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Comparison of Three Different Techniques for Follow-up of Free-living Amebae after Drug Therapy

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Abstract

The experiment compared fresh observation, staining, and tissue culture methods for detection of pathogenic free-living amebae after drug therapy. Fresh observations, using a compound microscope and phase-contrast optics were performed. Giemsa, Gram-chromotrope, and Thomas stains were used. The ameba trophozoites were inoculated into Hep-2 and primary chicken kidney cells and their survival was determined within 24 hours. Among the three methods, tissue culture could detect the ameba trophozoites from 24 hours' post-inoculation. Although atypical characters were demonstrated by fresh observation, and the survival of ameba trophozoites could not be determined by staining methods, tissue culture demonstrated the viability of ameba trophozoites. Therefore, tissue culture was the best technique for detecting the survival and viability of pathogenic free-living amebae after drug therapy.

Keywords: *Naegleria, Acanthamoeba*, phase-contrast, Giemsa stain, Gram-chromotrope stain, Thomas stain, tissue culture

Introduction

Primary amebic meningoencephalitis (PAM) and granolomatous amebic encephalitis (GAE) are potentially fatal diseases caused by *Naegleria fowleri* and *Acanthamoeba* spp, respectively. Diagnosis is based on patient history and the detection of ameba trophozoites from CSF or brain tissue sections. PAM is an acute infection occurring in healthy persons, while GAE is a chronic infection occurring in immunocompromised hosts [1-2]. Observation of pathogenic free-living amebae by fresh preparation was recommended [3]. The criteria for differentiating both parasites relied on the typical character of their movement and pseudopodia. Differential diagnoses of ameba trophozoites from leukocytes were quite important. In fresh specimens of severe infection, numerous trophozoites could be observed, but in mild infection or after drug treatment only a few ameba trophozoites may be misdiagnosed; it was also quite difficult to identify or determine live or dead trophozoites. Staining was an alternative method for detecting ameba trophozoites [4]. Trophozoites could be well-differentiated from leukocytes by expert personnel. However, this method could not specify the viability of the trophozoites. Culture methods were also reported [5]. Several media were suggested to promote the growth of *Naegleria* and *Acanthamoeba* spp, but

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some isolated specimens could not be grown in those media. Inoculation of parasites to cell lines was proposed to test cytopathic effects (CPE) [6]. Damage to cell lines caused by ameba trophozoites was determined as the degree of damage. Since fresh observation could not be used to determine the typical characters and survival of ameba trophozoites, and staining methods could only demonstrate the detailed structures of dead trophozoites, an alternative method was needed to clarify whether the trophozoites were alive or dead after treatment. We report here a comparison of fresh observation, staining and culture methods for detecting *Naegleria* and *Acanthamoeba* trophozoites from nasal lavage specimens after drug therapy.

Materials and methods

A patient came to the Hospital for Tropical Diseases with symptoms of severe headache and mild fever for a week. He had a history of diving into a pond for a few days. Before onset, he had no disease and was HIV-negative. He was fully conscious at first admission. Nasal exudates were collected and sent to the Department of Protozoology, Faculty of Tropical Medicine, Mahidol University. Fresh observation was performed under a compound microscope and phase-contrast optics, and numerous ameba trophozoites were observed. The active movements and typical characters of the pseudopodia of both *Naegleria* and *Acanthamoeba* spp were present. The patient was treated and an appointment was made for follow-up. The patient returned to the hospital during the following week with no recovery. He had nasal lavage and the fluid was examined for ameba trophozoites. Numerous active trophozoites were still observed.

The patient was sent to Ramathibodi Hospital for an operation. After the operation, fluids from nasal lavage were collected in a sterile bottle and promptly sent to the Department of Protozoology, Faculty of Tropical Medicine. The specimen fluid was centrifuged at 3,000 rpm for 10 minutes. The sediment was freshly smeared and examined at 40 objective under a compound microscope and phase-contrast optics. Some parts of the specimens were also smeared, fixed in absolute methanol and stained by three different stains—20% Giemsa solution (Merck, CI Nr 45380 and CI Nr 52015) in pH 6.8 of phosphate buffer; Gram-chromotrope, which is a combination of Gram stain for bacteria and 0.6% chromotrope 2R [8]; and Thomas stain using 0.5% phloxine B and 0.25% methylene blue-azure B [9]. Part of the sediment was also inoculated into tissue-culture flasks containing a monolayer of Hep-2 and primary chicken kidney cell lines for cultivation. Observation of ameba trophozoites and the cytopathic effects (CPE) on the cell lines from culture was performed by inverted microscope. Fresh culture medium was changed daily and the culture fluid was transferred to centrifuge tubes and centrifuged at 3,000 rpm for 10 minutes. The sediment was smeared and stained with three stains, as previously described. The specimens were collected weekly for two months to detect trophozoites and/or cysts during treatment.

Results

Immediately after the operation, the sample collected from the nasal cavity of the patient showed bloody fluid containing red blood cells, many ameba trophozoites with typical characters of both *Naegleria* and *Acanthamoeba* spp. Directional movements by the lobopodia of *Naegleria* spp and multiprogressive movement by the filopodia or acanthopodia of *Acanthamoeba* spp were clearly demonstrated by fresh observation (Fig 1). The trophozoites were active, and some demonstrated ingested red blood cells by fresh observation (40). Giemsa stain revealed numerous trophozoites with pale blue ectoplasm, dark pink nucleus and endoplasm (Fig 2). Trophozoites with pale blue cytoplasm and dark blue nucleus presented by Gram-chromotrope, and only ingested reticulocytes in food vacuoles of ameba trophozoites were clearly demonstrated (Fig 3). Some polygonal cysts with prominent nuclei of *Acanthamoeba* spp were demonstrated by Thomas staining (Fig 4). In the first week post-operation, numerous trophozoites were still observed in both fresh and stained specimens. Trophozoites were not active compared with the previous week. *Acanthamoeba* trophozoites predominated, and bacterial infection was

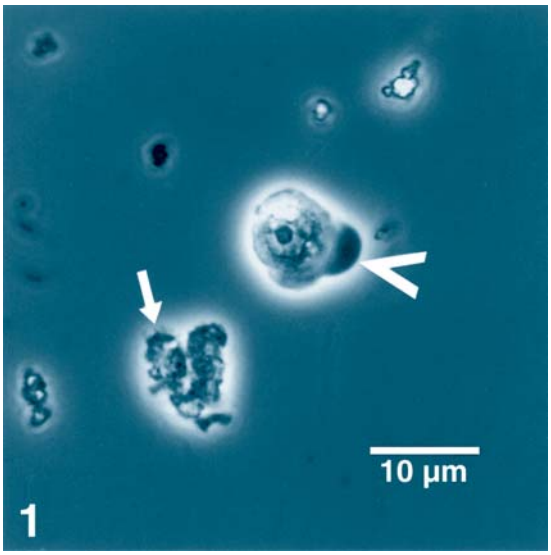

Fig 1 Typical characteristics of lobopodia of *Naegleria* sp (arrow head), and filopodia of *Acanthamoeba* spp (arrow), fresh preparation, phase-contrast optics, 400x, (bar = 10µm).

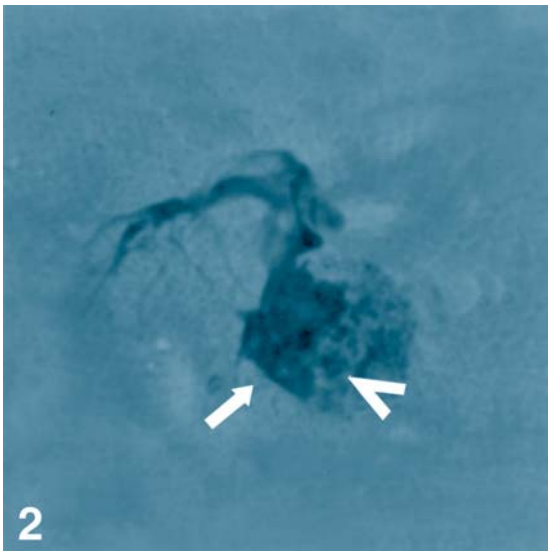

Fig 2 Trophozoite with pale blue ectoplasm (arrow), and dark pink nucleus (arrow head), Giemsa stain, 400x.

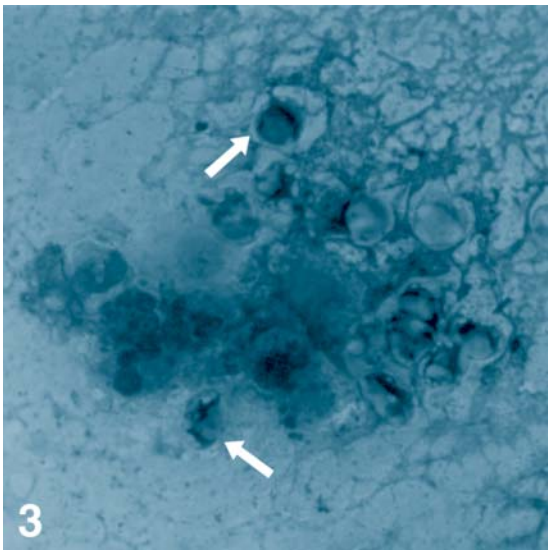

Fig 3 *Acanthamoeba* trophozoite within ingested reticulocytes in food vacuoles, Gram-chromotrope stain, 1,000x.

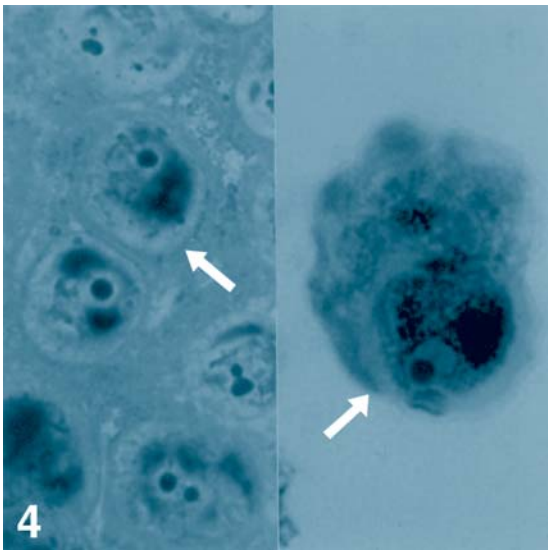

Fig 4 Polygonal cysts with prominent nuclei of *Acanthamoeba* spp, Thomas stain, 1,000x.

detected by fresh and staining methods. Both *Naegleria* and *Acanthamoeba* trophozoites could be grown in Hep-2 and primary chicken kidney cell lines, but more trophozoites were observed in Hep-2 over 24 hours (Fig 5). It was observed that bacterial infection and ameba growth caused cell

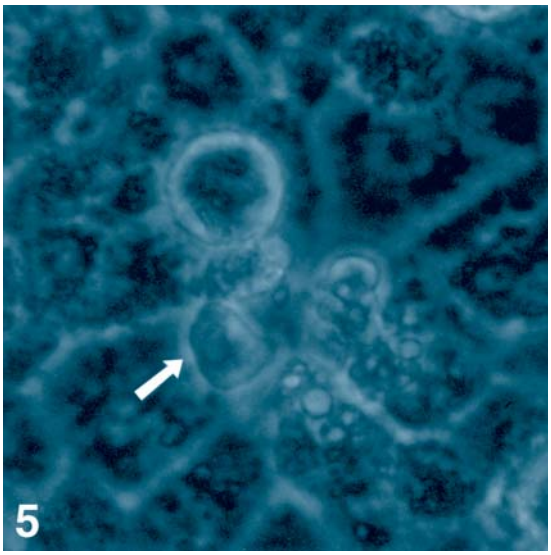

Fig 5 ***Acanthamoeba*** **trophozoites in Hep-2 cell line 24 hours post-inoculation, inverted microscope, 320x.**

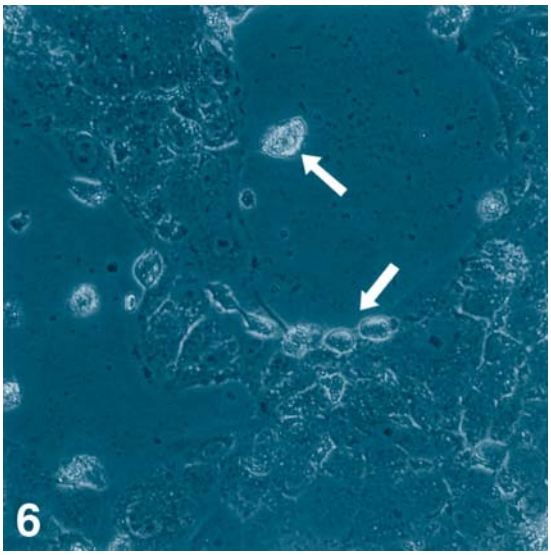

Fig 6 Cell damage, caused by bacterial infection and ameba growth 48 hours post-inoculation, inverted microscope, 200x.

damage after 48 hours (Fig 6). By the second and third week post-operation, fewer ameba trophozoites had been detected by fresh observation, but several of them were detected by staining and culture methods. From the fourth to sixth weeks, only two to three trophozoites per field (400) were detected by fresh observation and staining methods. However, numerous trophozoites could be detected from culture after 24 hours. By the seventh week, ameba trophozoites could not be detected by direct smear, but they could be observed after centrifugation of nasal fluid at 3,000 rpm for 10 minutes, and smeared stain. The growth of trophozoites in culture appeared abundant after 48 hours. By the eighth week, only a few trophozoites could be detected after centrifugation and observed by fresh preparation and staining methods. The trophozoites were not active; no movement presented by fresh observation. The culture was still positive at 24 hours. By the ninth week post-operation, which was the last week of drug treatment, the ameba trophozoites could not be detected by fresh observation after centrifugation. The trophozoites did not move and looked similar to neither *Naegleria* nor *Acanthamoeba* typical features. Few ameba trophozoites with atypical characters were observed by staining methods. However, growth of ameba trophozoites was demonstrated within 24 hours. In addition, bacterial infection was observed throughout cultivation.

Discussion

From the experiment, it was noted that fresh observation was the most appropriate method for detecting ameba trophozoites before drug treatment. Active movement and the typical characters of the pseudopodia were distinct. From observation, the deformities of the ameba trophozoites due to the effects of the drugs caused difficulty in determining whether the trophozoites were viable or not. Although few ameba trophozoites could be detected by centrifugation and staining methods, the survival of ameba trophozoites could not be determined. Giemsa or Wright stains are valuable in general laboratories, and both stains are used for hematological and blood parasite detection [7]. Giemsa stain is available in most laboratories. Although the staining of ameba trophozoites was not well-defined, they could be differentiated from

leukocytes by experiences and technique. Gram-chromotrope staining has been suggested for detecting *Microsporidium* spp, which are opportunistic protozoa in HIV-infected patients [8]. Thomas stain has been used for staining malaria parasites in tissue section [9]. These methods are simple and less time-consuming. Trichrome stain has been recommended for staining free-living amebae [4], and a prominent nucleus with a halo was described as the typical character. However, this technique needs a specific fixative, such as Schaudinns, which is not available in general laboratories, and it is time-consuming. Considering the simplicity of the methods, the availability of stains, and the clarity of the stained specimens, we suggest Giemsa, Gram-chromotrope and Thomas stains for detecting *Naegleria* and *Acanthamoeba* trophozoites. The morphology of ameba trophozoites, ingested red blood cells, and the typical characters of the nuclear structure of the amebae were well-defined by different staining methods that had not been described before. Each stain showed different character detail, but the combination of characters is useful for distinguishing parasites from other artifacts in specimens. These techniques were helpful in detecting ameba trophozoites from both nasal specimens and cultures. These stains could be considered for a general laboratory without an available specific stain.

The inoculation of amebae into cell lines was recommended to determine the CPE of *Naegleria* sp from patient specimens [6]. Cell damage was observed in only *Naegleria fowleri*, which is pathogenic to humans. In our study, bacterial infection was associated; therefore, we could not conclude that CPE was only caused by ameba trophozoites. In nature, free-living amebae feed on living organisms, such as bacteria or fungi. The cultivation of *Naegleria* and *Acanthamoeba* spp in various media was reported. Most media are composed of digested proteins, ameba saline and serum. A basic medium that supported the growth of *Acanthamoeba* spp consisted of proteose peptone or peptone, yeast extract and glucose [10-12]. In culture, the trophozoites fed on some kinds of bacteria, such as *Escherichia coli* or *Enterobacter aerogenes*. In our culture, both *Naegleria* spp and *Acanthamoeba* spp could be cultivated in either Hep-2 or primary kidney cell lines, but their growth was better in Hep-2 because of the properties of Hep-2, which is a carcinoma cell of the larynx, and its growth is much more rapid than primary kidney cell lines. Therefore, there were sufficient cells to support the growth of amebae. Bacterial association is an important factor in enhancing prolonged ameba growth. Most specimens were collected from the brain or CSF, which were free of bacterial contamination [13]. Our specimens were collected from the nasal cavity, which contained several kinds of bacteria resistant to various common antibiotics, *ie* penicillin, streptomycin, gentamicin, and kanamycin. Overgrowth of bacteria in this case destroyed most culture cells. Therefore, prolonged cultivation could not be maintained due to cell damage. In the last week of treatment, the use of antibiotics was reduced. Bacteria could not be detected by fresh preparation and were very rare by staining, but they were confluent after 48 hours of culture. In conclusion, we propose tissue inoculation and cultivation, which is a sensitive method for detecting pathogenic free-living amebae and bacterial contamination from patient specimens after drug therapy.

Summary

Specimens from the nasal cavity after operation and treatment were collected weekly from a symptomatic patient. Fresh observation, staining and inoculation into cell lines were described and compared with one another for detecting ameba trophozoites. The cultivation technique was the most sensitive method, compared with fresh observation and staining methods. Atypical ameba trophozoites due to drug effects were observed by fresh preparation grown in culture cells; their survival could be detected within 24 hours. Giemsa, Gram-chromotrope and Thomas stains were described. The typical characters of the nucleus were observed by Thomas staining, while Gram-chromotrope showed distinct ingested reticulocytes within the food vacuoles of the ameba trophozoites.

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References

1. Fowler M, Carter RF. Acute pyogenic meningitis probably due to *Acanthamoeba* sp.: a preliminary report. *Br Med J* 1965;5464:740-2.
2. Carter RF. Primary amoebic meningo-encephalitis. An appraisal of present knowledge. *Trans R Soc Trop Med Hyg* 1972;66:193-213.
3. Callicott JH Jr, Nelson EC, Jones MM, dos Santos JG, Utz JP, Duma RJ, *et al*. Meningoencephalitis due to pathogenic free-living amoebae. Report of two cases. *JAMA* 1968;206:579-82.
4. Alger N. A simple, rapid, precise stain for intestinal protozoa. *Tech Bull Regist Med Technol* 1966;36:43-4.
5. Balamuth W. Nutritional studies on axenic cultures of *Naegleria gruberi*. *J Protozool* 1964;11 suppl:19-20.
6. Visvesvara GS, Callaway CS. Light and electron microscopic observations on the pathogenesis of *Naegleria fowleri* in mouse brain and tissue culture. *J Protozool* 1974; 21:239-50.
7. Fleck SL, Moody AH. Diagnostic techniques in medical parasitology. London: Butterworth; 1988. p. 23-4.
8. Moura H, da Silva JLN, Sodre FC, Brasil P, Wallmo K, Wahlquist S, *et al*. Gram-chromotrope: a new technique that enhances detection of microsporidial spores in clinical samples. *J Eukaryot Microbiol* 1996;43:94S-95S.
9. Lee G, Luna HT. Manual of histologic staining methods of the Armed Forces Institute of Pathology. 3rd ed. American Registry of Pathology. NY: Mc Graw-Hill; 1968. p. 129.
10. De Jonckheere JF, Pernin P, Scaglia M, Michel R. A comparative study of 14 strains of *Naegleria australiensis* demonstrates the existence of a highly virulent subspecies: *N. australiensis italica* n. spp. *J Protozool* 1984;31:324-31.
11. Neff RJ. Purification, axenic cultivation, and description of a soil amoeba. *Acanthamoeba* sp. *J Protozool* 1957;4:176-82.
12. Schuster FL. Cultivation of pathogenic and opportunistic free-living amebas. *Clin Microbiol Rev* 2002;15:342-54.
13. Culbertson CG, Ensminger PW, Overton WM. Pathogenic *Naegleria* sp.–study of a strain isolated from human cerebrospinal fluid. *J Protozool* 1968;15:353-63.