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First Isolation of Pathogenic *Naegleria fowleri* in Japan

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Abstract

Thermophilic *Naegleria* spp. were isolated from geothermal waters and industrial warm waters in Japan. The isolates were identified to species by isoenzyme analysis after agarose isoelectric focusing. The majority of isolates belonged to *N. lovaniensis,* while *N. fowleri* were identified in two places. The pathogenicity of the *N. fowleri* strains was confirmed by intranasal instillation into mice. The pathogens were isolated from an industrial warm water and the water discharge of a geothermal bath.

Key words: *Naegleria fowleri,* geothermal waters, industrial warm waters, Japan, isoenzymes, pathogenic

Introduction

Free living amoeboflagellates of the genus *Naegleria* are found worldwide in different kinds of water, but they cannot live in seawater. One species, *N. fowleri,* causes primary amoebic meningoencephalitis (PAM) in man, leading to death within a week. Cases of PAM have been reported from every continent but there are no confirmed cases in Japan. Although there is one published report on a PAM case in Japan (Nakamura, *et al.*, 1979), the causative agent was later proven to be an *Acanthamoeba* sp. (Akai, *et al.*, 1980). No attempts have been made to investigate the presence of *N. fowleri* in water in Japan, while on all continents *N. fowleri* has been found, especially in warm waters (for a review see De Jonckheere, 1987b).

Since *N. fowleri* is invariably found in warm waters, it seemed important to investigate the geothermal pools that are so common in Japan. Most of the pools are kept at around 43°C which is very favorable for *N. fowleri* as this pathogen can grow in waters at temperatures up to 45°C. Another *Naegleria* sp. that also grows at this temperature, *N. lovaniensis,* is nonpathogenic to man and experimental animals, but its presence in the water is considered as an indication that the conditions are ideal for the growth of pathogenic *N. fowleri* (De Jonckheere, 1987a). Among other *Naegleria* spp. that are thermophilic, *N. australiensis* (up to 42°C) is pathogenic to experimental animals, but has never been identified until now in man (De Jonckheere, 1987a), while *N. andersoni* (up to 40°C) is nonpathogenic (De Jonckheere, 1988). We have also sampled industrial warm waters, as these waters are the preferred niche of *N. fowleri* in temperate climates (De Jonckheere, 1987b). If *N. fowleri* would indeed be isolated, *N. fowleri* should be considered as possible causative agent when meningoencephalitis is diagnosed in Japan. Furthermore, retrospective PAM cases should then be looked for in medical records.

Materials and Methods

During October to December 1990 twenty six samples from 18 different geothermal waters in Kanto area and in Kyushu area and 12 warm water samples from 8 different industries from

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Kanto area were taken. The sampling, consisted of 500 ml water and, where possible, swabbing the pool or discharge wall with sterile cotton tips. The temperature of each water was measured at site. The samples were processed the same day in the laboratory. Water samples of 50 ml were centrifuged at 1,000 rpm for 10 min. After discarding the supernatant, the sediment was spread on nonnutrient agar plates previously coated with *Escherichia coli* (NNE). One ml samples and the cotton swabs were inoculated directly on NNE. All samples were treated in duplicate for incubation at 42°C and 45°C respectively. The NNE plates were screened twice daily for the appearance of clearing zones in the *E. coli* lawn. Amoebae in these clearing zones with the morphology of *Vahlkampfiidae* were transferred to fresh NNE by cutting out the agar layer with the clearing zone and putting it upside down on the fresh NNE. These NNE were incubated at 37°C. The identity of *Naegleria* spp. was in most cases confirmed by the amoeba to flagellate transformation which occurred in the liquid accumulating at the interface of the transferred agar block and the agar layer of the fresh NNE plate. After one or 2 days incubation at 37°C, a piece of agar supporting the migrating zone of multiplying *Naegleria* isolates was transferred to Serum-Casein-Glucose-Yeast-Extract-Medium (SCGYEM) for axenic growth of *Naegleria* (De Jonckheere, 1977). It is known that pathogenic *N. fowleri* adapt much easier and faster to axenic growth in SCGYEM than any other *Naegleria* sp. and other genera. For specific identification water soluble protein extracts from axenically growing *Naegleria* isolates were separated by agarose isoelectric focusing in pH 3.5 to 10 gradient gels and stained for acid phosphatase (AP) and propionyl esterase (PE) activity (De Jonckheere, 1982). The banding patterns were compared to those obtained with *Naegleria* reference strains run on the same gel. Isolates identified as *N. fowleri* by their AP and PE banding pattern were tested for their ability to kill 4 weeks old mice after intranasal (IN) instillation.

Results

Thermophilic *Naegleria* spp. were isolated from 14 out of 26 samples taken from geothermal waters (Table 1) and from 10 out of 12 samples of industrial warm waters (Table 2). When identified to species by AP and PE isoenzyme patterns (Fig. 1) the majority of isolates were identified as *N. lovaniensis*. In one case we identified *N. andersoni* and in another *Willaertia magna* (De Jonckheere *et al.*, 1984). Among strains isolated from the discharge of one geothermal bath (sample 16) and from one industrial warm water (sample 26), *N. fowleri* were identified. The AP patterns are identical for *N. fowleri* with different geographic origin and are only slightly different from the *N. lovaniensis* pattern. With the PE patterns *N. lovaniensis* can easily be differentiated from *N. fowleri* while small differences in banding are seen with *N. fowleri* strains with different geographic origin. The PE patterns of Japanese *N. fowleri* isolates corresponded to that of the Australian reference strain. The pathogenicity of these isolates was confirmed by IN instillation in mice. When a species name is indicated in Table 1 and 2, it means only one or a few isolates could be grown axenically and, therefore, identified by isoenzymes. Therefore, other *Naegleria* spp. might have been present as well. In case no species name is given in the tables, it means none of the isolates could be adapted to axenic growth, which excludes identification by isoenzyme analysis. The maximum number of *Naegleria* growing at 45°C was calculated to be around 2,000/L for geothermal water (sample 38), and 4,000 for industrial warm water samples (sample 11). In samples where *N. fowleri* were found the total number of *Naegleria* growing at 45°C was calculated to be around 180/L (geothermal bath water discharge sample 16) and 140/L (industrial warm water sample 26).

When different samples were taken from the same geothermal place, it was noted the original well water never contained thermophilic *Naegleria* spp., but they were only found in the bathing pool and the water discharge from the bath (Table 1). At the place where the pathogenic *N. fowleri* was isolated only the water discharge could be sampled, so we don't know whether the pathogen was also present in the bathing pool.

In the case chlorine was used for disinfection (sample 10) or in case of strong sulphur smell

Table 1 Isolation of *Naegleria* spp. from geothermal waters in Kanto and Kyushu areas.

Sample	Temp. (°C)	*Naegleria* isolated*
Kanto area		
1. bath A	43.0	+
2. bath B, indoor	42.5	*Naegleria* sp.
3. outdoor	42.0	*N. lovaniensis*
4. bath C	44.0	*N. lovaniensis*
5. bath D, outdoor	41.0	*Naegleria* sp.
6. indoor	43.0	*N. lovaniensis*
7. bath E	41.0	+
8. bath F	42.5	*N. lovaniensis*
9. bath G	41.5	*Naegleria* sp.
10. bath H†	43.0	–
14. spring I	28.0	–
16. discharge bath J	38.0	*N. fowleri*
17. bath K	27.5	+
18. bath L	32.0	–
19. bath M	23.0	–
Kyushu area		
30. bath N‡	43.0	–
31. bath O	44.0	*N. lovaniensis*
32. well O	32.0	–
33. bath P	51.0	*Naegleria* sp.
34. well P	30.0	–
35. discharge P	44.0	*Naegleria* sp., *Willaertia magna*
36. bath Q	18.0	*Naegleria* sp.
37. fish pond Q	30.0	+
38. bath R	43.0	*Naegleria* sp.
39. discharge R	39.0	*N. lovaniensis*
40. well R	44.0	–

* +: positive for amoebae, but not *Naegleria*.
–: negative for amoebae
Naegleria sp.: with this isolate, isoenzyme identification to the species level could not be performed, because it did not adapt to axenic growth
† treated with chlorine
‡ strong sulphur smell

(sample 30), no amoebae were isolated.

Discussion

We report for the first time the isolation of pathogenic *N. fowleri* in Japan. Only two places in the Kanto area were found to contain the pathogen, but many places in both the Kanto and Kyushu area contained *N. lovaniensis*. Although *N. lovaniensis* is nonpathogenic, it is considered an indicator organism for places that are suitable for the growth of *N. fowleri* (De Jonckheere, 1987a). *Naegleria fowleri* is much more difficult to isolate than *N. lovaniensis* because the latter grows much faster, but both species favor the same ecological niche. Both species have the same maximum temperature tolerance for growth and are antigenically closely related. The *N. fowleri*

Table 2 Isolation of *Naegleria* spp. from warm waters from industries in Kanto area

Sample	Temp. (°C)	*Naegleria* isolated*
11. Glass industry	39.5	*N. lovaniensis*
12. Glass industry	23.5	*Naegleria* sp.
13. Metallurgical factory	19.5	*N. lovaniensis*
20. Fabric factory, thank	31.0	*Naegleria* sp.
21. Fabric factory, discharge	35.0	*Naegleria* sp.
22. Metallurgical factory, treated water	28.0	+
23. , original water	32.0	+
24. Cleaning factory, chemical treatment	34.0	*Naegleria* sp.
25. , biological treatment	34.0	*N. andersoni*
26. Food supply factory	30.0	*N. fowleri*
27. Food supply factory	32.0	*N. lovaniensis*
28. Metallurgical factory	48.0	*N. lovaniensis*

*see table 1

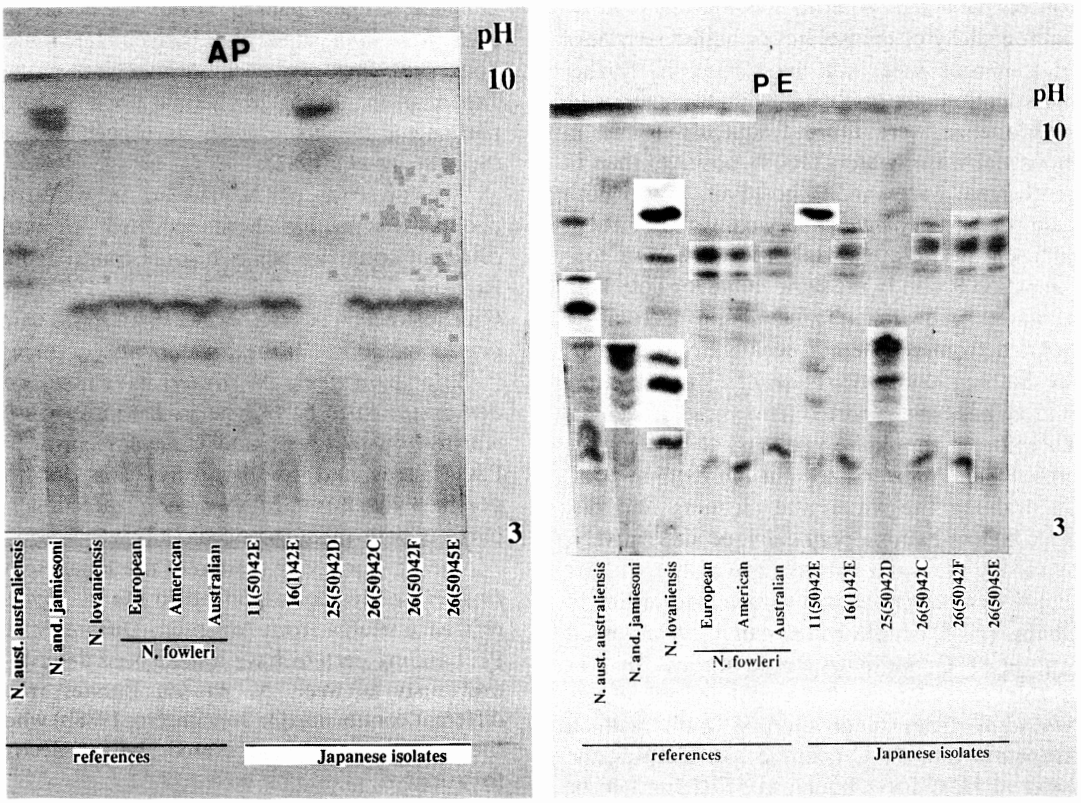

Fig. 1 Isoenzyme patterns of acid phosphatase and propionyl esterase after isoelectric focusing in pH gradient 3–10 of some Japanese isolates compared to *Naegleria* reference strains. Strains from place 11 correspond to *N. lovaniensis* and those from place 25 to *N. and. jamiesoni*. The isolates from place 16 and 26 are *N. fowleri* and the PE bands at the middle of the pH gradient indicate they correspond to the Australian *N. fowleri* type.

isolated in Japan did not adapt as easily to axenic growth as previously reported (De Jonckheere, 1977), but this could be due to variation in batch of fetal bovine serum or other medium ingredient. Because the medium appeared not to be optimal some *N. fowleri* might have escaped identification during this investigation. The other pathogenic *Naegleria* sp., *N. australiensis,* does not adapt very easy to axenic growth in SCGYEM (De Jonckheere, 1987a), and considering the fact the batch of medium used was not optimal even for *N. fowleri,* this could explain not a single *N. australiensis* strain has been identified. Therefore, it does not mean that *N. australiensis* is not present in Japan, as we have not much insisted in trying to find *Naegleria* spp. others than *N. fowleri.* Although the *N. fowleri* isolated were pathogenic to mice, we did not obtain 100% mortality as usually reported for this pathogen. Whether this was due to lower pathogenicity of the isolates or higher resistance of the mice used, will be subject to further investigation.

Amoebae were more frequently found in industrial warm waters (100% positive) than in geothermal waters (65% positive), but in both kind of waters *Naegleria* spp. consisted of about 80% of the isolates, while the maximum concentration is also in the same range for both kind of waters. However, *Naegleria* spp. were never isolated from geothermal well water that feeds the bathing pools. In New Zealand the same finding has been reported (Brown, *et al.*, 1983). Therefore it should be very easy to prevent the presence of *Naegleria* spp. in the bathing pool, by draining the water and cleaning and disinfecting the bathing pool daily, or alternatively, by raising the water temperature daily to a level that *Naegleria* spp. cannot survive. According to Chang (1978) trophozoites can survive for 30 min. at 51°C, while cysts even survive for 2 h. at this temperature. We could indeed isolate *Naegleria* from a geothermal bath with a temperature of 51°C (sample 33). Keeping the water at 51°C for 2 hours, at 55°C for 1 h. or at 65°C for 5 min. should eliminate all *Naegleria* (Chang, 1978). In case none of these procedures is effective, permanent disinfection with chlorine or adding salts to the water should be considered. *Naegleria* are known to be susceptible to chlorine and not to be able to grow in water with high mineral content (Brown, *et al.*, 1983).

Controlling the presence of *N. fowleri* in industrial cooling water is much more difficult even by increasing the temperature. Because of the permanent discharge a temperature gradient will always exist so that the *Naegleria* will always find an optimal temperature to proliferate. Also disinfecting by chlorine seems quite difficult because the chlorine will be readily neutralised by the organic matter in the industrial water.

Pathogenic *N. fowleri* have been previously found in geothermal waters in other countries as well, such as New Zealand (Brown, *et al.*, 1983), England (Aufy, *et al.*, 1986) and the USA (Seidel, *et al.*, 1982) and in all these instances, cases of PAM have been related to bathing in these geothermal pools. It is therefore surprising no attention has been paid to the possible occurrence of *N. fowleri* in geothermal baths in Japan until now, since they play such an important part in Japanese life. From geothermal pools in Italy, pathogenic *N. australiensis* have been isolated (Scaglia, *et al.*, 1983).

The presence of *N. fowleri* in industrial cooling waters has been reported in many different countries while in some countries like Belgium (Van Den Driessche, *et al.*, 1973) and Czechoslovakia (Cerva, *et al.*, 1980) PAM cases due to swimming in these waters also occurred.

Since pathogenic *N. fowleri* have now been demonstrated to be present in Japan, it will be worthwhile to investigate clinical records for PAM cases and to alert physicians for the possible diagnosis of *Naegleria* infection in patients with meningoencephalitis.

The PE isoenzyme pattern of the Japanese *N. fowleri* isolates corresponded to the *N. fowleri* reference strains from Australia. Differences in PE banding pattern have indeed been described previously between *N. fowleri* isolates from different continents (De Jonckheere, 1988b) when the proteins were separated by isoelectric focusing.

The *N. fowleri* strains isolated during this study are presently investigated by DNA restriction fragment length polymorphism to know whether the Japanese strains fit into the

hypothesis of origin and worldwide dispersal of this pathogen (De Jonckheere, 1988).

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