APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1983, p. 1282–1285
0099-2240/83/121282-04$02.00/0

Vol. 46, No. 6

Isolation and Identification of Pathogenic *Naegleria australiensis* (*Amoebida, Vahlkampfiidae*) from a Spa in Northern Italy

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Received 2 May 1983/Accepted 6 September 1983

Samples from therapeutic swimming pools and mud basins were cultured for free-living amoebae. Seven strains of pathogenic *Naegleria* species were isolated. Although some of the strains were as virulent as *Naegleria fowleri*, the etiological agent of primary amoebic meningoencephalitis, they were identified as *Naegleria australiensis* with the indirect fluorescent-antibody technique. The virulence of the isolates for mice corresponded with the cytopathic effect for Vero cells. The *N. australiensis* strains were isolated from swimming pools with water temperatures ranging from 32 to 35°C and from mud with temperatures from 25 to 43°C. The presence of pathogenic *N. australiensis* in the swimming pools did not correlate with bacterial indicators.

Since its description in 1970 (2), *Naegleria fowleri* is well known to be the cause of primary amoebic meningoencephalitis in humans. Until recently, this free-living amoeba has always been regarded as the only pathogenic species within the genus *Naegleria*. Immunological studies and determination of isoenzymes led in 1981 to the description of a new pathogenic species, *Naegleria australiensis* (6). The type strain of this species was isolated in 1973 in Australia (J. A. Jamieson, M.S. thesis, University of Adelaide, Adelaide, Australia, 1975). More recently, strains of *N. australiensis* were isolated in France (P. Pernin and J. De Jonckheere, Ann. Parasitol. Hum. Comp., in press), Germany (11), and India (R. Michel and J. De Jonckheere, Trans. R. Soc. Trop. Med. Hyg., in press).

We report here the isolation of pathogenic *N. australiensis* strains from therapeutic swimming pools and thermal mud basins of a spa in northern Italy. A long time ago, these waters were found to possess direct therapeutic effects. They are used, therefore, for the treatment of some respiratory and otorhinolaryngological afflictions, as well as gynecological disorders.

MATERIALS AND METHODS

Characteristics of collection sites. Thirty thermal swimming pools and mud basins in a spa in northern Italy were investigated. The hyperthermal radioactive bromine-iodine mineral waters (80 to 85°C) come from one geological layer located at a depth of ca. 300 m. The waters are mainly used for the maturation of kaolin muds. The muds are put into basins arranged in series in the open air. Over a period of several months, they undergo a slow and complex biological maturation process, stimulated by abundant proto- and metaphytic vegetation. When maturation is complete, they are employed for traditional crenotherapy (14, 15). The waters are also transferred into swimming pools where rehabilitation exercises are performed (hydrotherapy of arthritis, rheumatism, sequelae of traumatic lesions, etc.). To allow patients to bathe in this water, its temperature is reduced to about 32 to 35°C by serial passages into cooling tanks put outside. Water recycling, carried out by the method usually followed for swimming pools, keeps the thermal water in the pool at the desired temperature (14).

Physicochemical and bacteriological analyses. At the time of sampling, the outside ambient temperature and the temperature of the feedwater were measured in each swimming pool and mud basin. The pH of the water from swimming pools was measured in the laboratory with a glass electrode. Free chlorine was measured by the method of Palin (12).

The water from swimming pools was also investigated for bacterial load (total plate count) and the presence of coliforms.

Isolation of amoebae. By the use of sterile glassware, water samples of 500, 100, and 10 ml were collected from each pool. Mud samples from each mud basin were placed into sterile petri dishes. The samples were immediately taken to the laboratory and filtered through Millipore membranes (cellulose acetate; pore diameter, 1.2 μm). Filters were placed upside down on the agar medium. Mud samples were put directly onto the isolation medium. This medium consisted of 3% nonnutrient agar inoculated with a suspension of *Escherichia coli* K-12 (Farmitalia) on its surface. The petri dishes were incubated at 37 and 45°C to select only

TABLE 1. Isolation of pathogenic *Naegleria* strains from swimming pools

Characteristics	Swimming pool no.		
	2	6	21
Surface (m^2)	201	350	80
Capacity (m^3)	420	700	165
Filtration system	Sand and quartz	Quartz	Sand and quartz
Water turnover in 24 h	Continuous	Continuous	Continuous
Mean no. of users per day	35	60	60
Sampling date	9/26/82	9/26/82	9/27/82
Ambient temp (°C)	24	23	19
Water temp (°C)	35	32	32
Free chlorine (ppm [μg/ml])	0.34	0.60	0.22
pH	6.5	7	6.5
Bacterial load (CFU/ml)	28	28	2
E. coli	Absent	Absent	Absent
Naegleria strain designation	AB-T-2500	AB-T-6100/2	AB-T-2100

thermophilic species or strains, which are the most likely to show pathogenicity. The plates were controlled daily up to day 20 of incubation. The presence of any amoebic vegetative forms was checked with a Leitz-Diavert inverted microscope (×320), and their progressive encystment was followed. Isolates were cloned by transferring a single cyst onto fresh medium to obtain a homogeneous protozoan population from each strain.

Identification of isolates. The morphological characterization of the isolated strains was performed as follows: (i) by phase-contrast and interference-contrast study of the vegetative and cystic forms; (ii) by a flagellation test in distilled water at 37°C; and (iii) by the study of mitosis in slide microcultures by the method of Pussard (13). *Naegleria* strains were made axenic in Fulton's "A" medium (10) and CGVS medium (19).

The cytopathic effect of the isolates was tested in Vero cell cultures. The virulence for mice was investigated by intranasal and intracerebral inoculation of Swiss mice.

Pathogenic *Naegleria* isolates were serologically tested by the indirect fluorescent-antibody technique with antisera prepared against *N. fowleri* KUL (17) and the type strain of *N. australiensis* PP397 (ATCC 30958) (6).

RESULTS AND DISCUSSION

Of a total of 30 thermal swimming pools and mud basins, 7 yielded pathogenic *Naegleria* strains. The three swimming pools positive for pathogenic *Naegleria* differed in water volume, filtration system, and free chlorine levels (Table 1). The water of none of these swimming pools appeared to be bacteriologically bad; the total count was low (maximum, 28 CFU/ml), and *E. coli* was absent. The swimming pool water had a very high temperature (32 to 35°C). The four mud basins positive for pathogenic *Naegleria* strains had temperatures ranging from 25 to 43°C (Table 2).

All strains of pathogenic *Naegleria* isolates grew in Fulton's medium, whereas only four grew in CGVS (Table 3), but the growth without bacteria was satisfactory only for strains AB-T-F3 and AB-T-F4. Both strains also showed a more pronounced cytopathic effect for Vero cells and very high virulence for mice. It was thought, therefore, that they might be identified as *N. fowleri* (2). Although the other strains also killed mice when inoculated intranasally, the survival time of the mice was longer, and 100% mortality was not obtained. Also, it took these strains longer to destroy the Vero cell monolayer. Indirect fluorescent-antibody analysis of the isolates was performed with antisera against *N. fowleri* and *N. australiensis*, the only two species of the genus known to be pathogenic. The titers giving 2+ fluorescence on a scale from 0 to 4+ revealed that all isolates were *N. australiensis* (Table 4).

This is the first time pathogenic *N. australiensis* has been isolated so frequently from one area. Some of the isolates were shown to be much more virulent than the type strain of *N. australiensis* and the other known strains of this species isolated in France, Germany, and India. The virulence for mice of these particular strains is as high as that of *N. fowleri*, the etiological agent of primary amoebic meningoencephalitis in humans. A manuscript on a comparative study of the different *N. australiensis* strains available today is in preparation.

TABLE 2. Isolation of pathogenic *Naegleria* strains from mud basins

Mud basin no.	Sampling date	Ambient temp (°C)	Mud feedwater temp (°C)	*Naegleria* strain designation
1	5/27/82	24	40	PV-2891
3	9/25/82	22.5	43	AB-T-F3
4	9/26/82	24	39	AB-T-F4
9	10/13/82	22	25	AB-T-F9/2

TABLE 3. Growth and effects of pathogenic *Naegleria* isolates

Strain	Temp of isolation (°C)	Growth[a] at 37°C in:		CPE[b] at 37°C (day after inoculation)	Time of death of mice (day after inoculation)[c]	
		Fulton's medium	CGVS		IN	IC
PV-2891	45	+	−	6	8, 10, 10, S, S	3, 5, 5, 5, 7
AB-T-F3	45	+	+	2	5, 5, 5, 6, 8	2, 2, 2, 2, 3
AB-T-F4	45	+	+	3	4, 5, 6, 6, 6	2, 2, 3, 3, 3
AB-T-2500	45	+	−	8	8, 9, 9, 12, S	5, 5, 6, 6, 8
AB-T-6100/2	37	+	+	5	8, 9, 9, 12, S	4, 5, 6, 6, 8
AB-T-2100	45	+	−	6	10, 11, S, S, S	3, 5, 5, 5, 7
AB-T-F9/2	37	+	+	6	9, 10, 10, S, S	4, 4, 5, 6, 6

[a] +, Growth; −, no growth.
[b] CPE, Cytopathic effect on Vero cell line. Cultures were done in Corning flasks (surface area, 25 cm^2; ~6 × 10^5 amoebae).
[c] IN, Intranasal instillation (~6 × 10^4 amoebae); IC, intracerebral inoculation (~4 × 10^4 amoebae); S, survivor.

This is also the first time pathogenic *Naegleria* species were isolated from environments with therapeutical uses. Recently, De Jonckheere (7) showed that in hospital therapy pools treated with UV light, conditions are favorable for *N. fowleri*, as he was able to demonstrate the presence of large numbers of *Naegleria lovaniensis*, which occupies the same niche (17). Pathogenic strains, however, were not demonstrated in these pools (7). In the latter study, also, the bacteriological quality of the pools was very poor due to ineffective disinfection. In the swimming pools investigated here, the water was properly chlorinated (Table 1), resulting in total bacterial counts that were low and absence of fecal indicators. It is, therefore, surprising that *Naegleria* species were found, as the cysts and vegetative forms of these amoebae are very sensitive to chlorine (3, 4, 8).

In a chlorinated swimming pool in New Zealand where primary amoebic meningoencephalitis due to *N. fowleri* was contracted, the amoeba was thought to be introduced by the addition of untreated thermal bore water (5). Also, in England a case of primary amoebic meningoencephalitis infection occurred where *N. fowleri* was subsequently isolated from thermal spring water that supplied the pool (1). Our study provides further evidence that pathogenic *Naegleria* species can be present in water even though the water is bacteriologically safe. The results also confirm that high temperatures are favorable for the occurrence of pathogenic *N. australiensis*, as was found before for pathogenic *N. fowleri* (9, 18).

The isolation of highly pathogenic *N. australiensis* further illustrates the need to include antiserum prepared against *N. australiensis* when identifying pathogenic *Naegleria* species. The isolate from mud basin 1 (strain PV-2891) has already been reported as probably the first *N. fowleri* strain isolated in Italy (16). Serological examination showed instead that this strain belongs to *N. australiensis*.

TABLE 4. Indirect immunofluorescence results (reciprocal endpoint titers) of pathogenic *Naegleria* isolates compared with the homologous reactions of the antisera

Strain	Antiserum titer	
	N. fowleri KUL	*N. australiensis* PP397
PV-2891	<32[a]	1,024
AB-T-F3	<32	1,024
AB-T-F4	32	1,024
AB-T-2500	<32	1,024
AB-T-6100/2	<32	512
AB-T-2100	<32	1,024
AB-T-F9/2	64	512
PP397	32	1,024
KUL	1,024	128

[a] 1/32 was the lowest dilution tested.

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