INTERNATIONAL JOURNAL OF TECHNOLOGY

ISSN: 2086-9614

http://www.ijtech.eng.ui.ac.id

CORRIGENDUM TO:

Technique for Determining the Viability of *Acanthamoeba* Cysts Treated with a Cysticidal Agent Based on Membrane Integrity

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doi: 10.14716/ijtech.v12i4.4207

International Journal of Technology 12(4) 720-727 (2021)
Received July 2020 / Revised September 2020 / Accepted March 2021

International Journal of Technology

http://ijtech.eng.ui.ac.id

Technique for Determining the Viability of *Acanthamoeba* Cysts Treated with a Cysticidal Agent Based on Membrane Integrity

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Abstract. This study presents a straightforward and reliable method for determining the viability of *Acanthamoeba* cysts. A standard method for determining *Acanthamoeba* cyst viability in an *in vitro* cytotoxicity analysis is required to ensure that the double-walled and sturdy cysts are affected by the substance tested. In this study, a new approach was used to determine the cysticidal potential of redox Cleland's reagent, dithiothreitol (DTT), against *Acanthamoeba* cysts. This approach constitutes a significant breakthrough, as the cyst form of *Acanthamoeba* is known for its high resistance to various chemicals and drugs used to treat infections of the central nervous system and eyes caused by *Acanthamoeba*. Cyst viability was evaluated based on the intensity of the cyst population under fluorescence produced by propidium iodide (PI) dye and measured using an enzyme-linked immunosorbent assay (ELISA) reader at an absorbance of 636 nm. The results were validated using high-content screening (HCS). For analysis, an individual cell was imaged and examined for phenotypic changes in the *Acanthamoeba* cyst at the cyst population level. Fluorescence intensity of the cysts in each well in a 96-well plate was measured using Image J software. HCS is an automated technique that uses fluorescence microscopy to produce quantitative data.

Keywords: Dithiothreitol; Fluorescence intensity; High content screening; Keratitis; Propidium iodide

1. Introduction

Acanthamoebae are opportunistic protozoan parasites distributed in diverse environments, such as air, soil, freshwater, seawater, tap water, bottled mineral water, laboratory distilled water wash bottles, chlorinated swimming pools and sewage. Fish, reptiles, birds and mammals and are known to be one of the most ubiquitous organisms' host (Khan and Paget, 2002). Although *Acanthamoebae* exist primarily as free-living amoebae, they can infect the eye, brain and skin and can spread haemotogenously to the

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central nervous system (CNS) and various organs. *Acanthamoeba* spp. are dangerous ocular pathogens, particularly in wearers of contact lenses, as shown in a study that found 95% of reported cases involved contact lens wearers (Kilvington et al., 2004). Mazur et al. (1995) reported that the cysts of *Acanthamoeba* can survive in vitro for more than 20 years and under many unfavorable conditions, such as desiccation and starvation. Aksozek et al. (2002) reported that *Acanthamoeba* are resistant to a variety of chemicals, such as disinfectants and antimicrobials, and physical agents, including heat, freezing and UV radiation. The sturdy double-walled cyst consists of cellulose and proteinaceous elements, and a standard positive control with which to ensure the cysticidal effect of chemicals or agents on the *Acanthamoeba* cyst is necessary. A number of antimicrobials have shown efficacy against amoeba in vitro, but there is no evidence that those same drugs will be effective clinically (Schuster and Visvesvara, 2004). In addition, *Acanthamoeba* cysts are more resistant than trophic amoebae, which are sensitive to the antimicrobials used in treating eye infections caused by *Acanthamoeba*.

In the previous research, the fluorescence properties of samarium (Sm) and europium (Eu) have been reported by Usman et al. (2018) and Kusrini et al. (2014), respectively. Preparation of chitosan nanoparticles (CHN NPs) using potassium persulfate has also been reported by Kusrini et al. (2015). Furthermore, biocompatible chitin-encapsulated cadmium sulfide quantum dots (CdS@CTN) that synthesized using the colloidal chemistry method has also been reported by Lim et al. (2021). This CdS@CTN was also screened as antibacterial agent (Lim et al., 2021). This CdS@CTN compound is potential drug carriers and useful in biology, biomedical, fluorescent labelling, and diagnostic applications (Lim et al., 2021). Previously studies can be useful as references for preparation of complexes, nanoparticles and quantum dots.

In the cyst stage, *Acanthamoeba* is hard to eradicate, and its standard viability quantifications remain problematic. Cyst viability determination is necessary to establish the effectiveness of any substance on *Acanthamoeba* in the cyst stage. The potential of these substances as drugs to fight the diseases caused by *Acanthamoeba* at the cyst stage can be determined by the method presented in this study. In previous work by Lazuana et al. (2019) described the application of of trypan blue dye and a haemocytometer to evaluate the number of cysts affected by the cellulase enzyme. Assessing cell viability based on cell wall integrity and discriminating through staining with propidium iodide (PI) intensity in vitro is preferably because it allows for the reading of absorbance changes without bias. Absorbance detection using an enzyme-linked immunosorbent assay (ELISA) reader and HCS can be combined as an effective and simple method of determining the cytotoxicity of treatments for *Acanthamoeba* cysts. The data from this study show a reduction in *Acanthamoeba* cyst viability after treatment with DTT (also called Cleland's reagent) when analyzed using HCS and fluorescence intensity. To our knowledge, this is the first published study on *Acanthamoeba* cyst cytotoxicity induced by DTT in which viability was assessed by the amount of leakage from the endo- and ecto-cyst walls caused by DTT. This study can be useful as a reference for future research on *Acanthamoeba* cyst viability. These data will benefit the protozoologist, pharmacologist, cell biologist and microbiologist interested in studying the mechanism of cysticidal agents on *Acanthamoeba* cysts. The method described in this report can facilitate the study of *Acanthamoeba* cyst viability, as the mechanism of cysticidal activities is currently difficult to determine. The efficacy of this method was supported in this study by using it on images of increasing intensity and with increasing concentrations of the death agent.

2. Materials and Method

2.1. Acanthamoeba Cysts

Acanthamoeba sp. is well known for its dormant cyst form that is highly resistant to exposure to drugs or anti-amoebic compounds (Anwar et al., 2018). The experiments were performed using a 96-well plate with the initial number of *Acanthamoeba* trophozoites at 1×10^5 cells/mL. The cultured *Acanthamoeba* trophozoites were induced into cyst form by replacing the protease-yeast-glucose (PYG) medium (Nakisah et al., 2012) with 150 µL distilled water. Propidium iodide (PI) was then added, the intensity of each well was recorded and images were captured using HCS.

2.2. DTT Treatment

After complete transformation into cyst form, the *Acanthamoeba* were treated with DTT at various concentrations. The DTT was dissolved in sterile distilled water and diluted in a two-fold serial dilution, resulting in various concentrations ranging from 2.5 to 0.039 µM. DTT is a reducing agent that attacks the disulfide bond of protein molecules, which is the primary component of a cyst cell wall and causes them to disrupt (Tihonov et al., 2016). The experiment was done with five replicates and repeated three times.

2.3. Determination of Acanthamoeba Cyst Viability

After 24 hours of treatment with DTT, the cyst was stained with PI at 10 µg/mL in phosphate buffer saline (PBS). The plate was incubated for 10 minutes in the dark. Following that, the absorbance for each well of the plate containing PI stained -cyst of *Acanthamoeba* was examined. Subsequently, an image of the cyst population in each well was captured, and the intensity of each well was recorded using a HCS machine (Molecular Devices, San Jose, CA) at 636 wavelength emissions to validate the cyst population intensity. Red-colored cysts in the image captured by HCS indicate a non-viable cell or an *Acanthamoeba* cyst with a damaged membrane. Image J software was used to measure the intensity of the cyst population in the 96-well plate stained with PI dye. Then, the cyst population intensity was recorded on a plotted graph of cyst viability versus concentration of DTT to compare the trend of cell viability. The IC50 values obtained were used for further cytotoxicity analysis. Scheme for illustration of Acanthamoeba cyst viability determination with a 96-well plate (1), staining with PI dye (2), and staining identifying non-viable cysts (3) is shown in Figure 1.

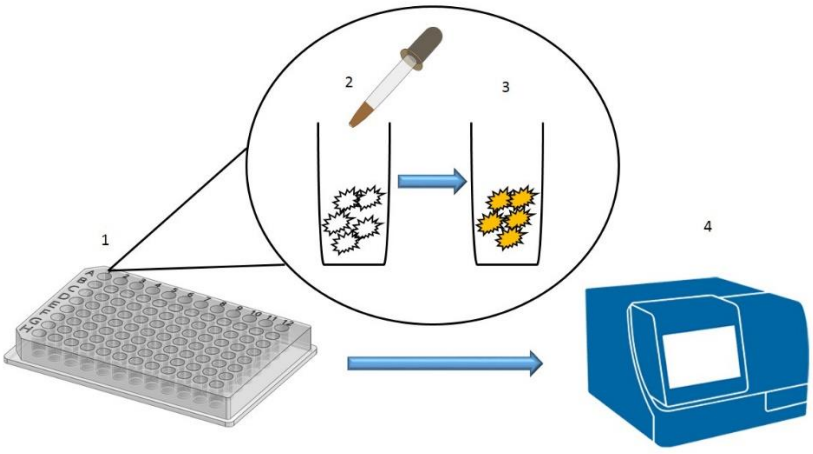


Figure 1 Work scheme for *Acanthamoeba* cyst viability determination with a 96-well plate (1), staining with PI dye (2), and staining identifying non-viable cysts (3). Absorbance was detected at 636 nm using an ELISA reader (4).

3. Results and Discussion

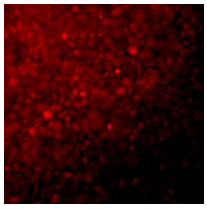
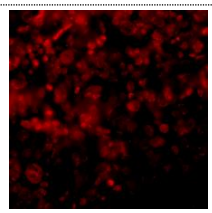
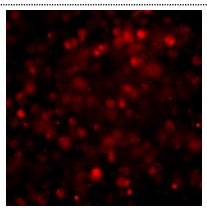
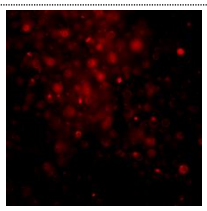
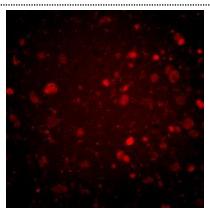
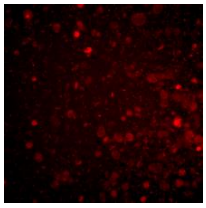
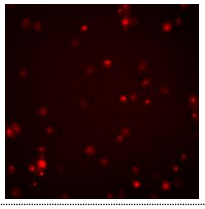
Viable (intact plasma membrane) and dead (damaged plasma membrane) of cultured eukaryotic cells can be identified using differential staining: Cells with disturbed plasma membrane (PM) permeability appear stained, whereas undamaged (viable) cells do not, as the PI dye does not penetrate the plasma membrane. The most frequently used dye for exclusion testing is trypan blue. However, PI becomes highly fluorescent after entering the cell and can be used in the same manner. Stained and unstained cells are counted using a standard microscope or flow cytometry. Experiments on *Acanthamoeba* cell membrane integrity at the trophozoite stage have been performed in numerous studies, such as in Kusrini et al. (2020), Kusrini et al. (2018), Hashim and Amin (2017) and Kusrini et al. (2016). In previous work by Kusrini et al. (2020) described diaqua-bis(picrato)(tetraethylene glycol)cerium(III)picrate complex with chemical formula of $[Ce(Pic)_2(H_2O)(EO4)](Pic).H_2O$ against *Acanthamoeba* sp. showed an excellent antiamoebic activity with IC50 values of 3 µg/mL with significant decrease ($P<0.05$) in *Acanthamoeba* sp. viability when the concentration was increased from 0 to 30 µg/mL. Kusrini et al. (2018) reported that the [bis(picrato)(pentaethylene glycol)samarium(III)] picrate complex with chemical formula of $[Sm(Pic)_2(EO5)](Pic)$ having the IC50 of 0.7 µg/mL. This value was ~ 10-fold lower than IC50 of the [bis(picrato)(18-crown-6)samarium(III)] picrate complex—referred to as $[Sm(Pic)_2(18C6)](Pic)$ with IC50 of 6.5 µg/mL. These values are smaller than those found for the terbium trinitrate.trihydrate.18-crown ether-6 complex with chemical formula of $Tb(NO3)_3(OH_2)_3.(18C6)$ complex (7 µg/mL) (Kusrini et al., 2016).

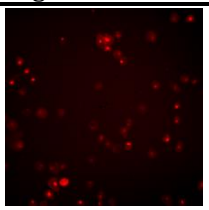
In the present study, PI fluorescence dyes were employed to distinguish between viable and dead *Acanthamoeba* cysts. This article describes a fast, simple and cost-effective method for examining the viability of *Acanthamoeba* sp. cysts (isolated from corneal scrapings from *Acanthamoeba* keratitis patient) following DTT treatment. This is an important assessment for confirming the cytotoxicity of any potential cysticidal agents toward *Acanthamoeba* cysts, as fluorescence excitation from the application of PI can confirm cell membrane leakage. PI is a light sensitive fluorescence impermeable dye, and it confirmed the accumulation of the PI dyes in the *Acanthamoeba* cyst when the image intensity recorded was synchronized to DTT concentration changes.

Table 1 is a summary of our findings, providing the concentration of DTT, images of the *Acanthamoeba* cyst population captured by HCS and the intensity of the cyst population after treatment with DTT. The images in Table 1 show the morphology of healthy, damaged and dead cells. The red fluorescence of the cysts is due to the entry of PI into the internal cyst cytoplasmic region through the injured ecto- and endo-cyst walls. Healthy cells and unstained cysts appear dark because the intact cyst cell wall prevents the penetration of PI into the cells.

The percentage of cell intensity was recorded and plotted on a graph according to DTT concentration. The 50 percent inhibition concentration for the *Acanthamoeb*a population can be used as a reference for further cytotoxicity analysis. This method can be used with a microplate reader at 636 wavelength emission. The IC50 value of the DTT indicates the concentration of the disulfide-reducing agent required to inhibit the specific biological process of *Acanthamoeba*. The images obtained show an increase in color intensity as the concentration of DTT increases. Figure 2 is a plotted graph of cell intensity at various concentrations of DTT analyzed by Graph Pad Prism (version 5.00).

Table 1 Red fluorescent images of *Acanthamoeba* cyst intensity after 24-hour treatment with DTT (2.5 – 0.039 μM). Images were captured of each individual well of a 96-well plate to observe the cell intensity changes after treatment.

DTT Concentration (μM)	Fluorescent Image (magnification 10×)	Intensity (Pixels; Image J)
2.5		3831435
1.25		2048266
0.625		1197840
0.3		1026432
0.15		811393
0.075		413543
0.039		317412

DTT Concentration (µM)	Fluorescent Image (magnification 10×)	Intensity (Pixels; Image J)
untreated		207978

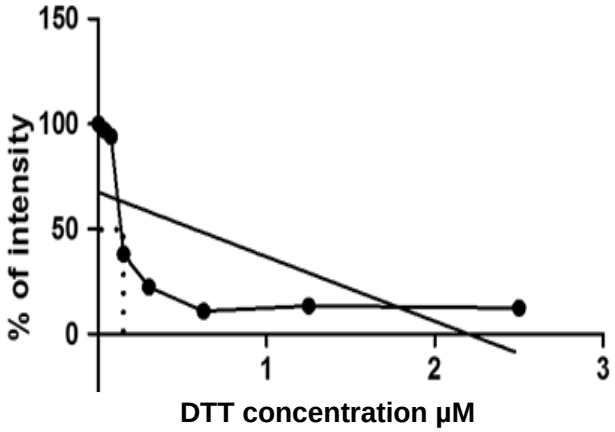


Figure 2 Percentage of *Acanthamoeba* cyst intensity at various DTT concentrations over 24 hours. The graph shows the IC50 value of the DTT, which is 0.1125 µM.

Features distinguishing dead from live cells include loss of transport function across the PM and loss of its structural integrity. Many assays of cell viability have been developed based on changes in the properties of the PM, including necrotic, late apoptotic and mechanically damaged cells (Riedy et al., 1991). Intact PMs of live cells exclude charged cationic dyes such as trypan blue, PI or ethidium bromide. Short incubation with these dyes resulted in selective labelling of dead cells, while live cells showed no dye uptake (Darzynkiewicz et al., 1997), as observed in the live *Acanthamoeba* cysts in the present study.

4. Conclusions

There have been many studies on toxicity to the trophozoite of *Acanthamoeba*, but studies on toxicity to the cyst are limited because a standard method for measuring mortality at the cyst stage was lacking. *Acanthamoeba* cyst viability determination based on membrane integrity, fluorescence intensity and absorbance is a simple, accurate and quick method for assessing *Acanthamoeba* cyst viability. It is hoped that this method will inspire future toxicity studies involving *Acanthamoeba* cysts that will identify potent substances to kill *Acanthamoeba* at the cystic stage.

Acknowledgements

This study was funded by UMT TAPE-RG 55115 (Talent and Publication Enhancement Research Grant). The facilities were supported by the Institute of Marine Biotechnology, Universiti Malaysia Terengganu.

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