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Research Article

Identification of *Acanthamoeba* sp. with Different Microscopes and Analysis of the Anatomical Changes from Trophozoite to Cyst form with an Electron Microscope

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Abstract

Background: *Acanthamoeba* is a free living ubiquitous protozoa, capable of causing *Acanthamoeba* keratitis (AK) of eyes and *Acanthamoeba* meningitis/ meningoencephalitis (AME) of central nervous system in human. Identification of amoeba is possible by a light microscope but, due to its poor resolution power, it is insufficient to distinguish the intimate sub cellular structures. As the resolution power of Differential Interference Contrast microscope (DIC) is more compared to light microscope, it can perfectly distinguish the trophozoite and cyst forms of amoeba on the basis of morphology, but the detailed understanding and characterization of the inner cell structure and sub cellular organelles is only possible with an electron microscope.

Methods: Corneal scraping was collected from a clinically suspected AK patient reporting to the outpatient department (OPD) of Dr RP. Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, India with prior consent and thorough clinical examination under a slit lamp. Specimen was plated carefully onto a 2% non-nutrient agar plate previously seeded with live *E. coli*. Growth of amoeba over the agar surface was confirmed with a light microscope. DIC microscope and electron microscope were implemented to study the morphology and anatomy of *Acanthamoeba*.

Results: Prominent spine like structures i.e. acanthapodia were clearly visible on the outer surface of *Acanthamoeba* trophozoites and prominent double layer cell wall (exocyst and endocyst) was seen in the mature cyst form with DIC microscope. Several oval mitochondrias, food vacuoles and water expulsion vacuoles were seen within the cytoplasm of trophozoite of *Acanthamoeba* sp. by electron microscopy. Number of Golgi bodies were more within the cyst and found close to the inner cell wall.

Conclusions: Although identification of *Acanthamoeba* sp. can be possible with light and DIC microscopes but analysis of the anatomical changes from trophozoite to cyst forms can be studied with an electron microscope.

Keywords: *DIC of Acanthamoeba; Electron Microscopy of Acanthamoeba; Light Microscopy of Acanthamoeba; TEM; SEM*

Introduction

Acanthamoeba is a free living ubiquitous protozoa, discovered by Castellani in 1930, as an eukaryotic cell culture contaminant and 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 [1,2]. *Acanthamoeba* sp. can exist either in trophozoite or cyst form depending on the situation of its surrounding medium. *Acanthamoeba* prefers to remain in active and dividing trophozoite form under non-stressful environmental conditions and when sufficient number of bacteria are available in the surrounding for phagocytosis. It prefers to undergo phenotypic change to inactive, resistant cyst form under stressful environmental conditions or deprivation of nutrients for prolonged period of time and are characterized by shrinking and rounding of cell and formation of two distinct cell walls i.e. endocyst and exocyst [3,4].

The use of in vivo confocal microscope to detect *Acanthamoeba* sp. in corneal tissue at distinct depths has been successfully used to diagnose AK infections [5]. High-contrast rounded bodies, indicative of amoebic cysts, are observed in the corneal tissue if infected with AK [6,7]. Light microscopic examination of the culture plate (2% non-nutrient agar) containing sample (corneal scrapings) spreaded over the *E. coli* overlay is considered as the gold standard for effective diagnosis of the diseases [8].

Laboratory identification of amoeba is possible by a light microscope, but due to its poor resolution power, it is insufficient to distinguish the intimate sub cellular structures. As the resolution power of DIC is more compared to light microscope, it can perfectly distinguish the trophozoite and cyst forms on the basis of morphology, but the detailed understanding and characterization of the inner cell structure and sub cellular organelles is only possible with an electron microscope. Electron microscope can analyze the intracellular changes, topological changes which either light microscope or DIC cannot analyze [9].

Transmission electron microscope (TEM) is essential for viewing internal structures of protozoa cell because most parasitic protozoa in human are less than 50 µm in size. An electron microscopy study of the ultrastructure of trophozoites and cysts of *A. castellanii* has been undertaken by Bowers and Korn [10].

Materials and Methods

Selection of cases and collection of clinical specimens

Corneal scraping was collected from a clinically suspected *Acanthamoeba* Keratitis (AK) patient reporting to the outpatient department (OPD) of Dr RP. Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi, India with prior consent and thorough clinical examination under a slit lamp.

Light Microscopy

Specimen was plated carefully onto a 2% non-nutrient agar plate previously seeded with live *E. coli*. Plate was incubated at 30°C and observed regularly under a light microscope (Nikon, Japan) till the growth of amoebas was seen over the agar surface.

Differential Interference Contrast Microscope (DIC)

Entire agar surface gets covered with *Acanthamoeba* sp. after a week. Amoebas were Scraped from the culture plate with a sterile loop and mixed with 100 µl of PBS buffer (pH 7.4), Amoebas were mixed properly with a vortex mixer (Hudson, India). 10 µl resuspended solutions of *Acanthamoeba* sp. was taken on clean glass slides. Coverslip was placed over the specimens and photomicrographs were taken by differential interference contrast microscope (DIC) (Nikon, japan) with 63X objective.

Culture of trophozoites in liquid medium and transformation into cysts

Acanthamoeba sp. grown on the culture plate was sub-cultured in a 75 cm2 cell culture flask (Nunc, USA) with 20ml of fresh PYG growth medium (proteose peptone 0.75%, yeast extract 0.75% and glucose 1.5%) (pH 7.4) with antibiotics (penicillin and streptomycin) in bactericidal concentrations. Flasks were incubated at 30°C for 5 days (Khan, 2009). After 5days of incubation, 20 ml was divided into 2 new tissue culture flasks (10 ml into each culture flask). One culture flask was processed for encystations in the standard laboratory conditions as per the Neffs encystment protocol (Khan, 2009) and incubated for 3 days while other flask was used for sample preparation for TEM and SEM from logarithmic phase of trophozoites. After 3 days of incubation the flask containing cysts was taken for sample preparation for TEM and SEM.

Scanning electron microscopy (SEM)

Acanthamoeba sp. was harvested from both PYG growth medium (trophozoites) and Neffs encystment medium at 500×g rpm for 10 mins. The pellet was suspended in 0.1 M sodium phosphate buffer (pH 7.4), centrifuged at 500×g rpm for 5 mins to remove the remaining mediums, followed by washing with 0.1 M sodium phosphate buffer (pH 7.4) at room temperature. Pellets of both trophozoites and cysts were fixed with 2.5% glutaraldehyde in 0.1M sodium phosphate buffer for 2hr at 4°C. Fixative was removed by washing the sample

twice with 0.1M phosphate buffer (pH 7.4). Postfixation was performed after primary fixation with 1% osmium tetraoxide at 4°C for 1 hr followed by washing twice with 0.1M phosphate buffer at 4°C. The samples were dehydrated with the increasing acetone concentrations starting from 30% followed by 50%,70%, 80%, 90%, 95% and finally with 100% for 15 mins. All the dehydration steps were carried out at 4°C for 15 mins each.

Both the samples were subjected to critical point drying (CPD), finally mounted on aluminum stubs for gold sputter coating of thickness around 35nm. The samples were observed with Zeiss Leo 435 VP scanning electron microscope (Leo Electron Microscopy Ltd Cooperation Zeiss Leica, Cambridge, England) at 15kV with magnification of 1500 to 2500k for image capturing.

Transmission electron microscopy (TEM)

Both trophozoites and cysts were harvested from their respective culture mediums at 500×g rpm for 10 mins. The pellets were washed twice with 0.1 M sodium phosphate buffer (pH 7.4) followed by fixing the cells in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 3 hrs at 4°C. The fixative was removed by centrifugation at 500×g rpm for 5 mins. The pellet was resuspended in 0.1 M sodium phosphate buffer (pH 7.4). Supernatant was removed after centrifugation at 500×g rpm for 5 mins, followed by washing in 0.1 M sodium phosphate buffer (pH 7.4). The samples (pellet of trophozoites and cysts) were postfixed for 1 hr in 1% osmium tetroxide at 4°C. Subsequently the pellets were rinsed once with 0.1 M sodium phosphate buffer (pH 7.4) to remove excess fixative. Pellets were dehydrated in ascending percentage concentrations (30%, 40%, 50%, 70%, 8o% and 90%) of acetone, infiltrated and embedded in araldite CY 212 (TAAB, UK). For transmission electron microscopic examination, thin sections (70 - 80 nm) were cut and mounted onto 300 mesh- copper grids. Sections were stained with alcoholic uranyl acetate and alkaline lead citrate, washed gently with distilled water and observed under a Morgagni 268D transmission electron microscope (Philips CM10 transmission electron microscope, Netherlands) at an operating voltage 80 kV. Images were digitally acquired using a CCD camera attached with the microscope.

Results

Isolation of *Acanthamoeba* sp. from clinical specimens

In the culture on 2% non nutrient agar plate overlaid with *E. coli. Acanthamoeba* sp. trophozoites were visible under a light microscope(Nikon, Japan) after 4 days and covered the entire agar surface after 7 days. Image was captured after 7 days of incubation of the plate with 10X objective of a light inverted microscope (Nikon, Japan) (Figure 1).

Figure 1: *Representative photograph showing the images of Acanthamoeba sp. isolates grown on 2% non-nutrient agar plate overlaid with E. coli.*

Differential Interference Contrast Microscopy (DIC)

Differential interference contrast microscope (DIC) increased the contrast of the image as compared to the light microscope. Prominent spine like structures i.e. acanthapodia were clearly visible on the outer surface of *Acanthamoeba* trophozoites (Figure 2). In the mature cyst form prominent double layer cell wall (exocyst and endocyst) and a space within the two walls was seen (Figure 3). The internal cytoplasm was devoid of any vacuoles. Operculum was noticed in the cysts where outer cell wall was very thin and close to the inner wall (Figure 3).

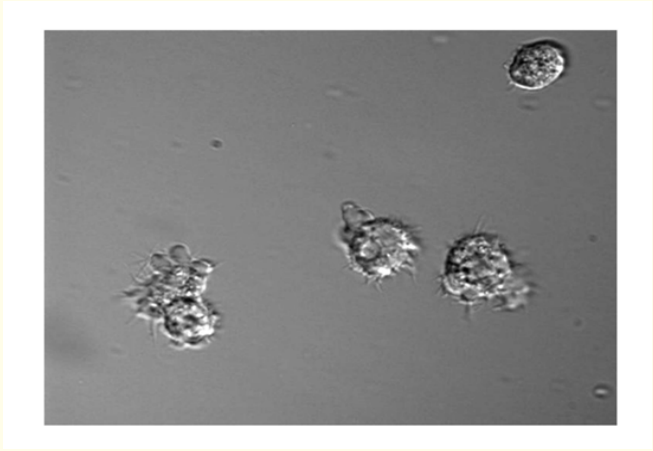

Figure 2: *Representative photograph showing the Differential Interference Contrast (DIC) microscopic image of trophozoites of Acanthamoeba sp.*

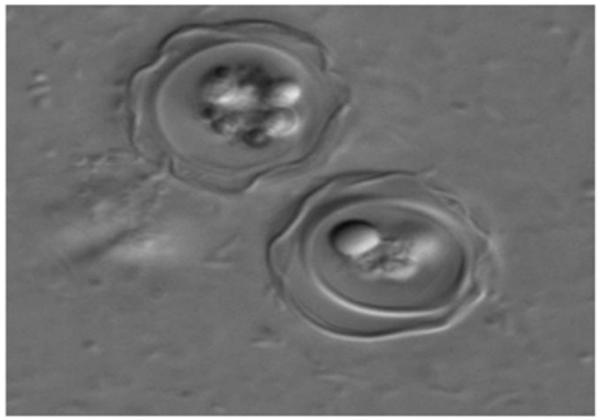

Figure 3: *Representative photograph showing the Differential Interference Contrast (DIC) microscopic image of cysts of Acanthamoeba sp.*

Scanning electron microscopy (SEM)

Trophozoite form of *Acanthamoeba* sp. feeding on *E. coli* by phagocytosis was observed in Figure 4. Several spine like acanthapodias was visualized emanating from outer wall. The cyst forms clumped together is shown in Figure 5. All the cysts had seized their movements and became almost spherical. The outer surface was noticed to be wrinkled.

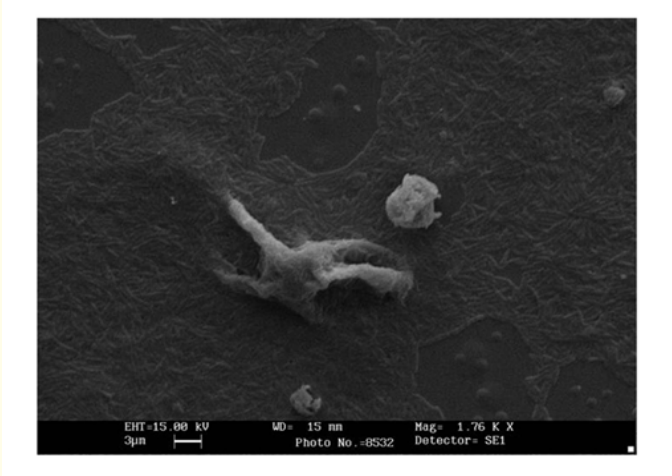

Figure 4: *Scanning Electron Micrograph (SEM) of trophozoite forms of Acanthamoeba sp.*

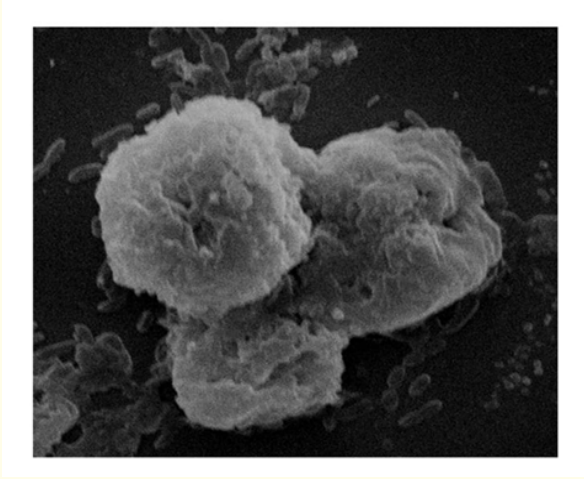

Figure 5: *Scanning Electron Micrograph (SEM) of trophozoite forms of Acanthamoeba sp.*

Transmission electron microscopy (TEM)

TEM images were taken for the *Acanthamoeba* sp. trophozoites in their logarithmic phase. Several oval mitochondrias, food vacuoles and water expulsion vacuoles were seen within the cytoplasm of trophozoite of *Acanthamoeba* sp. (Figure 6). Nuclear membrane with nuclear pore and a nucleolus was seen as a dense droplet within the nucleus (Figure 6). Ultra structure of cyst form of amoeba after incubation for 72 hrs in Neffs encystment medium was seen in Figure 7. Image revealed the outer wall (exocyst) and endocyst (inner wall) outside the plasma membrane with a space in between. Number of Golgi bodies were more within the cyst and found close to the inner cell wall. More number of fat droplets were visualized within the cytoplasm of cyst than the trophozoite. Food vacuoles and water exclusion vacuoles were not visible in the cyst.

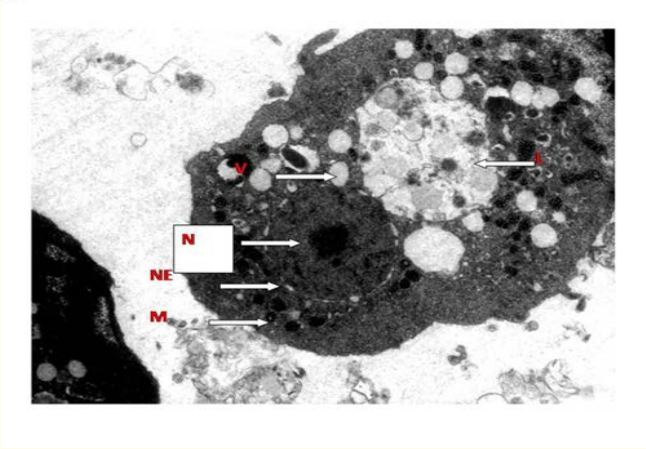

Figure 6: *Transmission electron micrograph (TEM) of trophozoite forms of Acanthamoeba sp.*

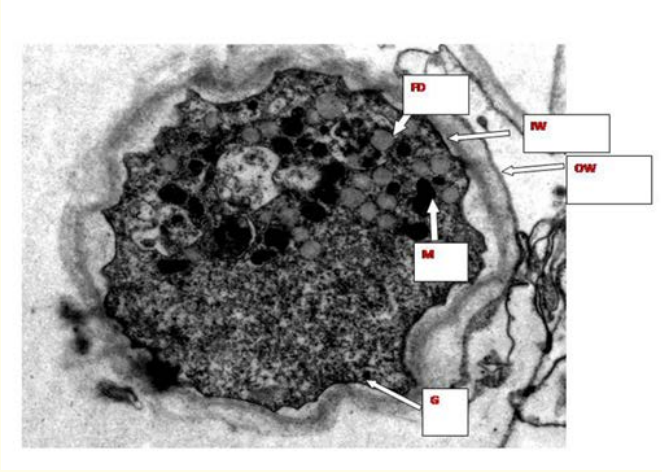

Figure 7: *Transmission electron micrograph (TEM) of cyst forms of Acanthamoeba sp.*

Discussions

Culture isolation of *Acanthamoeba* sp. is considered as the gold standard for routine laboratory diagnosis [8]. The commonest method is the culture of patient samples (corneal scrapings) on 2% non-nutrient agar plate overlaid with *E. coli*. or with 2% liquid 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 [11]. The non-nutrient agar medium contains minimal nutrients and thus inhibits the growth of unwanted microorganisms [12]. The amoeba can grow with several bacteria such as, *Klebsiella pneumoniae, Enterobacter* spp. (*Enterobacter aerogenes* and *Enterobacter cloacae*), and *Escherichia coli* [11]. Of all these non-pathogenic strains of *Escherichia coli* are commonly used in the laboratories for preparation of culture plates.

In the present study, two electron microscopic procedures were performed. Transmission electron microscopy (TEM) was performed for studying ultrastructural features in trophozoite and cyst forms of *Acanthamoeba* sp. Scanning electron microscopy (SEM) was performed for examining surface alterations in trophozoite and cyst forms of *Acanthamoeba* sp. In the present study, on the basis of SEM

result trophozoites were found to be of approximately 22 µm and cysts were of 15 µm in size. Trophozoites were seen to engulf the *E. coli* with acanthapodia spreading in all directions. Cysts had thick ridges over their entire outer surface giving compact appearance. Outer wall looked like covered with scales and devoid of any acanthapodias. As per the TEM result more number of vacuoles were seen in trophozoites than the cysts, similarly more number of fat droplets were seen in cysts than the trophozoites. Golgi cisternae in large numbers were seen close to inner cell wall of cyst form than the trophozoite form. This suggests there may be some role of fat droplets in the synthesis of cyst wall. In 1969 TEM was performed by Bowers and Korn on *Acanthamoeba* for the first time and they reported the presence of small dense vesicles of diameter of 70 nm budded from the Golgi cisternae. Later it was confirmed that, these Golgi cisternae play an important role in the formation of cell wall of cysts. Recently, Connell., *et al.* using confocal laser scanning microscopy and flow cytometry, reported the deposition of cellulose during encystation of *Acanthamoeba* [9]. Reiner., *et al*. working on another protozoa i.e. *Giardia lamblia*, had observed the formation of encystation specific vesicles (ESV) containing a precursor of filamentous material during cyst wall formation by immunoreactions [13]. There are major similarities in the composition of encystation specific vesicles (ESV) reported in *G. lamblia* with *Acanthamoeba* and other protozoas. Cha´vez-Munguı´a., *et al*. had worked on another amoeba i.e. *Entamoeba invadens,* where he showed that, the contents of ESV were stained with calcofluor white stain [14]. Later it was found that, the principal constituent of the *Acanthamoeba* cyst wall is cellulose-like material because, cyst wall (ectocyst and endocyst) fluoresce due to calcofluor white which has a specific affinity for polysaccharides such as cellulose [15,16].

Conclusions

In scanning electron microscopic (SEM) examinations trophozoites were found to be of approximately 22 µm and cysts were of 15 µm in size. In transmission electron microscopic (TEM) examination more number of vacuoles were seen within the trophozoites compared to cysts and more number of fat droplets were seen within the cysts compared to trophozoites of *Acanthamoeba* spp. Golgi cisternae in large numbers were seen close to inner cyst wall compared to trophozoite. Therefore, Golgi cisternae and fat droplets of cysts might have an important role.

Acknowledgement

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Ethical Approval

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institute and ethical clearance was obtained from the institute.

Informed Consent

Consent was obtained from all individual participants included in the study.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this work.

Funding and All Other Required Documents

There are no funding agencies to report for this submission.

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