

Viability and morphological changes of *Acanthamoeba* spp. cysts after treatment with Effective microorganisms (EM)

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Abstract *Acanthamoeba* is a free-living opportunistic protozoan parasite that is found in diverse environments. It can cause keratitis, mostly related to inappropriate use of contact lenses, as well as life threatening diseases including encephalitis, disseminated sinusitis, and skin ulcers. This study investigated morphological changes and fine structures of the cyst form of *Acanthamoeba* spp. after treatment with effective microorganisms (EMTM) using light and scanning electron microscopies. *Acanthamoeba* cysts treated with 1:2, 1:4, 1:6, and undiluted EMTM showed higher percentages of non-viable cysts than those treated with 1:8, 1:10, 1:100, 1:200, and 1:400 EMTM and at 5 days post-treatment developed from cystic stage to trophozoite stage. *Acanthamoeba* cysts treated at concentrations of 1:2, 1:4, 1:6, and undiluted EMTM exhibited cytoplasmic clumping and shrinkage of amoeba cells away from cyst walls. The effective EMTM concentration lethal

to *Acanthamoeba* spp. cyst could provide information to monitor the environmental control system.

Keywords *Acanthamoeba* spp. · Effective microorganisms · Viability and morphological changes

Introduction

Acanthamoeba are small free-living protozoa which can be existed to trophozoite and cyst forms (Visvesvara 1980). *Acanthamoeba* have the ability to survive in diverse environments and have been recovered from hospitals, dialysis units, eye wash stations, in human nasal cavities, pharyngeal swabs, lungs tissues, skin lesions, corneal biopsies, cerebrospinal fluid (CSF), and during brain necropsies (Meisler et al. 1985). *Acanthamoeba* keratitis has been recognized as a significant ocular microbial infection (Khan 2006). Cysts are resistant to biocides, chlorination and antibiotics (De Jonckheere and van de Voorde 1976; Khunkitti et al. 1998). There is no report that EMTM affect *Acanthamoeba* spp. cysts or trophozoites. The technology of EMTM was developed during the 1970s at the University of Ryukyus, in Okinawa, Japan (Szymanski and Patterson 2003). EMTM is a mixture of groups of organisms and has also been described as a multi-culture of coexisting anaerobic and aerobic beneficial microorganisms. The main species involved in EM include: Lactic acid bacteria; it has also been described such as cleaning septic tanks, algal control, and household uses (Higa and James 1994; Sangakkara 2002; Hirukawa et al. 1998). No such work has, to our knowledge, previously been carried out on any protozoan isolation. Use of EMTM solutions which are effective against a wide spectrum of *Acanthamoeba* strains would be desirable for the environmental contamination to

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contact lens wearers. The present study of lethal effects to the cyst form of *Acanthamoeba* spp. after treatment with EMTM were observed by light microscope using toluidine blue stains and electron microscope which producing various signals that can be detected and that contain information about the cyst's surface topography and composition to detect non-viable and viable cysts and reporting morphological change of cyst in appropriate concentration solutions.

Materials and methods

Acanthamoeba spp. was grown on non-nutrient agar plates enriched with heat-killed *Escherichia coli* (NNA-*E.coli*) for 7 days at room temperature. Cysts in the mature stage were harvested and observed. Normal saline solution was added to disperse the cysts and the suspended *Acanthamoeba* cysts were passed into normal saline solution in plastic tubes. Their amoebicidal effects of EMTM in the cyst assay by adding 200 µl of standard cyst suspension were obtained by resuspending the counted cysts in the normal saline at the final concentration of 1.5×10^6 cysts/mL. The wells were incubated at 37 °C for 24 h, and checked using cell counting slides under light microscope with toluidine blue stain, then the viable cysts (growth development and multiply to trophozoite) and non-viable cysts (no growth development to trophozoite or cell dead) were detected and percentage analyzed which the viable cyst appeared round or oval with double cyst walls, the ectocyst (Ec) and the endocyst (En). The Ec contracted into wrinkle and clearly separated from the En which was thin and smooth while the non-viable cyst exhibited double cyst walls that composed of wrinkle Ec and smooth En walls. Most showed shrinkage of the cytosol, or cytoplasmic clumps (Cy) which no typical nuclei were visible. Some cysts had empty double-walled cysts with a wrinkle Ec and a round En. *Acanthamoeba* trophozoites showed a prominent contractile vacuole (Cv).

EMTM was grown in *E.coli* bacterial culture media in tryptic soy broth (TSB) medium which is a nutritious medium that will support the growth of a wide variety of microorganisms, especially common aerobic and facultative anaerobic bacteria. The culture was washed with Page's amoeba saline and inactivated at 60 °C for 15 min before use. The turbidity of culture suspension was adjusted to be equal to 0.5 McFarland standards (approximately 10^8 CFU/mL). EMTM dilutions were prepared at concentrations of 1:2, 1:4, 1:6, 1:8, 1:10, 1:50, 1:100, 1:200, 1:400, and undiluted. The nine dilutions, as well as control and undiluted wells, were further tested for their amoebicidal effect in the cyst assay by adding 200 µl of the cyst suspension of 1.5×10^6 cysts/mL into each well;

EMTM was changed in the wells every day. The wells were incubated at 37 °C for 24 h and then observed for 7 days and checked microscopically to detect viable cysts daily for *Acanthamoeba* trophozoites (viable) which mostly presented on day 5th by their cyst ability to excyst and multiply, compared with the control plates by means of an inverted microscopy. *Acanthamoeba* cysts were recultured and observed everyday in *E. coli* bacterial culture media in TSB medium as mentioned for 7 days in order to confirm that they were dead.

Procedures for scanning electron microscopy

Cell suspensions of about 5 ml (treated and untreated as control) were centrifuged at 500 g for 2 min and washed twice with amoeba saline solution. The pellets were fixed in 2.5 % glutaraldehyde in sodium cacodylate buffer, 0.1 M, pH 7.4 and dehydrated with ethanol alcohol at 70, 80, 85, 90, and 95 %. The *Acanthamoeba* cyst pellets were dropped on covered glass, subjected to critical point drying, coated with gold, and viewed by scanning electron microscopy (LEO 1450 VP).

Results

The results revealed viable cysts at 27.80, 25.10, 33.60, 71.42, 74.62, 80.96, 78.54, 80.78, and 84.50 %, respectively to the list of EMTM concentrations of 1:2, 1:4, 1:6, 1:8, 1:10, 1:50, 1:100, 1:200, 1:400. The undiluted EMTM solution showed 26.06 % viable cyst and 73.94 % non-viable, while the control wells were not changed (Fig. 1).

The *Acanthamoeba* cysts were approximately 10–25 microm in diameter, round in shape and with a plica-like surface. The viable cysts had a round or oval shape with double cyst walls, i.e., the Ec and the En walls. The Ec contracted into wrinkles and clearly separated from the En which was thin and smooth (Fig. 2a). At day 5th post-treatment it developed to the trophozoite stage (Fig. 2b), and development of cyst to trophozoite stage (Fig. 2c). Cysts with two layers, a thick, wrinkled outer Ec and an inner polygonal En, and a wall pore with operculum were observed. The scanning electron micrographs of the normal cyst showed that the cysts have a round or polygonal shape. The ectocysts were wrinkled with thin high ridges over the surface. Some micrographs showed the operculum and the ostiole (the amoeba emerging through ostioles could turn into trophozoite and left an empty cyst).

Cysts treated with EMTM

Non-viable cysts exhibited double cyst walls composed of wrinkled Ec and smooth En walls were presented at 72.20,

Fig. 1 Percentages of viable assays of cysts treated with different EMTM concentrations

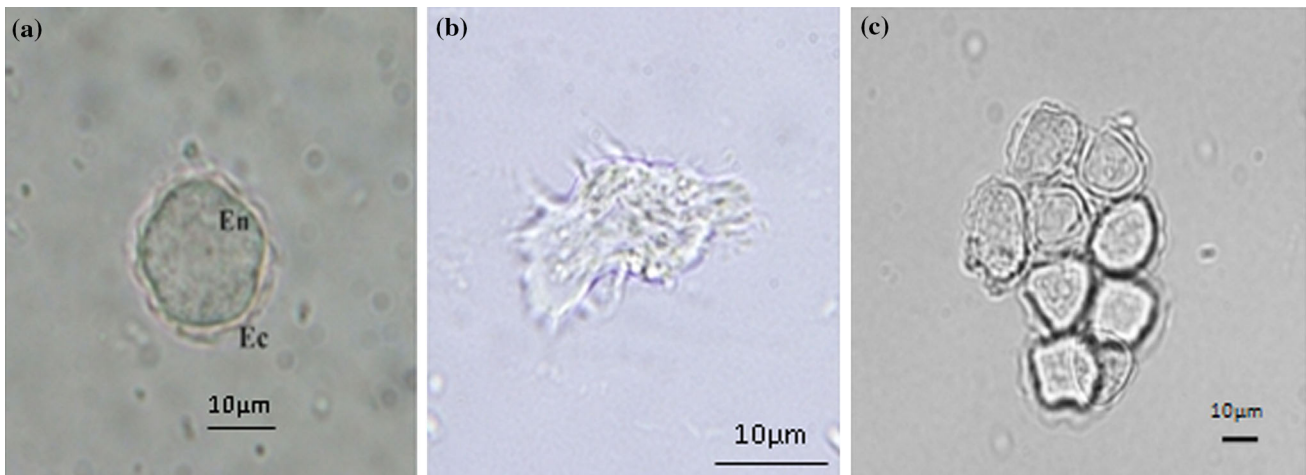
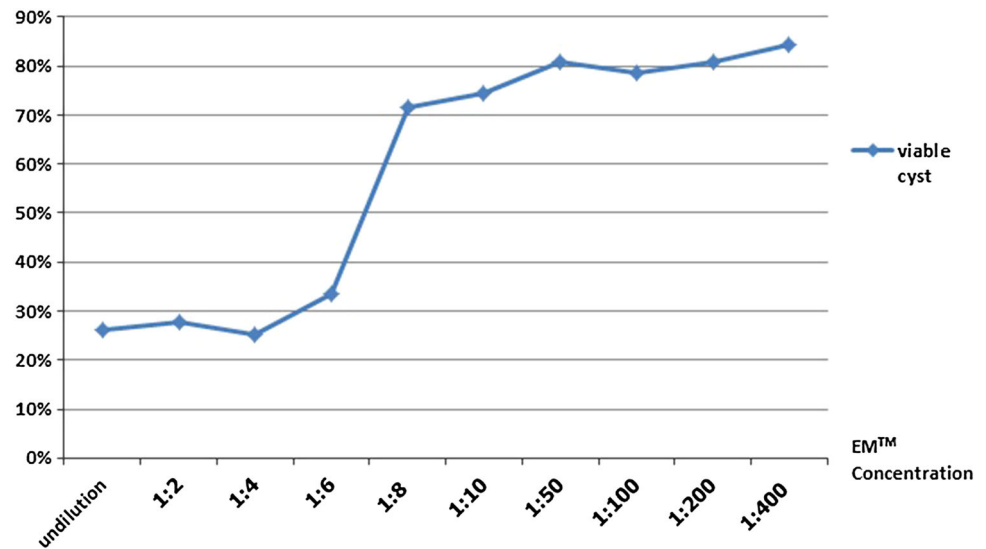


Fig. 2 Light micrograph of a normal *Acanthamoeba* cyst (a), trophozoite (40×) (b), and cysts in a group (c)

74.90, 66.40, 28.58, 25.38, 19.04, 21.46, 19.22 and 15.50 %, respectively to the list of EMTM concentrations of 1:2, 1:4, 1:6, 1:8, 1:10, 1:50, 1:100, 1:200, 1:400. Most non-viable cysts exhibited shrinkage of the cytosol or Cy and no typical nuclei were visible. Some cysts had empty double-walls with wrinkled Ec and round En (Fig. 3a–b). *Acanthamoeba* trophozoites showed a prominent Cv (Fig. 3c). Non-viable cysts appeared in 1:2, 1:4, 1:6 wells.

Viable cysts were round or oval with double cyst walls (Ec and En). Ectocyst wall contracted into wrinkles and were clearly separated from En wall which was thin and smooth. Viable cysts appeared in 1:8, 1:10, 1:50, 1:100, 1:200, 1:400 and undiluted wells (Fig. 3a).

The scanning electron micrographs of cysts treated with EMTM at concentrations of 1:2, 1:4, 1:6, and undiluted showed non-viable cysts which appeared as flat oval cysts with shrinkage and collapse of Ec walls. Some of these flat oval cysts had thick ridges on the wrinkled surface and

some had a decreased wrinkled surface. The viable cyst characteristics were similar in appearance to those of untreated cysts (Fig. 4a–c).

The scanning electron micrographs of the treated cysts showed that they were round or polygonal in shape. Ectocysts were wrinkled with thin high ridges on the surface. The operculum and ostiole were shrunken and narrow (Fig. 4d–e).

Discussion and conclusion

Acanthamoeba spp. is among the most prevalent protozoa found in the environment (Callicott 1968; Cerva and Novak 1968). They are distributed worldwide and have been isolated from soil, dust, air, natural and treated water, seawater, swimming pools, sewage, sediments, air-conditioning units, domestic tap water, drinking water treatment

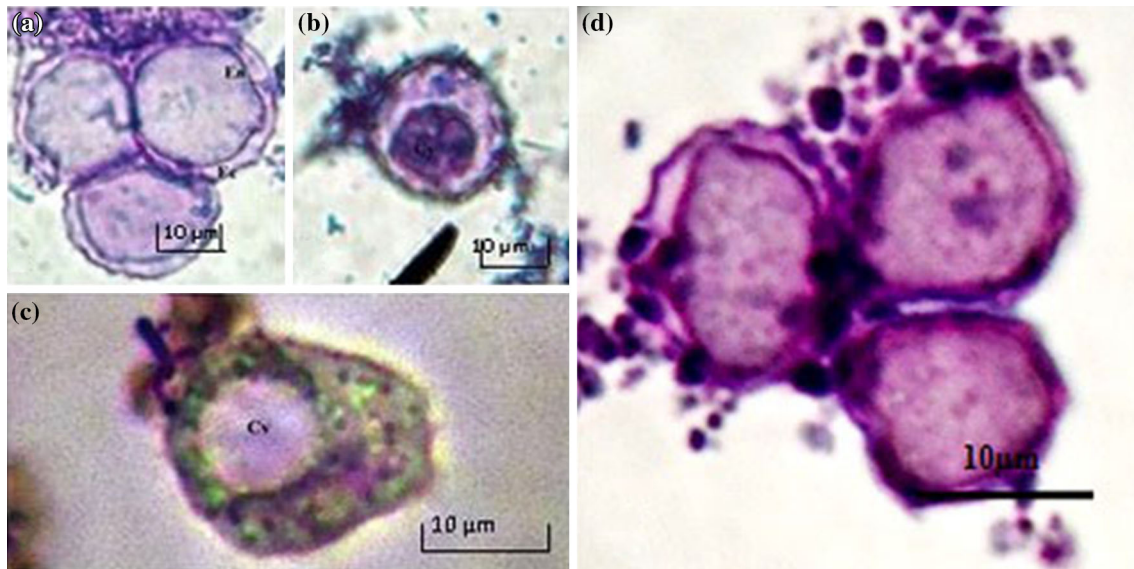


Fig. 3 Light micrograph of *Acanthamoeba* cyst after being treated with EMTM for each concentration: viable cyst (a), non-viable cyst (b), and trophozoite (40×) (c). *Acanthamoeba* viable cyst after

treatment with EMTM at 1:8, 1:10, 1:50, 1:100, 1:200, and 1:400 concentrations were observed (d)

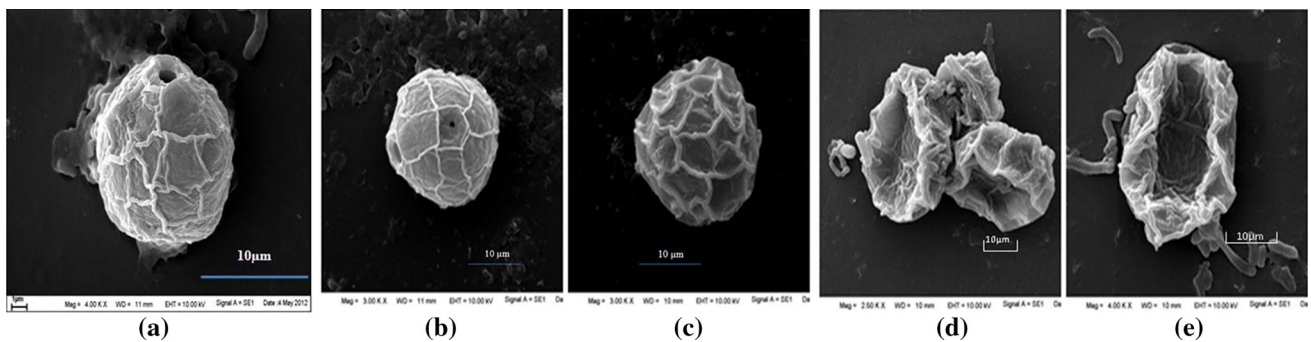


Fig. 4 Scanning electron micrograph of a normal *Acanthamoeba* cyst (a–c), top view and side view of a normal *Acanthamoeba* cyst. The ectocyst (Ec) exhibits a typical wrinkled appearance. Non-viable cyst with shrinkage, wall rupture, and decreased wrinkles at

mag = 2.50 KX, and mag = 4.00 KX, were observed, while the treated cysts with round or polygonal in shape. Ec were wrinkled with thin high ridges on the surface. The operculum and ostiole were shrunken and narrow (d–e)

plants, bottled water, dental treatment units, hospitals and dialysis units, eyewash stations, and contact lenses and lens cases and as contaminants in bacterial, yeast, and mammalian cell cultures (Fowler and Carter 1965). Cysts possess pores known as ostioles, which are used to monitor environmental changes. The trophozoites emerge from the cysts under favourable conditions leaving behind the outer shell and actively reproduce, thus completing the cycle. Cysts are airborne, which may help spread *Acanthamoeba* in the environment and/or carry these pathogens to the susceptible hosts. Several studies report that cysts can remain viable for several years while maintaining their pathogenicity, thus presenting a role in the transmission of *Acanthamoeba* infections (Martinez and Visvesvara 1997). A double-walled wrinkled cyst composed of an Ec and an En ranges in size from 13 to

20 µm and varies from species to species (Bowers and Korn 1968; Bowers and Korn 1969). Cyst formation occurs under adverse environmental conditions such as food deprivation, desiccation, and changes in temperature and pH (Byers 1979). Excystation stage occurs when trophozoites emerge from the cyst under suitable environmental conditions. In this study, mostly *Acanthamoeba* cysts at day 5th after cultured treatment with EMTM were developed and visible to be trophozoites by light micrographs, the viable cyst appeared round or oval with double cyst walls, the Ec and the En walls. The Ec appears wrinkled and clearly separated from the En which was thin and smooth. The non-viable cyst exhibited double cyst walls composing of wrinkled Ec and smooth En walls. Most showed shrinkage of the cytosol, or Cy while no typical nuclei were visible.

Acanthamoeba spp. has the ability to survive in diverse environments. However, the present study was undertaken to explain the mechanism of destroying cysts by EMTM. Non-viable and viable cysts exhibited different morphological changes according to exposure to EMTM, which can be lethal to the cyst form of *Acanthamoeba* spp. EMTM at high concentrations was more effective than at lower concentrations. Effective EMTM concentrations could be used to modify environmental contamination by *Acanthamoeba* management in solid and water sources. The establishment of the Saraburi Kyusei Nature Farming Center in 1988 also marked the start of EMTM propagation in Thailand and the other countries all over the world. EMTM is being applied in agriculture animal husbandry and shrimp cultivation. Similarly EMTM is used in a variety of areas such as environmental education, water treatment and rural development projects of the Thai Royal Army.

The results of this experiment may be useful in supporting the performance of EMTM during the encystment process of *Acanthamoeba* spp. The appropriate EMTM concentration lethal to the cyst form of *Acanthamoeba* spp. could provide information to develop a simple but effective solution for monitoring environmental control programs. However, the most proper concentration of EMTM in water solution is below ratio 1:6 may be appropriate effect and lethal to the *Acanthamoeba* spp. cyst in environmental treatment.

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