

Several staining techniques to enhance the visibility of *Acanthamoeba* cysts

Nagwa Mostafa El-Sayed · Wafaa Mohamed Hikal

Received: 23 July 2014 / Accepted: 14 October 2014 / Published online: 28 October 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract *Acanthamoeba* is one of the most common free-living amoebae. It is widespread in the environment and can infect humans causing keratitis. Delayed diagnosis or misdiagnosis leads to extensive corneal inflammation and profound visual loss. Therefore, accurate and rapid diagnosis of *Acanthamoeba* keratitis is essential for successful treatment and good prognosis. This study was designed to use different staining techniques to facilitate the identification of *Acanthamoeba* cysts. *Acanthamoeba* cysts were isolated by cultivation of either corneal scraping specimens or tap water samples onto non-nutrient agar plates seeded with *Escherichia coli*. Subcultures were done from positive cultures until unique cysts were isolated. *Acanthamoeba* cysts were stained temporarily using iodine, eosin, methylene blue, and calcofluor white (CFW) stains and as permanent slides after processing for mounting using modified trichrome, Gimenez and Giemsa staining. These stains were compared on the basis of staining quality including clarity of morphological details, differentiation between cytoplasm and nuclei, color and contrast, and also other characteristics of the staining techniques, including ease of handling, time taken for the procedure, and cost effectiveness. The cysts of *Acanthamoeba* were recognized in the form of double-walled cysts: the outer wall (ectocyst) that was being differentiated from the variably stained surrounding background and the inner wall

(endocyst) that was sometimes stellated, polygonal, round, or oval and visualized as separate from the spherical, sometimes irregular, outline of the ectocyst. Regarding the temporary stains, it was found that they were efficient for visualizing the morphological details of *Acanthamoeba* cysts. In CFW staining, *Acanthamoeba* cysts appeared as bluish-white or turquoise oval halos although the internal detail was not evident. On the other hand, the results of permanent-stained slides showed the most consistent stain for identification of *Acanthamoeba* cysts was modified trichrome followed by Gimenez stain and lastly Giemsa stain that gave poor visibility of *Acanthamoeba* cysts due to the intense staining background and monochrome staining of parasite. In the present study, multi-attribute ranking of the used staining techniques showed the highest rank for iodine stain (92 %) followed by eosin stain (84 %), Gimenez stain (76 %), methylene blue (72 %), CFW (64 %), modified trichrome (56 %), and the least was Giemsa stain (44 %). In conclusion, the staining techniques enhance the overall visibility of *Acanthamoeba* cysts.

Keywords *Acanthamoeba* cyst · Stain · Gimenez · Trichrome · Calcofluor · Giemsa · Methylene blue

Introduction

Acanthamoebae are free-living amoebae, occurring worldwide in soil and water. They have been isolated from ponds, lakes, brackish water, sea water, filters on heating and ventilating air-conditioning units, medical equipments such as gastric wash tubing, contact lenses, and contact lens solutions, as well as vegetables, cell cultures, and even human and animal tissues (Shoff et al. 2008). *Acanthamoebae* have two stages in their life cycle: a vegetative or trophozoite stage that reproduces by binary fission and feeds voraciously on bacteria and detritus present in the environment, and a non-dividing

N. M. El-Sayed (✉)

Medical Parasitology Department, Research Institute of Ophthalmology, 2 El-Ahram St, P.O. Box: 90, Giza 12556, Egypt
e-mail: nag.elsaka@yahoo.com

N. M. El-Sayed

e-mail: nagelsaka@hotmail.com

W. M. Hikal

Water Pollution Research Department,
National Research Center, Dokki, Giza, Egypt
e-mail: wafaahikal@hotmail.com

cyst stage with a double cyst wall, providing it with a high resistance to unfavoured and adverse environmental conditions, desiccation and disinfecting compounds, e.g., chlorine (Scheid and Schwarzenberger 2012).

Acanthamoeba causes some painful and life-threatening diseases (Marciano-Cabral and Cabral 2003) on immunocompromised individuals (granulomatous encephalitis) or immunocompetent individuals (*Acanthamoeba* keratitis) (El-Sayed et al. 2012; Ertabaklar et al. 2007; Scheid et al. 2008). *Acanthamoeba* keratitis is a severe, potentially sight-threatening ocular infection characterized by progressive corneal inflammation and ulceration. Delayed diagnosis or misdiagnosis leads to extensive corneal inflammation; the corneal epithelium becomes ulcerated with stromal infiltration, perforation, and finally loss of vision (Marciano-Cabral and Cabral 2003; Visvesvara et al. 2007). *Acanthamoeba* keratitis can result from corneal trauma or improper use of contact lenses. It is widely believed that manipulation of contact lenses may result in epithelial breaks that transmit infectious *Acanthamoeba* trophozoites to the eye (Ibrahim et al. 2009).

Definitive diagnosis of *Acanthamoeba* keratitis is based on morphological detection of the both trophozoite and cyst stages, either directly or by in vitro cultivation of infective samples. The unstained characteristics of the *Acanthamoeba* stages are difficult to detect and sometimes are not identified because these stages are transparent and are surrounded by bacteria or other organisms (e.g., fungi) growing together on the agar surface. Therefore, staining is needed to provide a detailed structure of the cellular organelles (Ithoi et al. 2011).

Several stains were currently used in the identification of *Acanthamoeba* including Gram's stain, Gimenez stain, Giemsa stain, chlorazol black E stain, hematoxylin-eosin, periodic acid-schiff, Masson's trichrome, iron hematoxylin-eosin, lactophenol cotton blue, Field's stain, Gomori methenamine silver, calcofluor white, and acridine orange (Shen et al. 2005; Thomas et al. 2011; Sharma et al. 2001; Pirehma et al. 1999). Some of these staining techniques are tedious and time consuming. For routine diagnostic work, most laboratories select one of the shorter staining procedures. So, the present study was designed to use different staining techniques to facilitate the identification of *Acanthamoeba* cysts and compare them on the basis of staining quality and other characteristics of these techniques.

Materials and methods

Acanthamoeba cysts isolation

After taking informed consents, corneal scraping specimens were collected from keratitis patients attending outpatient clinic at the Research Institute of Ophthalmology (RIO), Giza, Egypt. Also, tap water samples from the same patients'

localities were collected. For each sample, approximately 1000 ml water was filtered through a 0.45- μ m pore-size filter. Both corneal specimens and water filters were inoculated onto non-nutrient agar plates seeded with *Escherichia coli*. Plates were incubated at 37 °C and observed daily for the presence of amoebae as previously described (Init et al. 2010). Examination of the agar plate surface for the presence of amoebic growth was carried out daily for up to 7 days with light and inverted microscopes using a $\times 400$ objective. *Acanthamoeba* were identified at the genus level, based on morphological characteristics of trophozoites and cyst. Subcultures were done after 2 weeks from positive cultures confirmed with amoebic growth until unique cysts were isolated. Agar culture plate containing *Acanthamoeba* cysts was irrigated with 5 ml of Page's amoeba saline and pipetted several times to detach the amoebae from the agar surface. The suspension was centrifuged at 2500g for 10 min. The supernatant was aspirated, leaving 0.5 ml above the pellet. The leftover supernatant was gently pipetted to resuspend the mixture (Ithoi et al. 2011).

Staining techniques

1. Temporary staining

- (a) Eosin, iodine, and methylene blue stains were used in wet mount staining. Eosin stain was prepared by adding 1 g in 100 ml distilled water, and 0.5 ml acetic acid was added to sharpen the stain. Lugol's iodine solution was prepared by adding 20 g potassium iodide to the 100 ml distilled water; when dissolved, 10 g iodine crystals were added. Methylene blue stain was prepared by dissolving 1 g of methylene blue powder in 100 ml physiological saline. The wet mount staining was prepared by spreading out two drops (25 μ l) of *Acanthamoeba* suspension on a glass slide; a drop of the stain was then added, and a coverslip was finally applied to enclose the material. Staining slides were examined under a compound microscope (Zeiss, Axio Scope.A1, Jena, Germany) at a magnification of $\times 400$. Photographs were obtained using a Zeiss AxioCam MRc5 digital camera. The cysts of *Acanthamoeba* were recognized in the form of double-walled cysts: the outer wall (ectocyst) that was being differentiated from the variably stained surrounding background and the inner wall (endocyst) that was sometimes stellated, polygonal, round, or oval and visualized as separate from the spherical, sometimes irregular, an outline of the ectocyst. Cysts are uninucleate, and the nucleus has a centrally placed dense nucleolus (Schuster 2002).
- (b) Calcofluor white stain (CFW) (comprising of 1 g/l CFW M2R and 0.5 g/l Evans blue). Staining

procedure was done according to the manufacturer (Sigma-Aldrich, St Louis, MO, USA). One drop of *Acanthamoeba* cysts suspension was put onto a clean glass slide. Then, one drop of 10 % potassium hydroxide and one drop of CFW were added, before a coverslip was applied and the slide was allowed to stand for 1–2 min. Lastly, the slide was viewed under a UV microscope (MOTIC BA400 fluorescence compound trinocular microscope with a D350/50× exciter filter at a wavelength of between 395 and 415 nm). *Acanthamoeba* cysts appeared as bluish-white or turquoise oval halos.

2. Permanent staining

For preparation of permanent-stained smear, *Acanthamoeba* cysts suspension (50 µl) was dropped on a glass slide and spread to make a smear. It was dried in a moist chamber for 30 min at 37 °C and then fixed in absolute methanol for 3 min (Ithoi et al. 2011). The fixed smears were subsequently stained with the following staining techniques.

- (a) Modified trichrome blue staining method (Ryan-Blue) (Ryan et al. 1993). Modified trichrome staining using Ryan modification, by substitution of fast/light green by aniline blue and changing the incubation time and temperature, was adapted for this study. The staining solution was prepared by dissolving 6.0 g of chromotrope 2R (Sigma; C-3143) with 0.5 g of aniline blue (Fisher, Fairlawn, NJ; A-969) and 0.7 g of phosphotungstic acid in 3 ml of glacial acetic acid. This solution stood at room temperature for 30 min, after which 100 ml of distilled water was added and 1.0 M HCl was added to generate a pH 2.5 solution. Methanol-fixed smears were stained in this chromotrope 2R solution for 30 min at 37 °C and then rinsed for 10 s with acid alcohol (4.5 ml of acetic acid in 99.5 ml of 90 % ethyl alcohol). The smears were then dehydrated with a 10-s rinse in 95 % ethyl alcohol, two 5-min incubations in 95 % ethyl alcohol, a 10-min incubation in 100 % ethyl alcohol, and a 5-min incubation in xylene (or xylene substitute). *Acanthamoeba* cysts appeared bright pink with blue background.
- (b) Gimenez staining (Gimenez 1964). The fixed smears were flooded with freshly filtered carbol fuchsin solution prepared from 2 ml of stock solution of basic fuchsin in 5 ml of phosphate buffer for 10 s. The smear was then rinsed in tap water and incubated in malachite green (counterstain) for 9 s. The smear was then rinsed again in tap water and was air-dried. Basic fuchsin stain in aqueous solution with phenol and ethanol stained *Acanthamoeba* cysts red/magenta against a greenish-blue background.

- (c) Giemsa stain (Ithoi et al. 2011). Fix smears were stained with 20 % Giemsa solution (Merck, Darmstadt, Germany) in pH 6.8 of phosphate buffer for 20 min. *Acanthamoeba* cysts appeared blue and surrounded by a clear halo against a violet background.

All permanently stained slides were examined under a compound microscope (Zeiss Axio Scope.A1, Jena, Germany) at a magnification of ×400. Photographs were obtained using a Zeiss AxioCam MRc5 digital camera.

Assessment of different stains

Different staining techniques were assessed according to staining quality, i.e., clarity of morphological details, differentiation between cytoplasm and nuclei, color and contrast, and other characteristics of these techniques, including ease of handling, time taken for the procedure, and cost effectiveness. The cost calculations for each procedure included material and reagent costs and the cost of the technologist's time. Comparing between the staining techniques was conducted utilizing the multi-attribute evaluation method (MacPherson and McQueen 1993). This method identifies, characterizes, and then combines different attributes (variables) to assess the true ranking of these techniques. The attributes were given a rank order from one to five, with one being less desirable and five being most desirable.

Results

Results are shown in the Figs. 1, 2, 3, 4, 5, 6, 7, and 8 and Tables 1 and 2.

Discussion

Laboratory diagnosis of *Acanthamoeba* keratitis relies on the demonstration of trophozoite or cyst in corneal scrapings under microscopic observation directly or isolated from the culture (Whitcher et al. 2003). Wet mount preparation has the advantage of demonstrating the trophozoite motility. However, the internal structures are often poorly visible making definitive diagnosis of cysts or trophozoites difficult leading to misdiagnosis of *Acanthamoeba* in 60–70 % of *Acanthamoeba* keratitis cases (Sharma et al. 2004; Yera et al. 2007). So, several staining techniques were used in this study to make *Acanthamoeba* cysts easy to recognize and identify. It was found that stained preparations gave variable results and the staining quality of *Acanthamoeba* cysts was affected by the stain type, the concentration of the staining solution, the temperature, and duration of staining. Additionally, the differences in the ability of

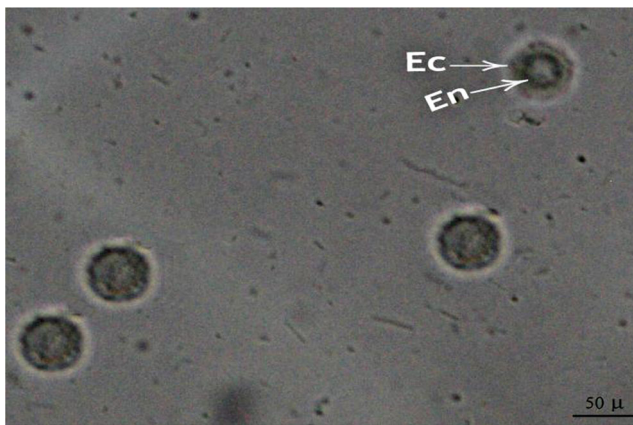


Fig. 1 Unstained *Acanthamoeba* cysts. The internal structures are poorly visible ($\times 400$). *Ec* ectocyst, *En* endocyst

stains to reveal *Acanthamoeba* cysts were likely to be due to the thickness of the cyst wall and the extent to which the stain is taken up by the *Acanthamoeba* depending on specimen processing and fixation.

In this study, the used staining techniques were classified according to their stability into temporary and permanent stains. Several staining solutions have been used for preparation of temporary stained wet mounts including Lugol's iodine, eosin, and methylene blue. These solutions were able to stain internal structures of *Acanthamoeba* cysts, thereby in the present study, the wet mount staining was considered as a simple, rapid method for visualization of *Acanthamoeba* cysts. Comparing these temporary stained wet mounts with the other permanent stains for the detection of *Acanthamoeba*, they are quick single-step procedures (the entire process taking about 1 min), use inexpensive stains easily available in any clinical parasitology laboratory, and do not require a fluorescent microscope or special filter for evaluation (Grossniklaus

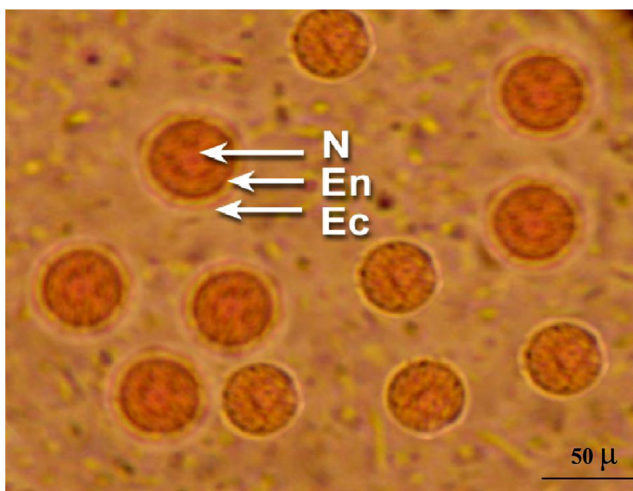


Fig. 2 Stained *Acanthamoeba* cysts with eosin wet mount stain. The cysts appeared reddish-pink with double walls ectocyst (*Ec*) and endocyst (*En*) and central nucleus (*N*) ($\times 400$)

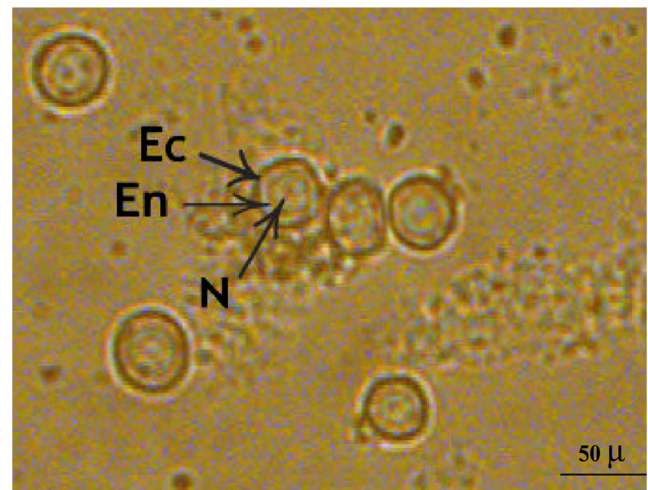


Fig. 3 Stained *Acanthamoeba* cysts with iodine wet mount stain. The cysts appeared yellowish-brown with well-defined ectocyst (*Ec*) and endocyst (*En*) and central nucleus (*N*) ($\times 400$)

et al. 2003). Disadvantage of wet mount staining is that the slide preparations are temporary; therefore, they are not available for future reference and the diagnosis relies on the experience of the technician only.

The other temporary staining method used in this study was calcofluor white which is a non-specific fluorochrome. This stain is able to bind cellulose in the cell wall of *Acanthamoeba* cysts. The results of the current study proved that CFW is a simple and fast method for detecting *Acanthamoeba* cysts as reported previously by Sharma et al. (2000, 2001). CFW stain was mixed with a potassium hydroxide mixture to clear up the specimen to facilitate visualization of *Acanthamoeba*. Also, Evans blue was used to act as a counter stain to diminish background fluorescence of tissues and cells when using blue light excitation (Harrington and Hageage 2003). Calcofluor

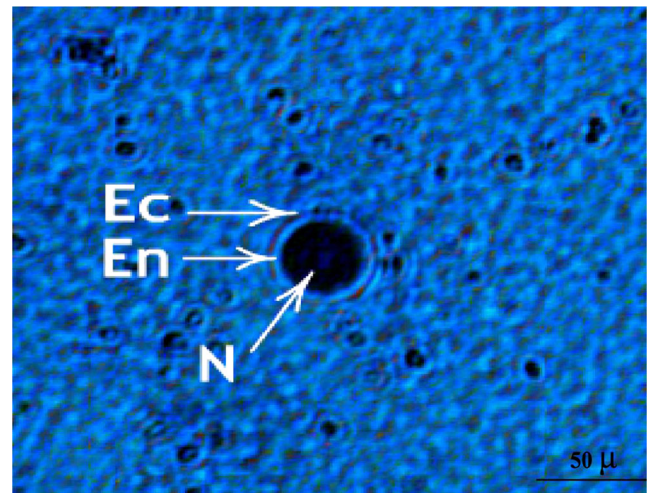


Fig. 4 Stained *Acanthamoeba* cysts with methylene blue stain. The cysts stained dark blue with faintly stained walls; ectocyst (*Ec*) and endocyst (*En*) and central nucleus (*N*) ($\times 400$)

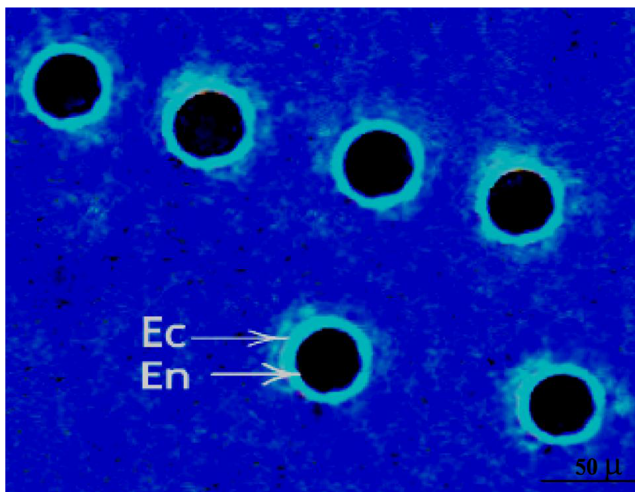


Fig. 5 Stained *Acanthamoeba* cysts with CFW. The cysts appeared as bluish-white or turquoise oval halos ($\times 400$). *Ec* ectocyst, *En* endocyst

staining procedure did not produce permanently stained slides for archiving however, if stored in the dark; the calcofluor-stained specimens may be viewed several months later with little loss of fluorescence (Didier et al. 1995). The disadvantages of this technique included its complexity, which required frequent quality control monitoring and technological expertise, and the fact that it is a non-specific stain. It is also important to note that in mixed fungi, *Acanthamoeba* infections, both pathogens will be stained, since both amoebic cysts and fungi cell walls are possible targets for CFW (Harrington and Hageage 2003). An added disadvantage is the need for a fluorescence microscope, an additional expense for the laboratories not equipped with one. The use of fluorescent dyes may lead to false-positive staining patterns of cell debris (Grossniklaus et al. 2003; Bharathi et al. 2006). Due to the cost involved with evaluating fluorescence staining of

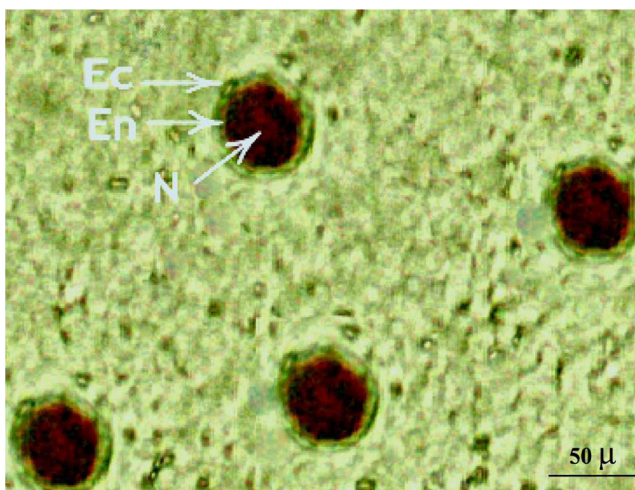


Fig. 6 Stained *Acanthamoeba* cysts with Gimenez stain. The cysts appeared reddish in color against a greenish-blue background ($\times 400$). *Ec* ectocyst, *En* endocyst, *N* central nucleus

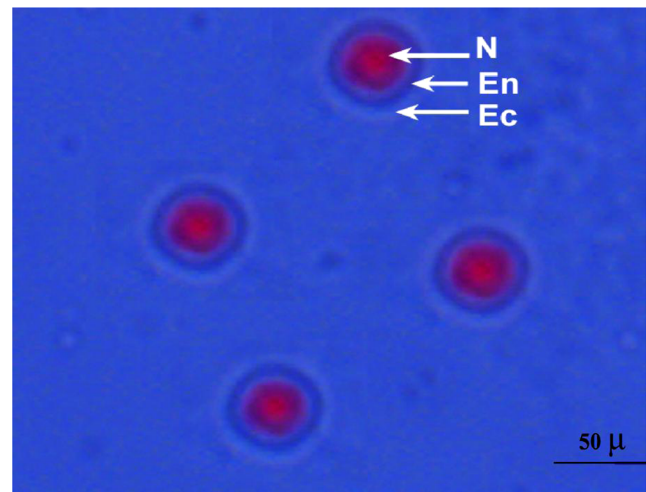


Fig. 7 Stained *Acanthamoeba* cysts with modified trichrome stain. The cysts appeared bright pinkish red in color on a blue background ($\times 400$). *Ec* ectocyst, *En* endocyst, *N* central nucleus

calcofluor white to diagnose *Acanthamoeba*, Waring et al. (2003) did not recommend its use.

Although an experienced microscopist can occasionally identify *Acanthamoeba* in a wet preparation both unstained and stained, most identification should be considered tentative until confirmed by the permanent stained slide. Permanent stained smears are reported to have many color ranges that either aided or hindered identification of organisms within the samples (Pietrzak-Johnston et al. 2000). The permanent stained smears not only provide the microscopic technician with a permanent record of protozoal organisms identified but also may be used for consultations with specialists when unusual morphologic characteristics are found (Garcia 2007) in which the morphology of *Acanthamoeba* cysts could be altered by culture age, pH, dryness, and lack of food (Ithoi et al. 2011; Walochnik et al. 2000).

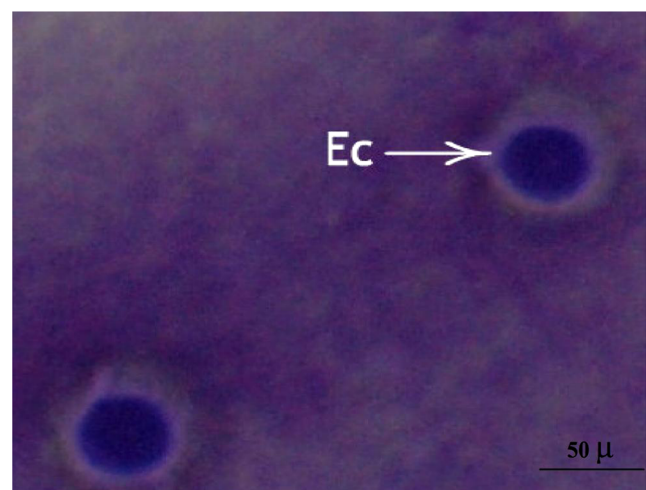


Fig. 8 Stained *Acanthamoeba* cysts with Giemsa stain. The cysts appeared blue and are surrounded by a clear halo against a violet background ($\times 400$). *Ec* ectocyst

Table 1 Assessment of the used stained techniques for identification of *Acanthamoeba* cysts regarding the staining quality

Stains	Morphological details		Color of cysts	Contrast
	Cyst walls	Differentiation between cytoplasm and nuclei		
Eosin	Well defined	Well differentiation	Reddish-pink	No contrast
Iodine stain	Well defined	Well differentiation	Yellowish-brown	Poor contrast
Methylene blue	Faintly stained	Not clear	Dark blue	No contrast
Calcofluor white	Well defined	Poor differentiation	Dark blue while the wall appears brightly blue fluorescent	Best contrast
Gimenez stain	Well defined	Well differentiation	Reddish color against a greenish-blue background	Good contrast
Modified trichrome	Well-defined	Well differentiation	Bright pinkish red color on a blue background	Very good contrast
Giemsa stain	Not defined	No differentiation	Blue and often are surrounded by a clear halo against a violet background	Poor contrast

Permanent staining by Gimenez stain was found to be effective for visualizing the morphological details of the parasite. *Acanthamoeba* cysts appeared as magenta/red-colored pleomorphic cysts against the greenish-blue background. The staining method was originally developed by Gimenez in 1964 (Gimenez 1964) to identify the bacteria. The primary stain for this technique is carbol fuchsin, and the secondary stain is malachite green. It is known that the basic dye carbol fuchsin is retained by the acid-fast organism which has a complex envelope composed of glycolipids and glycopeptidolipids (Brennan et al. 1990). The Gimenez staining technique had fewer steps than the other permanent staining methods and could easily be integrated into the workflow of a parasitology laboratory. It was moderately inexpensive, specimens could be used in batches, and the stained slide could be kept as a permanent record that can be archived. The result of this study was in agreement with Shen et al. (2005) who concluded that Gimenez staining is a rapid method for initial identification of *Acanthamoeba* cysts.

Alternatively, the slides stained with Giemsa's stain gave poor visibility of *Acanthamoeba* cysts due to the intense staining background and monochrome staining of parasite. In spite of this stain does not require time consuming or

special equipments for its evaluation (Grossniklaus et al. 2003); the poor color contrast leads to underestimation of *Acanthamoeba* cysts in positive cases.

In this study, modified trichrome stain was superior to other used stains for the identification of *Acanthamoeba* cysts; however, it is costly and time consuming (60 min) and it involved many steps. *Acanthamoeba* cysts stained with modified trichrome appeared bright pink with well-differentiated internal structures. In this method, aniline blue was used as a counterstain (Ryan et al. 1993) that provided good contrast to the pink-staining *Acanthamoeba*.

The process of accepting one staining procedure over another involves a complex decision analysis of several criteria related to those stains. In the present study, when comparing time efficiencies for performing the used staining methods, wet mount staining required the least time of approximately 1 min, followed by the calcofluor stain (5 min), Gimenez stain (10 min), Giemsa (20 min), and lastly, the modified trichrome blue stain, which required approximately 60 min. In addition, wet mount staining specimens were the easiest to read, requiring approximately 2 to 3 min per slide to view 200 fields while permanent staining specimens required approximately 5 to 10 min to carefully view 200 fields for detecting

Table 2 Multi-attribute evaluation of the used stained techniques for identification of *Acanthamoeba* cysts

Stains	Ranking for the following attributes					Rank % (total rank=25)
	Staining quality	Color and contrast	Ease of handling	Cost	Time consumed	
Eosin	3	3	5	5	5	21/25, 84 %
Iodine stain	4	4	5	5	5	23/25, 92 %
Methylene blue	3	3	4	4	4	18/25, 72 %
CFW	4	3	4	1	4	16/25, 64 %
Gimenez stain	5	5	3	3	3	19/25, 76 %
Modified trichrome	5	5	1	2	1	14/25, 56 %
Giemsa stain	1	1	3	3	3	11/25, 44 %

Acanthamoeba. Considering the total procedure cost, CFW was very expensive; that is why this stain was not suited for the identification of *Acanthamoeba* cysts in most laboratories (Waring et al. 2003). In addition, ease of handling and the ability to process large numbers of specimens were subjectively evaluated on the basis of normal procedures in a diagnostic laboratory performing parasitological examinations only and the ability to test batches of up to 25 specimens in the routine staining procedure. Adopting the method of multi-attribute evaluation (MacPherson and McQueen 1993), multi-attribute ranking of the used stained techniques showed the highest rank for iodine stain (92 %) followed by eosin stain (84 %), Gimenez stain (76 %), methylene blue (72 %), CFW (64 %), and modified trichrome (56 %) and the least was Giemsa stain (44 %).

In conclusion, the staining techniques enhance the overall visibility of *Acanthamoeba* cysts and laboratorians should be cautioned not to rely solely on the direct wet mount for detection or identification of this protozoan.

References

- Bharathi MJ, Ramakrishnan R, Meenakshi R, Mittal S, Shivakumar C, Srinivasan M (2006) Microbiological diagnosis of infective keratitis: comparative evaluation of direct microscopy and culture results. *Br J Ophthalmol* 90(10):1271–1276. doi:10.1136/bjo.2006.096230#_blank
- Brennan PJ, Hunter SW, McNeil M, Chatterjee D, Daffe M (1990) Reappraisal of the chemistry of mycobacterial cell walls, with a view to understanding the roles of individual entities in disease processes. In: Ayoub EM, Cassell GH, Branche WC Jr, Henry TJ (eds) *Microbial determinants of virulence and host response*. American Society for Microbiology, Washington, D.C, pp 55–75
- Didier ES, Orenstein JM, Aldras A, Bertucci D, Rogers LB, Janney FA (1995) Comparison of three staining methods for detecting Microsporidia in fluids. *J Clin Microbiol* 33(12):3138–3145
- El-Sayed NM, Ismail KA, Ahmed SAG, Hetta MH (2012) In vitro amoebicidal activity of ethanol extracts of *Arachis hypogaea* L., *Curcuma longa* L. and *Pancreatium maritimum* L. on *Acanthamoeba castellanii* cysts. *Parasitol Res* 110(5):1985–1992
- Ertabaklar H, Türk M, Dayanir V, Ertuğ S, Walochnik J (2007) *Acanthamoeba* keratitis due to *Acanthamoeba* genotype T4 in a non-contact-lens wearer in Turkey. *Parasitol Res* 100(2):241–246
- Garcia LS (2007) *Diagnostic medical parasitology* IASM press, Washington DC; part II, pp. 771–812
- Gimenez DF (1964) Staining Rickettsiae in yolk-sack cultures. *Stain Technol* 39:135–140
- Grossniklaus HE, Waring GO IV, Akor C, Castellano-Sanchez AA, Bennett K (2003) Evaluation of hematoxylin and eosin and special stains for the detection of *Acanthamoeba* keratitis in penetrating keratoplasties. *Am J Ophthalmol* 136(3):520–526
- Harrington H (2003) Calcofluor white: a review of its uses and applications in clinical mycology and parasitology. *Lab Med* 34(5):361–376
- Ibrahim YW, Boase DL, Cree IA (2009) How could contact lens wearers be at risk of *Acanthamoeba* infection? A review. *J Optom* 2(2):60–66
- Init I, Lau YL, Arin-Fadzlan A, Foad AI, Neilson RS, Nissapatorn V (2010) Detection of free living amoebae, *Acanthamoeba* and *Naegleria*, in swimming pools, Malaysia. *Trop Biomed* 27(3):566–577
- Ithoi I, Ahmad AF, Mak JW, Nissapatorn V, Lau YL, Mahmud R (2011) Morphological characteristics of developmental stages of *Acanthamoeba* and *Naegleria* species before and after staining by various techniques. *Southeast Asian J Trop Med Public Health* 42(6):1327–1338
- MacPherson DW, McQueen R (1993) Cryptosporidiosis: multi-attribute evaluation of six diagnostic methods. *J Clin Microbiol* 31(2):198–202
- Marciano-Cabral F, Cabral G (2003) *Acanthamoeba* spp as agents of disease in humans. *Clin Microbiol Rev* 16(2):273–307
- Pietrzak-Johnston SM, Bishop H, Wahlquist S, Moura H, Da Silva ND, Da Silva SP, Nguyen-Dinh P (2000) Evaluation of commercially available preservatives for laboratory detection of helminths and protozoa in human faecal specimens. *J Clin Microbiol* 38(5):1959–1964
- Pirehma M, Suresh K, Sivanandam S, Anuar KA, Ramakrishnan K, Kumar GS (1999) Field's stain—a rapid staining method for *Acanthamoeba* spp. *Parasitol Res* 85:791–793
- Ryan NJ, Sutherland G, Coughlan K, Globan M, Doultree J, Marshall J, Baird RW, Pedersen J, Dwyer B (1993) A new trichrome-blue stain for detection of microsporidial species in urine, stool, and nasopharyngeal specimens. *J Clin Microbiol* 31:3264–3269
- Scheid P, Schwarzenberger R (2012) *Acanthamoeba* spp. as vehicle and reservoir of adenoviruses. *Parasitol Res* 111(1):479–485
- Scheid P, Zöller L, Pressmar S, Richard G, Michel R (2008) An extraordinary endocytobiont in *Acanthamoeba* sp. isolated from a patient with keratitis. *Parasitol Res* 102(5):945–950
- Schuster FL (2002) Cultivation of pathogenic and opportunistic free-living amebas. *Clin Microbiol Rev* 15(3):342–354
- Sharma S, Grag P, Roa GN (2000) Patient characteristics, diagnosis and treatment of non-contact lens related *Acanthamoeba* keratitis. *Br J Ophthalmol* 84(10):1103–1108. doi:10.1136/bjo.2006.096230#_blank
- Sharma S, Athmanathan S, Ata-Ur-Rasheed M, Garg P, Rao GN (2001) Evaluation of immunoperoxidase staining technique in the diagnosis of *Acanthamoeba* keratitis. *Indian J Ophthalmol* 49(3):181–186
- Sharma S, Pasricha G, Das D, Aggarwal RK (2004) *Acanthamoeba* keratitis in non-contact lens wearers in India: DNA typing-based validation and a simple detection assay. *Arch Ophthalmol* 122(10):1430–1434
- Shen J, Jiang QW, Li QX, Chen HY, Li ZH (2005) Gimenez staining: a rapid method for initial identification of legionella pneumophila in Amoeba trophozoite. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* 23(4):240–242
- Shoff ME, Rogerson A, Kessler K, Schatz S, Seal DV (2008) Prevalence of *Acanthamoeba* and other naked amoebae in South Florida domestic water. *J Water Health* 6(1):99–104
- Thomas PA, Jesudasan CAN, Kaliamurthy J (2011) Rapid detection of *Acanthamoeba* cysts in corneal scrapings by chlorazol black E staining. *Can J Ophthalmol* 46(5):443–444
- Visvesvara GS, Moura H, Schuster FL (2007) Pathogenic and opportunistic free-living Amoeba: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. *FEMS Immunol Med Microbiol* 50(1):1–26
- Walochnik J, Obwaller A, Aspöck H (2000) Correlations between morphological, molecular biological and physiological characteristics in clinical and nonclinical isolates of *Acanthamoeba* spp. *Appl Environ Microbiol* 66(10):4408–4413
- Waring GO IV, Akor C, Castellano-Sanchez A, Grossniklaus H E (2003) Hematoxylin and eosin is superior to calcofluor white and acridine

- orange in detecting *Acanthamoeba* Keratitis. *Investig Ophthalmol Vis Sci* 44
- Whitcher JP, Srinivasan M, Upadhyay MP (2003) Microbial keratitis. In: Johnson GJ, Minassian DC, Weale RA, West SK (eds) *The epidemiology of eye diseases*, 2nd edn. Arnold, London, pp 190–195
- Yera H, Zamfir O, Bourcier T, Ancelle T, Batellier L, Dupouy-Camet J, Chaumeil C (2007) Comparison of PCR, microscopic examination and culture for the early diagnosis and characterization of *Acanthamoeba* isolates from ocular infections. *Eur J Clin Microbiol Infect Dis* 26:221–224