IN VITRO EFFECT OF ANTIFUNGAL DRUGS ON PATHOGENIC *NAEGLERIA* SPP

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Abstract. An ameba of the genus *Naegleria* causing fatal meningoencephalitis in human subjects was investigated for its sensitivity to antifungal drugs: amphotericin B, ketoconazole, fluconazole and itraconazole. The efficacy of these antifungal drugs for pathogenic *Naegleria* spp was investigated in three strains isolated from patients who had died of primary amebic meningoencephalitis infection at Siriraj Hospital (1986), Ramathibodi Hospital (1987) and Chachoengsao Hospital (1987). All of the isolates were maintained in axenic culture in the Department of Parasitology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Thailand. The sensitivities of the antifungal drugs (MIC_{50}) were: amphotericin B (0.05-0.5 μg/ml), ketoconazole (0.125 μg/ml), fluconazole (0.5-2.0 mg/ml), and itraconazole (10 mg/ml) ($p < 0.05$). It is important to explain that ketoconazole is slightly more effective than amphotericin B because its action is directed of the permeability of the amebic membrane. The amebae were more resistant ot fluconazole and itraconazole due to the action of the cytochrome P_{450} multienzyme (in the case of fluconazole) and the direct effect on heme-iron, blocking cytochrome P_{450}-dependent chitin synthesis (in the case of itraconzole). We conclude that amphotericin B and ketoconazole remain the main drugs with proven activity against pathogenic *Naegleria* spp.

INTRODUCTION

Primary amebic meningoencephalitis (PAM) is a rapidly fatal disease. The high mortality rate is caused by the normally free-living ameba, *Naegleria fowleri*. PAM usually affects previously healthy children and young adults, who contract the disease by swimming in warm, stagnant, fresh-water ponds. The amebae enter the human body by the nasal route and cause a rapidly fatal meningitis, similar to that of fulminating bacterial meningitis (Carter,1969). Because of the poor prognosis, clinicians try to treat the patients with a combination of drugs; successful treatment was reported in 6 cases during the period 1970-1992 ; amphotericin B was used as the first-line drug and was administered in all 6 cases. Other drugs that have been used are rifampicin, miconazole, sulphadiazine, sulfosoxazole, and chloramphenicol. In the sixth case mentioned above, a Chinese person living in Hong Kong received amphotericin B, rifampicin, and chloramphenicol for six weeks: the patient survived and has moderate disability (Wang *et al*, 1993). As only a few patients with PAM recovered, the chemotherapeutic agents that were used were tested for *in vitro* activity against *Naegleria fowleri*. Many drugs such as antimalarials (Peter, 1982; Gupta *et al*, 1995), ketoconazole, allopurinol riboside, BAYn 7133 (imidazole) (Ramond and David, 1984), cyclophosphamide (Zhang *et al*, 1988) and tetracycline showed *in vitro* efficacy (Thong *et al*, 1977). In addition, both the amebicins produced by *Bacillus licheniformis* (D12, D13) (Galvez *et al*,1993; 1994) and amphotericin B have shown *in vitro* activity against *Naegleria fowleri*. In 1999, amphotericin B and gentamicin were studied *in vitro* (Tiewcharoen and Lertlaiturn, 1999). Amphotericin B is the main drug in the treatment PAM; no other drug that have shown *in vitro* efficacy equal to that of amphotericin B. Although amphotericin B has greatest the effect on pathogenic *Naegleria* spp, very few cases of PAM have been treated successfully, hence the search for new drugs. Reported hear is an investigation into the effect of antifungals, which have a broad spectrum of activities, on pathogenic *Naegleria* spp. The

results of this study should be of use in the *in vitro* screening of drugs that have potential amebicidal activity.

MATERIALS AND METHODS

Culture of the amebae

Initial isolates of pathogenic *Naegleria* spp from a Thai patient who died in Siriraj Hospital were taken in 1986; additional isolates were taken from patients who died in Ramathibodi Hospital and Chachoengsao Hospital in 1987. These three samples were maintained in axenic culture in the Department of Parasitology, Faculty of Medicine, Siriraj Hospital, Mahidol University. Amebae were grown axenically in SCGYEM medium (Tiewcharoen and Lertlaiturn, 1999) and in Nelson medium (Cerva,1969), to which 10% heat-inactivated fetal calf serum at pH6 had been added. In order to study the sensitivity of the pathogenic *Naegleria* (Thai strain) to antifungal drugs and to make a comparison with the reference strain, *Naegleria fowleri* (CDC VO3081), 24-hours-old cultures of amebae in Nelson medium were inoculated into growth vessels (50 ml Erlenmeyer flasks) to give cell populations of around 10^4 cells/ml and incubated at 37ºC. One milliliter samples were removed daily for 7 days for counting; cell counts were made with a hemocytometer. Aliquots (1 ml) of this suspension were placed in the cells of a microtiter plate (Corning multiple well plate, Corning Costar Corporation). The microtiter plates were incubated at 37ºC. The control amebae, which had been cultured in axenic Nelson medium for 24 hours, numbered 15,000 cells/ml.

Drug preparation

Amphotericin B: working stock of amphotericin B diluted to 15 μg/ml was prepared from the white powdered drug (150 mg/vial, Bristol-Myers Squibb) using 5% dextrose in water as a diluent. Different dilutions of amphotericin B were prepared to yield final concentrations of: 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 μg/ml.

Ketoconazole: working stock of ketoconazole diluted to (50 mg/ml) was prepared from the white powdered drug using ameba saline pH 5.5 as a diluent. Different of dilution of ketoconazole were prepared to yield final concentrations of 0.03, 0.06, 0.125, 0.25, 0.5, 0.75 and 1.0 μg/ml.

Fluconazole: working stock of fluconazole IV infusion diluted to 2 mg/ml with a sterile aqueous solution rendered iso-osmotic by sodium chloride. Different dilutions of fluconazole were prepared to yield final concentrations of 0.25, 0.5, 1, 1.5, 1.75, 2 and 4 mg/ml.

Itraconazole: working stock suspension of itraconazole diluted to 2 mg/ml was prepared by adding 5% distilled water as a diluent. Different dilutions of itraconazole were prepared to yield final concentrations of 0.62, 1.25, 2.5, 5, 10 and 12 mg/ml.

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

Data collection and statistical analysis

The amebae of each of the treatment samples were exposed to one of the four antifungal drugs; each of the different concentrations of the antifungal drugs was tested five times. The control sample did not receive antifungal drugs. MIC_{50} was defined as the lowest concentration of a drug that kills 50% of 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 ($p<0.05$).

RESULTS AND DISCUSSION

To establish the effect of antifungal drugs, we used pathogenic Thai strains of *Naegleria* isolated from Thai patients who had died of PAM. The rates of multiplication of each of these strains were tested five times during propagation in the presence of one of the following: amphotericin B, ketoconazole, fluconazole, itraconazole; the drug concentra-

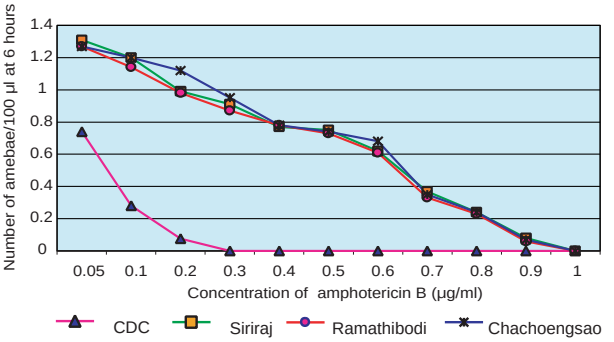

Fig 1–Sensitivity of the pathogenic *Naegleria* to amphotericin B.

MIC_{50} of amphotericin B for reference *Naegleria fowleri* (CDC V03081) = 0.05 µg/ml;

MIC_{50} of amphotericin B for pathogenic *Naegleria* (Siriraj, Ramathibodi, Chachoengsao strains) = 0.5 µg/ml.

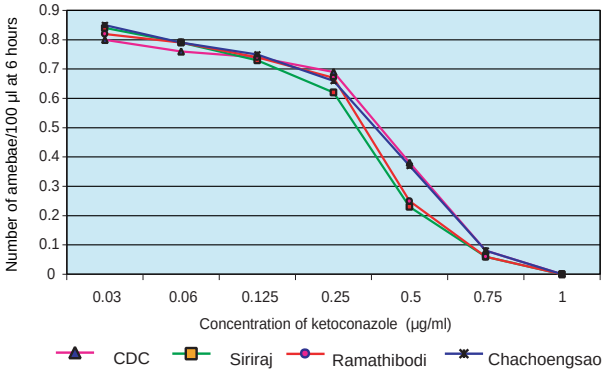

Fig 2–Sensitivity of the pathogenic *Naegleria* to ketoconazole.

MIC_{50} of ketoconazole for reference *Naegleria fowleri* (CDC V03081) and MIC_{50} of ketoconazole for pathogenic *Naegleria* (Siriraj, Ramathibodi, Chachoengsao strains) were equal (0.125 µg/ml).

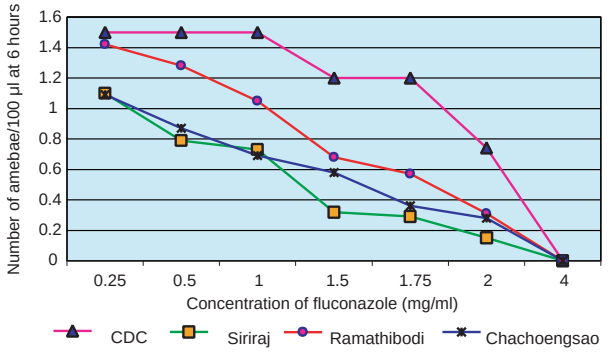

Fig 3–Sensitivity of the pathogenic *Naegleria* to fluconazole.

MIC_{50} of fluconazole for reference *Naegleria fowleri* (CDC V03081) and MIC_{50} of fluconazole for pathogenic *Naegleria* (Siriraj, Ramathibodi, Chachoengsao strains) were equal (2 mg/ml).

tions and the amebae counts are shown in Figs 1-4.

In fresh preparation, the amebae in the control group were active and progressed with unidirectional movement. Morphological study showed that the amebae had a pronounced polarity: the anterior end of the cell was characterized by a clear ectoplasmic pseudopod; the posterior end was characterized by a contractile vacuole. The amebae were sluggish and had hemispherical eruptions. The amebae exposed to the antifungal drugs were rounded, nonmotile, and showed no tendency to form a food vacuole; in addition, they did not settle on the test tube wall; they were presumed to be dead. This abnormal morphology was consistent with that reported previously (Nelson and Jones, 1970).

The sensitivities of the antifungal (MIC_{50}) were: amphotericin B (0.05-0.5 µg/ml), ketoconazole (0.125 µg/ml), fluconazole (0.5- 2 mg/ml), and itraconazole (10 mg/ml). The sensitivity to amphotericin B was similar to that found in earlier studies (Carter, 1969; Tiewcharoen, 1999). Amphotericin B and ketoconazole were more strongly active against pathogenic *Naegleria* spp (Thai strains) than fluconazole and itraconazole. The action of all these antifungals directly effects membrane permeability, allowing the leakage of intracellular contents and the inhibition of cell membrane biosynthesis (Schuster and Rechthand, 1975). This is important in determining the concentration of drugs obtained from the fluids of amebae. The amebae were more resistant to fluconazole than to itraconazole; fluconazole has a direct effect on cytochrome P_{450}-dependent enzymes in the nucleus of the cell (Goodman and Gilman, 1965), but it takes longer to affect membrane permeability and therefore gain access to the intracellular contents; itraconazole interferes with heme-iron, blocking cytochrome P_{450}-dependent chitin synthesis (Goodman and Gilman,

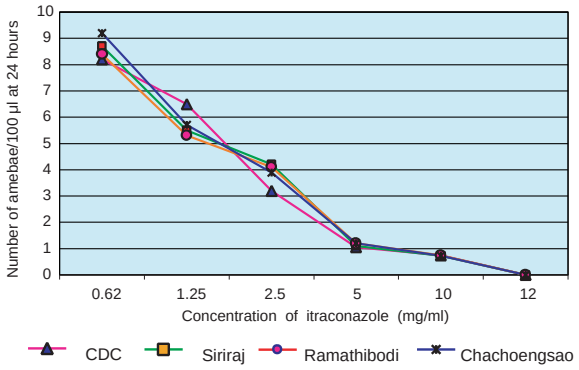

Fig 4–Sensitivity of the pathogenic *Naegleria* to itraconazole.
MIC_{50} of itraconazole for reference *Naegleria fowleri* (CDC V03081) and MIC_{50} of itraconazole for pathogenic *Naegleria* (Siriraj, Ramathibodi, Chachoengsao strains) were equal (10 mg/ml).

1965) -this means that itraconazole takes more time to react with the amebae.

The treatment of PAM remains ineffective for most patients since only six patients have been reported to survive (Wang *et al*, 1993). The poor prognosis is due to the rapid progression of the disease and to the lack of effective chemotherapeutic agents. Seidal *et al* (1988) found that amphotericin B and ketoconazole were the main drugs for that reached effective levels in the brain quickly enough to arrest and cure PAM; they also found that a combination of antifungals was more therapeutic than a single drug. An *in vitro* study of drug combinations should be carried out in order to find new treatments for PAM.

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