Effect of Antifungal Drugs on Pathogenic *Naegleria* spp Isolated from Natural Water Sources

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Abstract

Five of 16 strains of pathogenic *Naegleria* spp isolated from 350 natural water sources in Taling Chan District, Bangkok had similar molecular weights and zymogram patterns to those of *Naegleria fowleri* CDCVO 3081 and Thai strain. The *in vitro* effects of antifungal drugs (amphotericin B, ketoconazole, fluconazole and itraconazole) were tested at the following concentrations: amphotericin B 0.01-0.55 μg/ml, ketoconazole 0.01-0.3 μg/ml, fluconazole 0.75-3.5 mg/ml and itraconazole 4-12 mg/ml respectively. Aliquots (15,000 cells/ml) of the amoebae were placed in the cells of the microtiter plate and incubated at 37°C. Amoebae from each treatment sample were exposed to one of the four antifungal drugs. Statistical analysis was done by dependent *t*-test. The sensitivity of the antifungal drugs (MIC_{50}) was as follows : amphotericin B 0.03-0.035 μg/ml ketoconazole 0.05-0.15 μg/ml fluconazole 1.75 mg/ml and itraconazole 8-9 mg/ml respectively ($p < 0.005$). Conclusion : Amphotericin B and ketoconazole are more active against *Naegleria fowleri in vitro*. The results of the present study should be used as an *in vitro* screening test for drugs that have potential amebicidal activity.

Key word : Antifungal Drugs, Pathogenic *Naegleria* spp

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Naegleria fowleri is a free-living amebae found in moist soil fresh water and water around factory areas[1,2]. It enters the body *via* the nasal mucosa and migrates along the olfactory nerve to the brain when it causes fuminant meningoencephalitis that is generally fatal[3]. As only a few patients with primary amebic meningoencephalitis (PAM) have recovered, *in vitro* activity against *N. fowleri* has been tested in several chemotherapeutic agents[4]. The results of previous studies can be applied to the treatment of

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patients with PAM[5]. Although amphotericin B and ketoconazole are the two most chemically effective drugs, the mortality rate from PAM is still high. The results of the present study will provide a basis for further study of the *in vitro* activity of amebicidical drugs against *N. fowleri*.

MATERIAL AND METHOD

Amebae isolation from the environment

350 water samples were taken over one year (2001-2002) in five Tambons in Taling Chan District, Bangkok Metropolis. Sterile 500 ml glass containers were filled from the surface of the water. The temperature of the water was taken at the sampling sites, while the pH was measured in the laboratory with a glass electrode. *In vitro* to isolate pathogenic *Naegleria* spp 250 ml water samples were filtered with slight suction through sterile 5.0 µm cellulose acetate membranes (Gelman, Michigan), 47 nm in diameter then overlain with a thin pellicle of living *Escherichia coli* as a food source. The inverted membranes and solids were incubated at 37°C on non-nutrient agar.

Then, the method for isolation technique *N. fowleri* using a temperature of 45°C selectively favors the pathogenic *N. fowleri* over the known non-pathogenic variants, isolated from the same water sources.

Cultivation of the amebae

N. fowleri 3081 and three Thai strains of *N. fowleri* were maintained in Nelson media to which 10 per cent heat inactivated fetal calf serum had been added, in the Department of Parasitology, Faculty of Medicine Siriraj Hospital Mahidol University. Isolated *Naegleria* spp from natural water sources were cultured in Chang's medium (SCGYEM)[6], then inoculated into Nelson media[7] to give cell populations of around 10,000 cells/ml and were then incubated at 37°C.

Biochemical method tested

The amebae were prepared in sufficient quantity for polyacrylamide gel electrophoresis (PAGE) and zymogram identification. Crude antigen was extracted from the thermophilic amebae and compared with the four reference strains of *Naegleria fowleri* CDCVO 3081 and a local Thai strain[8].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weights of 16 thermophilic *Naegleria* strain were analyzed. SDS-PAGE was carried out in a vertical slab gel apparatus (Bio-Rad Labaratories, USA) according to the system of Laemmli. A 4 per cent acrylamide stacking gel and a 12 per cent acrylamide separating gel were used in the process. The crude antigenic extracts (CE) were run on SDS-PAGE. The low molecular weight marker ranged from 1.4-97.0 KD at 200 V for 45 minutes.

Isoenzyme identification

The amebae were first selected by SDS-PAGE, 5 out of 16 thermophilic *Naegleria* spp were identified by detecting twelve enzymes : aldehyde oxidase (ALDOX), aldolase (ALD), esterase (EST 1-7), glucose phosphate isomerase (GPI 1-5), isocitrate dehydrogenase (IDH), lactate dehydrogenase (LDH 1,2) malic enzyme (ME 1,2), a-glycerophosphate dehydrogenase (A-GPDH), glucose phosphomutase (GPM), xanthine dehydrogenase (XDH) malate dehydrogenase (MDH 1,2), leucine aminopeptidase (LAP 1-3). Allozyme electrophoresis was carried out using a vertical polyacrylamide slap gel following Richardson's method[9].

The crude antigenic extract (CE) was prepared and electrophoresis was performed at 5°C, then stopped when the indicator dye (bromophenol blue) had migrated about 10 cm into the separating gel. A reference sample of homogenous *Escherichia coli* was included in all gels to ensure that no isozyme of bacterial origin was scored as a *Naegleria* allozyme.

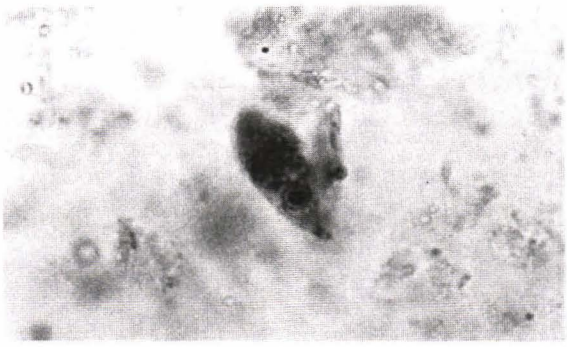

Fig. 1. Thermophilic *Naegleria* species isolations from water sources in Taling Chan District. Trophozite form in iron hematoxylin; cytoplasm appears fine and granular, with a clear nucleus, dense central nucleolus and halo. (Mag 1,250)

Drug preparation

Amphotericin B

Different dilutions of amphotericin B were prepared to yield final concentration of : 0.1, 0.15, 0.02, 0.025, 0.03, 0.035, 0.04, 0.045 and 0.05 μg/ml.

Ketoconazole

Different of dilutions of ketoconazole were prepared to yield final concentration of : 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 μg/ml.

Fluconazole

Different dilutions of fluconazole were prepared to yield final concentration of : 0.075, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5 and 2.75 mg/ml.

Itraconazole

Different dilutions of itraconazole were prepared to yield final concentration of : 4, 5, 6, 7, 8, 9, 10, 11 and 12 mg/ml.

All the stock drug solutions were stored at 4°C and were used within one week to minimize loss of potency.

Data collection and statistical analysis

MIC_{50} was defined as the lowest concentration of a drug that kills 50 per cent of the amebae in 24 hours. Amebae were counted at 0 (baseline), 6, 12, 18 and 24 hours; inverted and light microscopy were used. Statistical analysis was by dependent *t*-test ($p < 0.005$)

RESULTS

The results show sixteen thermophilic *Naegleria* spp isolated from natural water sources in the Taling Chan District (Fig. 1). In five species, thermophilic tolerance (45°C) was closely related to characteristic analysis by PAGE and positive to specific isoenzyme of *N. fowleri* (Malic enzyme). The effect of antifungal drugs was established and the rate of multiplication of the trophozoite stage was tested five times during propagation in the presence of one of the following :-amphotericin B, ketoconazole, fluconazole and itraconazole. The drug concentrations and the amebae counts are shown in Fig. 2-5, MIC_{50} of the antifungal drug were : amphotericin B (0.03-0.035 μg/ml), ketoconazole (0.05-0.15 μg/ml), fluconazole (1.75 mg/ml) and itraconazole (8-9 mg/ml) respectively ($p < 0.005$). The amebae exposed to all four antifungal drugs showed abnormal movements, and deformity of the extra and intrastructures, and they were therefore presumed to be dead.

DISCUSSION

N. fowleri is the causative agent of primary amebic meningencephalitis in young healthy patients.

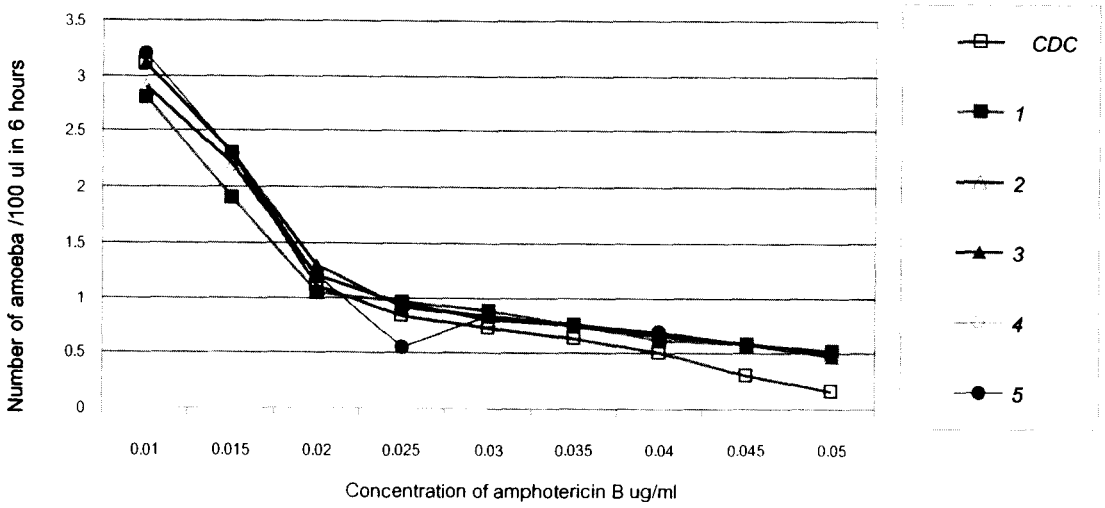

Fig. 2. Sensitivity of the pathogenic *Naegleria* spp isolated from natural water sources in the Taling Chan District (No 1-5) to amphotericin B.

MIC_{50} = 0.03-0.035 μg/ml ($p < 0.005$)

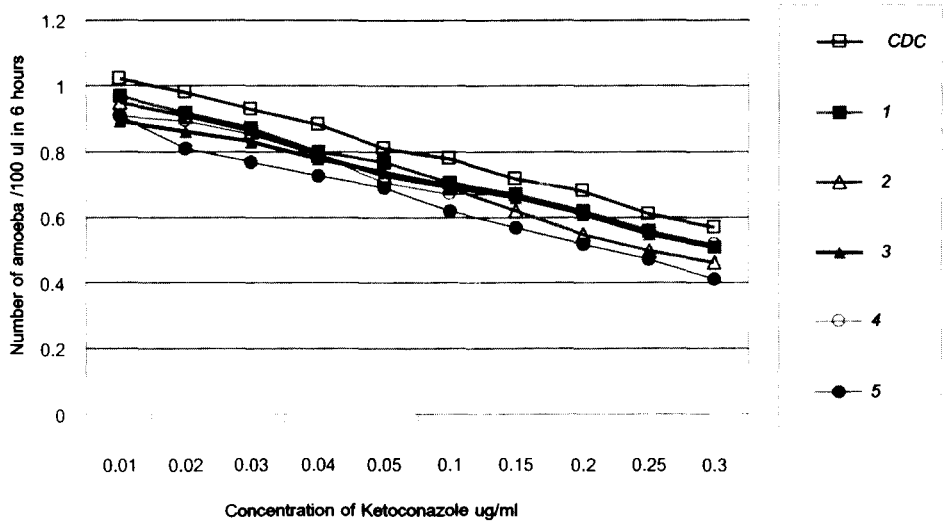

Fig. 3. **Sensitivity of the pathogenic *Naegleria* spp isolated from natural water sources in the Taling Chan District (No 1-5) to ketoconazole.**

MIC_{50} = 0.05-0.15 μg/ml ($p < 0.005$)

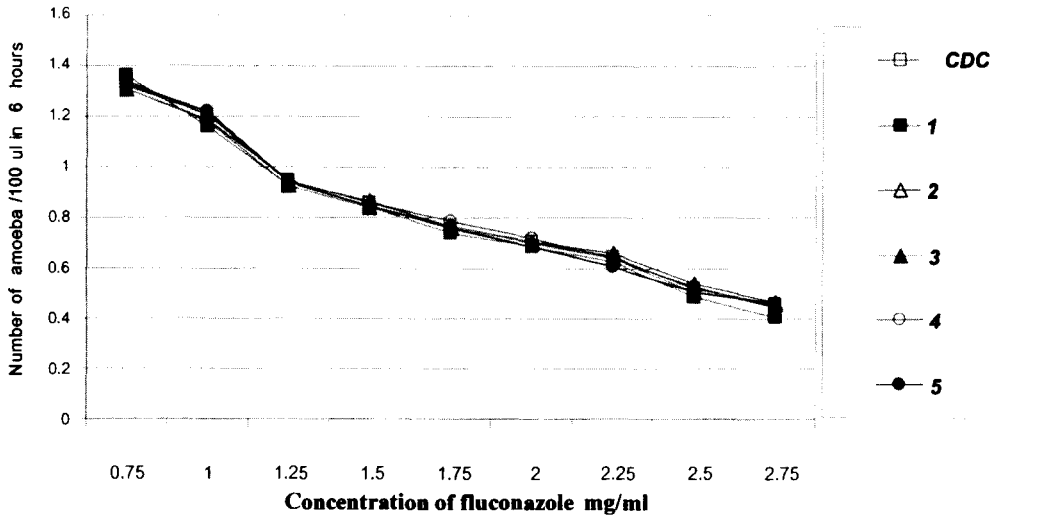

Fig. 4. **Sensitivity of the pathogenic *Naegleria* spp isolated from natural water sources in the Taling Chan District (No 1-5) to fluconazole.**

MIC_{50} = 1.75 mg/ml ($p < 0.005$)

The treatment of PAM is not yet successful as shown by poor patient survival despite treatment with antifungal drugs. For these reasons, an experiment to determine *in vitro* drug sensitivity has been carried out. The authors tested the sensitivity of pathogenic *Naegleria* spp isolated from water sources in the Taling Chan District. Identification of the amebae was performed using the morphological and biochemical

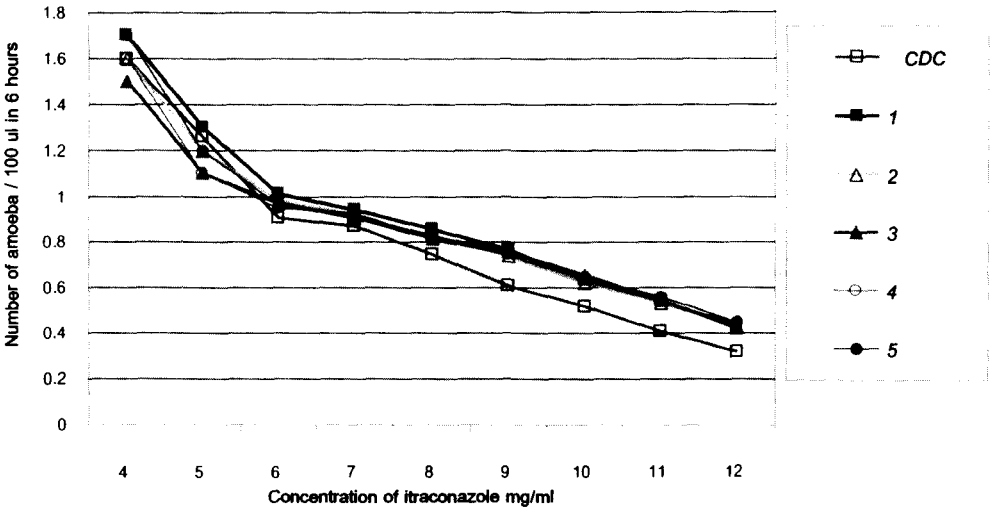

Fig. 5. Sensitivity of the pathogenic *Naegleria* spp isolated from natural water sources in the Taling Chan District (No 1-5) to itraconozole.

MIC_{50} = 8-9 mg/ml ($p < 0.005$)

methods that have been published in a previous study (10). 5 pathogenic *Naegleria* samples were identified out of 350 samples isolated with molecular weight and isoenzyme patterns similar to *N. fowleri*. CDC3081 and the Thai strain. In the present experiment, the *in vitro* susceptibility testing of the amebae to antifungal drug showed that amphotericin B and ketoconazole were more effective against *N. fowleri* than fluconazole and itraconazole. This might be explained by the mechanism of amphotericin B and ketoconazole which directly affects membrane permeability immediately and their sensitivities were similar to those found in a previous publication(11). It has also been found that a combination of amphotericin B and 5-fluorouracil was more effective than a single drug(12). Since a high dose is more likely to produce side effects a drug combination should be able to lower the side effects and may offer a new trend in treating PAM in the future.

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ผลของยาต้านเชื้อราต่อ pathogenic *Naegleria* spp ที่แยกจากแหล่งน้ำธรรมชาติ

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วัตถุประสงค์ : เพื่อศึกษาผลของยาต้านเชื้อราในการกำจัดเชื้อ pathogenic *Naegleria* spp ที่แยกจากแหล่งน้ำธรรมชาติ

วัสดุและวิธีการ : ศึกษาผลของยาต้านเชื้อราใน pathogenic *Naegleria* spp ที่แยกจากแหล่งน้ำธรรมชาติ เขตตลิ่งชัน โดยใช้ความเข้มข้นของยาต้านเชื้อราแตกต่างกัน : amphotericin B 0.01–0.55 μg/ml, ketoconazole 0.01–0.03 μg/ml, fluconazole 0.75–3.5 mg/ml itraconazole 4–12 mg/ml ตามลำดับ ในการวิจัยนี้ ได้นำผลวิจัยมาทำการวิเคราะห์โดยวิธี *t*–test

ผลการศึกษา : นำน้ำที่เก็บจากแหล่งน้ำธรรมชาติ 350 ตัวอย่าง นำมาแยก pathogenic *Naegleria* spp ได้ 5 strain โดยดูรูปร่างลักษณะและคุณสมบัติทางชีวเคมี คือ molecular weight และ isoenzyme identification ผลของการศึกษาได้เปรียบเทียบกับ *Naegleria fowleri* CDCVO 3081 และ *Naegleria fowleri* สายพันธุ์ประเทศไทย พบว่ามีความคล้ายกันของส่วนประกอบทางชีวเคมี จากนั้นได้นำเชื้ออมีบามาศึกษาความไวของเชื้อ โดยใช้ยาต้านเชื้อรา amphotericin B, ketoconazole, fluconazole, และ itraconazole ผลของการศึกษาพบว่า : MIC_{50} ของ amphotericin B 0.03–0.035 μg/ml, ketoconazole 0.05–0.15 μg/ml, fluconazole 1.75 mg/ml and itraconazole 8–9 mg/ml.ตามลำดับ ($p < 0.005$)

สรุป : พบว่า amphotericin B และ ketoconazole ยังคงเป็นยาที่มีประสิทธิภาพในการฆ่าเชื้อ pathogenic *Naegleria* spp

คำสำคัญ : ยาต้านเชื้อรา, *เชื้อนีเกอเรีย*ก่อโรค

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