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Identification of *Entamoeba histolytica* trophozoites in fresh stool sample: comparison of three staining techniques and study on the viability period of the trophozoites

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Abstract. *Entamoeba histolytica* causes about 50 million infections worldwide with a death rate of over 100,000 annually. In endemic developing countries where resources are limited, microscopic examinations based on Wheatley trichrome staining is commonly used for diagnosis of intestinal amoebiasis. Other than being a time-consuming method, it must be performed promptly after stool collection as trophozoites disintegrate rapidly in faeces. The aim of this study was to compare the efficacies of Eosin-Y, Wheatley trichrome and Iodine stains in delineating the diagnostic features of the parasite, and subsequently to determine the suitable microscopy observation period for detection of erythrophagocytic and non-erythrophagocytic trophozoites spiked in semi-solid stool sample. Wheatley trichrome staining technique was performed using the standard method while the other two techniques were performed on the slides by mixing the respective staining solution with the spiked stool sample. One million of axenically cultured non-erythrophagocytic *E. histolytica* and erythrophagocytic *E. histolytica* were separately spiked into 2 g of fresh semi-solid faeces. Percentage viability of the trophozoites in the spiked stool sample was determined at 30 minute intervals for eight hours using the 0.4% Trypan blue exclusion method. The results showed that Eosin-Y and Wheatley trichrome stained the karyosome and chromatin granules better as compared to Iodine stain. The percentage viability of non-erythrophagocytic trophozoites decreased faster than the erythrophagocytic form in the first 5 hours and both dropped to ~10% in the 6th hour spiked sample. In conclusion, Eosin-Y staining technique was found to be the easiest to perform, most rapid and as accurate as the commonly used Wheatley trichrome technique; Eosin-Y stained slide sealed with DPX could also be kept as a permanent record. A period not exceeding 6 hours after stool collection was found to be the most suitable in order to obtain good microscopy results of viable trophozoites.

INTRODUCTION

Entamoeba histolytica is an enteric anaerobic protozoan parasite that causes about 50 million infections with a death rate of over 100 000 worldwide annually (WHO, 1997; Jackson, 1998; Zlobl, 2001; Fotedar *et al.*, 2007). The amoebic infection is the third most common cause of death among parasitic diseases, after malaria and schistosomiasis (Tanyuksel & Petri, 2003). The disease is widely reported in developing countries like India and Bangladesh, tropical African countries and in some areas in Brazil and Mexico. The incidence is increasing in non-endemic and developed countries such as the USA and European countries, due to the ease of world travel and immigration of

people from endemic areas (Nari *et al.*, 2008). High risk people are those who travel to crowded endemic areas with low standards of hygiene and sanitation; and those who practice unnatural sexual activities such as direct anal-genital and/or oral-anal sex (Espinosa-Cantellano & Martinez-Palomo, 2000; Haque *et al.*, 2000; Zlobl, 2001; Fotedar *et al.*, 2007).

Entamoeba histolytica has a simple life cycle, in which the transmission is via the faecal-oral route. Infection occurs through ingestion of infective cysts (size 8-20 µm) or invasion of motile trophozoites (size 20-40 µm) (Martinez-Palomo, 1982; Lucas & Upcroft, 2001). The infection causes a variety of clinical presentations, from asymptomatic colonization to invasive amoebic dysentery and extraintestinal amoebiasis. Most infected individuals do not show clinical signs, and the problem is compounded by the lack of reliable and practical diagnostic tools (Martinez-Palomo, 1982; Huston *et al.*, 1999; Zlobl, 2001; Blessmann *et al.*, 2003; Huston, 2004; Fotedar *et al.*, 2007).

The routine diagnosis of amoebic dysentery is still based on identification of erythrophagocytic trophozoites in dysenteric specimens (Cheesbrough, 2005). This low- cost diagnostic technique is still the preferred method in developing countries although numerous molecular-based methods such as polymerase chain reaction and immunological-based methods such as enzyme-linked immunosorbent assay, have been reported to be effective in species-specific diagnosis of *E. histolytica* (Huston *et al.*, 1999; Tanyuksel & Petri, 2003; Visser *et al.*, 2006; Fotedar *et al.*, 2007). A major setback in microscopy is the requirement of freshly collected stool samples as the trophozoites had been reported to disintegrate in faeces from 30 minutes to 3 hours after collection (Gardner *et al.*, 1980; Tanyuksel & Petri, 2003; Fotedar *et al.*, 2007); nevertheless there is no conclusive published data to support this claim. Another disadvantage of microscopy is the time consuming Wheatley trichrome staining process, which requires at least 42 minutes to perform (Flournoy *et al.*, 1982).

As amoebiasis mostly occurs in resource-tight developing countries, microscopy technique will still remain the diagnostic method of choice. Laboratories worldwide reportedly used numerous successful staining methods such as Wheatley's trichrome, Iron hematoxylin, Giemsa, Wright's, Methylene blue, Chorazole Black E and Iodine-trichrome stains (Koontz & Weinstock, 1996; Tanyuksel & Petri, 2003; Fotedar *et al.*, 2007), but all are tedious and time-consuming. Hence, a simple, rapid and reliable staining technique is urgently needed. The objectives of this study were to compare the efficacies of Eosin-Y, Wheatley trichrome and Iodine in staining the characteristic features of the parasite; and subsequently to determine the most suitable microscopy observation period for detection of erythrophagocytic and non-erythrophagocytic trophozoites spiked in semi-solid stool sample.

MATERIALS AND METHODS

Staining of trophozoites

Approximately one million *E. histolytica* axenically cultured in TYI-S-33 medium was washed with 1X Phosphate Buffered Saline (PBS) and spiked in 2 g of fresh semi-solid stool sample obtained from a healthy volunteer. Then, spiked stool samples were stained separately with Wheatley trichrome, Iodine and Eosin-Y solution alcoholic with phloxine B (Sigma HT110316, USA) (Eosin-Y). Duplicate slide smears were prepared for each staining technique. Wheatley trichrome staining technique was performed based on the standard operating protocol (SOP) used at the Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia, Malaysia. An applicator stick was used to smear ~2 mg of stool sample on a clean slide. The smeared slide was then immersed in Schauddin's fixative for 2 hours. This was followed by soaking the slide in succession in tincture of iodine, 70% alcohol, Wheatley trichrome stain (REMEL Inc., Lenexa, USA), acid alcohol, absolute alcohol and xylene.

Finally, the slide was mounted with dibutyl phthalate xylene (DPX) and observed under a light microscope at 1000X magnification. The iodine stained smeared slide was prepared based on the protocol suggested by Koontz & Weinstock (1996). Briefly, an applicator stick was used to mix ~2 mg of stool sample with ~30 µL Lugol's Iodine solution on a clean slide. A cover slip was placed on the sample and sealed with DPX, then observed under a microscope. In Eosin-Y staining technique, an applicator stick was used to mix ~30 µL of Eosin-Y with ~2 mg of stool sample on a clean slide. A cover slip placed over the sample was sealed with DPX, then observed under a microscope at 400X and 1000X magnification. The images of the trophozoites stained by all three methods were captured using an Olympus Image Analysis System (Olympus System Microscope Model BX41, Japan). Comparisons were made among the images of the three types of stained trophozoites based on the clarity of their characteristic nuclear features.

Viability of non-erythrophagocytic and erythrophagocytic trophozoites in stool sample

About one million cultured trophozoites washed with 1X PBS were spiked into 2 g of fresh semi-solid stool sample. About 2 mg of the sample was mixed with 50 µl Trypan (0.4%) blue and the mixture was loaded into a Neubauer chamber to determine the viability of non-erythrophagocytic trophozoites by microscopy. The procedure was performed in duplicate and repeated every 30 minute intervals for eight hours.

In order to determine the viability of erythrophagocytic trophozoites, about 10 µL of blood was first added into a sterile microfuge tube containing 1×10^6 axenically cultured trophozoites. After 30 minutes, the trophozoites were washed with 1X PBS and spiked into 2 g of fresh semi-solid stool sample. Then, ~2 mg spiked stool sample was mixed with 0.4% Trypan blue, and the percentage viability was determined as described earlier.

Direct wet mounts were also prepared to observe the movement of motile trophozoites and their disintegration over time. An applicator stick was used to mix ~2 mg of spiked stool sample with ~30 µL normal saline (0.85% NaCl) on a clean slide. Then, a cover slip was placed on the sample and observed immediately under a light microscope.

RESULTS AND DISCUSSION

Staining of trophozoites

Images of the trophozoites were compared based on the detection of the characteristic features of trophozoites such as the chromatin granules that line the nuclear membrane and the small spherical karyosome at the centre of the nucleus. Nucleus of trophozoite has no fixed position in the cytoplasm, but moves freely and sometimes rotates rapidly (Martinez-Palomo, 1982). Thus, observation of the characteristic features of live trophozoites requires fine focusing of the optical microscope at 400X or 1000X magnification.

Permanent stains were much more effective than the direct wet mount for detection of trophozoites and/or cysts in stool specimens (Gardner *et al.*, 1980). Figure 1(a) shows the image of a Wheatley trichrome stained trophozoite; it was stained blue-purple with greenish background, with good delineation of the chromatin granules and karyosome. The stain provided a good contrast between the trophozoite and the background debris. However, an obvious disadvantage was the tedious protocol which required 2 hours fixation period and a total time of ~3 hours to complete. Appropriate fixation periods coupled with sufficient washing steps are pertinent in obtaining a well-stained nucleus, thus may require the preparation of a number of slides for each stool sample. Repeated use of acid alcohol in destaining trichrome stain will reduce its efficiency and subsequently require a longer destaining time although a better alternative is to use a fresh solution. The suggested fixation time with Schauddin's fixative is between 2 to 24 hours. Any increase in fixation time must be followed by an appropriate increase in washing time using

tincture of iodine. Moreover, Schauddin's fixative, which killed and fixed the trophozoites contains mercury compound which is not environmentally-friendly (Garcia & Shimizu, 1998; Amin, 2000). This staining technique demands technical skills of an experienced microscopist, and would be daunting to those unskilled personnel who have to perform the technique occasionally.

Iodine stain is mostly used to identify *E. histolytica* cysts in stool microscopic detection (Cheesbrough, 2005). However, Koontz & Weinstock (1996) reported that the stain could be used to delineate intestinal amoebas by negating the motility of the trophozoites. As shown in figure 1 (b), the nuclear chromatin granules were only faintly stained and the karyosome remained unstained.

Figures 1(c) and 1(d) show Eosin-Y stained non-erythrophagocytic and erythrophagocytic trophozoites, respectively. The former shows a trophozoite with its well-stained nuclear chromatin granules and karyosome; and the latter reveals well-stained characteristic features of the erythrophagocytic amoebic trophozoite and the engulfed erythrocytes. The whole trophozoite was stained light red, and both the chromatin granules and karyosome showed distinctly dark appearances. Eosin-Y also clearly stained the engulfed erythrocytes.

Various types of eosin stains are available commercially and some are used as counterstain to haematoxylin in Haematoxylin and Eosin (H&E) stain. Its acidic property stains the basic components of a cell, such as cytoplasm, light red in colour. Others used eosin as an exclusion dye to stain dead trophozoites light red in colour to distinguish them from the unstained viable trophozoites (Mirelman *et al.*, 1987; Behnia *et al.*, 2008). The stain was also reportedly used to facilitate the detection of motile trophozoites by staining the background pink without staining the live parasites (Cheesbrough, 2005). Interestingly, phloxine B in Eosin-Y was reportedly used to stain nuclear structures in histological sections (SPI.Supplies, 2009). Until now, there were no reports on the use of phloxine B to stain nucleus of *E. histolytica*. Here, we showed that Eosin-Y was just as accurate as Wheatley trichrome staining method in identification of trophozoites in stool samples. Besides staining the characteristic nuclear features of the trophozoites and/or the engulfed erythrocytes, it could be performed easily to give spontaneous results. The Eosin-Y used in this study is commercially available in its working dilution, thus can be applied directly onto the stool samples without fuss. Alternatively, Eosin-Y staining solution can also be prepared by mixing 1% (w/v) Eosin-Y, 1% (w/v) phloxine B, 95% ethanol and glacial acetic acid in appropriate volumes (Mayer's, 2009).

Another major advantage of Eosin-Y staining technique is that the stained trophozoites could easily be visualized under 400X magnification. At this magnification, it is very difficult to identify the Wheatley trichrome stained trophozoites. The rounded shape and immotile trophozoites left for 3 hours in stool sample were also easily stained by Eosin-Y [Figure 1(e)]. In fact, this stain was able to preserve the general morphology of the trophozoite for more than 24 hours. This was probably due to the presence of alcohol and glacial acetic acid in the stain, as these two chemicals are also used in Schauddin's fixative.

A major advantage of Wheatley trichrome staining technique is that it be used to prepare a permanent record of the stained amoebas. In contrast, the stained nuclear chromatin granules and karyosome of an Eosin-Y stained trophozoite gradually became fainter over time and almost indistinguishable from its cytoplasm after an hour [Figure 1(f)]. However, by sealing the edges of the cover slip to the slide with DPX, it prevented Eosin-Y from drying. This permanent record of the Eosin-Y stained slide could be stored longer if placed in a horizontal position (instead of a vertical position).

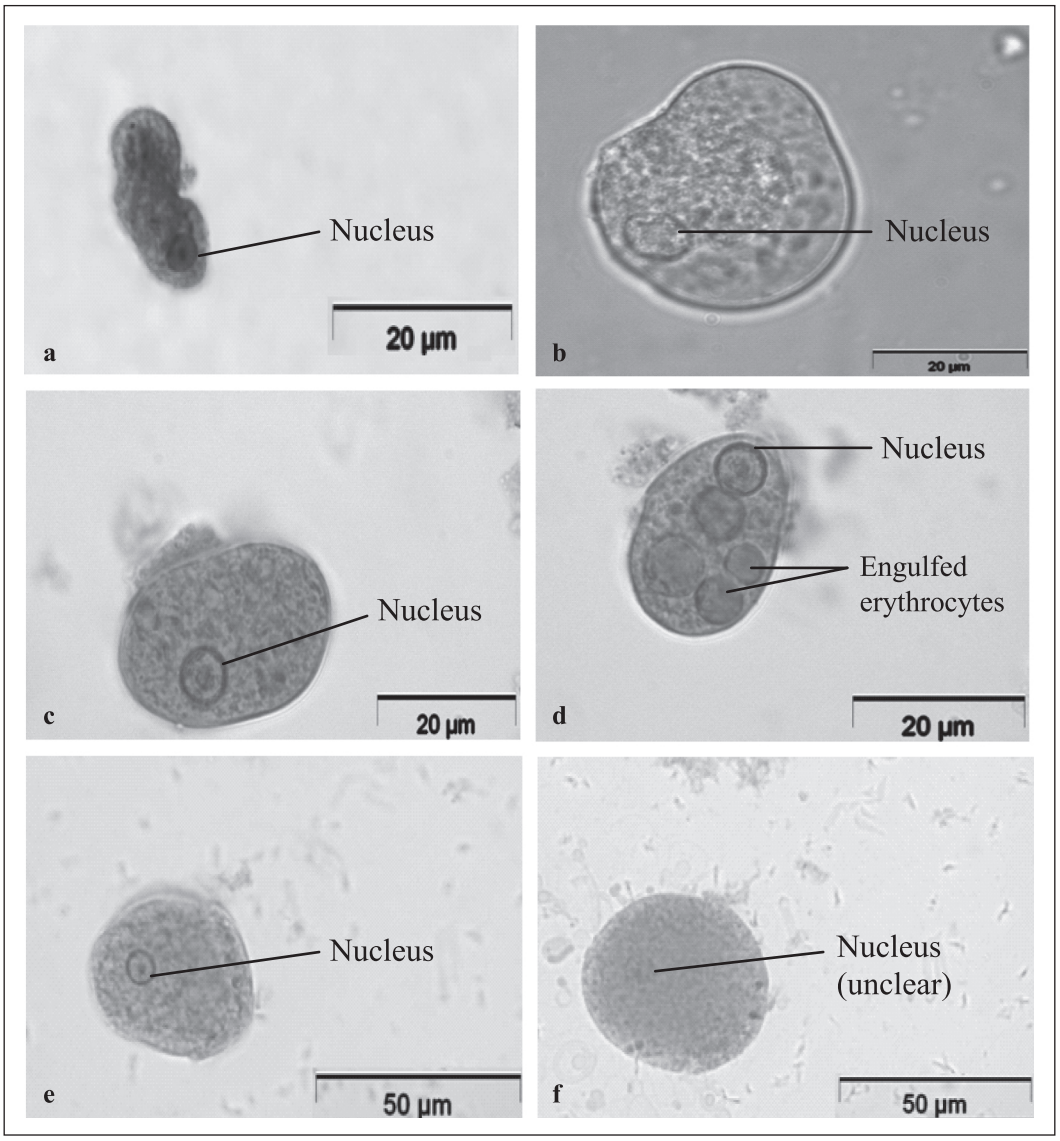

Figure 1. Stained trophozoites. (a) Wheatley trichrome stained trophozoite, 1000X magnification (b) Iodine stained trophozoite, 1000X magnification (c) Eosin-Y stained trophozoite, 1000X magnification (d) Eosin-Y stained erythrophagocytic trophozoite, 1000X magnification (e) Eosin-Y stained trophozoite showed clear chromatin granules and karyosome, 400X magnification (f) Eosin-Y stained trophozoite without DPX seal indicated unclear nuclear characteristics after an hour, 400X magnification.

Viability of non-erythrophagocytic and erythrophagocytic *E. histolytica* trophozoites in stool sample

Gonzalez-Ruiz *et al.* (1994) reported that trophozoites started to disintegrate rapidly as soon as they were in the faeces. However, the viability period of trophozoites outside its host was not studied. In the present study, the viability of trophozoites in stool sample was assessed by Trypan blue dye exclusion test whereby the dead trophozoites were stained blue and the live ones remained unstained (Figure 2). The viability chart of the non-erythrophagocytic trophozoites in spiked semi-solid stool is shown in (Figure 3). During the first hour, the percentage viability dropped rapidly and fluctuated at approximately 55%. This was probably due to the unfavourable conditions in the stool as compared to the optimal axenic

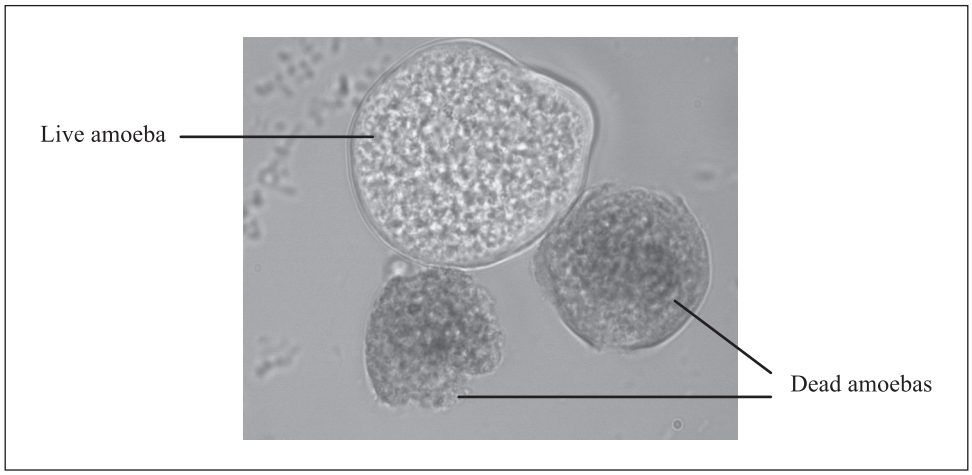

Figure 2. Trypan blue dye exclusion stained trophozoites, 1000X magnification.

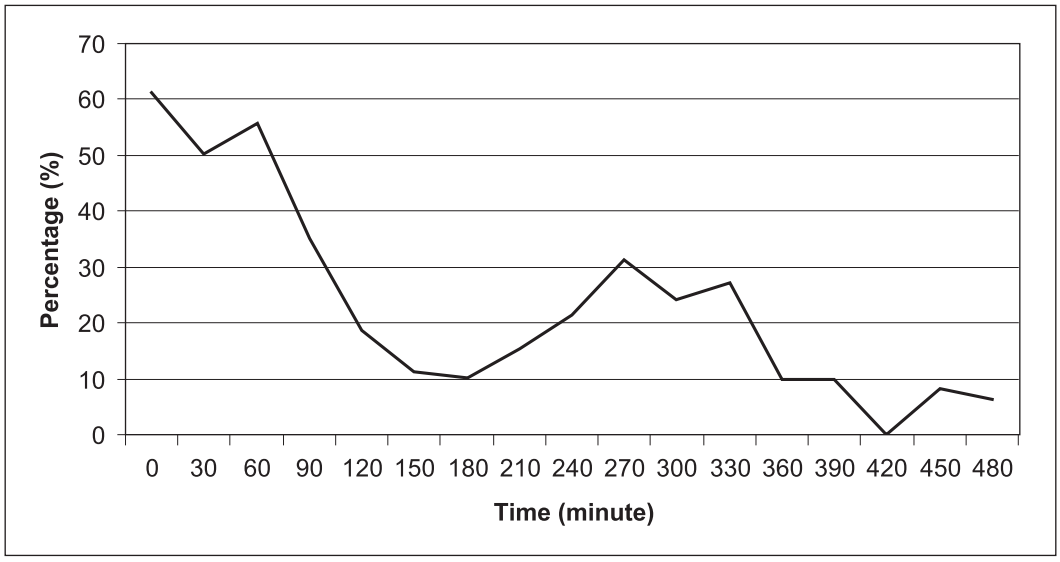

Figure 3. Viability chart of *E. histolytica* trophozoites in spiked semi-solid stool.

conditions of the trophozoites in TYI-S-33 medium at 36°C. Thereafter, the viability dropped to ~10% at the third hour. However, during the 3 to 5½ hours period, the percentage viability increased slightly, and then fluctuated around ~30%. At the 7th hour, none of the trophozoites was detected but ~10% viability was again observed at the 8th hour. This was probably due to the fact that *E. histolytica* in the stool samples was being challenged with a toxic high oxygen environment (30%) since it has been reported that amoebas can be supported in only less than 5% O_2 (Band & Cirrito, 1979). Figure 4 shows the viability chart of the erythrophagocytic trophozoites in spiked semi-solid stool. In comparison with figure 3, the percentages viability of erythrophagocytic trophozoites was higher (65% and 95%) during the first and third hours in stool sample. This was probably due to the antioxidant molecules (superoxide dismutase, catalase, glutathione, peroxirredoxin and vitamin E) present in the

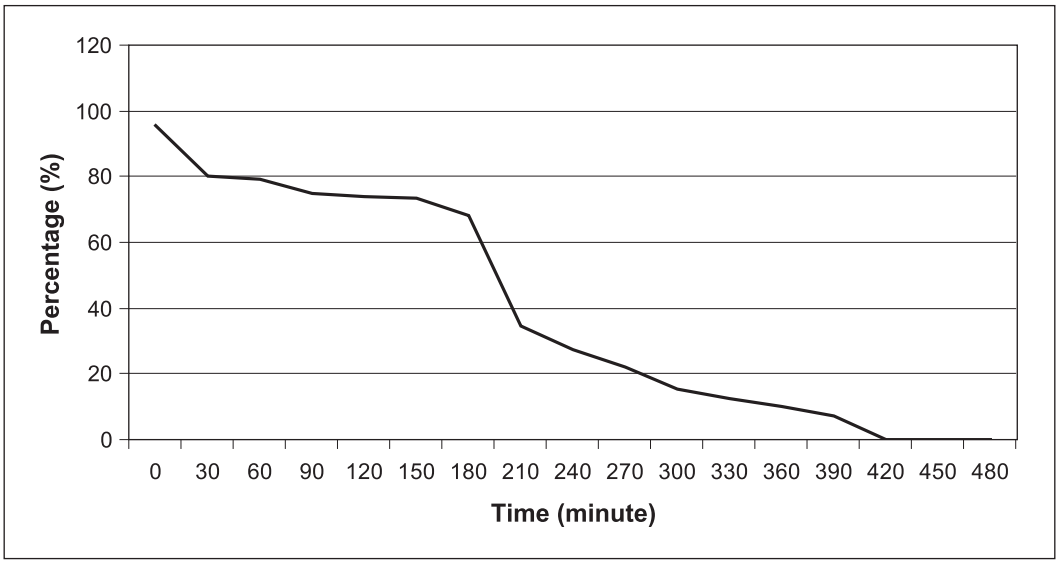

Figure 4. Viability chart of *E. histolytica* erythrophagocytic trophozoites in spiked semi-solid stool.

engulfed erythrocytes (Kuypers, 2007), which helped the amoeba to detoxify the reactive oxygen species generated during the oxygen reduction and/or because the erythrocytes were source of nutrients for the amoebas. Between the third and sixth hours, the mortality of the trophozoites increased gradually from about 65% to 90%, and none was detected from the seventh hour onwards. In general, the percentages viability of both forms of trophozoites dropped to ~10% at the sixth hour in semi-solid stool sample and none was detected from the seventh hour onwards. Since ~90% of the trophozoites were undetected at the sixth hour, microscopy detection to detect the amoebas should thus be performed within six hours after stool collection.

Observation of the direct wet mount slide preparation during the first hour in fresh semi-solid stool sample revealed that the trophozoites did not have fixed shape and were actively pushing out the ectoplasm to form pseudopodia, followed by the in-flowing endoplasm. In addition, the technique allowed the disintegration process of trophozoites to be observed over time (Figure 5a). Uroid of the amoeba was located at the posterior end of the live trophozoites. Faint engulfed erythrocytes were also visible but the characteristic nucleus was impossible to visualize without staining (Figure 5b). Thus the direct wet mount technique is neither sensitive nor reliable for detection of *E. histolytica* in stool samples.

All microscopy staining techniques (include those used in this study) cannot differentiate *E. histolytica* from the non-pathogenic *E. dispar*. However parasite identification by staining is still commonly used in developing endemic countries where resource are limited, as the costs of commercially available *E. histolytica* antigen detection tests are prohibitive. An important supportive evidence for microscopy is the detection of erythrophagocytic trophozoites in stool sample, although some non-pathogenic *Entamoeba* species may also ingest erythrocytes (Gonzalez-Ruiz *et al.*, 1994). Indiscriminate use of antiparasitic drugs may lead to development of drug-resistant. Thus, treatment should only be given to patients where the presence of *E. histolytica* in stool is confirmed, and no treatment should be administered if only *E. dispar* is found (WHO, 1997). Until now, light microscopic differentiation between the two amoeba species is not yet available and WHO has highlighted the urgent need in developing improved methods for the species-specific diagnosis of *E. histolytica* infection (WHO,

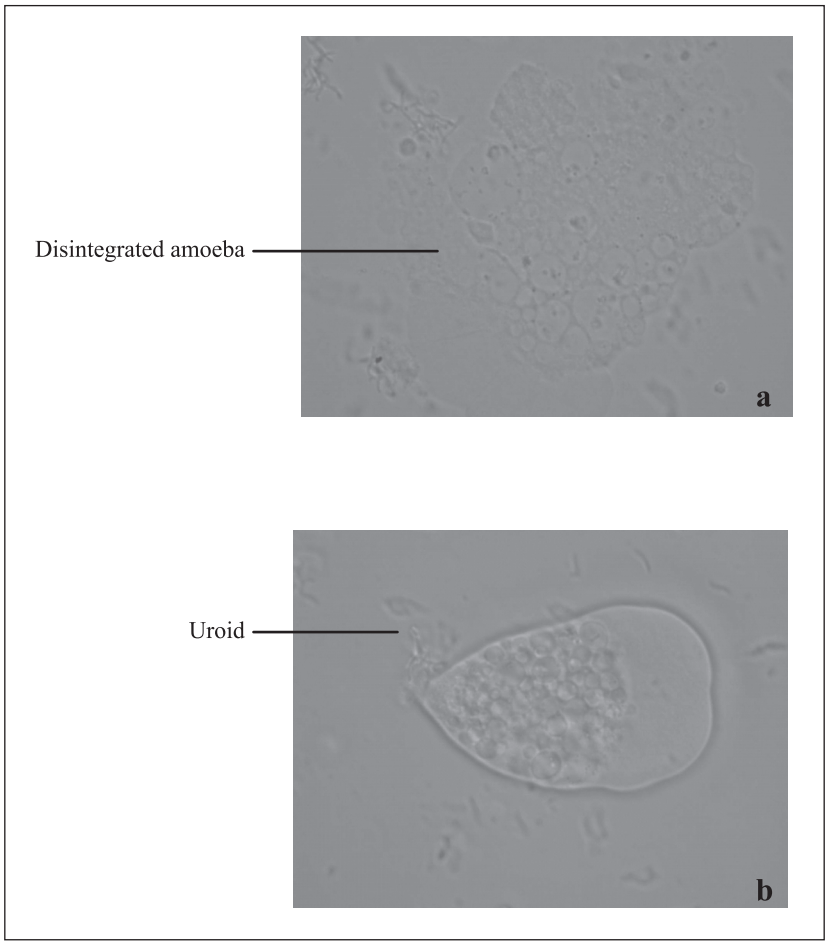

Figure 5. (a) Disintegrated trophozoite, 1000X magnification (b) Motile trophozoite with uroid at posterior end examined using direct wet mount, 1000X magnification.

1997). Hence efforts should also be on the search for stains which can specifically demonstrate structural compounds found in *E. histolytica* but absent in *E. dispar*.

In conclusion, this study showed that for microscopic identification of *E. histolytica* in patients' samples, Eosin-Y could stain the characteristic nuclear chromatin granules and karyosome of the trophozoites as accurately as the Wheatley trichrome, and better than the Iodine stain. Eosin-Y stained slide could also be kept as permanent record if the cover slip is sealed to the slide with DPX, however further studies are needed to determine the period of time before drying occurs. Nevertheless, Eosin-Y technique offers the added advantages of being rapid and easy to perform, thus is very useful for the purpose of identification of *E. histolytica* in patients' stool samples, especially in busy, and/or understaffed laboratories. The identification of *E. histolytica* was supported by the signs and symptoms presented by the patients and the detection of erythrophagocytic trophozoites. This study also suggests that the microscopy observation for viable trophozoites is best performed within the first 6 hours after stool collection.

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