

Culture, PCR and Different Types of Staining Procedures for Identification of *Acanthamoeba* Spp. from Patient Samples

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Abstract

Background: *Acanthamoeba* spp. are free living ubiquitous protozoa capable of causing *Acanthamoeba* keratitis (AK) of eyes and *Acanthamoeba* meningitis/ meningoencephalitis (AME) of central nervous system and fatal Granulomatous amoebic encephalitis (GAE) of brain in human. These must be diagnosed at an early stage to avoid any complications associated with the disease like blindness of eye and death due to GAE/ AME. This study aimed to focus on application of different diagnosis procedures like culture, staining and PCR assay for early diagnosis of the *Acanthamoeba* spp. in patient samples.

Methods: One corneal scraping and 1 CSF sample were collected by the Ophthalmologist and Neurologist from patients of culture positive *Acanthamoeba* Keratitis (AK) and *Acanthamoeba* meningitis/ meningoencephalitis (AME) using standard procedures. Samples were stained with Giemsa, PAS and Calcofluor white stain for direct examination. Simultaneously samples were cultured on 2% non-nutrient agar plates overlaid with *E. coli*. for culture isolation. Samples were further confirmed by molecular diagnosis with PCR assay using *Acanthamoeba* specific primers.

Results: *Acanthamoeba* spp. were visible under light microscope after direct staining such as Giemsa, PAS and Calcofluor white stain. In culture, *Acanthamoeba* spp. were visible on non-nutrient agar plate after 4 days and covered the entire agar surface after 7 days of incubation. In PCR assay clear band of ~ 461bp length (ASA.S1 region) was visualised in agarose gel.

Conclusions: It was concluded from the above study that, for early diagnosis of *Acanthamoeba* spp. in clinical specimens, staining and PCR assay could be used as compared to culture. Culture isolation takes about a week where as PCR assay takes nearly one or two days for identification of *Acanthamoeba* spp in a clinical setting. Staining takes only few minutes for reporting the *Acanthamoeba* spp. in clinical samples such as corneal scrapings and CSF.

Keywords: Diagnosis of *Acanthamoeba* spp.; PCR Assay; Culture; Staining; Giemsa Stain; Calcofluor Stain

Introduction

Acanthamoeba spp. are free living ubiquitous protozoa capable of causing *Acanthamoeba* keratitis (AK) of eyes and *Acanthamoeba* meningitis/ meningoencephalitis (AME) of central nervous system and fatal Granulomatous amoebic encephalitis (GAE) of brain in human. AK is an acute, sight threatening infection of cornea that can cause corneal ulcers or even permanent blindness if not diagnosed and treated at an early stage [1]. This condition occurs most often among contact lens wearers who do not properly disinfect their lenses, exacerbated by a failure to wash hands prior to handling the lenses. Multipurpose contact lens solutions are largely ineffective against

Acanthamoeba, whereas hydrogen peroxide based solution have good disinfection characteristics. Similarly, AME is a slowly progressive infection of the central nervous system which occurs mostly in immunocompromised individuals with HIV/AIDS, tuberculosis or undergoing cancer chemotherapy treatment and if not diagnosed and treated at an early stage becomes fatal [2-5].

For laboratory diagnosis of *Acanthamoeba* spp. from clinical specimens such as corneal scrapings or CSF, culture, staining, and PCR assay are commonly used. There are several staining procedures available for direct detection of parasite from clinical specimens such as calcofluor white, PAS and Giemsa staining etc. Although these tools are less time consuming but are not considered to be 100% sensitive for efficient laboratory diagnosis [6]. Light microscopic examination of the culture plate (2% non-nutrient agar) containing infected sample (corneal scrapings or CSF) spreaded over the *Escherichia coli* overlay is considered as the gold standard for effective laboratory diagnosis of the diseases [7]. With the advancement of research, several investigators across the world had demonstrated that, molecular diagnosis by PCR assay is more reliable, specific and sensitive than culture for effective laboratory diagnosis of *Acanthamoeba* spp. [6,8]. After the discovery of complete nucleotide sequence of the 18S ribosomal RNA gene (18S rRNA) “*Acanthamoeba* specific amplimer (ASA. S1)” a small region within it (~ 464 bp length) is widely used for molecular detection of *Acanthamoeba* spp. in all types of patient samples [9,10].

This study aimed to focus on application of different diagnosis methods like culture, staining and PCR assay for early diagnosis of the *Acanthamoeba* spp. in patients with AK and AME.

Materials and Methods

Isolation of *Acanthamoeba* spp. from the clinical specimens in culture

One corneal scraping and 1 CSF samples were collected by the Ophthalmologist and Neurologist from patients of culture positive *Acanthamoeba* Keratitis (AK) and *Acanthamoeba* meningitis/ meningoencephalitis patients using the standard procedures. All the clinical specimens were spreaded on 2% non-nutrient agar plates seeded with live *E. coli*, sealed and incubated at 30°C and observed daily under a light microscope (Nikon, Japan) till the growth of amoebas were seen on the agar surface.

Observation of *Acanthamoeba* trophozoites and cysts using different staining procedures Giemsa staining

Both corneal scraping and CSF samples collected from patients were placed over a clean slide; smear was prepared, air dried and fixed in methanol for 2 mins. Giemsa staining was performed following the protocol as described in Garcia, *et al.* 2001 [11]. Slides were flooded with diluted Giemsa stain (in buffered water, pH 7.2) and left for 30 minutes on a flat surface. After incubation, stain was removed and washed with the buffered distilled water till the extra stain wash away from the slide. Slides were air dried, visualized under a light microscope (Nikon, Japan) and photomicrographs were taken.

PAS staining

Both corneal scraping and CSF samples were placed over a clean slide; smear was prepared, air dried and fixed in methanol for 2 mins. 0.5% Periodic acid was added to the smear and incubated for 10 mins. Slides were washed with distilled water to remove the stain. Schiff reagent was added to the smear, incubated for 5mins followed by washing with distilled water. Smear was dehydrated in ascending alcohol concentrations (50%, 70%, 80% and 100%) [12]. Cover slip was mounted over the specimen, visualized under a light microscope (Nikon, Japan) and photomicrographs were taken.

Calcofluor white staining

Both corneal scrapings and CSF samples were placed on a clean glass slide. A drop of 0.1% calcofluor white fluorescent dye (Sigma, Germany) was added over the sample followed by addition of a drop of 10% KOH [13]. A cover slip was placed over the specimen and al-

lowed to stand for 2 mins at room temperature. Slides were examined under UV light (395 - 415 nm) in a Nikon eclipse 90i fluorescence microscope (Nikon, Japan) with 100X objective lens and photomicrographs were taken.

Detection of *Acanthamoeba* spp. by molecular method

Extraction of DNA from clinical specimens

DNA was extracted from the collected corneal scraping in PBS buffer (pH 7.4) and CSF specimen using QIAmp DNA Mini Kit (QIAGEN, USA) following the manufacturer's instructions. Pelleted DNA was dissolved with 50 µl of double distilled water and used for PCR assay.

PCR assay for amplification of ASA.S1 region of 18S rRNA gene

PCR amplification of genus specific region "Acanthamoeba specific amplicon" (ASA.S1 of 464 bp length) of 18S rRNA gene of *Acanthamoeba* spp. was performed from the isolated DNA using the primers JDP1 (5'GGCCAGATCGTTTACCGTGAA3') and JDP2 (5'TCTCA-CAAGCTGCTAGGGGAGTCA3') [9]. The temperature profile of the PCR assay was as follows, i.e. initial denaturation for 10 mins at 94°C, followed by 35 cycles of denaturation at 94°C for 1min, primer annealing at 61°C for 1min, strand elongation at 72°C for 1 min, with the final elongation at 72°C for 10mins. DNA isolated from known isolates of *Acanthamoeba* spp. was used as a positive control and reaction mixture with 5 µl of distilled water was used as a negative control in PCR reaction. Amplified PCR products were electrophoresed on 1.5% agarose gel, which was visualised under a Gel documentation system (Syngene, USA).

Results

Isolation of *Acanthamoeba* spp. from clinical specimens in culture

Acanthamoeba spp. were visible in the culture plate under light microscope (Nikon, Japan) after 4 days and covered the entire agar surface after 7 days of incubation. Figure 1 shows the image captured after 7 days of incubation of the plate with 10X objective of a light inverted microscope (Nikon, Japan).

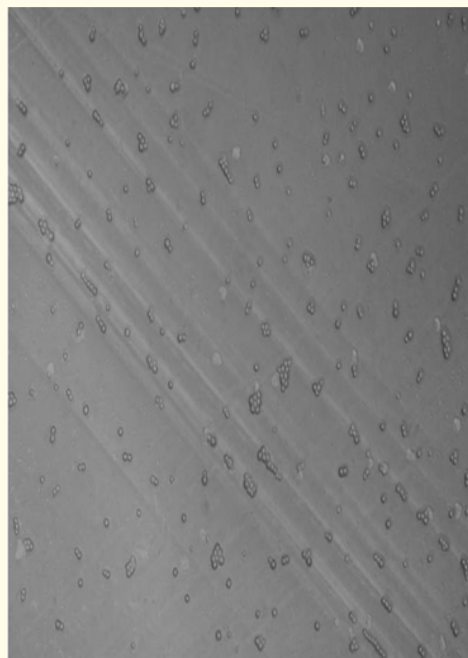


Figure 1: Representative photograph showing the images of *Acanthamoeba* spp. isolates after staining with Giemsa stain.

Giemsa staining

After Giemsa staining *Acanthamoeba* spp. (trophozoites) appeared blue in color. The organisms were clearly visible with inner nucleus with 40X objective lens of a light microscope (Nikon, Japan) (Figure 2). Giemsa staining stains the nucleus and cytoplasm of cell, but cannot stain the outer cell wall of cysts.

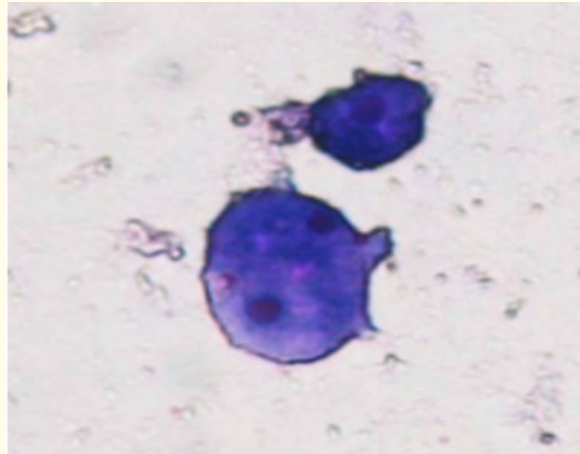
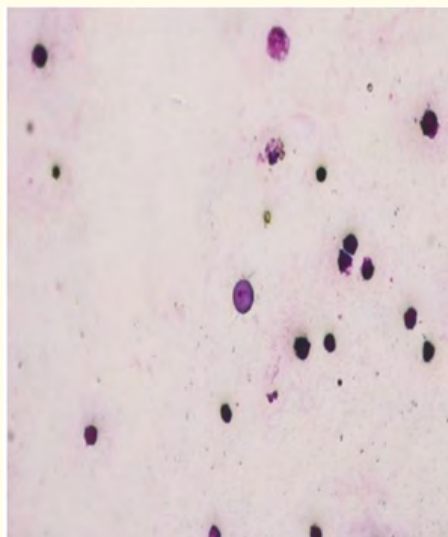


Figure 2: Representative photograph showing the images of *Acanthamoeba* spp. isolates after staining with PAS stain.

Periodic-acid Schiff's (PAS) staining

Acanthamoeba spp. appeared pink in color after PAS staining. Some were dark pink and some were lightly stained. Amoebas were clearly visualized with 10X objective lens of a light microscope (Nikon, Japan) (Figure 3).



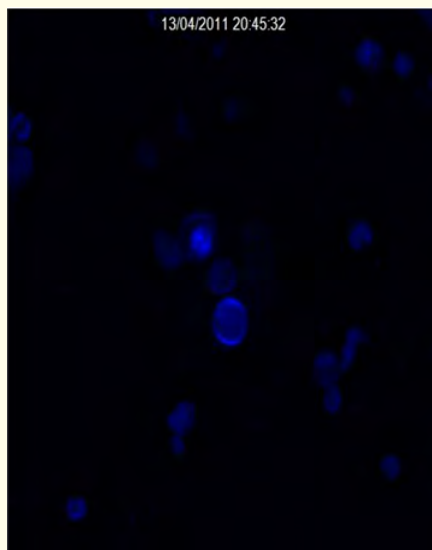


Figure 3: Representative photograph showing the images of *Acanthamoeba* spp. isolates after staining with Calcofluor white stain. A: image of cysts taken with 20X objective of a fluorescence microscope; B: image of cysts taken with 40X objective of a fluorescence microscope.

Calcofluor white staining

Acanthamoeba cysts appeared diffusely fluorescent with different fluorescent intensities depending on the thickness of cell wall (Figure 4). Image shows the mature cysts with double layer cellulose cell walls. The outer wall i.e. exocyst contained less amount of cellulose so appeared less bright while the inner wall i.e. endocyst contained more cellulose so appeared more bright.

PCR assay for amplification of ASA.S1 region of 18S rRNA gene

After gel electrophoresis of the PCR amplified products in 1.5% agarose gel clear band of ~ 461bp length was visualised in both the culture positive specimens (Figure 4).



Figure 4: Representative photograph of 1.5% agarose gel showing the amplified product of 461 bp region (ASA.S1) of 18S rRNA gene of *Acanthamoeba* spp. Samples loaded into the wells are mentioned on the right in a box.

Discussions

Culture isolation of *Acanthamoeba* spp. is considered as the gold standard for laboratory diagnosis [7]. The commonest method is the culture of patient samples (corneal scrapings or CSF) on 2% non-nutrient agar plate overlaid with *E. coli* or with 2% agar medium containing low concentrations of nutrients (e.g., proteose peptone 0.05%, yeast extract 0.05%, glucose 0.1%) in the presence of living or killed bacteria [14,15]. The non-nutrient agar medium contains minimal nutrients and thus inhibits the growth of unwanted microorganisms [16]. The amoeba can grow with several bacteria such as, *Klebsiella pneumoniae*, *Enterobacter* spp. (*Enterobacter aerogenes* and *Enterobacter cloacae*), and *Escherichia coli* [15]. Of all these non-pathogenic strains of *Escherichia coli* are commonly used in the laboratories for preparation of culture plates. *Acanthamoeba* feed on these bacteria by phagocytosis and grow over the agar surface.

Acanthamoeba trophozoites and cysts have variable staining characteristics due to the presence of cell wall in cyst form and its absence in trophozoite form. Different types of staining are used for direct detection of *Acanthamoeba* spp. in clinical samples like Giemsa, PAS, Calcofluor white etc. Giemsa stain is used to stain several protozoan trophozoites which differentiate the nuclear and cytoplasmic morphology of parasites. This stain stains the nucleus and cytoplasm of cell, but cannot stain the outer cell wall of cysts. Calcofluor white (CFW) is a fluorescent dye which has a sharp affinity for cellulose present in the cell walls of cysts (both inner and outer) of *Acanthamoeba* spp. thus has found widespread use for detection of cysts in clinical samples [17-19]. Calcofluor white staining is mostly used as a simple, rapid method for the laboratory diagnosis of *Acanthamoeba* keratitis and any CNS infections. In the present study when the mature cysts were stained with calcofluor white, fluorescence was observed both in the outer cell wall and inner cell wall of the cysts but, the fluorescence in the ectocyst was intense while in the endocyst it was faint.

Vodkin., *et al.* were the first to use PCR assay for the detection of various strains *Acanthamoeba* spp. in clinical samples, using primer pairs designed from an anonymous repetitive sequence of 18S and 5S ribosomal RNA genes of *A. castellanii* that amplifies a 272 bp region [20]. Lehmann., *et al.* in 1998 also tested with another set of primers to amplify a 253 bp region of 18S rDNA gene of *Acanthamoeba* spp. [21]. Later Schroeder., *et al.* in 2001 had shown that the above two primer pairs could also amplify 18S rDNA of related amoebas i.e., *Balamuthia* and *Hartmanella* along with *Acanthamoeba*. So, he designed a new set of genus specific primers called JDP1 and JDP2 which could amplify a 464 bp region of 18S rDNA of *Acanthamoeba* spp. specifically [9]. From that time, onwards these set of primers are used in laboratory diagnosis of *Acanthamoeba* spp. all over the world [6,22,23].

Conclusion

It was concluded from the above study that, for early and effective diagnosis of *Acanthamoeba* spp. in clinical specimens, staining and PCR assay could be used compared to culture. Staining takes very less time, while culture takes about a week for reporting. PCR assay takes nearly one or two days for identification of *Acanthamoeba* spp. in a clinical setting. We concluded from this study that, staining could be implemented for diagnosis of *Acanthamoeba* spp. in routine laboratory practices.

Bibliography

1. Shoff ME., *et al.* "Efficacy of contact lens systems against recent clinical and tap water *Acanthamoeba* isolates". *Cornea* 27.6 (2008): 713-719.
2. Martinez AJ and Visvesvara GS. "Laboratory diagnosis of pathogenic free-living amoebas: Naegleria, *Acanthamoeba*, and *Leptomyxid*". *Clinics in Laboratory Medicine* 11.4 (1991): 861-872.
3. Feingold JM., *et al.* "*Acanthamoeba* meningoencephalitis following autologous peripheral stem cell transplantation". *Bone Marrow Transplantation* 22.3 (1998): 297-300.

4. Steinberg JP, *et al.* "Review Disseminated acanthamebiasis in a renal transplant recipient with osteomyelitis and cutaneous lesions: case report and literature review". *Clinical Infectious Diseases* 35.5 (2002): e43-e49.
5. Satpathy G and Dada BK. "Acanthamoeba keratitis. A preliminary report". *Afro-Asian Journal of Ophthalmology* XII (1995): 102-105.
6. Pasricha G, *et al.* "Use of 18S rRNA gene-based PCR assay for diagnosis of Acanthamoeba keratitis in non-contact lens wearers in India". *Journal of Clinical Microbiology* 41.7 (2003): 3206-3211.
7. Sharma S, *et al.* "Patient characteristics, diagnosis and treatment of non-contact lens related Acanthamoeba keratitis". *British Journal of Ophthalmology* 84.10 (2000): 1103-1108.
8. Niyiyati M, *et al.* "Isolation of Balamuthia mandrillaris from urban dust, free of known infectious involvement". *Parasitology Research* 106.1 (2009): 279-281.
9. Schroeder JM, *et al.* "Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of Acanthamoebae from humans with keratitis and from sewage sludge". *Journal of Clinical Microbiology* 39.5 (2001): 1903-1911.
10. W Meersseman, *et al.* "Rapidly fatal Acanthamoeba encephalitis and treatment of cryoglobulinemia". *Emerging Infectious Diseases* 13.3 (2007): 469-471.
11. Garcia LS. "Diagnostic Medical Parasitology, ed. 4". *ASM Press, Washington, D.C* (2001).
12. NCCLS. "Laboratory Diagnosis of Blood-Borne Parasitic Diseases. Approved Guideline M15-A". National Committee for Clinical Laboratory Standards, Villanova, PA (2000).
13. Calcofluor White Stain Sigma-Aldrich USA
14. Khan NA. "Pathogenesis of Acanthamoeba infections". *Microbial Pathogenesis* 34.6 (2003): 277-285.
15. Frederick L Schuster. "Cultivation of Pathogenic and Opportunistic Free-Living Amebas". *Clinical Microbiology Reviews* 15.3 (2002): 342-354.
16. Khan NA, *et al.* "Molecular and physiological differentiation between pathogenic and non-pathogenic Acanthamoeba". *Current Microbiology* 45.3 (2002): 197-202.
17. Connell C, *et al.* "Encystation of Acanthamoeba castellanii: dye uptake for assessment by flow cytometry and confocal laser scanning microscopy". *Journal of Applied Microbiology* 90.5 (2001): 706-712.
18. Maeda H and Ishida N. "Specificity of binding of hexopyranosyl polysaccharides with fluorescent brighterer". *Journal of Biochemistry (Tokyo)* 62.2 (1967): 276-278.
19. Ramaswamy S, *et al.* "Identification and characterization of Myxococcus xanthus mutants deficient in calcofluor white binding". *Journal of Bacteriology* 179.9 (1997): 2863-2871.
20. Vodkin M H, *et al.* "Identification of Acanthamoeba at the generic and specific levels using the polymerase chain reaction". *Journal of Protozoology* 39.3 (1992): 378-385.
21. Lehmann J O, *et al.* "Polymerase chain reaction analysis of corneal epithelial and tear samples in the diagnosis of Acanthamoeba keratitis". *Investigative Ophthalmology and Visual Science* 39.7 (1998): 1261-1265.
22. Ge Zhao, *et al.* "Genotyping of Acanthamoeba isolates and clinical Characteristics of patients with Acanthamoeba keratitis in China". *Journal of Medical Microbiology* 59.4 (2010): 462-466.

23. Booton GC., *et al.* "Identification and distribution of Acanthamoeba species Genotypes associated with nonkeratitis infections". *Journal of Clinical Microbiology* 43.4 (2005): 1689-1693.

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