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Effect of Amphotericin- B on Pathogenic Strain of *Naegleria fowleri*

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Abstract: The amoebicidal effect of anti- amoebic drug Amphotericin- B was tested on pathogenic strains of aerobic amphizoic amoebae. The anti-amoebic activity of drug was expressed as the minimal inhibitory concentration (MIC), i.e. the lowest concentration of anti-amoebic drug at which more than 50% of amoebic population showed stunted growth or formed the clumps and lost the property to stick to the glass surface.

The minimal drug concentration which completely destructs amoebic population called minimal amoebicidal concentration (MAC) was also recorded. Two pathogenic *Naegleria fowleri* specimens (Strain MA-1 and HS-1) were studied, isolated from municipal water supply of Aliganj and Gomti River (Lucknow, India). The amoebae were cultured in non-nutrients Agar plates, incubated at 35 C for 24 h. The anti-amoebic activity of drug was tested and the observations compared after 24, 48 and 72 h of incubation.

Working stock of Amphotericin- B dilution (15 µg/ml) was prepared from powdered drug, using 5% dextrose in water to solubilize. Different dilutions of Amphotericin-B were prepared to yield final concentration in the range of 0.25 µg/ml, 0.50 µg/ml and 1.0 µg/ml. All the concentrations were duplicates and the experiments were repeated twice. The results were recorded by using light microscope.

Keywords: Amphotericin-B, *Naegleria fowleri*, MIC, MAC

Introduction

The realization that small free-living aerobic amoebae could also produce serious diseases in human being was first detected in 1958 when Dr. Clyde Culbertson and his associates at Eli-Lilly Research Laboratories, U.S.A. discovered the presence of highly pathogenic *Acanthamoebae sp.* containing a 'batch' of killed polio virus vaccine (Culbertson *et al.,* 1958). At first these investigators thought that the vaccine contained live virus, because its inoculation into monkeys kidney cell culture produced plaques with cytopathic effects. As a part of routine safety test, mice and monkeys were inoculated with it, which later produced

a paralytic, central nervous system (CNS) infection and finally death from meningoencephalitis. At early stage of disease, *N. fowleri* does not involve lungs and blood vessels as the path to central nervous system (CNS). Later on, the amoebae enter the veins of CNS and bone marrow to spread the disease. Histopathological studies of brain of mice and monkey revealed that an amoebae was growing and producing plaques, which was originally thought to be of viral origin (Culbertson *et al.,* 1959). This amoeba was originally identified as *Acanthamoeba castellanii*, a common small free-living amoeba, thought to be saprophytic in nature.

Free-living aerobic amoebae were also observed to occur as air borne contaminants of tissue culture in other countries (Castellanii, 1930; Shinn and Hadly, 1936; Nakamura, 1951; Jahnes *et al.,* 1957; Casemore, 1969; Mc Kellar *et al.,* 2006).

Small free-living amoebae are both pathogenic and non-pathogenic. They are ubiquitous in nature and are widely dispersed in our environment (John, 1993; Visvesvera and Stehr Green, 1990). These amoebae have been isolated from rivers, fresh water ponds, lakes, domestic water supply, thermal discharge of power plants, hot springs, swimming pools, heating ventilation and air condition units. They have also been isolated from soil, sewage and even nasal passage of healthy children (Im and Shin, 2003; Siripanth, 2005; John, 1993; Hoffmann and Michel, 2001).

Amphizoic amoebae have also been isolated from CFS from different countries (Culbertson, 1968; Pisani *et al.,* 2003; Shenoy *et al.,* 2002; Jain *et al.,* 2002; Ahmad *et al.,* 2007; Kaushal *et al.,* 2008; Rai *et al.,* 2008). These amoebae have also been isolated from tears, corneal scrapings, eyes of patients by Sharma *et al.* (2000), Schroeder *et al.* (2001), Parija (2001), Hammersmith (2006), De Jonckheere (2003) and Kirwood (2007). *In vitro* cytotoxicity and pathogenicity of *N. fowleri* also depends on the culture medium. PAM caused by *Naegleria* is a medical emergency, only few patients have survived till date. Early diagnosis and treatment may have played a role in their survival. Several drugs like Amphotericin-B, Miconazole and Rifampicin have been used (Carter, 1969). Several molecular techniques which include polymerase chain reaction (PCR) and real time PCR play an important role in detection of *N. fowleri* in clinical and environmental specimen (Mushtaq and Shinwari, 2016). Amphotericin-B at a dose of 1-1.5mg/kg/day alone or in combination with Miconazole, Rifampicin and Sulpha drugs may be used (Loschiavo *et al.,* 1993; Burri *et al.*, 2010). Granulomatous Amoebic Encephalitis (GAE) due to *Acanthamoeba sp.* or some other free-living amoebae cause chronic, sub-acute encephalitis with CNS infection with granuloma formation (Martinez and Visvesvara, 1997). It occurs predominantly in patients who are immunosuppresed with diabetes or alcoholism or those receiving radiation therapies (Reed *et al.,* 1997; Rowen *et al.,* 1995; Galarza *et al.,* 2002). According to Cardenas-Zuniga *et al.* (2017) ultra-structural changes increase in reactive oxygen species and decrease in intracellular potassium.

Many amoebicidal drugs have been found to possess *in vitro* amoebicidal action against pathogenic free-living amoebae, but they also have limited therapeutic use as they fail to negotiate the blood brain barrier. Thus the therapeutic agents against pathogenic free-

living amoebae are very rare. Prophylactic efficacy and cure of disease due to pathogenic free-living amoebae have been reported. Amphotericin-B was used as a prophylactic agent by Carter (1969), Schuster (1975), Ferrante (1984) and John (1993). Amphotericin-B is a polygene compound that acts on the plasma membrane disrupting its selective permeability and causing leakage of cellular compound (Kobayashi and Medoff, 1977). After exposure to Amphotericin-B, amoebae round up and fail to form pseudopodia. Amphotericin-B induces morphological, chemical and genetic changes of apoptosis like programmed cell death (PCD) in the genus *Naegleria*.

N. fowleri is very sensitive to Amphotericin- B *in vitro*, only a few patients recovered following intrathecal or intravenous injections of drug alone or combination with miconojole (Schuster and Visvesvara, 2004; Maaty and Hamja, 2012). Many other antimicrobial, antifungal and antiparasitic drugs have been screamed for therapeutic activity against *N. fowleri in vitro* and *in vivo* with varying degrees of efficacy (Tiewcharoen *et al.,* 2003). Azithromycin has been described for the effective treatment of PAM in mice. Phenothiazine compounds, which accumulated in the CNS , also had inhibitory effects on *N. fowleri in vitro* (Omar *et al.*, 2003). A recent study reported that chloropromazine is highly potent and rapidly active than Amphotericin-B and Variconazole against *N. fowleri* throphozoites (Tiewcharoen *et al.*, 2011).

Materials and Methods

Two pathogenic *Naegleria fowleri* specimens (Strain MA-1 and HS-1) were employed in this study, isolated from municipal water supply of Aliganj and Gomti River (Hanuman setu). The amoebae were cultured in non-nutrients agar plates, incubated at 35 C for 24 h. The anti-amoebic activity of drug was tested and the observations compared after 24, 48 and 72 h of incubation.

Amphizoic amoebae were isolated from all the collected water samples of different sources in Lucknow city, India. For isolation of amoebae, 15-20 ml of sterilized (2.5% w/v) non-nutrient agar (Hi- Media lab, Mumbai, India) with NaCl (pH 6.6 to 6.8) is poured into pre-sterilized petri dish (Borosil 9-10 cm diameter) and allowed to set for 24 h. Escherichia coli culture of 24 h old, grown on the surface of nutrient agar slants (pH 7.2) was used as food for amoebae. Bacterial culture was then scraped with nicrome wire loop and spread as thick suspension on the solidified non-nutrient agar surface in the form of circular patch or bacterial circle of about 20-25 mm in diameter aseptically. Two to three such bacterial circles, well separated from each other, were made in each petri dish before making it ready for inoculation of substrate for the growth of amoebae.

About two liters of water from each source was collected aseptically in sterilized bottles. This was then filtered through sterile filter paper (Whatmann No. 1). After filtration; the inner narrow end of the filter paper with collected sediments was aseptically cut into 3-4 small pieces and these were placed in the centre of each bacterial patch in a petri dish ready for inoculation. These plates were incubated at 28 C and 37 C for 10 to 12 days for the amoebae to grow and form cyst. The plates were examined microscopically daily for the presence of trophic and cystic stage of amoebae.

Amoebae isolated from water samples contained a mixed population of different genera and species. These amoebae were subcultured 3 to 4 times by cutting a small square piece of agar, containing plenty of amoebae and placing it facing downwards on the fresh bacterial circle in a non-nutrient agar plate. For obtaining clonal monobacterial culture from this mixed population, the method of single cyst picking was used. The mature cysts from mixed population were scrapped with the help of sterilized nicrome wire loop and put in glass cavity slides containing sterile distilled water. The number of cysts per cavity were reduced by serial dilution. When cysts settled down on glass surface in cavity, these were then separated from each other. Single cyst was picked up with the help of fine sterilized micropipette; made of soft glass, under low power of microscope and transferred to non-nutrient agar plates already seeded with Escherichia coli. These plates were incubated at 28 C for 5 to 10 days. After the strains were cloned, the cysts were put on a streak of Escherichia coli at one end on non-nutrient agar plates. Trophozoites that had migrated far away from the bacterial streak were put on a fresh bacterial streak. This process was repeated at intervals in order to get monobacterial cultures of different strains of amoebae (Singh and Hanumaiah, 1979).

Working stock of Amphotericin-B dilution (15 µg/ml) was prepared from powdered drug, using 5% dextrose in water to solublize. Different dilutions of Amphotericin-B were prepared to yield the final concentrations in the range 0.25 µg/ml, 0.50µg/ml, 0.75 µg/ml and 1.0 µg/ml in 5% dextrose water. All the concentrations were duplicate and the experiments were repeated twice, these stock solutions of drug were stored at 4 C and were used within one week to minimize loss of potency.

Results

The amoebicidal effect of anti-amoebic drug was tested on pathogenic strains of aerobic amphizoic amoebae. The anti-amoebic activity of drug was expressed as the -- amoebic drug at which more than 50% of amoebic population showed minimal inhibitory concentration (MIC), i.e. the lowest concentration of anti-stunted growth or formed the clumps and lost the property to stick to the glass surface. The minimum drug concentration at which complete destruction of amoebic population occurred called minimal amoebicidal concentration (MAC) was also recorded.

The number of control *Naegleria fowleri* amoebae is 15,000 amoebae/ml cultured in mono-bacterial culture medium for 24 h. In a fresh preparation, the amoebae in the control groups were active and progressed with unidirectional movement (Plate I). The morphological study of amoebae showed a pronounced polarity; the anterior end of the cell was characterized by a clear ectoplasmic pseudopodia and the posterior end was characterized by a contractile vacuole. The pseudopodia were sluggish, with a hemispherical eruption. In the experimental group, appropriate concentrations of the drug used in this study were determined after initial screening to select drug levels. The amoebae exposed to Amphotericin-B for 6 h became rounded, non-motile and showed no tendency to form a food vacuole. In addition, they did not settle on the glass cavity slide and were presumably dead (Plate II).

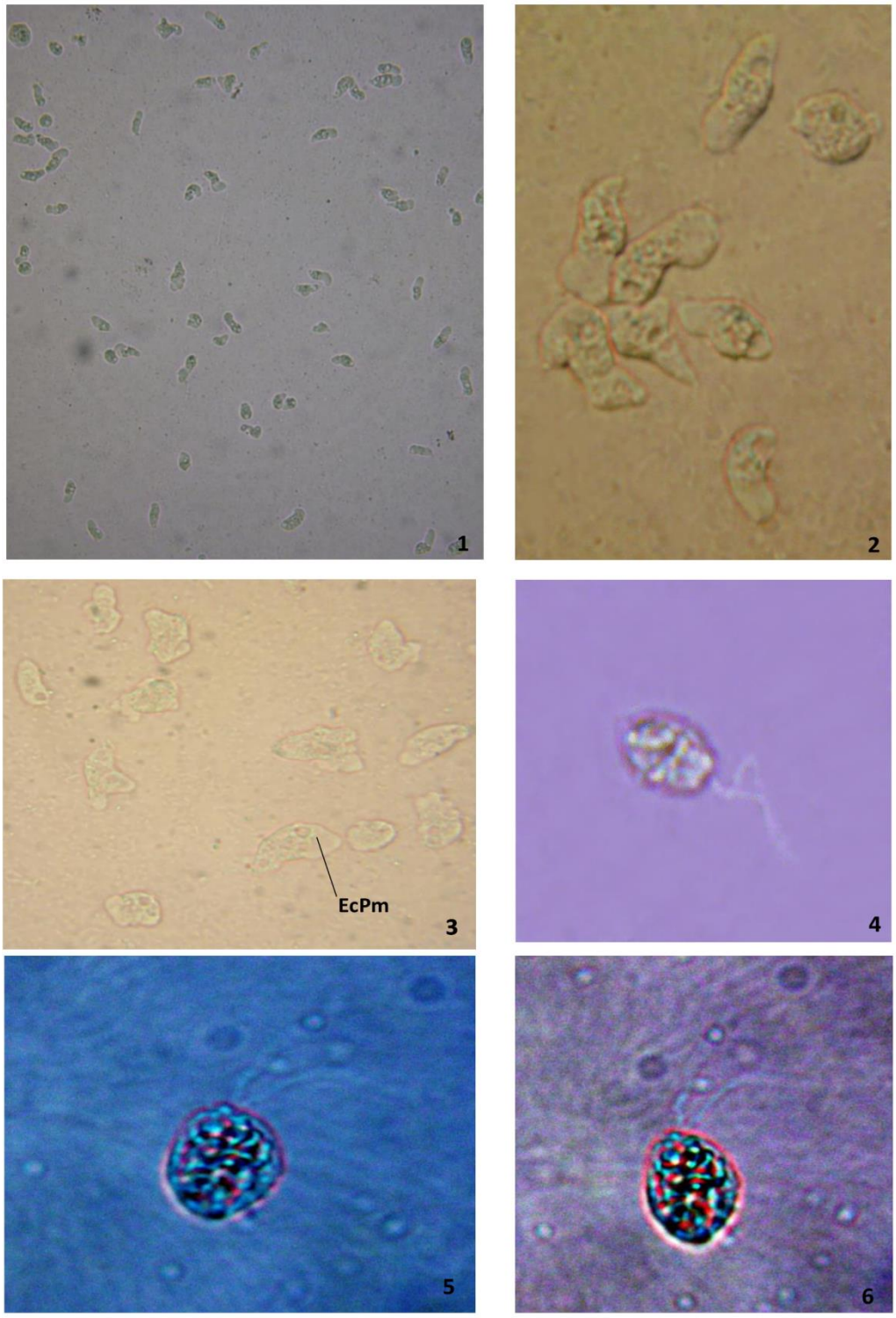

Plate I: Fig. 1-3: Control Trophozoite of *Naegleria fowleri* (10x40 Magnification)
Fig. 4-6: Amoeboflagellate stage of *Nagleria fowleri* (10x40 Magnification)

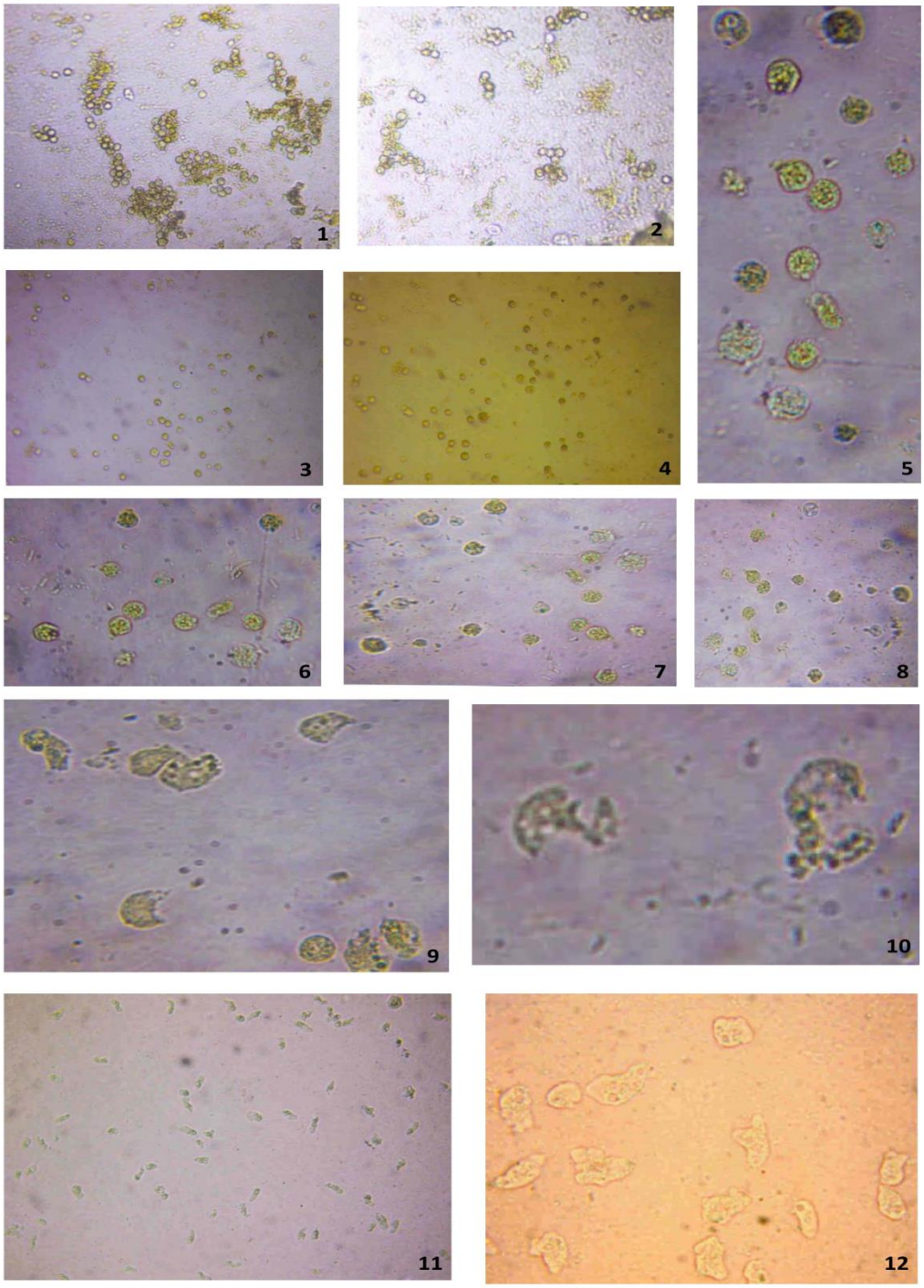

Plate II: Photograph showing *in vitro* effects of antiamoebic drug on amphizoic amoebae
Fig. 1: Formation of big clumps of Amphotericin B (10x10 Magnification)
Fig. 2: Formation of small clumps (10x10 Magnification)
Figs.3-4: All the trophozoite become rounded (10x10 Magnification)
Fig.5: All the trophozoite in rounded condition (10x40 Magnification)
Fig. 6-8: A stage before effect of Amphotericin B (10x40 Magnification)
Fig.9: Lysis in all after Amphotericin B (10x40 Magnification)
Fig.10: Complete lysis after Amphotericin B (10x40 Magnification)
Fig.11: Control showing all trophozoite in their original shape and size (10x10 Magnification)
Fig.12: Control in high power (10x40 Magnification)

The Minimum Inhibitory Concentration (MIC) of Amphotericin-B to pathogenic *Naegleria fowleri* isolated from Gomti River (HS-1) were 0.25 µg/ml and 0.50 µg/ml in 24 h and 0.75 µg/ml and 1.0 µg/ml in 48 h and 72 h, respectively. The major application of Amphotericin-B has been used for systemic fungal infection, but it also has activity against protozoa of the genus *Naegleria* (Ghosh and Ghosh, 1963; Horvath and Zierdt, 1974). According to available information, *Naegleria* trophozoites were inhibited by a concentration of ≤1 µg/ml. This value varied (0.1-0.7 µg/ml) in the different strains (Tiewchaloren *et al.,* 1998).

Some of the variation noted in the susceptibility of pathogenic *Naegleria* to Amphotericin-B could be dependent on strain differences, the duration of exposure to Amphotericin-B and the amoebae population. All these factors probably influence drug response to some degree (Carter, 1969).

Peter (1982) reported the effect of various anti-microbial drugs on the growth of *Naegleria fowleri*; one was that of Neomycin for which the MIC was 100 µg/ml. Amoebae of the genus *Naegleria* have been shown susceptible to Amphotericin-B (Carter, 1970; Apley *et al.,* 1970; Duma, 1971; Jain *et al.,* 2002).

Chang (1971) noted that *Naegleria* amoebae exposed to Amphotericin-B showed a degree in motility, with cells rounding up. This was also observed in the present study. Chang (1971) applied the drug at concentrations of 1 or 2 µg/ml to the culture medium and found that two to eight transfers were required before amoebae were killed at the lower concentration, with pathogenic *Naegleria* appearing more susceptible to Amphotericin-B than the non-pathogenic *N. gruberi.*

Duma (1971) reported rounding, granulation and disintegration of *Naegleria* amoebae within 3 h after exposure to Amphotericin-B concentrations of ≤1.25 µg/ml.The haemoflagellate *Trypanosome cruzi* exhibited granularity and loss of mobility after *in vitro* exposure to Amphotericin-B concentrations as also highly effective against *Naegleria fowleri*, when used alone (Thong *et al.,* 1977) and also in combination with Rifampicin and Tetracycline (Thong *et al.,* 1977a, b).

Scaglia *et al.* (1988) reported *in vitro* sensitivity of Amphotericin-B on *Naegleria australiensis* and *Naegleria fowleri*. Studies have demonstrated *in vitro* and *in vivo* activity of Amphotericin-B against various strains of *N. fowleri* (Lee *et al.,* 1979; Seidel *et al.,* 1982; Smego and Durack, 1984; Stevens *et al.,* 1981) and at least seven patients with primary amoebic meningoencephalitis have been successfully treated with Amphotericin-B alone or in combination with other drugs (Anderson and Jamieson, 1972; Poungvarin and Jariya, 1991; Wang *et al.,* 1993; Goswick and Brenner, 2003; Ahmad *et al.,* 2007; Kaushal *et al.,* 2008; Rai *et al.,* 2008). Amphotericin-B showed promising results against *Naegleria fowleri in vitro* and *in vivo*, but the success rate of drug in PAM patients remained low (Mushtaq and Shinwari, 2016). Tiewcharoen *et al*. (2011) reported that chlorpromazine has high potent and rapidly active than Amphotericin-B against *Naegleria fowleri* trophozoites.

Conclusion

Amphotericin-B has 300-600 times higher efficacy and it is still the main drug for curing PAM. The occurrence of a reproducible set of ultrastructural changes in pathogenic amoebae may be useful in monitoring effectiveness of *in vitro* drug susceptibility in chemotherapeutic studies involving *Naegleria*. *N.Fowleri* is susceptible to chorine in water; amoeba proliferation can be controlled by adequate chlorination of heavily used swimming pools especially during summer months.

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