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Characterization of *Naegleria fowleri* strains isolated from human cases of primary amoebic meningoencephalitis in Mexico

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ABSTRACT

The protozoon *Naegleria fowleri* (*N. fowleri*) is a free-living amoeba that produces primary amoebic meningoencephalitis (PAM), which is an acute and frequently fatal infection of the central nervous system. We characterized the strains of *N. fowleri* isolated from the cerebrospinal fluid (CSF) of two cases presented in northwestern Mexico. The strains were isolated and cultured in 2% bactocasitone medium. Enflagellation assays, ultrastructural analysis, protein and protease electrophoresis patterns, and PCR were performed as confirmatory tests. Virulence tests were done using in Balb/c mice. Light microscopy analysis of brain tissue showed amoebae with abundant inflammatory reaction and extensive necrotic and hemorrhagic areas. The enflagellation assay was positive and the electron microscopy showed trophozoites with morphologic features typical of the genus. Protein and protease profiles of the isolated strains were identical to the reference strain. Finally, a 1500-bp PCR product was found in all three strains. Based on all the analyses performed, we concluded that the etiologic agent of both PAM cases was *N. fowleri*. The need for better epidemiological information and educational programs about basic clinical and pathological aspects of free-living amoebae provided by the health authorities are emphasized.

**Caracterización de cepas de
Naegleria fowleri *aisladas de casos de*
meningoencefalitis amibiana primaria en México**

RESUMEN

El protozoario Naegleria fowleri *(*N. fowleri*) es una amiba de vida libre que produce la meningoencefalitis amibiana primaria (MAP), la cual es una infección aguda del sistema nervioso central, generalmente fatal. En el presente trabajo se reporta la caracterización de cepas de* N. fowleri *aisladas del líquido cefalorraquídeo de dos casos que se presentaron en el noroeste de México. Las cepas fueron aisladas y cultivadas en medio de Bactocasitona al 2%. Los estudios de flagelación, ultraestructura, patrones proteicos y de proteasas, así como PCR se realizaron como pruebas confirmatorias. Además se utilizaron ratones Balb/c para estudios de virulencia. El análisis histopatológico mostró amibas con reacción inflamatoria intensa y extensas áreas de necrosis. Los ensayos de flagelación fueron positivos y los estudios de ultraestructura mostraron trofozoítos con morfología característica del género. Los patrones de proteínas y proteasas fueron similares a la cepa de referencia, y el producto del PCR de 1,500 bp se encontró en las tres cepas. Basados en los estudios realizados, se determinó que el agente etiológico en los dos casos de meningitis fue* N. fowleri*. La necesidad de mejores informaciones epidemiológicas y programas de educación sobre los aspectos clínicos y patológicos sobre las infecciones proporcionadas por las autoridades de salud se enfatizan.*

Key words. Free-living amoebae. *Naegleria fowleri*. Primary amoebic meningoencephalitis. Case reports. Diagnosis.

Palabras clave. *Amibas de vida libre.* Naegleria fowleri. *Meningoencefalitis amibiana primaria. Casos clínicos. Diagnóstico.*

INTRODUCTION

The amoebae of genus *Naegleria* are widely distributed and have been isolated from soil and water samples throughout the world. The species *N. fowleri* is a free-living amoeboflagellate that produces a disease known as primary amoebic meningoencephalitis (PAM), an acute and fatal infection of the central nervous system.[1] Clinically, the diagnosis of PAM is difficult and an effective treatment is still lacking at present. The infection is acquired by exposure during recreational activities in parasite-polluted water bodies. Most PAM reported cases have occurred in healthy young people with a recent swimming history. The parasite gains access to the brain by penetrating the olfactory neuroepithelium and migrating trough the cribiform plate, eventually reaching the olfactory bulbs, the meningeal layers and the cerebral parenchyma.[2]

In Mexico, only about 30 cases of PAM have been reported to date, and these are mainly from the northwestern region of the country and during the hottest months of the year.[3,4] Only one case has recovered successfully after treatment.[5] In this work, we report the complete morphological, ultrastructural, biochemical, cell biological and molecular features of amoeba isolates obtained from the cerebrospinal fluid (CSF) of two suspected cases of PAM.

MATERIAL AND METHODS

Human cases

- **Case 1.** A 9-year-old male presenting headache, nausea, vomiting, photophobia and somnolence was admitted to the Hospital. Seven days prior to the onset of symptoms, the child had a history of swimming in a ditch of Villa Zapata, Mexicali, Baja California (Mexico). A computed tomography scan study was reported normal. Three days later a sample of CSF was taken for diagnostic purposes. Motile amoebic forms were observed in fresh samples at light microscopy, and treatment with Fuconazole (200 mg/day) and Amphotericin B (1mg/kg/day) was started immediately. However, the patient went into a coma, soon after had episodes of apnea and died on the next day. The same CSF sample was submitted to Mexico City for further analysis.
- **Case 2.** A 20-year-old male from Lagunitas, Sonora (Mexico), started his illness with an intense headache, nausea and vomiting. Two days later, the patient presented with lethargy, poor response to stimulus, dyspnea, sweating, distant-audible rasping breath and reduced motility of the extremities. Although no evidence of previous swimming activities was reported, it was made known that the patient used to swim frequently. CSF was taken and submitted to Mexico City for analysis. Prior to final diagnosis, treatment was initiated with Ceftriaxone, kanamycin and dexametasone; however, patient died the next day.

Amoeba cultures

Both strains isolated from CSF of patients were labeled as Mexicali (Case 1) and JFF (Case 2). A reference strain, *Naegleria fowleri* (ATCC 30808) was used for comparative purposes. All strains were axenically cultured in bactocasitone medium supplemented with 10% fetal bovine serum, at 35.5°C. For the different assays, trophozoites were chilled and harvested during the logarithmic phase of growth (48 h).

Enflagellation assay

Trophozoites were harvested and washed twice with phosphate buffer saline (PBS), and then incubated in a 25 cm^2 culture flasks with isotonic saline solution for 2 to 4 h, at 35.5°C. A sample of these cultures was withdrawn, fixed with 2% paraformaldehyde and stained with Giemsa for light microscopy analysis.

Virulence test and immunohistochemistry.

The isolated strains were tested for in vivo virulence and comparison was made with the reference strain. Balb/c mice were inoculated with 2.5 x 10^4 trophozoites by the nasal route and euthanized 5 days later. Representative fragments of brain tissue were taken for light microscopy analysis. *N. fowleri* trophozoites were identified by immunohistochemistry using a rabbit polyclonal anti *N. fowleri* antibody (1:50). A secondary antibody, anti rabbit IgG labeled with peroxidase, was used to complete the reaction, and the diaminobenzidine kit (Pierce, Rockford, Illinois USA) was used for color development.

The animal management protocol was approved by the institutional committee (IACUC, ID number 244/05). Our institution fulfills all the technical specifications for the production, animal care and use of laboratory animals and is certified by a national law (NOM-062-ZOO-1999). All mice were euthanized with CO_2 at the end of the experiments and were ba-

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sed on the guidelines of the 2000 AVMA Panel of Euthanasia.

Electron microscopy

For ultrastructural analysis, trophozoites were harvested in the log phase of growth. They were then fixed with a mixture of 1% paraformaldehyde/2.5% glutaraldehyde in cacodylate buffer for 1 h, and post-fixed with 1% osmium tetroxide, for 1 h. Next, the amoebae were centrifuged and cell pellets were processed for embedding in epoxy resin. Thin sections were contrasted with uranyl acetate and lead citrate and examined with an EM-10 Zeiss transmission electron microscope.

Protein and protease patterns

Crude extracts were prepared by disrupting the trophozoites using four freeze-thaw cycles in phosphate buffer. Protein was quantified by Bradford method.[6] Protease activities were determined by electrophoresis of crude cell extracts in SDS-PAGE, copolymerized with 0.1% of porcine gelatin as substrate. Ten μg of crude protein extracts of each strain were loaded per well. Electrophoresis was performed at 4°C on an ice bath and constant voltage (100v) for 1 h; gels were washed twice for 30 min under orbital agitation in 2.5% Triton X-100 solution. The gels were then incubated overnight with 100 mM Tris-OH (pH 7.0) 2 mM $CaCl_2$. For protein profiles, total extracts were prepared with the following protease inhibitors: 10 mM p-hydroxymercuribenzoic acid, 5 mM N-ethylmaleimide (NEM), 5 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM ethylenediaminetetracetic acid (EDTA). Extracts were electrophoresed as performed for proteases, but using 12% SDS-PAGE non gelatin-copolymerized gels. Finally, gels were stained with 0.5% Coomassie blue R-250 for 30 min.

DNA isolation and PCR

One million amoebae were suspended in 29 μL phosphate buffer and mixed with 10.5 μL of cell lysis buffer (Tween 20, 2.5% in PCR buffer 5X) and 0.5 μL of proteinase K (20 mg/mL). After one hour at 55°C, proteinase K was inactivated by 5 min boiling.

The primers used were: p3f; 5'-GCTATCGAATGGATTCAAGC- 3', and p3r; 5'-CACTACTCGTGGAAGGCTTA-3' described by Kilvington and Beeching[7,8] and based on the DNA probe pB2.3 of the genomic library from *N. fowleri* cloned into the lambda phage vector EMBL3. One μL of the extracted DNA was used for PCR in 50 μL volume reactions using the following conditions: 1x change PCR buffer (10mM Tris-HCl, 50mM KCl, pH 8.3) 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphates (dATP, dGTP, dCTP and dTTP), 1 μM of each primer, 1% dimethyl sulfoxide, 1% glycerol, and 1.5 units of Taq DNA polymerase (Roche Applied Science, Penzberg, Germany). Cycling programs were as follows: denaturing at 95°C for 5 seconds, annealing at 55°C for 5 seconds and extension at 72°C for 1.5 minutes for 35 cycles, with a final 2 min extension at 72°C. Amplicons (5 μL aliquot of the PCR reaction) were analyzed by electrophoresis on 1% agarose gel in TBE buffer, stained with ethidium bromide (0.5 $\mu g/\mu L$) and visualized under UV light.

RESULTS

After amoebic forms were found in CSF of fresh samples of the two patients, they were cultured in axenic medium. Once the amoebic cultures were well established, the enflagellation test was performed. This assay was positive for all strains. After 2 to 4 h of culture in isotonic saline solution, motile biflagellate forms were observed. These were fixed and examined under the light microscope. Several flagellate forms were observed; parasites had in general two posterior flagella (Figure 1).

In the virulence test, Balb/c mice sacrificed at 5 days post-inoculation showed severe cerebral damage in all animals. Nervous tissue with soft consistency and hemorrhagic areas were localized mainly at the olfactory bulbs. The histological analysis of
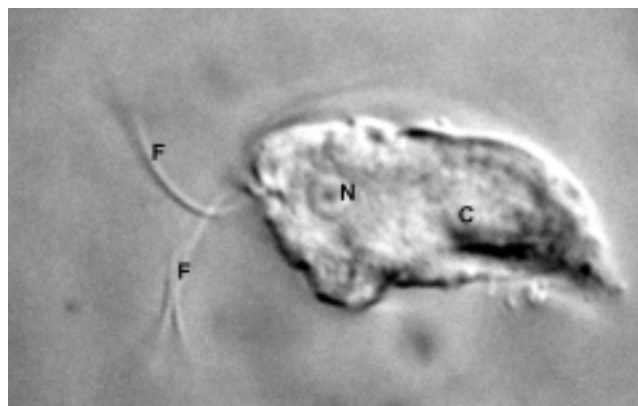

Figure 1. Light microscopy of N. fowleri *flagellate (Mexicali) showing a large pyriform structure with two flagella (F), a conspicuous nucleus (N) and cytoplasm (C). Reference and JFF strains displayed similar shapes. Nomarski. Original magnification x400.*

brain samples showed abundant trophozoites surrounded by a great number of inflammatory cells, mainly neutrophils and macrophages, with different grades of damage, characterized by the presence of cellular debris and pyknotic nuclei. The amoebae were associated with extensive lytic areas (Figure 2).

The fine structure of the three strains analyzed showed in general features typical for the *Naegleria* genus. The reporting case strains (Mexicali and JFF) had rounded nucleus with a dense central nucleolus similar to reference strain (ATCC 30808). Differen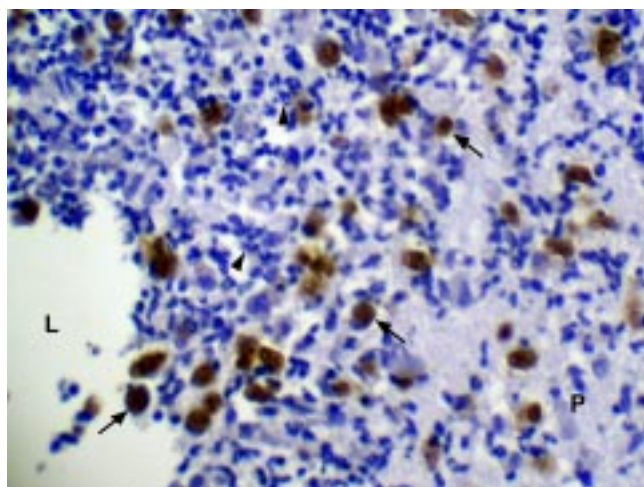ces between the problem strains were mainly noted in the amount and shape of the mitochondria, which were more abundant and round in the Mexicali strain. This showed also the presence of lipid inclusions in the cytoplasm, and the JFF strain presented several lysosome-like dense bodies of unknown origin that was rarely seen in the Mexicali and the reference strains. Vacuoles containing homogeneous electron dense material and others with irregular fibrillar material were common in Mexicali and ATCC 30808 (Figure 3).

No significant differences were noted in the protein patterns of all three strains. The main bands were observed at 26, 39 and 72 kDa, and only a little change in the intensity of some bands was observed (Figure 4A). The protease profiles were also similar

Figure 2. Immunohistochemistry of olfactory bulb of balb/c mouse at 5 days post-nasal inoculation of N. fowleri *trophozoites. Extensive lytic (L) areas are observed in the nervous tissue. Trophozoites (arrows) are located inside the necrotic tissue of olfactory bulb parenchyma and surrounded by inflammatory cells (arrowheads). Cerebral parenchyma (P). Polyclonal anti* N. fowleri *antibody. Hematoxylin counter-stain. x400*
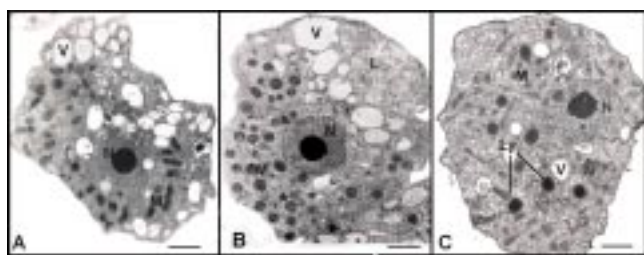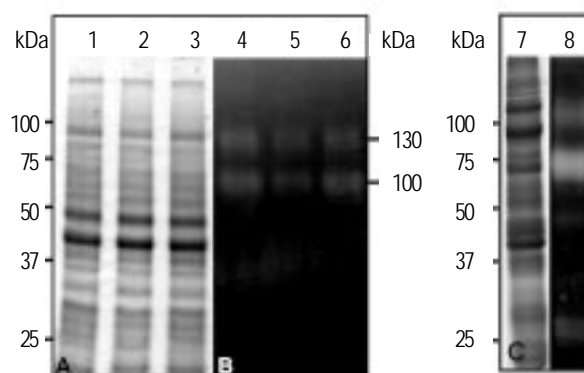

Figure 4. Comparison of total protein and protease extracts of three Naegleria *strains. Lanes 1-3: 12% SDS-PAGE of total extracts. Lanes 4-6: 0.1% gelatin copolymerized 7.5% SDS-PAGE of total extracts. Lanes 1 and 4:* N. fowleri *ATCC 30808 extract (reference strain); lanes 2 and 5: case 1 strain (Mexicali) extract; lanes 3 and 6: case 2 strain (JFF) extract. Lane 7: SDS-PAGE of total crude extract of* E. histolytica; *lane 8: gelatin copolymerized SDS-PAGE of* E. histolytica *total extract.*

Figure 3. Electron micrographs of N. fowleri *ATCC 30808 reference strain (A) and both Mexicali (B) and JFF (C) isolated strains. The typical* N. fowleri *nucleus (N) and spherical nucleolus are shown in all three strains. The mitochondria (M) are round in Mexicali and elongated in JFF strain. The number of vacuoles (V) was less common in the JFF strain. Lipid droplets (L) and lysosome-like (Ly) structures are seen in the Mexicali and JFF strains respectively. Bar = 2 μm.*
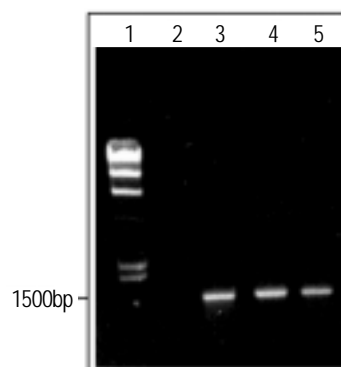

Figure 5. PCR with specific primers for N. fowleri. *Lane 1: MR, lambda/Hind III; lane 2: negative control (Without DNA); lane 3:* N. fowleri *(ATCC 30808); lane 4: case 1 strain (Mexicali); lane 5: case 2 strain (JFF).*

for all strains. Gelatin-copolymerized gels incubated at pH 7.0 showed two bands with proteolytic activities of 130 and 100 kDa (Figure 4B). For comparison purpose, *E. histolytica* protein and proteolytic patterns were determined. These patterns showed important differences compared with *N. fowleri* strains (Figure 4C).

Finally, PCR analysis showed a predictable 1,500 bp Amplicon in the tested strains as well as in the reference strain (Figure 5).

DISCUSSION

Although free living amoebae, such as *N. fowleri,* are ubiquitous with a worldwide distribution, most of the cases of PAM caused by this protozoon in Mexico, have been detected in the northwestern provinces of the country (Baja California and Sonora states) and mainly during the summer months.[4] These geographic areas constitute intense agricultural zones in Mexico, and most of the irrigation systems come from a network of canals that originate from the Colorado River. Depending on the annual cycle of irrigation, these canals may form stagnant ditches that may be contaminated by free living amoebae. During the hot summer times, a common recreational activity of young people is swimming in these ditches, and a close association of PAM with a previous history of swimming in these water bodies has been reported. Two cases presented here were no exception, since both had swimming histories.

In this work we reported the characterization of two PAM-isolated *N. fowleri* strains. This characterization was made using light microscopy analysis of CSF, transmission electron microscopy, enflagellation assay of isolates, virulence test in Balb/c mice, and cellular and molecular biology. The results obtained, for two isolated strains, using the techniques above mentioned were similar when compared with the reference strain (ATCC 30808). Only slight differences were seen in electron microscopy analysis. Ultrastructural differences observed among the trophozoites of the three strains studied were basically related to the cytoplasmic lipid inclusions, vacuoles and lysosome-like bodies. In general, these differences are mainly attributed to the age of culture in axenic conditions in the laboratory; so, Mexicali strain that is an older than JFF culture, shows more vacuoles and lipid droplets, and contrarily lysosome-like structures are more common in fresh or recently cultured strain (JFF strain). Differences in the shape and number of mitochondria among strains have not a clear explanation yet.

Clinically, the similarity of PAM symptoms with other more common cases of meningitis, produced by viruses or bacteria, requires a high level of suspicion on the part of the physician, who must communicate with the clinical laboratory for a rapid study and diagnostic examination of the CSF sample. Unfortunately, limited knowledge of this illness by the medical community, combined with its great severity and rapid progression are important factors related to the high mortality of the disease. Amphotericin B has shown effectiveness in the treatment of few cases of PAM. However, an accurate diagnosis is often slow, and consequently, the delay in starting treatment using this drug, constitute additional factors for the elevated number of deaths due to this parasitic infection.

In conclusion, the present report constitutes the first 2 cases of PAM in Mexico in which the etiologic agents (*N. fowleri*) were undoubtedly determined by using both, the conventional as well as the modern cellular and molecular biology techniques currently available. However, these procedures are not easily accessible in most hospitals, and in turn of little practical benefits for the patients; so, it seems essential that health authorities provide the necessary laboratory facilities and educational programs to the medical community about clinical, pathological and epidemiological aspects of free-living amoebae infections in probable endemic areas in Mexico.

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