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Isolation of *Naegleria fowleri* from a domestic water tank associated with a fatal encephalitis in a 4 month-old Venezuelan child

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Abstract. This study describes the association of household water system contamination with the pathogenic Free-Living Amoeba (FLA) *Naegleria fowleri* and a case of fatal Primary Amoebic Meningoencephalitis (PAM) in a child from the state of Monagas in Venezuela. Amoebae were initially identified by microscopy from a sample of cerebrospinal fluid (CSF) from the child. Direct DNA extraction and specific PCR/sequencing for *N. fowleri* was also carried out from the same CSF sample. In order to determine a possible environmental source of infection, water samples from the water tank of the child's home and also water bodies recently visited by the child and his family, were examined for the presence of *N. fowleri* by culture and PCR/sequencing. The results obtained from the collected water samples revealed that only the water tank of the house was positive for *N. fowleri*. PCR/sequencing showed that the strains isolated from the patient and the water tanks were 100 % identical. Therefore, the house water tank was confirmed as the source of infection in this case, possibly as a result of the occasional immersion of the child´s head under the water while bathing. This case highlights a novel source of thermally polluted water and another threat of *N. fowleri* infection.

INTRODUCTION

Naegleria fowleri, a Free-Living Amoeba (FLA) species is distributed worldwide mostly in warm water related sources. This amoebic species is the causative agent of an acute and fatal disease known as Primary Amoebic Meningoencephalitis (PAM) (De Jonckheere, 2011). *Naegleria fowleri* amoebae penetrates through the cribiform plate until it reaches the olfactory bulb of the central nervous system. The incubation period for PAM ranges from 5 to 7 days, and infection leads to death within a week. Onset of symptoms is abrupt, with bifrontal or bitemporal headaches, fever, and stiff neck, followed by nausea, vomiting, irritability, and fatigue. The mortality rate is as high as 95% with only a few cases of survival reported so far (Shenoy *et al*., 2002; Moussa *et al*., 2013; Abrahams-Sandi *et al*., 2015).

In the Americas, PAM cases have been reported in Venezuela, Brazil, Cuba, Mexico, Costa Rica and the United States where contact with heated water bodies is the main associated risk factor for infection (De Jonckheere, 2011; Abrahams-Sandi *et al.*, 2015). Venezuela has reported seven fatal

cases of PAM so far, although the isolation of the pathogen from the environment has not previously been achieved in any of these cases (García-Tamayo *et al*., 1980; Rodríguez *et al*., 1998; Ávila *et al*., 2001; Cermeño *et al*., 2006; Petit *et al*., 2006; Caruzo and Cardozo, 2008; De Jonckheere, 2011). Nevertheless, the epidemiology of most reported cases of PAM indicates an association between recreational aquatic activity and infection. Swimming, diving, and immersion in hot springs, spas, and warm freshwater bodies have been related to *N. fowleri* infection (De Jonckheere, 2011; Moussa *et al*., 2013).

In this work, the environmental isolation of a strain of *Naegleria fowleri* was achieved and associated to a fatal case of PAM in a 4-month old child in the state of Monagas in Venezuela by using culture and specific PCR/sequencing to confirm the amoebic identity. This is the first infection from a domestic water tank.

METHODS

Samples

CSF Sample

In May 2014, cerebrospinal fluid (CSF) from a 4 month-old boy who died 13 days previously was submitted to the Laboratorio de Amibiasis-Cátedra de Parasitología, Escuela de Bioanálisis, Facultad de Medicina, Universidad Central de Venezuela, Caracas, Venezuela in order to check for the presence of FLA, as PAM was suspected to be the cause of the child´s death. Since the sample of CSF was insufficient for both culture and PCR analyses, the sample was centrifuged and use directly for DNA extraction. Before using it for that purpose, CSF was centrifuged at 1000 rpm and the sediment observed at the microscope directly and after staining with Romanowski Giemsa.

Water samples

Water samples of 1 L volume from different places reported to be visited by the child and his family were collected in order to check for the presence of *N. fowleri*: 9 samples from a large recreational swimming pool (1G to 9G); 4 samples from a pool for children (1P to 4P); 1 sample from the water storage tank of the child's house (1C); 3 samples from the main water supply of the child's neighborhood (1PS to 3PS) and 1 sample from a drinking water bottle used by the child's mother to bath him, 1B.

All samples mentioned above were filtered through 0.45-µm pore diameter nitrocellulose filter membranes using a vacuum manifold system. Each filter was cut in three pieces and placed over 2% Non-nutrient Agar (NNA) plates supplemented with a layer of heat killed *Escherichia coli*. After that plates were incubated at 26ºC, 37ºC and 42ºC for 7 days and monitored daily. Amoebic strains which showed compatible morphology with *Naegleria* genus, grew at temperatures of 37ºC and 40ºC for 7 days and were positive for the enflagellation test, were then checked by specific PCR for *Naegleria fowleri*.

DNA extraction

DNA from CSF and cultures identified as putative *Naegleria* spp. strains by microscopy was extracted as previously described (Reyes-Batlle *et al*., 2014) by placing 1–2 ml of amoebic cultures directly into the Maxwell® 16 Tissue DNA Purification Kit sample cartridge (Promega, Madrid, Spain). Amoebic genomic DNA was purified using the Maxwell 16 Instrument as described in the Maxwell 16 DNA Purification Kits Technical Manual #TM284 (Promega). DNA yield and purity were determined using the NanoDrop® 1000 spectrophotometer (Fisher Scientific, Madrid, Spain).

Polymerase chain reaction (PCR) reaction and molecular characterization of isolates

For molecular identification of the genus *Naegleria*, the complete ITS region (ITS1, 5.8S, and ITS2) was amplified using the NFITSFW and NFITSRV primer pair which is specific for *N. fowleri* as previously described (Moussa *et al*., 2013] which amplifies a fragment of around 300–400 bp depending on the species of *Naegleria*. Briefly, DNA amplification reactions were performed using a volume of 30 µl containing

40 ng template DNA, Buffer (1X) without $MgCl_2$, 2.5 mM $MgCl_2$, 200 µM dNTP, 2.5 pmol of each primer pair, and 1.25 units of Taq DNA polymerase (Applied Biosystems, New Jersey), pH 8.3 was used for amplification in a Artik Cycler Thermocycler (Thermo Scientific). The cycling conditions were: initial denaturation of 95ºC for two minutes; 35 repetitions of denaturation at 95ºC for 1 minute, annealing phase at 55ºC for 1 minute and 30 seconds and elongation at 72ºC for 2 minutes; and final elongation at 72ºC for seven minutes. Amplification products were fractionated using 2% agarose electrophoresis stained with a solution of 20.000X of REALSAFE Nucleic Acid Staining Solution (Durviz, Madrid, Spain) and visualized under the UV light. A type strain from the American Type Culture Collection (ATCC) *Naegleria fowleri* ATCC 30894 was used a positive control in the PCR reactions and distilled water added to the reaction mixture (instead of DNA) as the negative control.

The obtained PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced using a MEGABACE 1000 automatic sequencer (Healthcare Biosciences, Barcelona, Spain) in the University of La Laguna Sequencing Service (Servicio de Secuenciación SEGAI, University of La Laguna). Sequences were obtained twice from both strands. The obtained sequences were aligned using Mega 5.0 software program (Tamura *et al*., 2011). Moreover, nucleotide similarity search was performed by BLAST search (Basic Local Alignment search tool) of the sequenced amplicons against amoeba species.

RESULTS

Morphological examination

After direct examination of CSF sediment, degenerated host cells and other stationary structures without morphology suggestive of protozoa (Fig. 1A), fungi or bacteria were observed. In Giemsa staining, lymphocytes and other mononuclear cells were detected. (Fig. 1B). Nevertheless, some suspicious structures with blunt pseudopodia and a nucleus with a large central karyosome were observed (Fig. 1C).

In the case of the tested water samples and after cultivation of all water samples in NNA plates, only the sample identified as 1C showed growth of amoebae at 26ºC, 37ºC and 42ºC after a week. Observed amoebae presented a morphology compatible with *Naegleria* genus.

Molecular identification

PCR using the NFITSFW and NFITSRV primer pair specific for *Naegleria fowleri* was positive for the CSF and the water identified as 1C (Fig. 2). Moreover, nucleotide similarity search was performed by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) of the sequenced amplicons which revealed more than 98% homology with other *N. fowleri* strains available in GenBank such as KT375442.1 and KF709536. Therefore, the presence of *N. fowleri* was confirmed both in the CSF and 1C samples, being both strains 100% identical in their sequence.

DISCUSSION

Naegleria fowleri infections are rare, however more than 95% of the cases are fatal. Most cases of PAM are related to contact with contaminated water bodies, water sports or other recreational activities requiring contact with warm water bodies (De Jonckheere, 2011; Abrahams-Sandi *et al*., 2015).

In this study, a 4-month year old child was affected. This child from the state of Monagas in Venezuela, showed symptoms of PAM around 19 days after bathing with his family in a recreational swimming pool in this region. Patient's condition became worse and was then being admitted in hospital where he was administered antibiotics and corticoids but died at the hospital. CSF was collected and checked for the presence of any bacterial or viral agent but were negative in all cases. As it was mentioned above, microscopy and direct DNA extraction and further PCR/DNA sequencing revealed that the CSF was positive for the presence of *Naegleria*

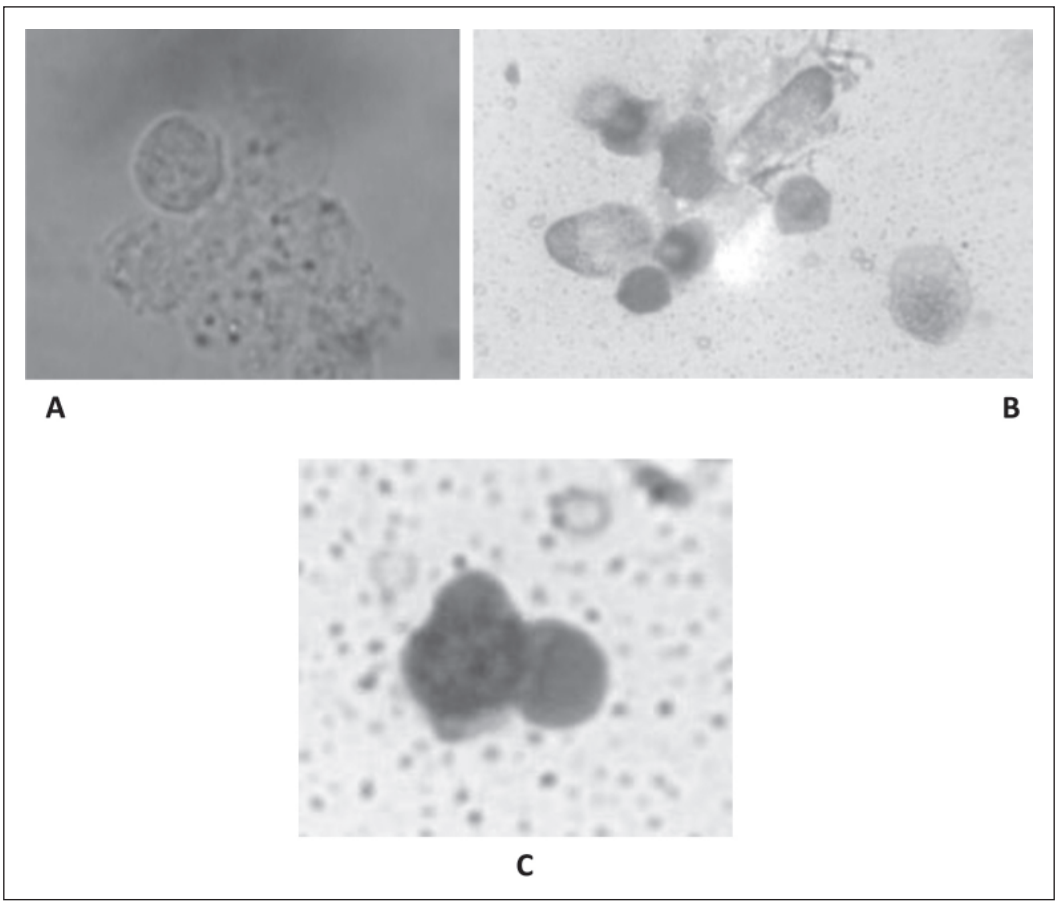

Figure 1. A) Degenerated cells, direct examination, 100 x. B) Mononuclear cells and lymphocytes, Giemsa staining, 40 x. C) Suspicious cell with blunt pseudopodia, Giemsa staining, 100 x.

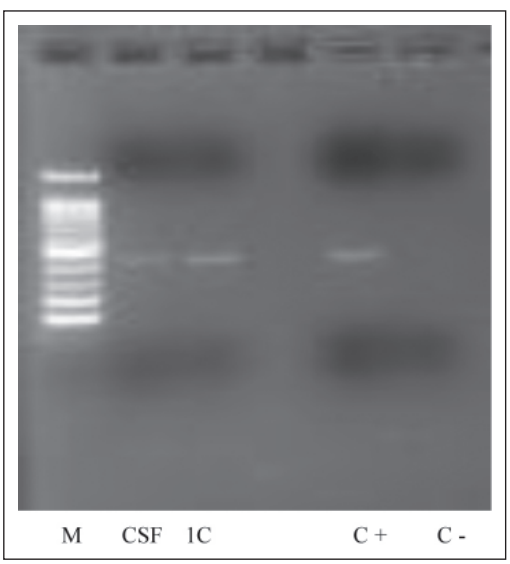

Figure 2. *Naegleria fowleri* PCR for CSF sample and water sample 1C.

fowleri. Nevertheless, it is important to mention that the CSF sample analyzed for the presence of *Naegleria fowleri* was 13-day old which was kept at 4°C until it reached our laboratory. Therefore, even though amoebic forms were observed under the microscope, it should be noted that in such conditions not viable amoebic forms were kept. The only possible approach with such material was to try to identify amoebic DNA. Storage conditions however, may affect the quality of DNA and subsequently have impact on amplification and BLAST analysis since only 98% of homology was observed in the obtained sequences when compared to the available ones in Genebank. When a case of PAM is suspected, CSF should be collected and processed for microscopy and PCR/ sequencing as soon as possible in order to

avoid issues such as the ones described in this study.

In the case reported in this study, the patient's mother reported to have recently visited a swimming pool in a hotel nearby their home. Therefore, samples were collected from the water sources of this hotel and also from the patient's house in order to elucidate the infection source. After performance of culture and specific PCR, only the water sample collected in the patient's house was positive for *Naegleria fowleri* (sample 1C). Interestingly, the water source from the house was reported to be chlorinated enough to be able to be used for household purposes which highlights an important epidemiological data in cases of PAM as previously reported (Abrahams-Sandi *et al*., 2015). Moreobver, this is the first time that the environmental infection source of PAM has been identified from the 7 previously reported cases of this infection in Venezuela (García-Tamayo *et al*., 1980; Rodríguez *et al*., 1998; Ávila *et al*., 2001; Cermeño *et al*., 2006; Petit *et al*., 2006; Caruzo *et al*., 2008, De Jonckheere, 2011).

Although we believe this is the first case of PAM where the source of infection has proven to be a domestic water tank, cases of other FLA infections such as *Acanthamoeba* keratitis from a domestic water supply are known (Jeong and Yu, 2005). Nevertheless, the present case is rather different in that *N. fowleri* requires constant warm water otherwise other FLA could grow and compete for the habitat (Griffin, 1983). Importantly, the water tank source in the present case was exposed to the sun becoming warm constatly which may have encouraged the specific growth of *N. fowleri* and the reported infection with a fatal end.

CONCLUSIONS

In the current study, both the environmental source of infection and the pathogen in the patient were isolated and identified by morphological and molecular methods. To the best of our knowledge, this is the first time that the epidemiology of a case of infection by *N. fowleri* is achieved in Venezuela and the first where the source was the domestic water tank anywhere. Awareness should be risen in the region in order to check municipal water sources and avoid further cases of PAM in the area in general and more specifically domestic water tanks should be protected against becoming warm for extended periods.

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Declaration of Interest. None

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