Identification of Environmental Isolates of Pathogenic *Naegleria* Amebae by Indirect Immunofluorescence

David T. John, Marsha J. Howard, and Kenneth R. Watson

Department of Biochemistry and Microbiology, Oklahoma State University, College of Osteopathic Medicine, Tulsa, OK 74107.

The species identity of 19 environmental isolates of pathogenic *Naegleria* amebae, obtained from Tulsa area waters, was determined by the indirect immunofluorescence antibody technique. Results showed that 12 of the isolates were *N. australiensis,* six were *N. fowleri,* and one was *N. lovaniensis.* Although both *N. fowleri* and *N. australiensis* cause disease in humans and mice, respectively, *N. lovaniensis* is not known to produce disease. ***©1998 Oklahoma Academy of Science***

INTRODUCTION

Naegleria is a genus of free-living amebae that contains two pathogenic, or opportunistic, species (*1*). Pathogenic amebae are able to invade the nasal mucosa, migrate through the cribiform plate, and produce a fatal central nervous system (CNS) infection. *N. fowleri* is the cause of primary amebic meningoencephalitis (PAM), a rapidly fatal infection involving the CNS. The disease, which occurs naturally in humans, can be produced experimentally in mice. *N. australiensis* causes a similar disease in experimentally infected mice; however, *N. australiensis* has not yet been isolated from humans. Nonetheless, because it is pathogenic to mice, it should be considered a potential human pathogen.

During a previous environmental survey in the Tulsa area, we obtained 19 pathogenic *Naegleria* isolates from water and swab samples (*2*). Pathogenicity was determined by the intranasal inoculation of mice. The purpose of this current study was to determine the species identification of the 19 *Naegleria* isolates using an indirect immunofluorescence (IIF) antibody assay.

MATERIALS and METHODS

Amebae: The 19 pathogenic *Naegleria* isolates used in this study were obtained during a survey of opportunistic amebae in Tulsa area waters (*2*). Ten of the isolates were from water samples and nine were from cotton-tipped swab samples of objects near the water's edge. Samples from a variety of sources produced nine isolates from a golf course pond; seven from a stock pond; and one each from a woodland pond, a stream, and the cooling tower of an institutional-sized air-conditioning system.

Amebae were cultivated in Mix ameba medium (*2*), consisting of 0.55% liver digest, 0.50% proteose peptone, 0.25% yeast extract, and 0.30% glucose in Page ameba saline (0.12 g NaCl, 0.004 g $MgSO_4 \bullet 7H_2O$, 0.004 g $CaC1_2 \cdot 2H_2O$, 0.142 g Na_2HPO_4, and 0.136 g KH_2PO_4 per liter of distilled water) (*3*), supplemented with 4% bovine calf serum and 1 μg hemin/mL.

Pathogenicity was determined by the intranasal inoculation of mice. Amebae were harvested by centrifugation (1,200 *g*, 5 min, 20°C) and inoculated intranasally into 21-day-old male CD-1 mice (Charles River Laboratories, Wilmington, Massachusetts). While mice were under anesthesia (Metofane, Pitman-Moore, Inc., Washington Crossing, New Jersey), a 10-μL drop containing the desired inoculum was introduced into a single naris using an Eppendorf pipet. Brain tissue from dead or dying mice was cultured for amebae in Mix medium.

Antisera: Polyclonal antisera against *Naegleria* species, *N. australiensis* (PP 397 strain), *N. fowleri* (LEE-M strain), *N. gruberi* (EGB strain), and *N. lovaniensis* (Aq/9/1/45/D strain), were produced in male New Zealand white rabbits (Middlefork Kennels, Salisbury, Missouri) weighing approximately 2.5 kg each. Amebae were emulsified

with a syringe using either complete or incomplete Freund's adjuvant and injected intramuscularly in the thigh muscle of a hind leg. The priming immunization contained complete Freund's adjuvant and an equal volume of ameba suspension, and 0.5 mL of the emulsion (approximately 500 μg ameba protein) was injected. Booster immunizations, given 4, 6, and 8 wk after the priming immunization, contained incomplete Freund's adjuvant and were prepared and injected in the same manner as for the priming immunization. Ten days after the final booster immunization, blood was drawn from anesthetized rabbits to prepare the antisera used in the indirect immunofluorescence assay.

Indirect immunofluorescence (IIF) test: Approximately 1×10^4 amebae in Mix medium were placed in each well of multiwell slides and incubated in a moist chamber at 37 °C for 30 min. Slides were removed, the medium absorbed with a pad, and the adherent amebae fixed in a solution of 2% formalin-anhydrous methyl alcohol. Slides were rinsed three times in phosphate buffered saline (PBS), a final rinse in distilled water, dried, and stored at -20 °C until assayed.

Rabbit anti-ameba serum was diluted serially two fold beginning at 1:2. A 10-μL drop of each serum dilution was added to the fixed amebae in the multiwell slides and incubated in a moist chamber at 37 °C for 30 min. Slides were then rinsed three times with PBS and dried. A 10-μL drop of fluorescein-conjugated goat anti-rabbit immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania), diluted 1:50 in PBS, was added to each well and the slides again incubated in a moist chamber at 37°C for 30 min. The slides were again rinsed three times with PBS, counterstained with Evans blue diluted 1:1200, rinsed, and dried. The slides were examined by epifluorescence using a Leitz Orthoplan fluorescence microscope equipped with an Osram HBO short arc mercury vapor lamp. Fluorescence was scored from 1+ to 4+, with 4+ denoting brightest apple-green fluorescence. The endpoint titer was the final dilution of antiserum producing positive (1+) fluorescence.

TABLE 1. Indirect immunofluorescence antibody titers of environmental isolates of pathogenic *Naegleria* amebae.

Isolate (EPA-)	Antiserum titer[a] *N. a.* [b]	*N. f.* [c]	*N. g.* [d]	*N. l.* [e]	Ameba
1194	512	16	4	8	*N. a.*
1260	512	8	4	8	*N. a.*
1265	1024	4	8	8	*N. a.*
1368	256	4	8	8	*N. a.*
1425	256	2	2	2	*N. a.*
1877	128	2	4	4	*N. a.*
1879	256	4	8	2	*N. a.*
1892	256	4	4	8	*N. a.*
1908	128	4	2	2	*N. a.*
1912	1024	4	2	4	*N. a.*
1919	1024	16	16	4	*N. a.*
1968	256	8	4	8	*N. a.*
409	8	512	2	32	*N. f.*
988	4	256	2	128	*N. f.*
1020	2	128	2	64	*N. f.*
1027	2	256	2	8	*N. f.*
1102	4	512	4	128	*N. f.*
1911	4	512	2	64	*N. f.*
741	64	32	8	512	*N. l.*
Control					
N. a.[b]	1024	4	8	2	*N. a.*
N. f.[c]	8	1024	2	64	*N. f.*
N. g.[d]	64	8	1024	8	*N. g.*
N. l.[e]	4	4	0	1024	*N. l.*

[a] Reciprocal endpoint titers. Assays were performed in triplicate at least twice for each isolate, and the endpoint titer of greatest frequency was recorded.
[b] *N. a.* = *N. australiensis* (PP 397).
[c] *N. f.* = *N. fowleri* (LEE-M).
[d] *N. g.* = *N. gruberi* (EGB).
[e] *N. l.* = *N. lovaniensis* (Aq/9/1/45/D)

RESULTS and DISCUSSION

The results of the indirect immunofluorescence antibody assay of the 19 pathogenic *Naegleria* isolates are given in Table 1. Twelve of the isolates were identified by IIF as *N. australiensis,* six as *N. fowleri,* and one as *N. lovaniensis.*

The amebae of *N. fowleri* normally live as phagotrophs in aquatic habitats where they feed on bacteria, but as opportunists they are

able to invade the CNS of humans and produce a fatal disease. The term "amphizoic" (Gr. *amphi*, on both sides) has been proposed to describe the ability of the opportunistic amebae to live in two worlds, as free-living organisms and as endoparasites (*4*).

PAM, caused by *N. fowleri*, was first described in 1965 from Australia (*5*). Since then, cases have been reported worldwide. Most of the reports have been from the developed rather than the developing nations, probably because of greater awareness rather than greater incidence. Australia, the Czech Republic, and the United States have reported 75% of all cases of PAM. In the United States, most of the reported cases have been from the coastal states of Virginia, Florida, and Texas, accounting for 67% of cases. The majority of victims of PAM have had a history of recently swimming in freshwater during hot summer weather.

N. fowleri has been isolated from a variety of environmental sources worldwide. Although the three ponds in the present study were sampled year-round (*2*), *N. fowleri*, identified by IIF, was isolated only from the golf course pond, the pond with the least amount of visible organic matter, during the hot summer months of July and August.

The range of titers for the *N. fowleri* isolates was four fold, from 1:128 to 1:512, with 1:512 the most frequently occurring titer. In contrast, the *N. australiensis* titers ranged eight fold, from 1:128 to 1:1024, with 1:256 being the titer of greatest frequency. The *N. fowleri* isolates, however, showed greater cross reactivity than did the *N. australiensis* isolates. *N. fowleri* amebae cross reacted with *N. lovaniensis* antiserum to a titer of 1:128, whereas *N. australiensis* amebae cross reacted with *N. fowleri* antiserum only to a titer of 1:16. It may be that *N. fowleri* has more surface antigens, or better exposed antigens, than *N. australiensis.*

N. australiensis was described in 1981 from an isolate obtained from a water sample in Australia (*6*). The first environmental isolations of *N. australiensis* in the Western Hemisphere were described from Oklahoma (*7*). Although pathogenic to mice, via intranasal inoculation, *N. australiensis* is not as virulent for mice as *N. fowleri,* and it loses virulence in axenic culture more rapidly than *N. fowleri* (*7*).

In the present study, there were twice as many environmental isolates identified as *N. australiensis* as there were *N. fowleri.* Unlike *N. fowleri,* which was obtained only from the cleanest water, *N. australiensis* was recovered from all three ponds and was the species most frequently isolated from the stock pond, the pond with the greatest amount of organic content (*2*). *N. australiensis* appears to be more widely distributed in the environment than *N. fowleri.*

One isolate, EPA-741, obtained from the cooling tower of an air-conditioning system (*2*), was identified by IIF as *N. lovaniensis.* Originally described as a nonpathogenic variant of *N. fowleri* (*8*), *N. lovaniensis* was later described as a new thermotolerant species (*9*). *N. lovaniensis* grows at 45°C, as does *N. fowleri,* but is not pathogenic to mice. *N. lovaniensis* is an indicator of the potential presence of *N. fowleri* in the environment. Our isolate EPA-741 was recovered from the brain tissue of a mouse after intranasal inoculation. On subsequent intranasal inoculations, amebae could not be recovered from brain tissue; however, amebae were cultivated from lung tissue 2 wk after intravenous inoculation (*2*). Further study is needed to resolve the pathogenicity of the EPA-741 isolate of *N. lovaniensis*.

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