**Original Article**

# A new product, BioCool®, to combat free-living amoebae in water

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***Acanthamoeba* is a suitable model to study eukaryotic cells as regards disinfectants, survival and growth. It could be a general model for such studies and can represent several eukaryotic human parasites. In the present study a new disinfectant product BioCool® was tested for ability to kill *Acanthamoeba castellani* a free-living amoebae present in the environment. By count of the viable cells 2% of final concentration of BioCool®substance inhibits viability of *A castellani,* after 1, 2 and 24 hour according to the *p* value of t-test which was 0.002, 0.049 and 0.001 respectively.**

***Key words*: *water, amoeba, parasits***

## Introduction

Bacteria, viruses and yeasts can be found as endosymbionts of amoebae (1). An endosymbiont is an organism that lives inside the body of another organism. Some can be called obligate endosymbionts, since neither the host nor the endosymbiont will survive independently of each other. For *Acanthamoebae* the precise role of endocymbionts is unclear. For example, it is not known whether the amoebae gain from being a host to bacteria, or whether *Acanthamoebae* pathogenicity is enhanced by endosymbionts (2). The ability of *Acanthamoebae* to act as host for a number of bacteria, including human pathogens, has been established (3). If bacteria are present in the amoeba as well as being able to multiply, the amoeba can be described as a reservoir (2). The interaction between bacteria and amoeba takes place for different reasons. For the amoeba, the search for food, *i.e.* bacteria, is indubitably a factor. Bacteria, on the other hand, may use the amoeba host as shelter during harsh environmental conditions to survive before and during transmission from one host to another (4). It has been noted that during intracellular growth, bacteria change properties, becoming less sensitive to antibiotics and capable of better environmental survival and increased virulence (5-8).

*Acanthamoe abae*are free-living amoebae distributed worldwide, and are among the most prevalent protozoa found in the environment (2, 9-12). These amoebae can survive in a variety of environmental conditions and have been isolated from public water supplies, swimming pools, bottled water, seawater, pond water, stagnant water, freshwater lakes, salt water lakes, river water, distilled water bottles, ventilation ducts, the water-air interface, air-conditioning units, sewage, compost, sediments, soil, beaches, vegetables, air, surgical instruments, contact lenses, dental treatment units, hospital and dialysis units and mammalian cell cultures (9, 13-21). *Acanthamoeba* spp. has also been isolated from the nasal mucosa and throat of apparently healthy humans, from infected brain and lung tissue, from skin lesions of immunocompromised patients and from corneal tissue of patients with keratitis (9, 22-27).

The life cycle of *Acanthamoeabae* consists of two stages: a vegetative, dividing trophozoite stage and a dormant, protective cyst stage. The trophozoites are of various sizes, ranging from 25 to 40 μm in diameter. The trophozoites have spine-like structures known as acanthopodia on their surface. Important functions of these acanthopodia include adhesion to surfaces, cellular

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movements and capturing prey. During the trophozoite stage, *Acanthamoebae* actively feed on bacteria, algae, yeasts or small organic particles and several food vacuoles can be seen in the cytoplasm of the cell. *Acanthamoebae* divide asexually by binary fission, and can be maintained in the trophozoite stage with an adequate food supply, neutral pH, appropriate temperature (30 ºC) and an osmolarity between 50- 80 mOsmol. Harsh conditions such as lack of food, hyper- or hypo-osmolarity and extremes in pH and temperature induce the transformation of trophozoites into cysts (28). The cysts are normally 12-20 µm in diameter. Such cysts can be airborne, consequently promoting the spread of *Acanthamoebae* in the environment. The *Acanthamoeba* cyst can remain viable for several years, due to the fact that the walls of the cysts contain cellulose accounting for 10% of the total dry weight of the cyst, rendering them impenetrable (29). The cysts contain pores known as ostioles, which are used to monitor environmental changes, and trophozoites emerge from the cysts under favourable conditions, leaving behind the outer shell (30-32).To harmonise with previous test in which a model bacterium was tested (33), a eukaryotic model microorganism was used.

## Material and Methods

***Acanthamoeba castellanii***(ATCC 30234) has been obtained from the American Type Culture Collection, 10801 University Blvd. Manassas, VA 20110-2209. USA and will be used as control for amoebae. **Substance:** The test substance was obtained from the company BioCool AB, Skellefteå, Sweden, hereafter eferred to as the test substance. The test substance has been described on the open market as BioCool ®. The mechanism by which the microorganism is killed is a chemical reaction resulting in singlet oxygen. Singlet oxygen is known from other biological systems to have killing effect on microorganisms(34). The substance has previously been tested and found to kill *Escherichia coli*(33). The governmental own company FOA-test performed the test. In present study test substance was tested at concentration of 2% and 4% final concentration. The substance was added to a solution of amoebae at an initial concentration of one million per ml.

**Microscopy analysis.** *A. castellanii* cells was counted in a Bürker chamber (Merck Eurolab) under a light microscope (Zeiss, Carl Zeiss)using erythrosine B stain (ATCC), which stains dead amoebae only. To examine the abundance and distribution of bacteria in *A. castellanii* cells, 2-ml samples of cell suspension from each co-culture flask will be diluted in 8 ml PBS and centrifuged for 10 min at 300x g in Labofuge GL centrifuge from VWR International. The resulting pellets were prepared for microscopy by scraping part of pellet on a slide following fixation for 1 min in methanol. The slides was stained with 10% Giemsa stain in PBS for 10 min prior to examination by microscopy to determine the localisation of bacteria in *A.castellanii* cells.

**Statistical analysis.**Chi-square test used to examine for significant differences in growth between control, 2% and 4% of the substance.

## Results.

### Effect of the test substance on viability of *Acanthamoeba*

By count of the viable cells 2% of final concentration of substance inhibits viability of ***Acanthamoeba castellanii*** after 1, 2 and 24 hour according to the *p* value of t-test which was 0.002, 0.049 and 0.001 respectively. Effect of 4% was not statistically significant on the viability of ***A*.castellanii** after 1 and 2 hour incubation. However, the effect was statistically significant after 24 hours of incubation since *p* value of t-test was 0.001(table 1). Lower concentration of chemicals down to 0.02 % killed amoebae although at a much slower time frame. At lower concentrations of amoebae all amoebae were killed and no detectable amoebae could be found after 1 hour (data not shown). In conclusion a concentration of 2% of the substance is proper to significantly rapidly kill this eukaryotic parasite.

## Discussion

The test substance obtained from BioCool® has previously been tested for killing of the bacterium *E.coli* with significant killing results (33). The tests were performed by FOA-test a governmental owned organisation. It was found that bacteria were rapidly killed but no comments on the biological action were given in the report (33). It is known by personal communication with the owner of BioCool® that the action of the substance is release of $H_2O_2$ and breakdown of this molecule to active $O_2$cleavage, which is generally known to kill microorganisms (34) including waterborne parasites such as *Cryptosporidium*(35). *Cryptosporidium* is also heat sen-

**Table 1 Effect of 2% and 4% of the test substance on viability of *A. castellanii.* By count of the viable cells, 2% of final concentration of substance inhibits viability *of A. castellanii*after 1, 2 and 24 hours. No significant effect was seen by use of 4% after 1 and 2 hours but after 24 hours a significant decrease in viable microorganisms was disclosed.**

| Hours | Control | 2% substance | *P*- value | 4% substance | *P*-value |
|---|---|---|---|---|---|
| 1 | 5.1E+05 ± 5.0E+04 | 2.2E+05 ± 3.2E+04 | 0.002 | 4.5E+05 ± 4.2E+04 | 0.223 |
| 2 | 6.5E+05 ± 2.0E+05 | 1,5E+05 ± 3.1E+04 | 0.049 | 3.2E+05 ± 2.0E+04 | 0.106 |
| 24 | 2.0E+05 ± 2.0E+04 | 2. 7E+04 ± 1.2E+04 | 0.001 | 3.0E+04 ± 1.0E+04 | 0.001 |

sitive (35), which not cyst forming amoebae are. Therefore *Acanthamoeba* with its connection to water in nature seems to be an excellent and relevant eukaryotic model for this study. The test substance at a concentration of 2% significantly killed *Acanthamoeba* within 1hour and at a concentration of 4% a significant killing was reached after 24 hours. It can be speculated that water could obtain a non-pleasant smell and taste, which is not common and has not been reported, from the use of 2% chemicals the amount of chemicals can be decreased to a hundred times less. By doing this it must be considered that the time for action of chemicals must be extended. Nevertheless, compared to other chemicals such as chlorine or silver for disinfecting water from amoebae (2) the test substance has advantages form an environmental point of view. From this it can be concluded that the test substance can be utilized to kill parasites of eukaryotic nature in normal drinking water.

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