MORPHOLOGICAL CHARACTERISTICS OF DEVELOPMENTAL STAGES OF *ACANTHAMOEBA* AND *NAEGLERIA* SPECIES BEFORE AND AFTER STAINING BY VARIOUS TECHNIQUES

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Abstract. Seven stains were studied to determine the best color and contrast for staining the developmental stages of free living pathogenic *Acanthamoeba* and *Naegleria* species. The acid-fast bacilli stain (AFB) produced a blue color without contrast; trichrome-eosin and modified Field's showed various color contrasts; Giemsa, iron-hematoxylin, modified AFB and Gram produced only one color which distinguished the nucleus, nucleolus, cytoplasm, food- and water-vacuoles. The motile organs (acanthopodia, pseudopodia, lobopodia and flagella) were also clearly differentiated but produced a similar color as the cytoplasm. These motile organelles were first induced by incubating at 37°C for at least 15 minutes and then fixing with methanol in order to preserve the protruding morphology prior to staining. The trichrome-eosin and iron-hematoxylin stains showed good color contrast for detecting all three stages, the trophozoite, cyst and flagellate; Giemsa and Gram stained the trophozoite and flagellate stages; the modified Field's and modified AFB stains stained only the trophozoite stage. Depending on the purpose, all these stains (except the AFB stain) can be used to identify the developmental stages of *Acanthamoeba* and *Naegleria* for clinical, epidemiological or public health use.

Keywords: *Acanthamoeba*, *Naegleria*, stains, developmental stages

INTRODUCTION

Free living amebae (FLA) of the genera *Acanthamoeba* and *Naegleria* are ubiquitous in nature and can be found in nearly all environments worldwide. *Acanthamoeba* cause Acanthamoeba keratitis (AK) which is reported in 1-2 cases per million contact lens wearers annually (CDC, 2010), and is capable of causing skin lesions and granulomatous amebic encephalitis (GAE) in individuals with compromised or competent immune systems, who inhale infective cysts or develop indolent, granulomatous skin lesions in soil-contaminated wounds (CDC, 2010). Unlike *Acanthamoeba*, only one species of *Naegleria*, *Naegleria fowleri*, is known to infect humans by causing an

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acute, fulminant, usually lethal, central nervous system (CNS) infection, known as primary amebic meningoencephalitis (PAM). Definitive diagnosis of PAM, GAE and *Acanthamoeba* keratitis is based on morphological detection of the trophozoite stage, either directly or by *in vitro* cultivation of infective samples. The cyst stage of *Acanthamoeba* can also be found in corneal scrapings (McClellan *et al*, 1988; Ishibashi *et al*, 1990). It is difficult to detect the amebae in unstained specimens because of their transparent cytoplasm and the acanthopodia or lobopodia do not protrude in passive conditions. Therefore, staining is needed to detect these motile organs, as well as their developmental stages in specimens from patients.

MATERIALS AND METHODS

Preparation of amebae

Several isolates of *Acanthamoeba* and *Naegleria* isolated from swimming pools during a previous study (Init *et al*, 2010) were used in this study. An agar plate culture containing ameba was irrigated with 5 ml of cold PAS solution [0.12g NaCl, 0.004g $MgSO_4.7H_2O$, 0.004g $CaCl_2.2H_2O$, 0.142g Na_2HPO_4 and 0.136g KH_2PO_4; from Sigma (St Louis, MO) diluted in 1,000 ml distilled water] and pipetted several times to detach the amebae from the agar surface. The suspension was then transferred to a tube and centrifuged at 2,500 rpm for 10 minutes. The supernatant was removed, leaving 0.5 ml above the pellet. The left over supernatant was gently pipetted to resuspend the mixture. The wet-smear was made by dropping 25 l of the suspension on the well of teflon-coated microscope slides and left for 5 minutes in a 30°C incubator. A proper size cover slip was applied and the slide was immediately viewed, photographed using an Olympus BX51 microscope which was attached to a photo adapter and a computer with imaging software. Similarly to making a fixed smear, the resuspended solution (50 l) was dropped on a glass slide and spread to make a smear. It was dried in a moist chamber for 30 minutes at 37°C and then fixed with methanol (Caledon) for 3 minutes. The fixed smears were subsequently used for the staining techniques.

Staining procedures

The Giemsa, modified Field's, trichrome, iron hematoxylin stains were carried out according to the standard procedures of the Parasitology Department, Faculty of Medicine, University of Malaya, Malaysia; whereas the original acid-fast bacilli (AFB) kit (Merck, Darmstadt, Germany), modified AFB kit and Gram kit (Merck, Darmstadt, Germany) stains were carried out according to manufacturer instructions. The procedures are described below.

Giemsa stain

The fixed smear was immersed in Giemsa (Merck, Darmstadt, Germany) solution (1: 45 ml of dH_2O) in a staining container for 60 minutes. The slide was then rinsed with dH_2O, dried at 37°C for 60 minutes (or overnight at room temperature), mounted with neutral mounting medium (NMM, Gurr) and viewed under a microscope and photographed.

Modified Field's stain

The slide was dipped in 1% methanolic eosin for 2 minutes, and then covered with 3 drops of 3% acid-alcohol for 1-3 seconds (just enough to discolor the eosin). It was then quickly rinsed under running tap water for 10 seconds. The smear was then stained with 3 drops of 1% Field "A" solution for 1-3 seconds and washed 3 times with dH_2O to discard the

excess stain. The slide was air-dried then photomicrographed.

Trichrome-eosin stain

The slide was dipped in 1% methanolic eosin for 2 minutes and trichrome stain for 10 minutes, followed by 90% acid-alcohol [0.5 ml of acetic acid (Mallinckrodt Phillipsburg, NJ) mixed with 99.5 ml of 90% ethanol] for 1-3 seconds. The slide was then dipped in 100% EtOH twice at an interval of 3 minutes and in xylene twice at an interval of 5-10 minutes. The Trichrome solution was prepared by adding 1 ml acetic acid to a mixture of 3 chemicals from Sigma (0.6 g chromotrope 2R, 0.3 g light green SF yellowish and 0.7 g phosphotungstic acid) and incubated for 15-30 minutes at room temperature. It was then added to 100 ml dH_2O (giving a purple color) and used immediately or stored in a dark glass bottle for subsequent use.

Iron hematoxylin stain

The slide was placed in 70 % ethanol [70 ml of 100% EtOH (Surgipath) mixed with 30 ml of distilled water] for 5 minutes. It was then washed under running tap water for 10 minutes and placed in iron hematoxylin stain [10 g of hematoxylin (Sigma, ST Louis, MO) mixed with 1,000 ml of 100% EtOH and allowed to stand in a lighted room for at least 1 week before use] for 4-5 minutes. The slide was then washed as above for another 10 minutes and further dehydrated in 70% and 95% ethanol (95 ml of 100% EtOH mixed with 5 ml of distilled water) for 5 minutes. The slide was then dipped twice in 100% EtOH and xylene (Fluka) at an intervals of 5 minutes. The slide was then photomicrographed.

Original and modified acid-fast bacilli (AFB) stain

Acid-fast staining was carried out by staining the smear with Solution 1 (carbol fuchsin) for 5 minutes and then rinsing with tap water to discard the excess stain. The smear was then covered with Solution 2 (hydrochloric acid in ethanol) for 30 seconds, then rinsed with tap water. The smear was then stained with Solution 3 (malachite green) for 1 minute, rinsed with water and dried for 1 hour at 37°C or overnight at room temperature. The slides were then photomicrographed. The modified AFB stain was carried out as in the original procedure above except instead of Solution 2 (hydrochloric acid in ethanol) it was stained with Gram's decolorizing solution (Merck, Darmstadt, Germany).

Gram stain

The smear was stained with Solution 1 (crystal violet) for 1 minute, washed with tap water, then Solution 2 was applied (Lugol's iodine) for 1 minute and rinsed again with tap water. The slide was then subjected to Solution 3 (Gram's decolorizing solution) for 30 seconds, rinsed and finally stained with Solution 4 (Safranin) for one minute, then rinsed again. The slide was air-dried then photomicrographed.

RESULTS

The morphological characteristics are shown in Fig 1. The trophozoites of *Naegleria* (1n-2n) and *Acanthamoeba* (8a-11a) species had a variety of shapes when they placed in cold buffered saline (3n and 7a). The flagellate stage of *Naegleria* had active movement in liquid buffer with the aid of flagellum (4n-5n) that emerged from the anterior part of the pear-shaped organism. The rounded cyst stage of *Naegleria* (6n) had a double smooth wall, and the *Acanthamoeba* cyst (12a-15a) had a variety of shapes with a thicker double wall.

The morphological characteristics of trophozoite, flagellate and cyst stages

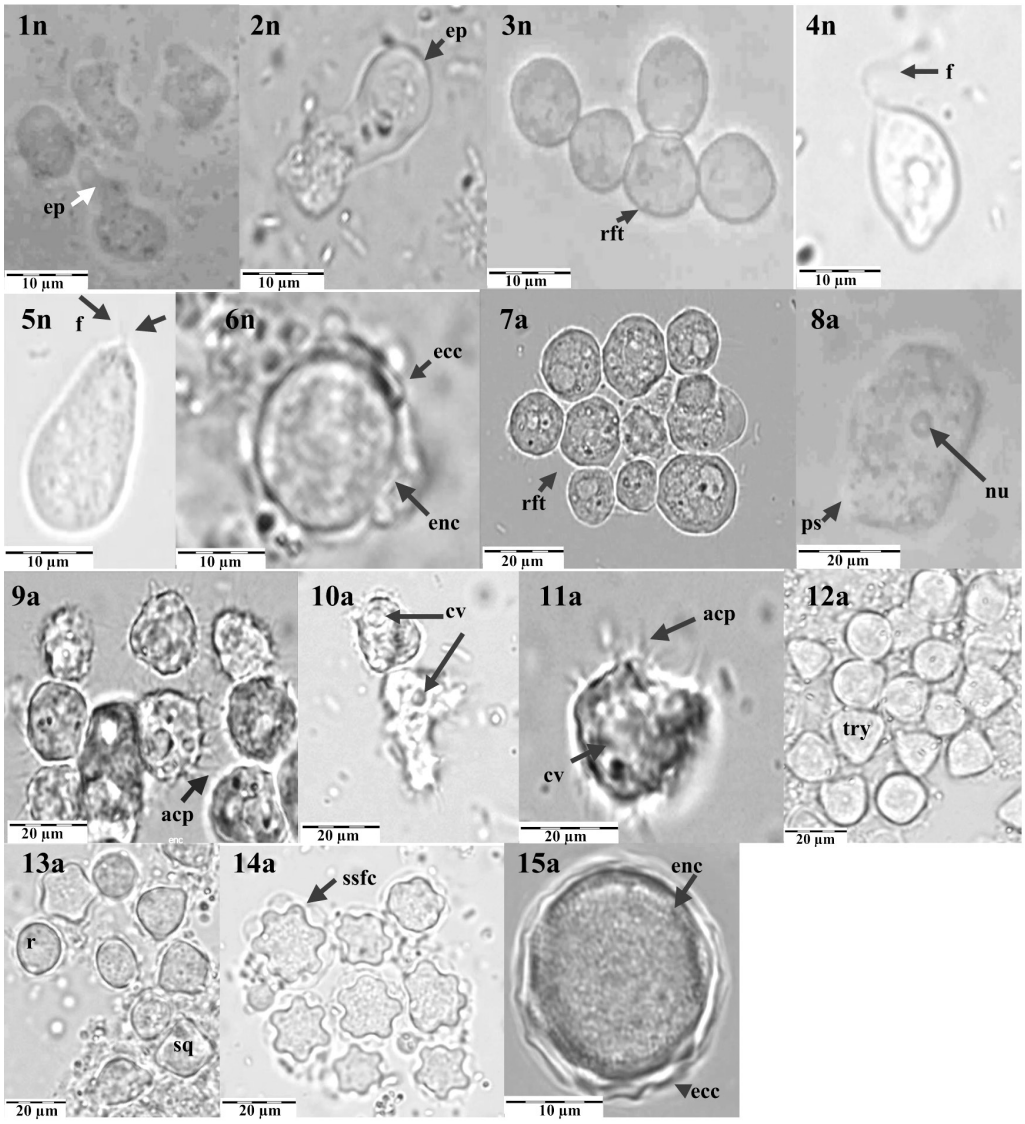
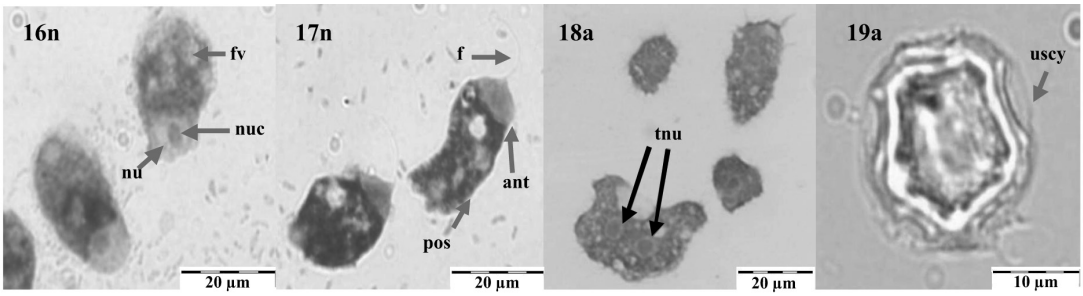


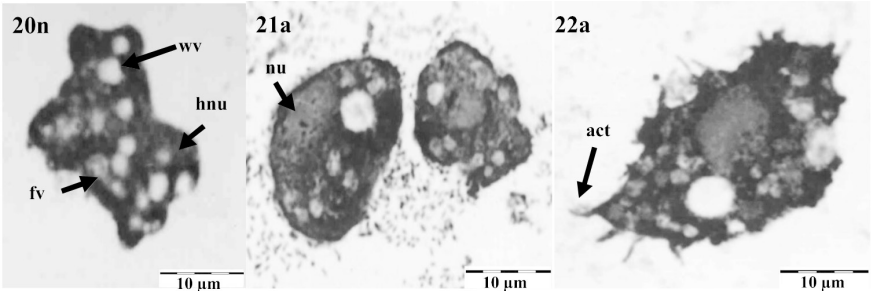
Fig 1–Unstained morphology of *Naegleria* (n) and *Acanthamoeba* (a) stages under a light microscope. 1n (InM) and 2n, x400 (*Naegleria* trophozoites showing typical eruptive pseudopodia/ lobopodia); 3n, x400 (rounded form of trophozoites in cold buffer after being detached from agar surface); 4n, x400 and 5n, x1,000 (flagellate stage showing one/two visible flagella); 6n, x400 (a single cyst showing a smooth ectocyst and endocyst); 7a, x400 (*Acanthamoeba* trophozoites showing a rounded form in cold buffer after being detached from the agar surface); 8a, x1,000 (a single trophozoite showing pseudopodia and nucleus); 9a, 10a, x400 and 11a, x1,000 (various shapes of trophozoites after being detached from the agar surface showing acanthopodia and prominent contractile/water vacuole); 12a, 13a, 14a x400 [cysts with multiple shapes, round, triangular and square (5-7) protrusions]; 15a, x1,000 (a rounded cyst exhibiting a wrinkled ectocyst and endocyst).

InM, inverted microscope; ep, eruptive pseudopodia; rft, rounded form trophozoite; f, flagella; ecc, ectocyst; enc, endocyst; ps, pseudopodia; nu, nucleus; acp, acanthopodia; cv, contractile vacuole; r, round; try, triangle; sq, square; ssfc, star shape form cyst

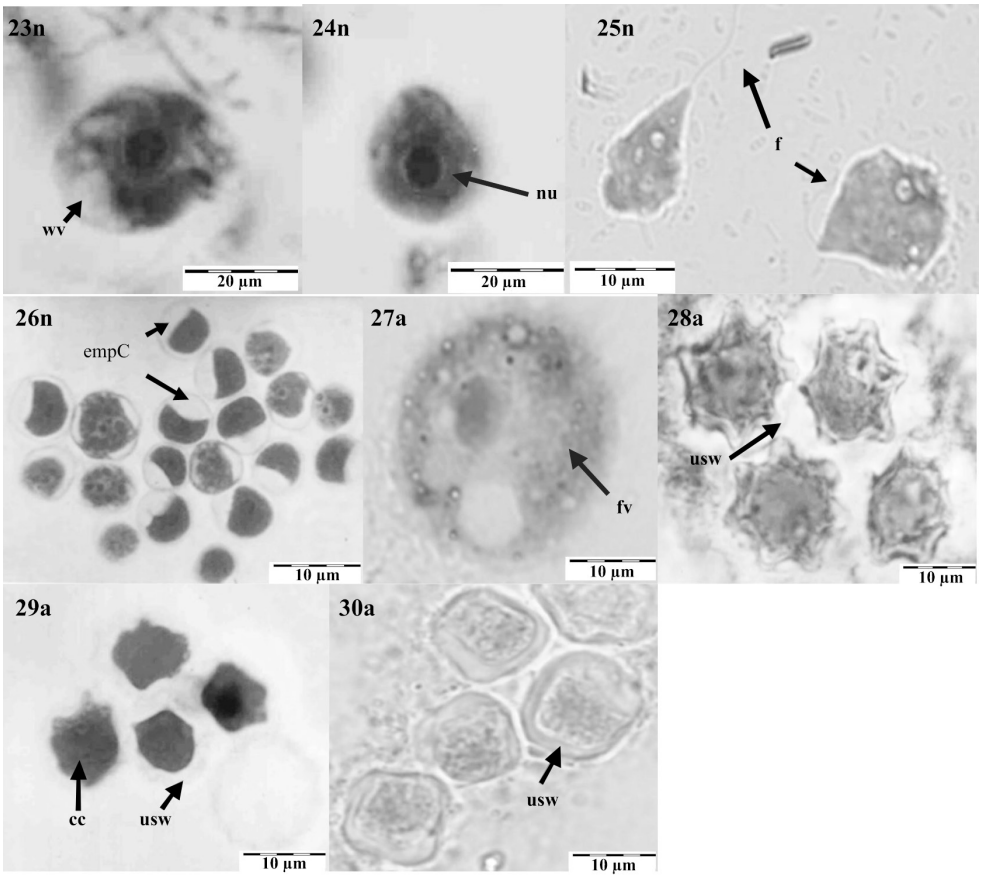
1.Giemsa stain



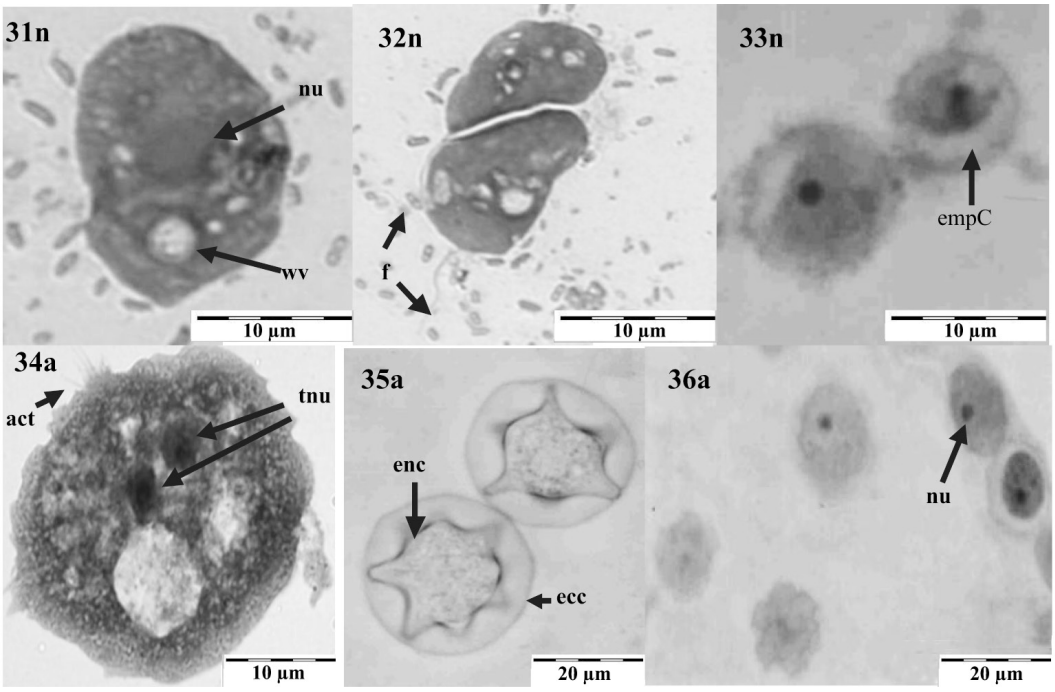
2. Modified Field's stain



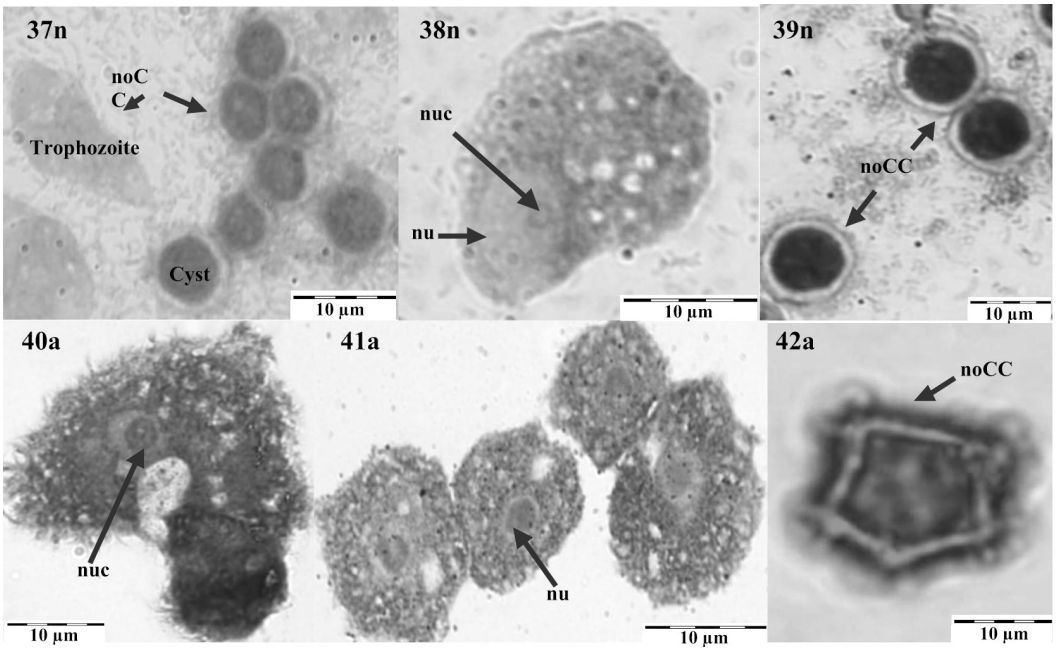
3.Trichrome-eosin stain



4. Iron hematoxylin stain



5. Original and 6. Modified acid-fast bacilli (AFB) stain



7. Gram's stain

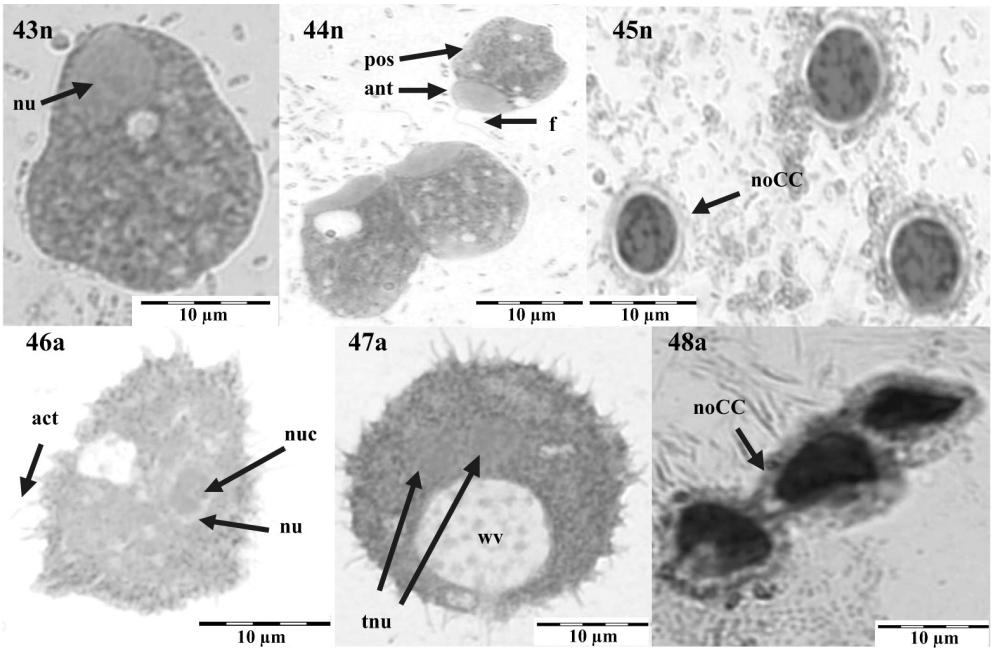


Fig 2–Stained morphological stages of *Naegleria* (n) and *Acanthamoeba* (a) under a light microscope. 16n, 20n, 23n, 24n, 31n, 37n, 38n and 43n, x1,000 (trophozoite stage of *Naegleria*); 17n, 25n, 32n and 44n, x1,000 (flagellate stage of *Naegleria*); 26n, 33n, 37n, 39n and 45n, x1,000 (cyst stage of *Naegleria*); 18a, 21a, 22a, 27a, 34a, 40a, 41a, 46a and 47a, x1,000 (trophozoite stage of *Acanthamoeba*); 19a, 28a, 29a, 30a, 35a, 42a and 48a, x1,000; 36a, x400 (cyst stage of *Acanthamoeba*).

fv, food vacuole; nuc, nucleolus; nu, nucleus; f, flagella; ant, anterior part; pos, posterior part; tnu, two nuclei appeared before cell division; uscy, unstained cyst; wv, water/contractile vacuole; hnu, nucleus with halo; act, acanthopodia; empC, empty compartment; usw, unstained wall; cc, compact cytoplasm; enc, endocyst; ecc, ectocyst; noCC, no color contrast

stained with Giemsa (16n-17n, 18a-19a), modified Field's (20n, 21a-22a), trichrome-eosin (23n-26n, 27a-30a), iron-hematoxylin (31n-33n, 34a-36a), acid-fast bacilli (37n-39n, 40a-42a) and Gram's (43n-45n, 46a-48a) stains are seen in Fig 2. The nuclear and cytoplasmic staining of these two amebae, as well as the anterior and posterior parts of the flagellate stage of *Naegleria* are described in Table 1.

The morphological identification of *Acanthamoeba* and *Naegleria* trophozoites in agar culture was carried out using an inverted microscope (Olympus CKX41) or a light microscope (Olympus BX51). The amebae can be observed directly under an inverted microscope if the thickness of the agar does not exceed 3.0 mm. Amebae on the agar surface need to be transferred to a teflon coated slide prior to observation under a light microscope.

DISCUSSION

Acanthamoeba trophozoites displayed two specific motile organs, hyaline pseu-

Table 1
Colorization effect of stains against various stages of *Acanthamoeba* and *Naegleria* isolates.

Species/ Stage/ Organ/ Staining method	*Acanthamoeba*				*Naegleria*					
	Trophozoite		Cyst		Trophozoite		Cyst		Flagellate	
	Nu	Cy	Nu	Cy	Nu	Cy	Nu	Cy	An	Pos
Giemsa	blue	blue	NS	NS	L-blue	blue	NS	NS	L-blue	D-blue
Field's	purple-red	D-blue	NS (PNSF)	NS (PNSF)	purple-red	D-blue	NS (NSF)	NS (NSF)	ND	ND
Trichrome-eosin	D-red	red	D-red	red	D-red	red	D-red	red	red-bluish	red-bluish
Iron hematoxylin	D-grey	D-grey	D-grey	L-grey	D-grey	D-grey	D-grey	L-grey	D-grey	D-grey
Acid-fast bacilli (AFB)	Bl-nsp	Bl-nsp	Bl-nsp	Bl-nsp	Bl-nsp	Bl-nsp	Bl-nsp	Bl-nsp	Bl-nsp	Bl-nsp
Modified AFB	purple	purple	nsp	Red-bluish-nsp	purple	purple-bluish	Bl-nsp	Bl-nsp	purple-bluish-nsp (PNSF)	Purple-bluish-nsp (PNSF)
Gram	red	red	Bl-nsp	Bl-nsp	L-red	red	red-nsp	red-nsp	L-red	D-red

Nu, nucleus; Cy, cytoplasm; An, anterior; Pos, posterior; NS, not stained; ND, not done; L-, light in respective color; D-, dark in respective color; -nsp, no recognition of specific organ due to no color contrast where the whole cell was stained red, grey (Gr) or blue (Bl); PNSF, photograph not shown in Fig 2.

dopodia and acanthopodia. Hyaline pseudopodia are seen with sluggish movement on a broad front without direction, while acanthopodia are seen as numerous spine-like structures or filamentous pseudopodia that surround the cell with inactive movement by extending and retracting from the trophozoite's surface. Acanthopodia are used to differentiate *Acanthamoeba* and *Naegleria* trophozoites (Schuster and Visvesvara, 2004). Acanthopodia have the ability to bind to corneal epithelial cells during *Acanthamoeba* infection (Pettit *et al*, 1996). They grip the surface of agar during culture. Cold (4ºC) distilled water or normal saline with extensive flushing is used to detach them. *Naegleria* trophozoites only have one motile protruding organ, the lobopodium or bluntly eruptive pseudopodium. It had active movement in a unidirectional manner (Marciano-Cabral, 1988). The lobopodium does not grip like the acanthopodia, thus *Naegleria* trophozoites are easily removed from the agar surface by adding cold buffer or by gentle pipetting. *Naegleria* trophozoites can easily transform into the flagellate stage when the agar surface is wet. This could happen when there is an accumulation of moist-vapor during incubation of culture plates, or after adding distilled water or non-nutrient buffer (PAS, normal saline, PBS, etc). Enflagellation can be induced by several factors, such as nutrient depletion, temperature, phase of growth, and culture agitation (Fulton, 1977; Woodworth *et al*, 1982). The flagellate stage involves changes in cell shape and synthesis of the flagellar apparatus, which is seen as a blunt elongated-cigar or pear-shaped cell with two flagellae or multiflagellates emerging from beneath the anterior rostrum (Fulton, 1977). The capability of *Naegleria* trophozoites to enflagellate is an important indicator to identify *Naegleria* species. However, there are some *Naegleria* species (*eg*, *N. chilensis* and *N. indonesiensis*) which are unable to enflagellate (De Jonckheere *et al*, 2001).

In a cold environment, trophozoites of both amebae have a rounded morphology because their motile organs are not moving. Once the trophozoites are incubated for 30 minutes at room temperature or 37ºC, the amebae begin to protrude lobopodium (*Naegleria*), hyaline pseudopodia or acanthopodia (*Acanthamoeba*), showing their irregularity in shape; sizes range from 15 to 25 m for *Naegleria* and 20 to 30 m for *Acanthamoeba* (Init *et al*, 2010). In this study, the morphology of these motile organelles was first preserved by fixing before applying any stain. The smears were dried slowly on a teflon slide in a moist chamber then incubated at 37ºC for at least 30 minutes before fixation with methanol.

The morphology of non-motile cyst stages of these amebae can be maintained in its original form because they are protected by a double wall. The cyst is a dormant or resting stage which appears under unfavorable conditions, such as food depletion, desiccation or changes in temperature or pH (Stratford and Griffith, 1978; Marciano-Cabral, 1988). The cysts of *Naegleria* are uniformly spherical with smooth, thin double walls and are clumped closely together, forming a colony on the agar surface (Carter, 1970; Init *et al*, 2010). The cysts of *Acanthamoeba* exhibit various shapes with double thick walls consisting of outer (ectocyst) and inner (endocyst) layers. The ectocyst is wrinkled and the endocyst is stellated, polygonal, round or oval (Fig 1, 12a-15a). The cyst stages for both amebae were detected 7 days after cultivation of the original samples. However, they appeared on day 4 or 5 after sub-cultivation.

In this study, the *Acanthamoeba* cysts of several isolates were first seen with 3-5 protrusions (Fig 1, 12a and 13a) but slowly changed their morphology to become rounded (Fig 1, 15a) after 12 weeks of several sub-cultivations. Several authors have reported the morphology of cysts could be altered by factors such as culture age, pH, dryness and lack of food (Visvesvara *et al*, 1975; Walochnik *et al*, 2000).

The unstained characteristics of the ameba stages are difficult to detect and sometimes are not identified because these stages are transparent and are surrounded by bacteria or other organisms (*eg*, fungi) growing together on the agar surface. The stained amebae provided a detailed structure of the cellular organelles. The motile organelles were clearly demonstrated after the staining process. Of the 7 staining procedures, trichrome-eosin and iron hematoxylin stains showed good color contrast during all three stages (trophozoite, cyst and flagellate); Giemsa and Gram stains stained the trophozoite and flagellate stages well and modified Field's and modified AFB stains stained only the trophozoite stage. The original AFB stain failed to show good color contrast at any of the stages and the modified Field's stain could not be used to stain the flagellate stage of *Naegleria*.

Using the trichrome-eosin stain, the cytoplasm, nucleolus, food and water vacuoles were clearly distinguished from each other based on several contrasting colors, but the iron hematoxylin stain stained these organelles with a grey color only. Both of these staining methods showed the *Acanthamoeba* cysts were more compact than the *Naegleria* cysts. The *Acanthamoeba* cysts had an unstained double thick-wall with stained compact cytoplasm and a rarely seen nucleolus, while the *Naegleria* cyst had a thin wall surrounding non-compact cytoplasm and nucleolus had empty compartments.

Giemsa and Gram stains stain motile stages blue and red, respectively, but did not show a color contrast against the cyst stage for both amebae. The stains produced differences in intensity demonstrating the different organelles in the trophozoite stage. The nucleolus, stained darker, was located inside the lighter colored nucleus. The stains demonstrated dividing nuclei in *Acanthamoeba* trophozoites. The flagellate stage of *Naegleria* was darker posteriorly and lighter anteriorly where the flagella emerged. The acanthopodia and flagella had a similar color as the respective cytoplasm.

The modified Field's technique stained the nucleolus and particles in the food vacuole purple-red, and the cytoplasm and acanthopodia dark-blue. This demonstrated the water vacoules of the *Acanthamoeba* trophozoite. Apart from that, the nucleolus of *Naegleria* trophozoites consisted of non-stained compartments which suggests their nucleolus is less than the nucleolus of *Acanthamoeba* trophozoites. Pirehma *et al* (1999) reported modified Field's stain produced a good color contrast in *Acanthamoeba* trophozoites. We found this stain also produced good color contrast in *Naegleria* trophozoites but failed to stain the cyst stage of both amebae.

The original AFB stain produced a blue coloration without contrast against all stages. The modified AFB stain gave a color contrast with purple cytoplasm and nucleoli of trophozoites. Food and water vacuoles could not be differentiated from cytoplasm, but acanthopodia could be demonstrated. There was no color contrast between cysts.

All stains described in this study can

be used to identify *Acanthamoeba* and *Naegleria*. The time needed to complete the modified Field's stain was the shortest (less than 5 minutes), followed by modified AFB and Gram stains (less than 10 minutes), trichrome-eosin (less than 45 minutes), Giemsa stain (less than 65 minutes) and the longest time taken was with the iron-hematoxylin stain (less than 80 minutes). Decolorizing was the most crucial step of the staining procedures. Insufficient time for decolorizing results in a darker color, while excessive decolorization produces a lighter color, giving poor color contrast among the important organelles in the cell.

Morphological identification of *Acanthamoeba* and *Naegleria* was carried out by detecting specific organelles in the trophozoite, cyst and flagellate stages. The trophozoites of both amebae were commonly used for the diagnostic stage where motile organs (acanthopodia, pseudopodia or lobopodia), the nucleus with a large nucleolus without chromatin and water- and food-vacuoles are easily identified. The cyst stage is detected with a characteristic shape and double-wall. The flagellate stage is only present with *Naegleria*. These stages were detected without staining under light microscopy with difficulty, especially when the amebae are in the xenic culture of non-nutrient agar lawned with *Escherichia coli*. The stained parasite gave better discrimination of the morphology due to the color contrast and stained organelles.

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