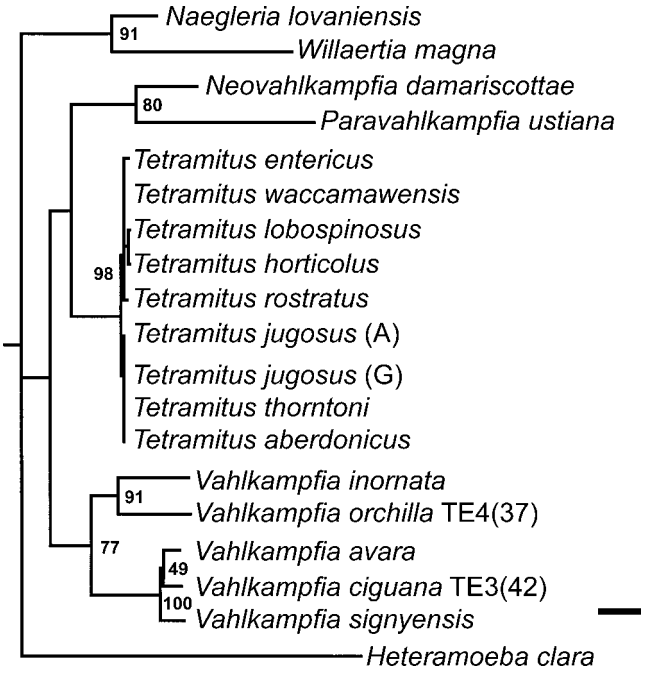
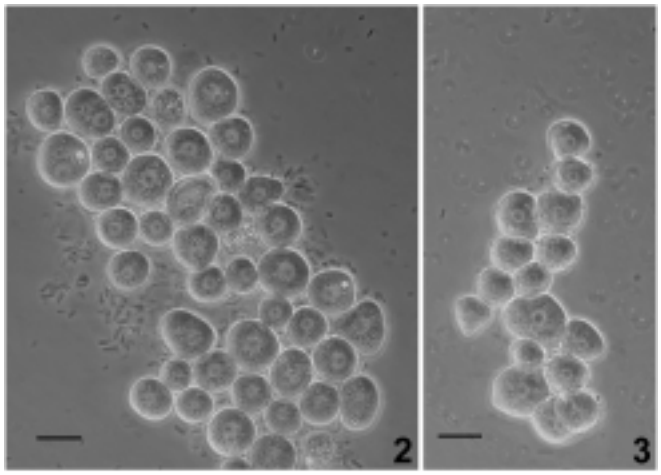


Fig. 1. Phylogenetic tree inferred from comparisons of the 5.8S rDNA sequences using the Kimura two-parameter correction and the neighbor-joining method. Only bootstrap values higher than 50% are indicated at the nodes, except for *Vahlkampfia avara - V. ciguana*. The distance corresponding to 10 changes per 100 nucleotide positions is indicated by the bar.



Figs 2, 3. 2 - cysts of *Vahlkampfia ciguana* n. sp. with phase contrast; **3** - cysts of *Vahlkampfia orchilla* n. sp. with phase contrast. Scale bars: 10 µm.

ITS2 (Table 6). I propose the name *V. orchilla*, denoting the name of the place from where the strain was isolated, Rio Orchilla in Tenerife, Spain.

Light microscopy of the two *Naegleria* isolates. Both isolates T1(37) and T3(37) have typical *Naegleria* cysts, round with pores (not shown). No angular type cysts are observed. The cysts become empty and non-viable very quickly on the agarose plates. When grown at 42°C (strain TE3(37) only) the cysts are much more variable in shape and size.

Light microscopy of the two *Vahlkampfia* isolates. Isolate T3(42) has mostly circular cysts (diameter 8.0-16.5 µm) with a smooth outer wall to which bacteria stick (Fig. 2). Cysts are often loosely aggregated, embedded in detritus and bacteria. Amoebae sedimented by centrifugation show a pink colour. Isolate TE4(37) has cysts (diameter 8.0-20 µm) that are more variable in form, circular and reniform, to angular, and size (Fig. 3) with a smooth outer wall. In the biggest cysts two or three nuclei are frequently observed. Cysts are often loosely aggregated. Amoebae sedimented by centrifugation show a pink colour.

Naegleria canariensis n. sp.

Diagnostic summary. The trophozoites have the typical appearance of *Naegleria* spp. and they can be induced to transform into flagellates. The cysts have a round shape with pores in the walls as for most other described *Naegleria* spp. The strain grows at 37°C but not at 42°C.

Because of the morphological similarity of the cysts with those of other *Naegleria* spp. molecular identification is required. The species can be identified from the ITS2 sequence, which differs by 2 bp substitutions from that *N. gallica.* The ITS1 and 5.8S rDNA sequence is identical to that of *N. gallica*. The sequence is available from EMBL under accession N° AJ973124.

The type strain TE1(37) was isolated from a water reservoir in Tenerife, one of the islands of the Canary Islands, from which the name was chosen.

Naegleria tenerifensis n. sp.

Diagnostic summary. The trophozoites have the typical appearance of *Naegleria* and they can be induced to transform into flagellates. The cysts have a round shape with pores in the walls as for most other described *Naegleria* sp. The strain grows at 42°C.

Because of the morphological similarity of the cysts with those of other *Naegleria* spp. molecular identification is required. The species can be identified from the ITS2 sequence, which differs by 2 bp substitutions and

3 bp deletions from that *N. philippinensis*. The ITS1 and 5.8S rDNA sequence is identical to that of *N. philippinensis*. The sequence is available from EMBL under accession N° AJ973125.

The type strain TE3(37) was isolated from a water reservoir in Tenerife, from which the name was chosen.

***Vahlkampfia ciguana* n. sp.**

Diagnostic summary. The trophozoites have the typical appearance of vahlkampfiid amoebae and could not be induced to transform into flagellates. The cysts have a round shape without pores in the walls. The strain grows at 42°C. A pink colour is observed.

Because morphology does not allow identification to genera within the Vahlkampfiidae, except for *Naegleria*, molecular identification is required. The species can be identified from the ITS1, 5.8S and ITS2 sequences. The 5.8S rDNA sequence demonstrates the strain belongs to the genus *Vahlkampfia* and that its closest relative is *V. avara*. The sequence is available from EMBL under accession N° AJ973126.

The type strain TE3(42) was isolated from a water reservoir in Tenerife, Embalso de ciguana, from which the name was chosen.

***Vahlkampfia orchilla* n. sp.**

Diagnostic summary. The trophozoites have the typical appearance of vahlkampfiid amoebae and they can not be induced to transform into flagellates. The cysts have a round to irregular shape without pores in the walls. The strain grows at 37°C but not at 42°C. A pink colour is observed.

Because morphology does not allow identification to genera within the Vahlkampfiidae, except for *Naegleria*, molecular identification is required. The species can be identified from the ITS1, 5.8S and ITS2 sequences. The 5.8S rDNA sequence demonstrates the strain belongs to the genus *Vahlkampfia* and that its closest relative is *V. inornata*. The sequence is available from EMBL under accession N° AJ973127.

The type strain TE4(37) was isolated from a river in Tenerife, Rio Orchilla, from which the name was chosen.

DISCUSSION

It has been shown that in the genus *Naegleria* strains within the same species have identical ITS2 sequences (De Jonckheere 2004). Only in two species, *N. byersi* and *N. andersoni*, a difference of one bp is detected in the ITS2 of one strain in each species. Two *Naegleria* spp. have been described which differ by only two bp in the ITS2 sequence. *Naegleria australiensis* has a closely related species, which was called originally a sister species, later the spa variant, and then a subgroup of *N. australiensis*. It was finally given species status, *N. tihangensis* , and differs by an insert of only two T's in the ITS2 compared with that of *N. australiensis* (De Jonckheere 2002). Unlike *N. australiensis*, which is pathogenic in experimental animals, there is no indication that *N. tihangensis* is pathogenic.

It is, therefore, through the sequence of the ITS2 that it can be concluded that the two *Naegleria* isolates from the island of Tenerife are new species, as they differ from described species by 2 bp (1.2%) and 5 bp (4.5%) respectively. Both have round cysts with pores which are not distinguishable from those of their closest relatives *N. gallica* strain Capd60 (Pussard and Pons 1979) and *N. philippinensis* strain RJTM (Matias *et al.* 1990) respectively. The cysts of strain Capd60 were also reported to need frequent subculture in order to survive, just as these two new isolates from Tenerife. The max. growth temperature of *N. gallica* is 37°C, and also the new species *N. canariensis* grows at this temperature, not at 42°C. The max. growth temperature of *N. philippinensis* is 40°C, while the new species *N. tenerifensis*, its closest relative, grows at 42°C.

Also in the case of the two new *Vahlkampfia* isolates it is very difficult to see the differences in cyst morphology between the new isolates and those of the three established *Vahlkamfia* spp., i.e. *V. avara*, *V. inornata* (Page 1967) and *V. signyensis* (Garstecki *et al*. 2005). Page (1967) reported that the cysts of *V. avara* collect more debris, as is found in the related *V. ciguana*, and that the cysts of *V. inornata* are more variable in shape, as is found in the related *V. orchilla*. Only the sequence results disclose that these two new isolates are to be considered two new species. Based on rDNA sequences, *V. avara* and *V. inornata* were the only two *Vahlkampfia* spp. of the 7 investigated, which remained within the genus (De Jonckheere and Brown 2005). The cysts of the only other molecular valid *Vahlkampfia* sp., *V. signyensis*, are always spherical and are often loosely aggregated and/or embedded in detritus (Garstecki *et al*. 2005). *Vahlkampfia avara* grows at 37°C while the new species *V. ciguana*, strain TE3(42), grows at 42°C, but *V. signyensis*, within the same cluster, grows at 20°C only. The latter was isolated from Antarctica, which probably explains this low temperature tolerance.

In the cluster of *V. avara*, *V. ciguana* and *V. signyensis* all have a 5.8S rDNA length of 155 bp and the latter two differ by almost the same number of bp from *V. avara* (8.5% sequence divergence). In the other *Vahlkampfia* cluster *V. inornata* has a 5.8S rDNA length of 154 bp and *V. orchilla* 153 bp (27.5% sequence divergence). There is also a lot more sequence difference in the ITS1 and ITS2 between the latter two species than there is between *V. ciguana* and *V. avara*. In general, the new *Vahlkampfia* spp. are more divergent from their nearest relatives than the new *Naegleria* spp. are to their closest relatives. The latter is probably due to the already high number of described *Naegleria* spp. in contrast to the low number of *Vahlkampfia* spp. discovered until now

Acknowledgements. I thank B. Vandevijver (Research Unit Polar Ecology, Limnology and Paleobiology, University of Antwerp; head L. Beyens) for help in microphotography. The family Brugmans is acknowledged for the technical support during sampling.

REFERENCES

De Jonckheere J. F. (1998) Sequence variation in the ribosomal internal transcribed spacer, including 5.8S, of *Naegleria* spp. *Protist* **149:** 221-228

De Jonckheere J. F. (2002) A century of research on the amoeboflagellate genus *Naegleria*. *Acta Protozool*. **41:** 309-342

De Jonckheere J. F. (2004) Molecular definition and the ubiquity of species in the genus *Naegleria*. *Protist* **155:** 89-103

De Jonckheere J. F., Brown S. (2005) The identification of vahlkampfiid amoebae by ITS sequencing. *Protist* **156:** 89-96

Felsenstein J. (1989) PHYLIP: phylogenetic inference package (version 3.2). *Cladistics* **5:** 164-166

Garstecki T., Brown S., De Jonckheere J. F. (2005) Description of *Vahlkampfia signyensis* n. sp. (Heterolobosea), based on morphological, ultrastructural and molecular characteristics. *Europ. J. Protistol.* **41:** 119-127

Hugo E. R., Stewart V. J., Gast R. J., Byers T. J. (1992) Purification of amoeba mtDNA using the UNSET procedure. In: Protocols in Protozoology, (Eds. A. T. Soldo, J. J. Lee). Allen Press, Lawrence, Kansas

Jaffar-Bandjee M. C., Alessandri J. L., Molet B., Clouzeau J., Jacquemot L., Samperiz S., Saly J. C. (2005) Primary amebic menigoencephalitis: 1st case observed in Madagascar. *Bull. Soc. Pathol. Exot*. **98:** 11-13

Lorenzo-Morales J., Ortega-Rivas A., Foronda P., Martinez E., Valladares B. (2005) Isolation and identification of pathogenic *Acanthamoeba* strains in Tenerife, Canary Islands, Spain from water sources. *Parasitol. Res*. **95:** 273-277

Matias R. R., Enriquez G. L., Schottelius J. (1990) Surface lectin receptors on a *Naegleria* species from the Philippines. *Lectins, Biol., Biochem., Clin. Biochem.* **7:** 329-333

Page F. C. (1967) Taxonomic criteria for limax amoebae, with descriptions of 3 new species of *Hartmannella* and 3 of *Vahlkampfia*. *J. Protozool*. **14:** 499-521

Page F. C. (1988) A new key to freshwater and soil gymnamoebae. Freshwater Biol. Assoc., Ambleside

Pussard M., Pons R. (1979) Etudes des pores kystiques de *Naegleria* (Vahlkampfiidae-Amoebida). *Protistologica* **15:** 163-175

Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F., Higgins D. G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res.* **24:** 4876-4882

Visvesvara G. S., De Jonckheere J. F., Marciano-Cabral F., Schuster F. L. (2005) Morphologic and molecular identification of *Naegleria dunnebackei*, n. sp. isolated from a water sample. *J. Eukaryot. Microbiol.* **52:** 523-531

Received on 10th August, 2005; revised version on 12th September, 2005; accepted on 22nd September, 2005