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# MOLECULAR DETECTION OF VIBRIO CHOLERAE AND PROTOZOA FROM WATER AND INTERACTION OF V. CHOLERAE CLINICAL ISOLATES WITH ACANTHAMOEBA CASTELLANII

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# Molecular detection of Vibrio cholerae and protozoa from water and interaction of V. cholerae clinical isolates with Acanthamoeba castellanii

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By


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To my Family
In the memory of my mother

# ABSTRACT

Microbial contamination of water is a major public concern due to outbreaks of cholera, giardiasis and cryptosporidiosis. Despite this, a large number of bacteria, protozoa and viruses can be found in water. This thesis analysed and identified protozoal species and *Vibrio cholerae* in water samples from Sudan and investigated the interactions of Swedish clinical isolates of *V. cholerae* with the free-living amoeba *Acanthamoeba* species. The effect of outer membrane protein A (OmpA) and vesicles on the survival of *V. cholerae* was also investigated, using molecular methods, cell culture, viable cell count, gentamicin assay, vital staining, light and electron microscopy and statistical analysis.

Analysis of natural water samples collected from different cholera endemic areas in Sudan showed that most *V. cholerae* bacteria occurred together with *Acanthamoeba* species in the same samples. The percentage of samples containing *V. cholerae* only was 11% and that of *V. cholerae* together with Acanthamoeba was 89%. Moreover, sequencing identified 66 protozoa species, of which 19 (28.8%) were amoebae, 17 (25.7%) apicomplexa, 25 (37.9%) ciliates and 5 (7:6%) flagellates.

Examination of the role of OmpA and outer membrane vesicles (OMVs) in survival of *V. cholerae* alone and during its interaction with *A. castellanii* showed that OmpA suppressed survival of wild-type *V. cholerae* cultivated alone. Co-cultivation with *A. castellanii* enhanced survival of wild-type and *OmpA* mutant strains. However, in co-cultivation, OmpA had no effect on attachment, engulfment or intracellular growth of *V. cholerae* interacting with the amoeba, although the *OmpA* mutant released more OMVs and inhibited viability of the amoeba more than the wild type. Surprisingly, treatment of amoebal cells with OMVs isolated from the *OmpA* mutant significantly decreased their viable counts.

The Swedish clinical isolates *V. cholerae* O3, *V. cholerae* O4, *V. cholerae* O5, *V. cholerae* O11 and *V. cholerae* O160 *V. cholerae* were able to grow and survive outside and inside the amoeba and the presence of the amoeba enhanced the survival of all bacteria strains, indicating that all strains can be considered facultative intracellular bacteria. It can be concluded that the presence of *Acanthamoeba* species supports the presence of *V. cholerae*. In co-cultivation, Swedish clinical isolates *V. cholerae* and other strains grew and survived inside *A. castellanii*. The *OmpA* gene of *V. cholerae* suppressed production of OMVs, which decreased the viability of *A. castellanii*. Thus *V. cholerae* might be adapted to survive better in association with eukaryotes and *Acanthamoeba* might be a biological factor enhancing survival of *V. cholerae* in nature.

# LIST OF SCIENTIFIC PAPERS

I. **Shanan S**, Abd H, Hedenstrom I, Saeed A, Sandstrom G. Detection of *Vibrio cholerae* and *Acanthamoeba* species from same natural water samples collected from different cholera endemic areas in Sudan. BMC Res Notes. 2011; 4:109. Epub 2011/04/08

II. **Shanan S**, Abd H, Bayoumi M, Saeed A, Sandstrom G. Prevalence of protozoa species in drinking and environmental water sources in Sudan. BioMed research international. 2015;2015:345619. Epub 2015/03/20.

III. Valeru SP, **Shanan S**, Alossimi H, Saeed A, Sandstrom G, Abd H. Lack of outer membrane protein A enhances the release of outer membrane vesicles and survival of *Vibrio cholerae* and suppresses viability of *Acanthamoeba castellanii*. International Journal of Microbiology. 2014; 2014:610190. Epub 2014/05/07

IV. **Shanan S**, Magdi Bayoumi, Amir Saeed, Gunnar Sandström and Hadi Abd. Swedish isolates of *Vibrio cholerae* enhance their survival when interacted intracellularly with *Acanthamoeba castellanii* (Manuscript)

# LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THESIS

I. **Salah Shanan**, Samia A. Gumaa, Gunnar Sandström and Hadi Abd. Significant association of *Streptococcus bovis* with malignant gastrointestinal diseases. International Journal of Microbiology Volume 2011, Article ID 792019, 5 pages doi:10.1155/2011/792019.

II. Hadi Abd, **Salah Shanan**, Amir Saeed and Gunnar Sandström. Survival of *Vibrio cholerae* inside *Acanthamoeba* and detection of both microorganisms from natural water samples may point out the amoeba as a protozoal host for *V. cholerae.* J Bacteriol Parasitol 2011, S1 http://dx.doi.org/10.4172/2155-9597.S1-003.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| CTX | Cholera toxin |
| TCP | Toxin co-regulated pilus |
| US | United States |
| TTSS | Type three secretion system |
| OmpA | Outer membrane protein A |
| LPS | Lipopolysaccharide |
| OMVs | Outer membrane vesicles |
| FLA | free-living amoebae |
| UV | Ultra violet |
| LB | Luria-Bertani |
| TBE | Tris-Borate/EDTA |
| bp | base pair |
| PBS | Phosphate-buffered saline |
| DNA | Deoxyribonucleic acid |
| RNA | Ribonucleic acid |
| PCR | Polymerase chain reaction |
| ATCC | American Type Culture Collection |
| PBS | Phosphate buffered saline |

# 1 INTRODUCTION

*Vibrio* is a genus of Gram-negative bacteria found in water. The genus comprises nearly 70 species such as *V. mimicus*, *V. vulnificus* and *V. cholerae* (1). *Vibrio cholerae* species are straight or curved rods widely distributed in aquatic environments (2). Evidence suggests that *V. cholerae* is a component of the autochthonous flora of brackish water, estuaries and salt marshes in coastal areas of the temperate zone, posing an ongoing hazard to public health (3, 4). Various *V. cholerae* O1 strains have become endemic in many regions in the world, including Australia and the Gulf Coast region of the United States (5, 6). Such cholera outbreaks are thought to have resulted from consumption of raw, undercooked, contaminated or re-contaminated seafood, since *V. cholerae* is transmitted primarily by the faecal-oral route, indirectly through contaminated water supplies (4, 7-10). Food supplies may be contaminated by the use of human faeces as fertiliser or by freshening vegetables for market with contaminated water (4, 7, 8, 11). Any infected water and any foods washed in that water, as well as shellfish living in the affected waterway, can cause an infection. The causative agent of cholera is rarely spread directly from person to person.

*Vibrio cholerae* occurs naturally on the zooplankton of fresh, brackish and salt water, attached primarily to their chitinous exoskeleton (12). The bacterium has been isolated routinely from many aquatic environments throughout the world, often in association with plankton, plants, invertebrates and fish, and there are some reports of its presence in water birds, seals and diseased farm animals (13).

*Vibrio cholerae* species are subdivided into 206 serogroups (14), but only *V. cholerae* O1 and *V. cholerae* O139 produce cholera toxin and cause cholera. The other serogroups can cause vibriosis symptoms such as gastroenteritis, open wound infections and septicaemia. Vibriosis is increasing globally, with an estimated 80,000 cases and 300 deaths annually in the United States (15). Epidemic and pandemic cholera affects many millions annually, creating a worldwide health problem (5, 16-18).

*Vibrio* species can produce multiple extracellular cytotoxins and enzymes that are associated with extensive tissue damage and which may play a major role in the development of sepsis (19). The major virulence factors of *V. cholerae* are cholera toxin (CT) and an intestinal colonisation factor known as toxin co-regulated pilus (TCP). *Vibrio cholerae* responds to environmental changes by altering the protein composition of its outer membrane (OM), which is composed of protein and lipopolysaccharide (LPS) (20, 21). Outer membrane protein A (OmpA) is a β-barrel protein in the membrane and is highly

conserved among Gram-negative bacteria (22). It is expressed to very high levels and is tightly regulated at the post-transcriptional level. It can function as an adhesin and invasin, participate in biofilm formation, act as both an immune target and evasin, and serve as a receptor for several bacteriophages (23, 24). It has been shown that *Escherichia coli* utilises OmpA for adhesion to Hela epithelial cells and Caco-2 colonic epithelial cells (25).

Outer membrane vesicles (OMVs) are produced by most Gram-negative bacteria, including *Vibrio* species (26). The vesicles contain outer membrane proteins, lipopolysaccharides and phospholipids and, as they are being released from the surface, the vesicles entrap some of the underlying periplasm. They can deliver toxins and other virulence factors to the host at relatively high concentrations, without requiring close contact between the bacterial and target human cells, and are believed to represent a key factor in effecting an inflammatory response in the host to bacterial pathogens (26-31).

OMVs are released by Gram-negative bacteria as a novel stress response (29, 32), whereas Omps play a major role in adherence to mucosal membrane in the small intestine and possible protective antigens (29).

Despite the fact that amoebae are significant predators of bacteria in soil and aquatic environments (33), many biological factors affect the survival of *V. cholerae* in aquatic environments, such as loss to predators (34), regulating the level of viable cells of *V. cholerae* (35) able to infect humans.

The bacteria need a biological reservoir as a training ground in order to grow and survive in concentrations high enough to infect humans. It has been stated that humans are the only known hosts and reservoirs for *V. cholerae* outside its aquatic environment (36), but based on the need for high numbers of bacteria to cause infection, it seems likely that the bacterium has an environmental host besides humans and that such a host may support growth and survival of the bacterium in nature. Finding aquatic reservoirs for *V. cholerae* is an important factor in the epidemiology of cholera (reviewed in (37).

The protozoa are eukaryotic cells distributed worldwide in nature and are receiving increasing attention as human and animal pathogens and potential vehicles for the transmission of bacteria in the environment. Unfortunately, around 50% of the world's population is affected by waterborne or foodborne parasites (38). The protozoa are among the most common parasitic pathogens present in environmental samples. They have multi-stage life cycles, consisting of an active trophozoite stage and a resistant stage (oocyst or cyst) excreted in faeces that is capable of infecting new hosts (39).

The protozoa are subdivided into four phyla depending on their method of locomotion. Mastigophora (flagellates) move by using one or more flagella. Sarcodina (amoebae) have extensions of the cytoplasm called pseudopodia that assist phagocytosis and motion in the organisms. Ciliophora (ciliates) move by means of cilia. Sporozoa (apicomplexa) have no locomotion (40).

Over the past few decades, pathogenic enteric protozoa have been increasingly implicated in compromising the health of millions of people, mostly in developing countries. These protozoa contribute significantly to the staggering caseload of diarrhoeal disease morbidity encountered in these regions and are also a significant concern in industrialised countries, despite improved sanitation (41).

*Acanthamoeba* is a genus of free-living amoebae (FLA), which are environmental eukaryotic cells distributed worldwide in nature (42, 43). The life cycle of *Acanthamoeba* includes two stages, a feeding trophozoite and a dormant cyst stage. *Acanthamoeba* supports bacterial growth and survival and saves the bacteria from chlorination (44-46), increasing the risk of human illness caused by bacteria or *Acanthamoeba*. *Acanthamoeba* species are currently playing an increased role as human pathogens, causing encephalitis, keratitis, pneumonitis and dermatitis (47, 48) across the globe, and the infection routes are mostly from the environment.

*Cryptosporidium* species and *Giardia intestinalis* are major pathogens in the waterborne transmission of infections and they are able to persist in the environment due to the robustness of their oocysts and cysts. The occurrence of these microorganisms in different types of water has been confirmed, and a considerable number of waterborne outbreaks has been reported worldwide (49-51).

In Sudan, *Cryptosporidium* species is an important cause of diarrhoea in children, and it is suggested that intrafamilial spread occurs (52). The highest prevalence of diarrhoeal diseases has been recorded among Port Sudan children (15.5%), followed by children where people draw water from unrectified *hafirs* (13.5%), and children where people draw water from rectified hafirs (6.0%) (53). However, there is no information about the presence of protozoa species other than *Cryptosporidium*.

*Vibrio cholerae* and FLA are present in aquatic environments, including drinking water (54-56). The vibrios may be carried by sea-living animals and the combination of increased water temperature and salinity may contribute to increased association rates of the bacteria with sea-living animals or protozoa (57). The ability of FLA to act as reservoirs for many bacteria has been studied (reviewed in (37). However, output of the interaction between

bacteria and amoeba is dependent on whether the interacting bacterium is extracellular or intracellular and on whether it possesses a Type Three secretion system (TTSS), since TTSS effector proteins are observed to strongly affect output of the interaction. The amoeba may become a host or predator to the interacted bacteria but, on the other hand, many bacterial species are able to kill the amoeba (37). In 2004, more than 50 cases of vibriosis were reported in Sweden after exposure to water from the Baltic Sea or swimming outdoors in summer (58-60). In addition, recent studies have shown that *V. cholerae* O1, *V. cholerae* O139 and *V. mimicus* have developed a survival strategy to grow and survive inside *Acanthamoeba* species (44, 61-64).

# 2 AIM OF THE PROJECT

The overall aim of this doctoral project was to study the interaction between *V. cholerae* and protozoa.

**Specific aims were to:**

**Paper I**

- Examine the occurrence of both *V. cholerae* and *Acanthamoeba* species in the same natural water samples through detection by the polymerase chain reaction (PCR) method in 400 water samples collected from different cholera endemic areas in Sudan.

**Paper II**

- Detect protozoa in 600 water samples collected from different cholera endemic areas in Sudan by PCR using primers (P-SSU-342f and Medlin B) and identify species of the protozoa by sequencing.

**Paper III**

- Examine the role and influence of OmpA and OMVs in the survival of *V. cholerae* alone and its interaction with *Acanthamoeba castellanii*.

**Paper IV**

- Investigate the interactions of the Swedish clinical isolates *V. cholerae* O3, *V. cholerae* O4, *V. cholerae* O5, *V. cholerae* O11 and *V. cholerae* O160 with *A. castellanii*.

# 3 MATERIALS AND METHODS

## 3.1 MICROORGANISMS USED IN THE STUDY

The bacterial strains used in this thesis were wild-type *Vibrio cholerae* strain A1552 O1 El Tor Inaba (65) and its *OmpA* mutant by internal in-frame deletion of the *OmpA* gene, kindly provided by Dr. S.N. Wai, University of Umeå, Umeå, Sweden. It has been proven that the *OmpA* mutant bacterium fails to produce OmpA protein, unlike the wild-type strain (23). The clinical isolate strains *V. cholerae* 03, *V. cholerae* 04, *V. cholerae* 05, *V. cholerae* 011 and *V. cholerae* 0160 were obtained from the Public Health Agency of Sweden. *Acanthamoeba castellanii* ATCC 30234 was obtained from the American Type Culture Collection, Manassas, VA, USA.

## 3.2 SAMPLE COLLECTION

A total of 1000 water samples were collected from different places in Sudan previously known as foci of *V. cholerae*. The samples were collected over a period of 10 months, from February to November 2008, in sterile 50-mL tubes and transported to the laboratory at the Microbiology Department, University of Medical Sciences and Technology, Khartoum, Sudan.

The sources of the collected water samples were zeers, hafirs, tanks, lakes and streams in the areas of Gadarif, Khartoum, Kordofan, Juba and Wad Madani. Hafirs (simple dams for storing water) are one of the most common sources of water in peripheral areas and different types of materials (contaminants) accumulate in them (due to erosion), as well as the faeces from wild and domestic animals. Zeers are traditional Sudanese storage jugs made of baked clay, commonly used to keep drinking water.

**For Paper I**, 400 water samples collected from four states in Sudan, Gadarif, Juba, Kordofan and Khartoum. Of these, 128 samples were from zeers (home pots), 167 from hafirs, 66 from water tanks and 39 from lakes.

**For Paper II**, 600 samples were collected from water sources (zeers, hafirs, tanks, lakes and streams) in different areas (Gadarif, Khartoum, Kordofan, Juba and Wad Madani).

## 3.3 DNA EXTRACTION

The 50-mL water samples were centrifuged for 10 min at 4000 rpm. The supernatant was removed and the sediment was used for DNA extraction using routine proteinase K procedures and the Qiagen DNA mini kit (Qiagen, Valencia, CA, USA).

## 3.4 DNA AMPLIFICATION (PAPERS I AND II)

### 3.4.1 Detection of *Vibrio cholerae* and *Acanthamoeba* species from same natural water samples collected from different cholera endemic areas in Sudan. (Paper I)

In this amplification, two reactions were performed. In the first reaction, two primer sets were used, the AcU primer 5`- GGC CCA GAT CGT TTA CCG TGA A-3` and the Ac L primer 5`-TCT CAC AAG CTG CTA GGG GAG TCA-3`. In the second reaction, two primers sets were also used, the VCT-1 primer 5` -ACA GAG TGA GTA CTT TGA CC-3` and the VCT-2 primer 5` ATA CCA TCC ATA TAT TTG GGA G-3`. PCR was carried out for both reactions in a final volume of 20 μL containing each primer at a concentration of 0.3 μM, 1.0x PCR golden buffer, 200 μM deoxyribonucleoside triphosphate, 1.2 mM $MgCl_2$ and 1.25 U/50 μL of Ampli Taq Gold (Sigma, Saint Louis, USA). PCR conditions were: 32 cycles of 95°C (denaturation) for 4 min, 55°C (annealing) for 20 s and 72°C for 10 s (extension).

### 3.4.2 Prevalence of protozoa species in drinking and environmental water sources in Sudan (Paper II)

A protozoa-specific forward primer (P-SSU-342f) and eukarya-specific reverse primer (Medlin B) targeting 18S rRNA gene were used. The forward primer was P-SSU-342f (5′ CTTTCGATGGTAGTGTATTGGACTAC-3′) and the reverse primer was Medlin B (5′-TGATCCTTCTGCAGGTTCACCTAC- 3′ (66) in PCR, to obtain a product of about 1,360 bp. The PCR reaction performed at 95°C for 10 min, 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Final extension was at 72°C for 10 min with 0.4 μM of each primer. The PCR amplification step was carried out and the final PCR reaction mixture was divided into two parts. One part was used for 2% agarose gel electrophoresis with ethidium-bromide staining and the other part was used for DNA sequencing. *Acanthamoeba castellanii* was used as a positive control.

## 3.5 GEL ANALYSIS OF PCR PRODUCT (PAPERS I AND II)

The PCR products were analysed by electrophoresis on agarose gel in 1x TBE buffer (Tri base, boric acid and EDTA), pH 8.0. The gel was stained in 0.1% SYBR Green bath, visualised by UV translumination and photographed using Polaroid films. DNA fragment 487 bp for *Acanthamoeba* was obtained in the first reaction and DNA fragment 370 bp for *V. cholerae* toxin was obtained in the second reaction.

## 3.6 SEQUENCING (PAPER II)

After sample collection, DNA extraction and PCR analysis, direct sequencing of the protozoa 18S rRNA gene was performed using purified nested PCR products. The sequencing was carried out by MWG operon (Ebersberg, Germany). The nucleotide sequences were then edited and aligned using the Blast programme. The sequencing confirmed protozoal species according to the GenBank accession numbers for their nucleotide sequences.

## 3.7 CULTURE MEDIA AND GROWTH CONDITIONS (PAPERS III AND IV)

*Vibrio cholerae* strains were stored frozen in Luria-Bertani (LB) medium with 15% glycerol at -80ºC. Both bacterial strains were grown overnight at 37ºC on LB plates. In order to infect the amoeba, *V. cholerae* were grown in LB broth, with shaking to an absorbance$_{600}$ of 0.6. *Acanthamoeba castellanii* was grown without shaking at 30°C to a final concentration of $10^6$/mL in ATCC medium no. 712.

## 3.8 CO-CULTIVATION (PAPERS III AND IV)

The co-cultivation assay was based on a method presented previously (62). The amoeba was grown at 30°C to a final concentration of $2x10^6$ cells/mL in ATCC medium, as described above. Co-cultivations of *V. cholerae* with *A. castellanii* were incubated in NUNC tissue culture flasks (75 mL) purchased from VWR International (Stockholm, Sweden). Each flask received 50 mL ATCC medium 712 containing *A. castellanii* at a concentration of $2x10^5$ cells/mL and the particular *V. cholerae* species at a concentration of $2x10^6$ cells/mL. Control flasks containing bacteria or amoebae only were prepared in the same way and with the same initial concentration as the co-culture flasks. All flasks were prepared in triplicate and incubated at 30°C. Samples were taken and plated on blood agar plates regularly to study the growth and survival of *V. cholerae*.

Co-cultures of each bacterial strain and *A. castellanii* were incubated in 75 mL cell culture flasks (Corning Incorporated Costar , Sigma, Saint Louis, USA) filled with 50 mL of ATCC medium 712 containing an initial concentration of $10^5$ cells $mL^{-1}$ of *A. castellanii* and $10^6$ cells $mL^{-1}$ of each bacterial strain. Control flasks with bacteria cultured in the absence of amoeba were prepared in the same way and with the same initial concentration as those with amoeba. The flasks were incubated statically at 30°C. Samples were withdrawn regularly for microscopy, cell counts and culturable counts.

## 3.9 BACTERIAL ADHERENCE (PAPER III)

Co-cultures of the *V. cholerae* strains with *A. castellanii* were incubated in 75 mL cell

culture flasks containing 50 mL ATCC medium no. 712 with an initial concentration of $10^5$ cells *A. castellanii*/mL and $10^6$ cells of each *V. cholerae* strain/mL. The flasks were incubated without shaking at 30°C and samples were withdrawn after 1 h to determine the percentage of bacteria adhering to the amoeba cells. This was done by dividing the number of amoeba with adhered bacteria by the total number of amoeba with and without adhered bacteria and multiplying by 100.

### 3.10 BACTERIAL UPTAKE, GROWTH AND INTRACELLULAR SURVIVAL (PAPER III)

The ability of *A. castellanii* to take up *V. cholerae* A1552 strains and the effect of the *OmpA* mutant on uptake and intracellular growth of the bacterial strains were examined by comparing the interactions of wild-type A1552 and *OmpA* mutant with the amoeba.

Co-cultures of each bacterial strain with *A. castellanii* were incubated in 75 mL cell culture flasks containing 50 mL ATCC medium no. 712 with an initial concentration of $10^5$ cells *A. castellanii*/mL and $10^6$ cells each bacterial strain/mL. The flasks were incubated without shaking at 30°C for 2 h. Each cell suspension was centrifuged for 10 min at 300 *g* in a Labofuge GL centrifuge (VWR International, Stockholm, Sweden) and washed six times in PBS to remove non-adhered extracellular *V. cholerae*. The pellets were re-suspended in 1 mL PBS and incubated with 500 mg gentamicin/mL for 1 h at room temperature. The samples were then diluted in 9 mL PBS and centrifuged for 10 min at 300 *g*. Each pellet was re-suspended in a volume of 50 mL ATCC medium in a 75 mL culture flask to analyse uptake, intracellular growth and survival of *V. cholerae* strains. A 1 mL portion from each flask was centrifuged for 10 min at 300 *g* and each pellet was diluted twofold with 0.1% sodium deoxycholate to permeabilise the amoeba cells. A series of 10-fold dilutions of the sample from $10^1$ to $10^{10}$ was prepared and spread on blood agar plates. All plates were incubated overnight at 37°C and viable counts were performed for the engulfed bacteria. The re-culture flasks were incubated without shaking at 30°C to investigate the intracellular growth and survival of *V. cholerae* strains using a gentamicin assay and viable counts for 15 days.

### 3.11 GROWTH OF *V. CHOLERAE* STRAINS IN THE ABSENCE OR PRESENCE OF AMOEBA (PAPER IV)

To estimate the growth and survival of *V. cholerae* strains in the absence or presence of *A. castellanii* by viable counts, 1 mL samples from each bacterial control flask and from flasks containing both bacteria and amoeba were withdrawn. The samples were prepared by 10-

fold dilution from $10^{-1}$ to $10^{-10}$ and spread on blood agar plates. All plates were incubated at 37°C overnight. Thereafter, the numbers of colonies were counted.

### 3.12 GROWTH AND SURVIVAL OF *V. CHOLERAE* STRAINS INSIDE *A. CASTELLANII* (PAPER IV)

To examine the growth and survival of *V. cholerae* strains inside *A. castellanii* cells by viable count assay, 1 mL of cell suspension from flasks each containing one of the bacterial strains and the amoeba were diluted in 9 mL of PBS, centrifuged for 10 min at 300 g and washed three times in PBS to minimise extracellular *V. cholerae* contamination. The pellets were resuspended in 1 mL of PBS and incubated with 500 mg $mL^{-1}$ of gentamicin for 1 h at room temperature. The samples were then diluted in 9 mL of PBS and centrifuged for 10 min at 300 g. A 100 µL portion of each supernatant was spread on blood agar plates, and each pellet was diluted twofold with 0.1% sodium deoxycholate. Series of 10-fold dilutions from $10^{-1}$ to $10^{-4}$ of the sample were prepared and spread on blood agar plates. All plates were incubated at 37°C overnight and viable counts were performed.

### 3.13 GROWTH OF *A. CASTELLANII* IN THE ABSENCE OR PRESENCE OF *V. CHOLERAE* STRAINS (PAPER IV)

*Acanthamoeba castellanii* was grown without shaking at 30°C to a final concentration of $10^6$ cells/mL in ATCC medium. To study the effect of *V. cholerae* on *A. castellanii*, growth of *A. castellanii* in the presence or absence of *V. cholerae* strains was studied by means of viable amoeba cell counts. The initial concentration of the amoeba in the presence or absence of *V. cholerae* strains was $2\times10^5$ cells/mL.

### 3.14 MICROSCOPY ANALYSIS (PAPER IV)

*Acanthamoeba castellanii* cells, in the absence and presence of bacteria, were counted in a Bürker chamber (Merck Eurolab, Täby, Sweden) under a light microscope (Carl Zeiss, Stockholm, Sweden). Eosin staining was used to detect dead amoeba cells, which were stained red, in contrast to the viable amoeba cells, which remained unstained. The intracellular localisations of *V. cholerae* were analysed by electron microscope, for which 5 mL samples from culture flasks containing the amoeba in the presence of bacteria were centrifuged for 10 min at 300 g in a Labofuge GL centrifuge (VWR International). The resulting pellets were washed with PBS. Each pellet of infected amoeba was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.3, with 0.1 M sucrose and 3 mM $CaCl_2$, for 30 min at room temperature. Samples were then washed in sodium cacodylate

buffer and postfixed in 2% osmium tetroxide in the same buffer for 1 h. The samples were centrifuged and the pellets were dehydrated and embedded in epoxy resin, LX-112. The embedded samples were cut into ultra-thin sections, placed on grids and stained with uranyl acetate and lead citrate. Sections were examined with a transmission electron microscope (SEM, Philips 420).

## 3.15 ISOLATION OF OUTER MEMBRANE VESICLES AND ESTIMATION OF PROTEIN CONCENTRATION (PAPER III)

Outer membrane vesicles were isolated by ultracentrifugation, as described previously (31). *Vibrio cholerae* strains were grown in broth culture to late exponential phase. Broth cultures were then centrifuged at 8,000*g* (30 min, 4°C) in a JA-25.50 rotor (Beckman Instruments Inc., Fullerton CA, USA). Filtered (0.22 µm; Millipore) supernatants were centrifuged at 85,000 *g* (2 h, 4°C) in a 70 Ti rotor (Beckman Instruments Inc.) to collect OMVs. Pellets were washed twice with PBS, suspended in PBS to a total volume of 500 µL and used as the OMVs preparation. Concentration of total protein in the OM vesicles was measured spectrophotometrically by the Bradford assay (Bio-Rad, Sundbyberg, Sweden).

The effect of outer membrane vesicles on the viability of *A. castellanii* was examined by incubation of 50 µL of amoeba cell suspension containing $10^6$ cells/mL with 50 µL OM vesicle preparation from each bacterial strain or with 50 µL BPS for controls. Triplicate experiments were performed and the viability of the amoeba was examined after 2 h by viable count utilising erythromycin B stain (ATCC).

## 3.16 STATISTICAL ANALYSIS

The *t*-test and the $\chi^2$ test were performed to search for significant differences between bacteria cultivated/detected in the absence or presence of the amoeba and verify differences in the existence of protozoa in water samples collected from different sources.

# 4 RESULTS

## 4.1 DETECTION OF *VIBRIO CHOLERAE* AND *ACANTHAMOEBA* SPECIES FROM THE SAME NATURAL WATER SAMPLES COLLECTED FROM DIFFERENT CHOLERA ENDEMIC AREAS IN SUDAN

Of the total of 400 water samples examined by PCR to detect *V. cholerae* toxin gene (*toxA*) and *Acanthamoeba* 18S RNA gene, eight water samples (numbers 8, 117, 121, 150, 156, 160, 193 and 213) were found to contain both *V. cholerae* and *Acanthamoeba*. Furthermore, it was found that the number of water samples containing only *V. cholerae* was one (number 54), while 13 samples (numbers 24, 46, 70, 84, 87, 128, 177, 202, 259, 266, 287, 319 and 397) contained *Acanthamoeba* only (Table 1). Representatives of PCR-positive samples are shown in Figure 1.

**Table 1.** PCR results showing all samples testing positive for *Vibrio cholerae* and/or *Acanthamoeba*

| **Sample number** | **Region** | **Source** | ***V. cholerae*** | ***Acanthamoeba*** |
|---|---|---|---|---|
| 8 | Gadarif | Zeer | +ve | +ve |
| 24 | Gadarif | Zeer | -ve | +ve |
| 46 | Gadarif | Hafir | -ve | +ve |
| 54 | Gadarif | Hafir | +ve | -ve |
| 70 | Gadarif | Hafir | -ve | +ve |
| 84 | Gadarif | Tank | -ve | +ve |
| 87 | Gadarif | Tank | -ve | +ve |
| 117 | Gadarif | Tank | +ve | +ve |
| 121 | Gadarif | Tank | +ve | +ve |
| 128 | Gadarif | Tank | -ve | +ve |
| 150 | Juba | Lake | +ve | +ve |
| 156 | Juba | Lake | +ve | +ve |
| 160 | Juba | Lake | +ve | +ve |
| 177 | Juba | Zeer | +ve | +ve |
| 193 | Juba | Zeer | +ve | +ve |
| 202 | Juba | Zeer | -ve | +ve |
| 213 | Khartoum | Lake | -ve | +ve |
| 259 | Khartoum | Zeer | -ve | +ve |
| 266 | Khartoum | Zeer | -ve | +ve |
| 287 | Kordofan | Hafir | -ve | +ve |
| 319 | Kordofan | Hafir | -ve | +ve |
| 397 | Kordofan | Hafir | -ve | +ve |

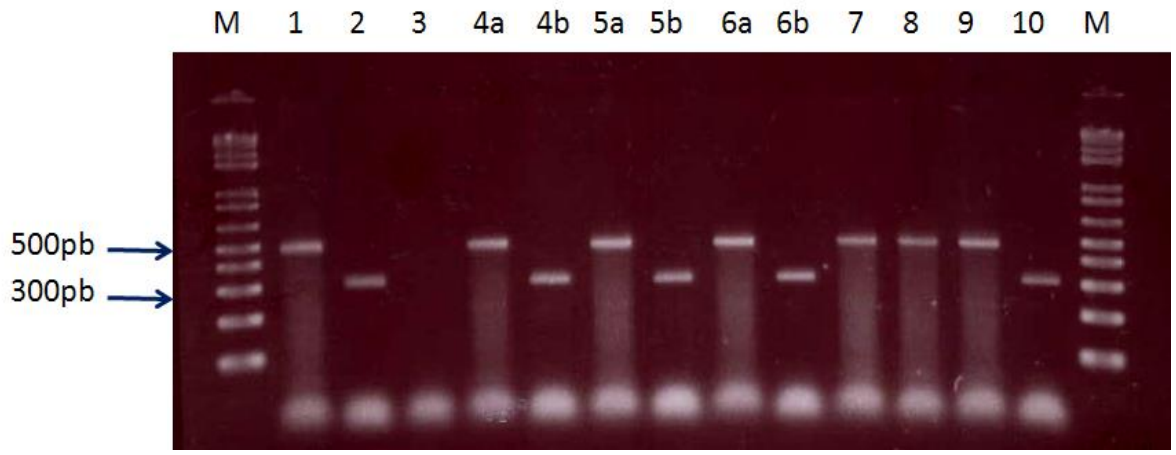


**Fig. 1.** Agarose gel electrophoresis of PCR products of cholera toxin gene (*ctxA*) and *Acanthamoeba* 18S rDNA gene. M are molecular mass markers (1500 bp); lane 1, amoebic positive control (approximately 450 bp); 2, bacteria-positive control (308bp); 3, negative control; 4-6, samples containing both *Acanthamoeba* (a) and *V. cholerae* (b); 7-9, samples containing amoeba only; 10, sample containing *V. cholerae* only.

Analysis of the association of detected microorganisms showed that the detected number of associated and alone microorganisms (amoeba and bacteria) (Fig. 2) was significantly different ($\chi^2$ test, $p<0.05$). *Vibrio cholerae* apparently needs to be associated with other microorganisms such as *Acanthamoeba*, since 89% of detected *V. cholerae* co-existed with *Acanthamoeba*, compared with 11% occurring alone. As regards amoeba, 38% of *Acanthamoeba* existed with *V. cholerae* and 62% existed alone. Moreover, prevalence of *V. cholerae* alone was 0.25% and that of *Acanthamoeba* alone was 3.25%, while prevalence of both *Acanthamoeba* and *V. cholerae* was 2% (Table 2).

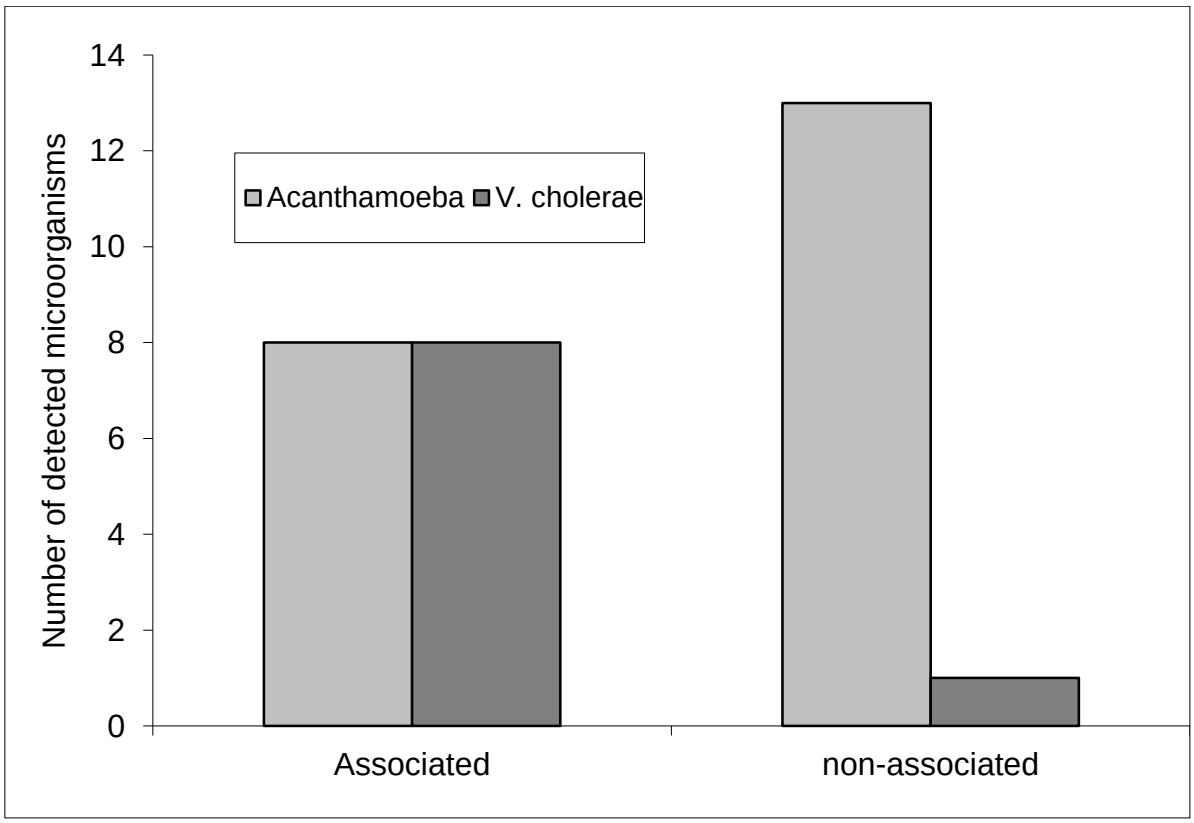


**Fig. 2.** Number of associated and non-associated microorganisms detected by PCR.

**Table 2.** Prevalence of detected microorganisms.

| **Detected microorganisms** | **Positive** | **Prevalence, %** |
|---|---|---|
| *Acanthamoeba* and *V. cholerae* | 8/400 | 2 |
| *Acanthamoeba* only | 13/400 | 3.25 |
| *V. cholerae* only | 1/400 | 0.25 |

## 4.2 PREVALENCE OF PROTOZOA SPECIES IN DRINKING AND ENVIRONMENTAL WATER SOURCES IN SUDAN

Protozoa have been associated with the outbreak of waterborne infections and may be present in water due to direct or indirect contamination with human or animal faecal matter. The PCR amplification of the genomic DNA extracted from water samples produced a single band of the expected size of 1360 bp (Fig. 3). Of 600 water samples, 57 positive samples were detected by PCR and gel electrophoresis utilising primer targeting 18S rRNA gene of the protozoa (Table 3).

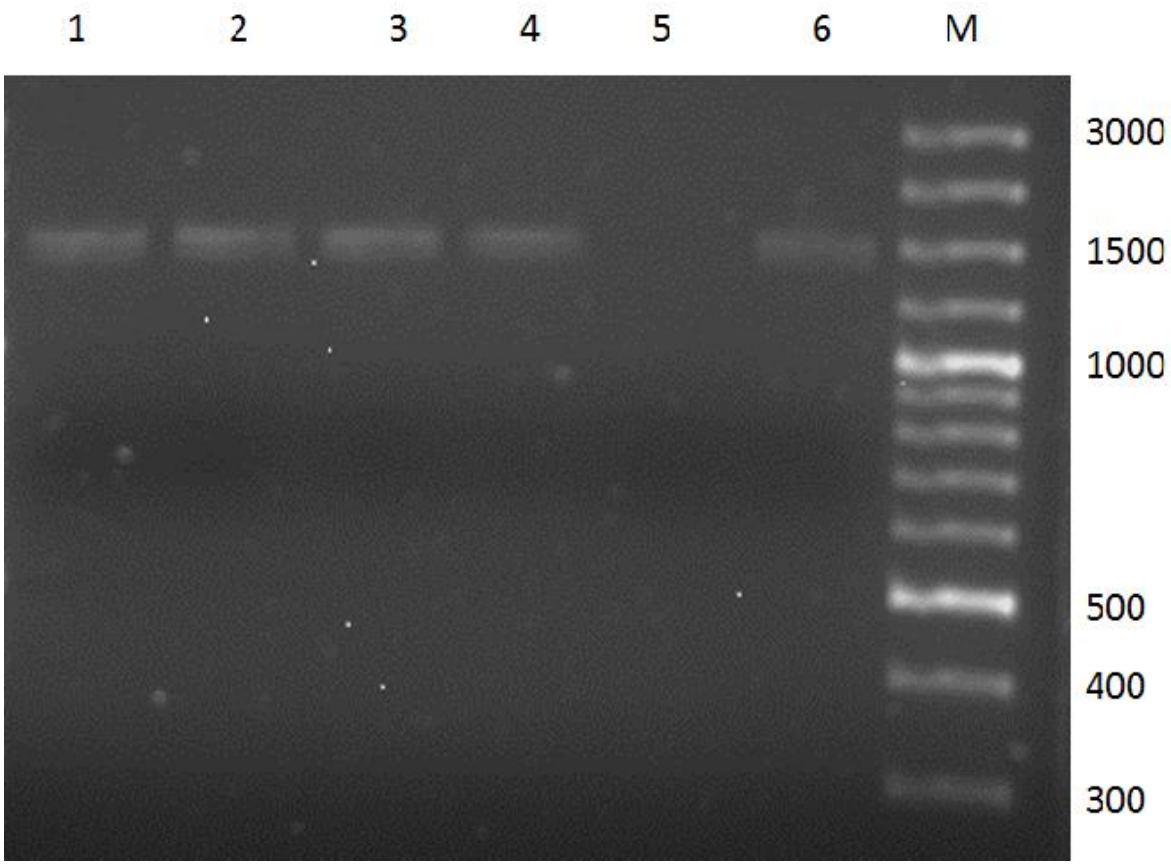


**Fig. 3.** Representative agarose gel (2%) electrophoresis of PCR products of protozoal 18S rRNA. Lanes 1-4, samples containing protozoa; 5-6, negative and positive controls (approximately 1,360 bp). M is molecular mass markers (1bp).

**Table 3.** Number of positive samples by PCR utilising primer targeting protozoal 18S rRNA gene

| Location | Positive samples | Negative samples | Total | Prevalence |
|---|---|---|---|---|
| Gadarif | 19 | 114 | 133 | 14.3% |
| Juba | 12 | 74 | 86 | 14.0% |
| Khartoum | 6 | 60 | 66 | 9.1% |
| Kordofan | 14 | 86 | 100 | 14.0% |
| Wad Madani | 6 | 209 | 215 | 2.8% |
| Total | 57 | 543 | 600 | 9.5% |

The PCR-positive samples were sequenced, with 31 samples passing sequencing and 26 samples failing to pass sequencing. The sequenced samples yielded 49 species of protozoa belonging to the four phyla: amoebae (36.7%), apicomplexa (30.6%), ciliates (28.6%) and flagellates (4.1%).

The identified amoebae were *A. castellanii*, *Casaspora owczarzaki*, *Dictyostelium purpureum*, *Entamoeba dispar* and *Naegleria gruberi*. The apicomplexa were *Ascogregarina taiwanensis*, *Blastocystis hominis*, *Cryptosporidium muris*, *Cryptosporidium parvum*, *Neospora caninum*, *Theileria parva* and *Toxoplasma gondii*. The ciliates were *Ichthyophthirius multifiliis*, *Oxytricha trifallax*, and *Stylonychia lemnae*. The flagellates were *Perkinsus marinus* and *Trichomonas vaginalis* (Table 4).

**Table 4.** Locations and sources of the water samples containing protozoa.

| **Location** | **Source** | **Number of identified protozoa** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **Amoebae** | **No** | **Apicomplexa** | **No** | **Ciliates** | **No** | **Flagellates** | **No** |
| Gadarif | Zeer | *A. castellanii* | 2 | *T. gondii* | 1 | | 0 | | 0 |
| | Hafir | *C. owczarzaki*<br>*D. purpureum*<br>*A. castellanii* | 1<br>1<br>2 | *C. muris*<br>*N. caninum* | 1<br>1 | *I. multifiliis*<br>*O. trifallax*<br>*S. lemnae* | 1<br>4<br>3 | *P. marinus* | 2 |
| | Tank | *A.castellanii*<br>*E. dispar* | 1<br>1 | *C. muris*<br>*T. parva* | 2<br>1 | *S. lemnae*<br>*O. trifallax* | 2<br>2 | | 0 |
| Juba | Lake | *A. castellanii* | 3 | *C. muris*<br>*N. caninum*<br>*A. taiwanensis* | 2<br>1<br>1 | *S. lemnae*<br>*O. trifallax* | 1<br>3 | | 0 |
| | *Zeer* | *C. owczarzaki*<br>*A. castellanii* | 1<br>1 | *B. hominis* | 1 | *O. trifallax* | 1 | | 0 |
| Khartoum | Lake | *A. castellanii* | 1 | *T. gondii* | 1 | *O. trifallax* | 2 | | 0 |
| | *Zeer* | 0 | 0 | *C. muris* | 1 | 0 | 0 | *T. vaginitis* | 1 |
| Kordofan | *Hafir* | *D. purpureum*<br>*N. gruberi*<br>*E. dispar* | 1<br>1<br>1 | *T. gondii*<br>*A. taiwanensis* | 2<br>1 | *O. trifallax*<br>*S. lemnae* | 4<br>2 | *P. marinus* | 2 |
| Wad Madani | Stream | *A. castellanii*<br>*C. owczarzaki* | 1<br>1 | *C. parvum* | 1 | 0 | 0 | | 0 |
| **Total** | | | **19** | | **17** | | **25** | | **5** |

## 4.3 LACK OF OUTER MEMBRANE PROTEIN A ENHANCES THE RELEASE OF OUTER MEMBRANE VESICLES, SURVIVAL OF *V. CHOLERAE* AND SUPPRESSES VIABILITY OF *A. CASTELLANII*

### 4.3.1 Adherence, uptake and intracellular growth

To estimate adherence of *V. cholerae* wild-type and *OmpA* mutant strain to amoeba cells, the percentage of each bacterial strain adhering to *A. castellanii* was determined and found to be 83.3 ± 2.1% and 73.3 ± 3.5%, respectively. The difference in adherence between the wild-type and *OmpA* mutant of *V. cholerae* was not statistically significant (t-test, p=0.29).

To estimate growth and survival of the engulfed bacteria following gentamicin treatment and re-cultivation, the number of intracellular bacteria was estimated by viable counts. The viable count of uptaken wild-type and *OmpA* mutant of *V. cholerae* was 3.2 x $10^3$ ± 1.6 x $10^3$ cells/mL and 4.0 x $10^3$ ± 5.0 x $10^2$ cells/mL, respectively (Fig. 4). The viable count of intracellular wild-type and *OmpA* mutant of *V. cholerae* after 2 h was 1.7 x $10^3$ ± 1.2 x $10^3$ cells/mL and 1.4 x$10^3$ ± 2.0 x $10^2$ cells/mL, respectively, while after 24 h it was 3.0 x $10^5$ ± 1.0 x $10^5$ cells/mL and 1.5 x$10^5$ ± 5.0 x $10^4$ cells/mL, respectively (Fig. 4). Uptake and intracellular growth of the wild-type and *OmpA* mutant *V. cholerae* were not significantly different (*t*-test, *p*=0.68).

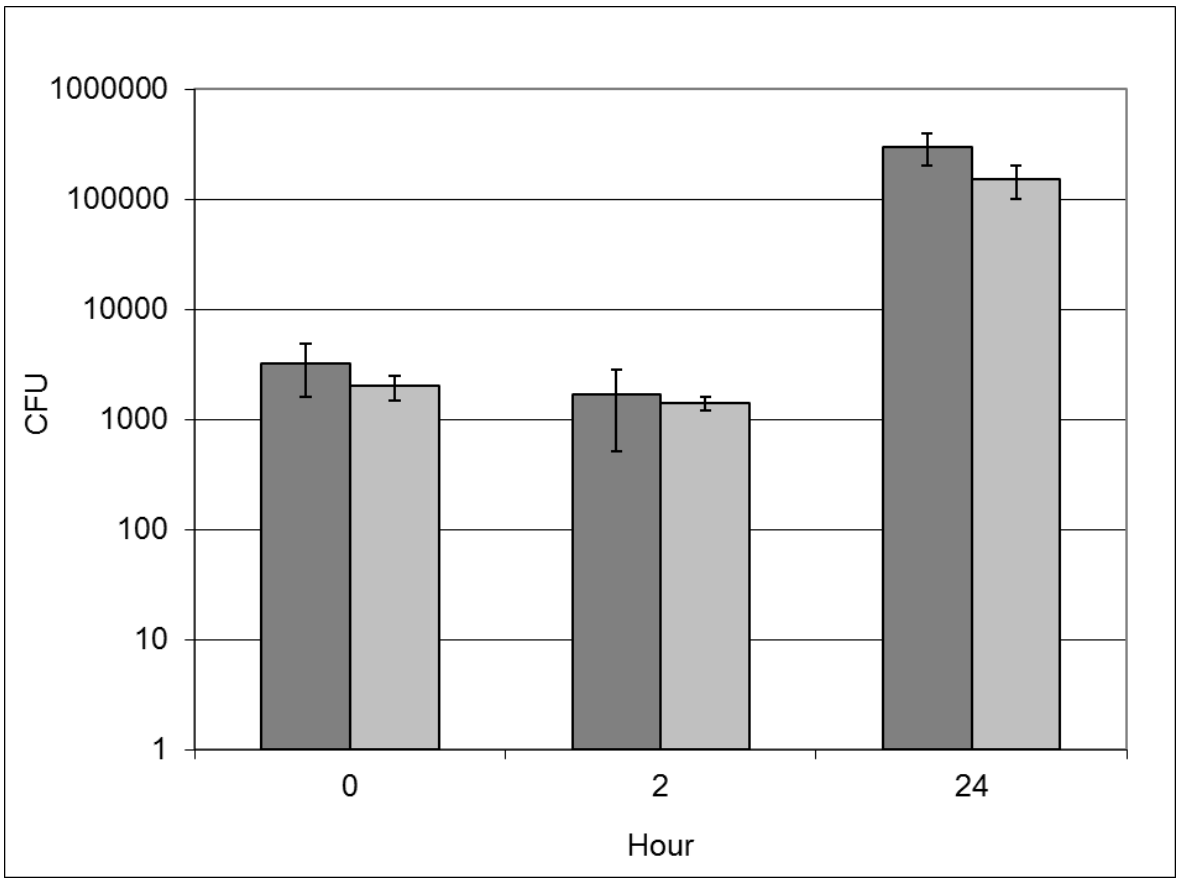


**Fig. 4.** Uptake, intracellular growth and survival of wild-type *V. cholerae* (dark grey bars) and *OmpA* mutant strains (light grey bars). Zero time is uptake of the bacteria by amoeba cells. Data represent mean ± SD of three different experiments.

### 4.3.2 Growth and survival of wild-type and *OmpA* mutant *V. cholerae* cultivated alone

Viable counts of wild-type and *OmpA* mutant *V. cholerae* cultivated alone in the absence of *A. castellanii* showed $10^6$-fold increases after 1 day for both. Surprisingly, the wild-type strain survived for only 3 days, while the *OmpA* mutant of *V. cholerae* survived more than 2 weeks, with a viable count of $1.7 \times 10^3 \pm 2.1 \times 10^2$ cells/mL at day 15 (Fig. 5). The survival rates of the wild-type and *OmpA* mutant of *V. cholerae* were significantly different (*t*-test, $p$=0.005).

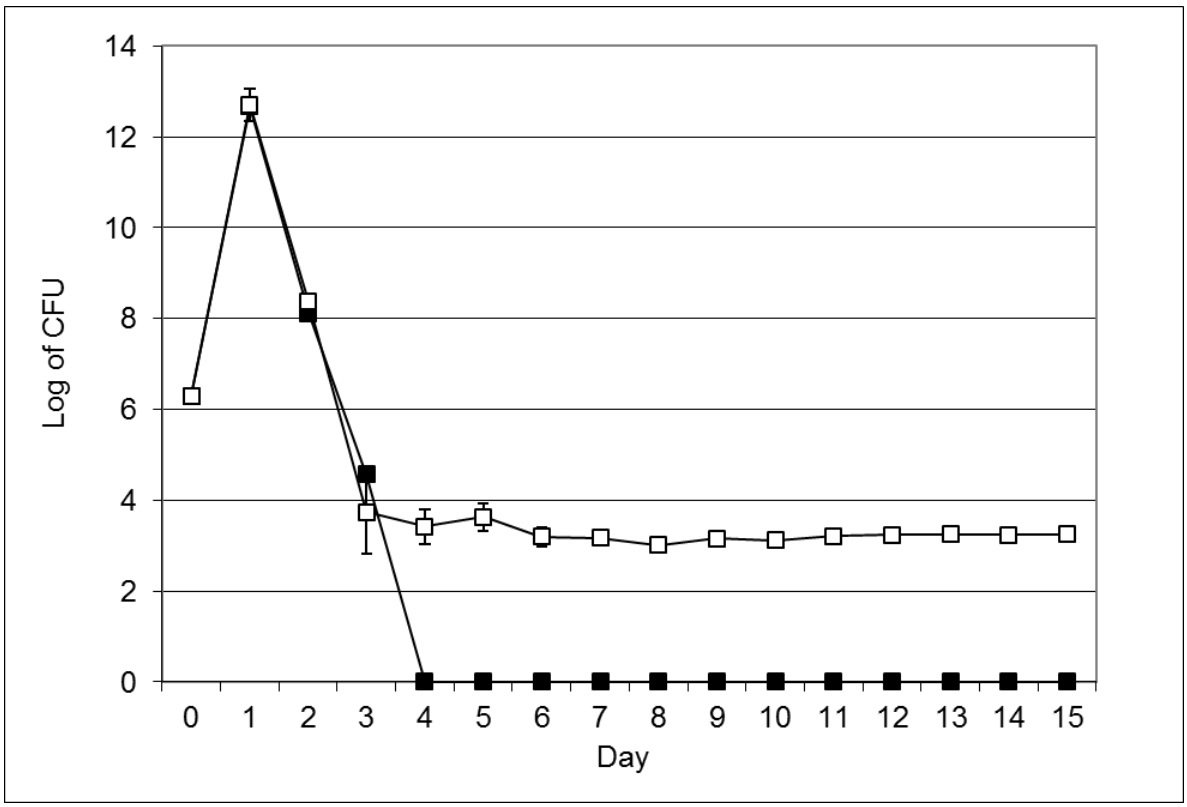


**Fig. 5.** Growth and survival of wild-type *V. cholerae* (filled squares) and the *OmpA* mutant strain (empty squares) cultivated alone. Data represent mean ± SD of three different experiments.

### 4.3.3 Growth and survival of wild-type and *OmpA* mutant *V. cholerae* co-cultivated with *A. castellanii*

Viable counts of wild-type and *OmpA* mutant *V. cholerae* co-cultivated with *A. castellanii* showed $10^3$-fold increases after 1 day for both strains. Surprisingly, both wild-type and *OmpA* mutant *V. cholerae* survived more than 2 weeks, but their viable counts were different ($2.0 \times 10^5 \pm 1.0 \times 10^5$ cells/mL and $4.1 \times 10^8 \pm 2.6 \times 10^8$ cells/mL at day 15, respectively) (Fig. 6).

The presence of *A. castellanii* enhanced survival and growth of both wild-type and *OmpA* mutant stains of *V. cholerae* (Fig. 5) compared with that in the absence of *A. castellanii* (Fig. 5). Growth of the co-cultivated wild-type and *OmpA* mutant strains of *V. cholerae* differed significantly (*t*-test, $p$=0.0004), with growth of the *OmpA* mutant being higher than that of the wild-type strain (Fig. 6).

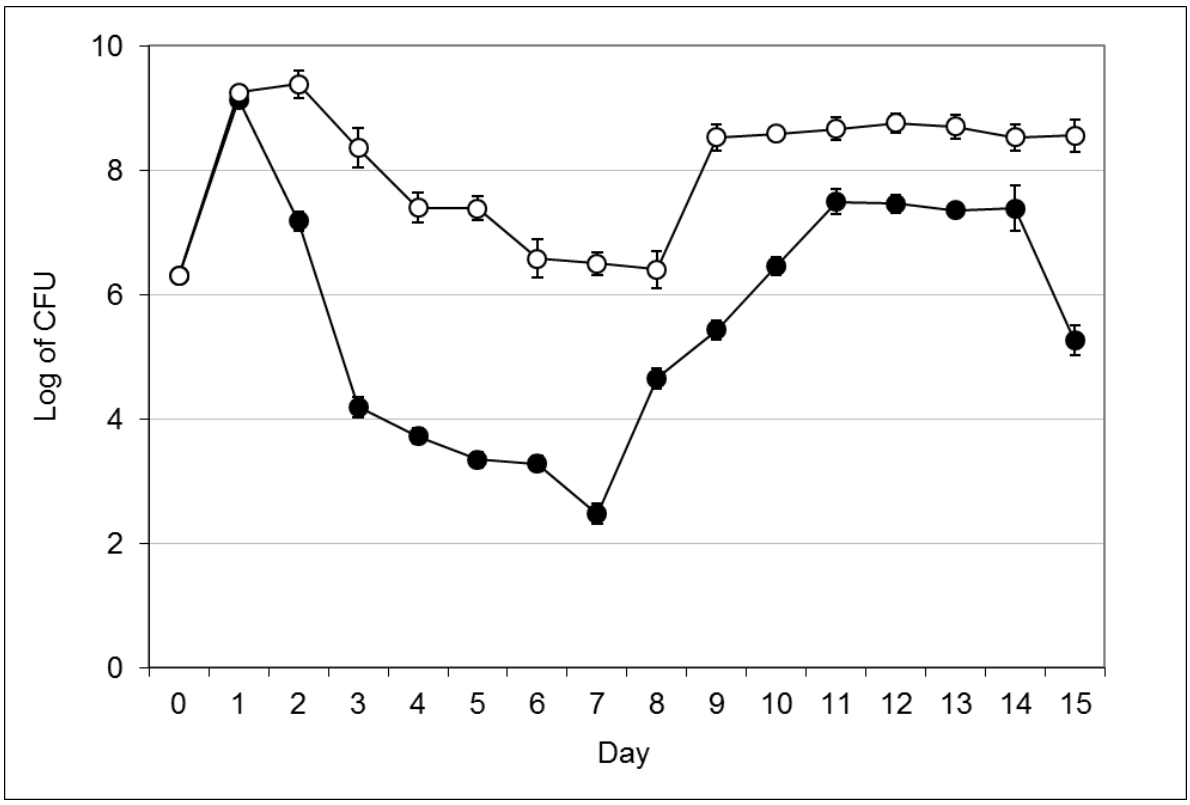


**Fig. 6.** Growth and survival of wild-type *V. cholerae* (filled circles) and the *OmpA* mutant strain (empty circles) when co-cultivated with *A. castellanii*. Data represent mean ± SD of three different experiments.

### 4.3.4 Growth and survival of *A. castellanii* alone or co-cultivated with wild-type and *OmpA* mutant strains of *V. cholerae*

The number of viable *A. castellanii* alone and co-cultivated with wild-type *V. cholerae* increased from 2.0 x $10^5$ ± 0.0 cells/mL to 1.8 x $10^6$ ± 4.2 x $10^5$ and 9.3 x $10^5$ ± 1.8 x $10^5$ cells/mL, respectively, after 15 days. In contrast, the number of viable *A. castellanii* co-cultivated with the *OmpA* mutant *V. cholerae* decreased from 2.0 x $10^5$ ± 0.0 cell/mL to 1.3 x $10^4$ ± 1.0 x $10^3$ cells/mL after 15 days (Fig. 7).

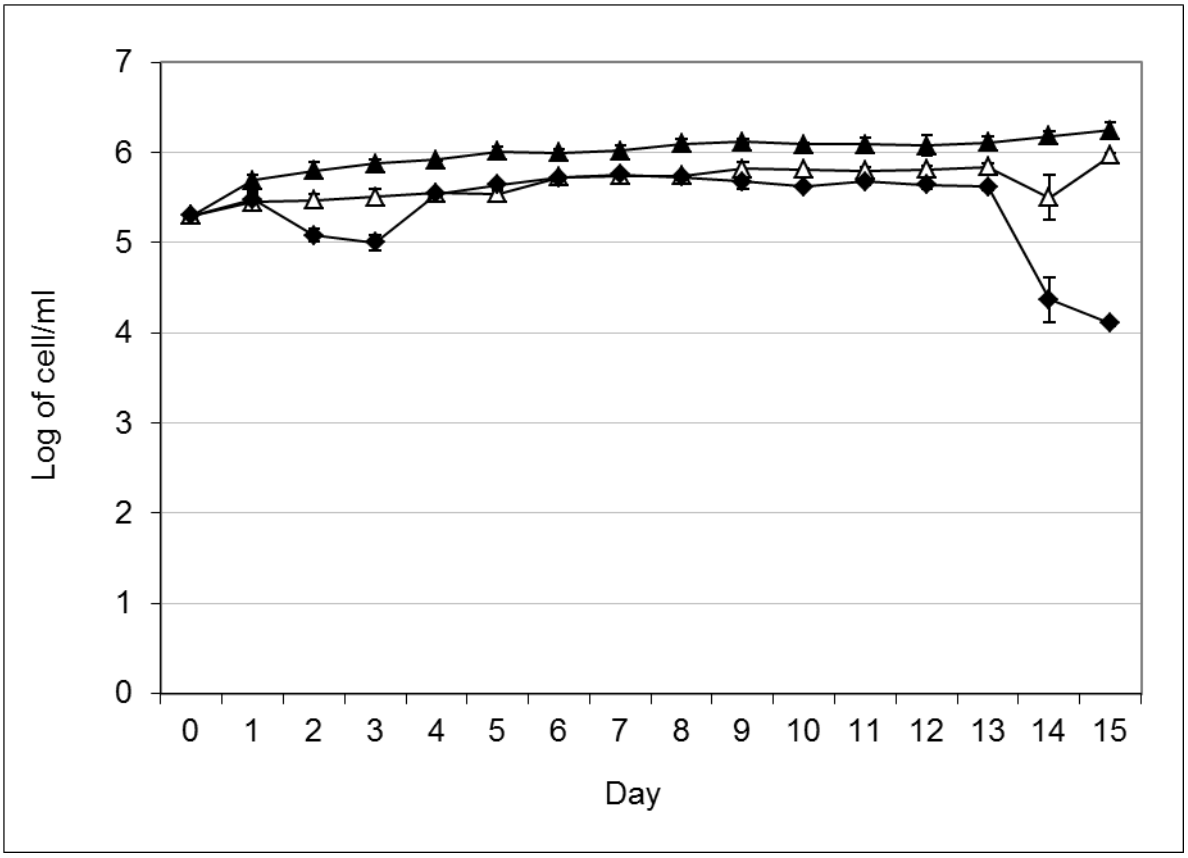


**Fig. 7.** Growth and survival of *A. castellanii* cultivated alone (filled triangles), with wild-type *V. cholerae* (empty triangles) and with the *OmpA* mutant strain of *V. cholerae* (filled diamonds). Data represent mean ± SD of three different experiments.

Co-cultivation of the amoeba with *V. cholerae* strains revealed that the growth rate of *A. castellanii* alone and in the presence of wild-type or *OmpA* mutant *V. cholerae* was significantly different (*t*-test, $p$=0.00020 and $p$=0.00024, respectively). However, the difference between the growth rate of *A. castellanii* co-cultivated with wild-type or *OmpA* mutant of *V. cholerae* was less significant ($p$=0.04).

### 4.3.5 Production of outer membrane vesicles by *V. cholerae* strains and effect of OMVs on *A. castellanii* viability

Outer membrane vesicles were isolated from the wild-type and *OmpA* mutant strains as described in the Methods section. The amount of vesicles released from the two strains was compared by measuring protein concentration, which was found to be 510 ± 24 µg/mL for the wild-type and 1550 ± 51 µg/mL for the *OmpA* mutant strain (Fig. 8). This difference in protein concentration was statistically significant (*t*-test, $p$=0.0001).

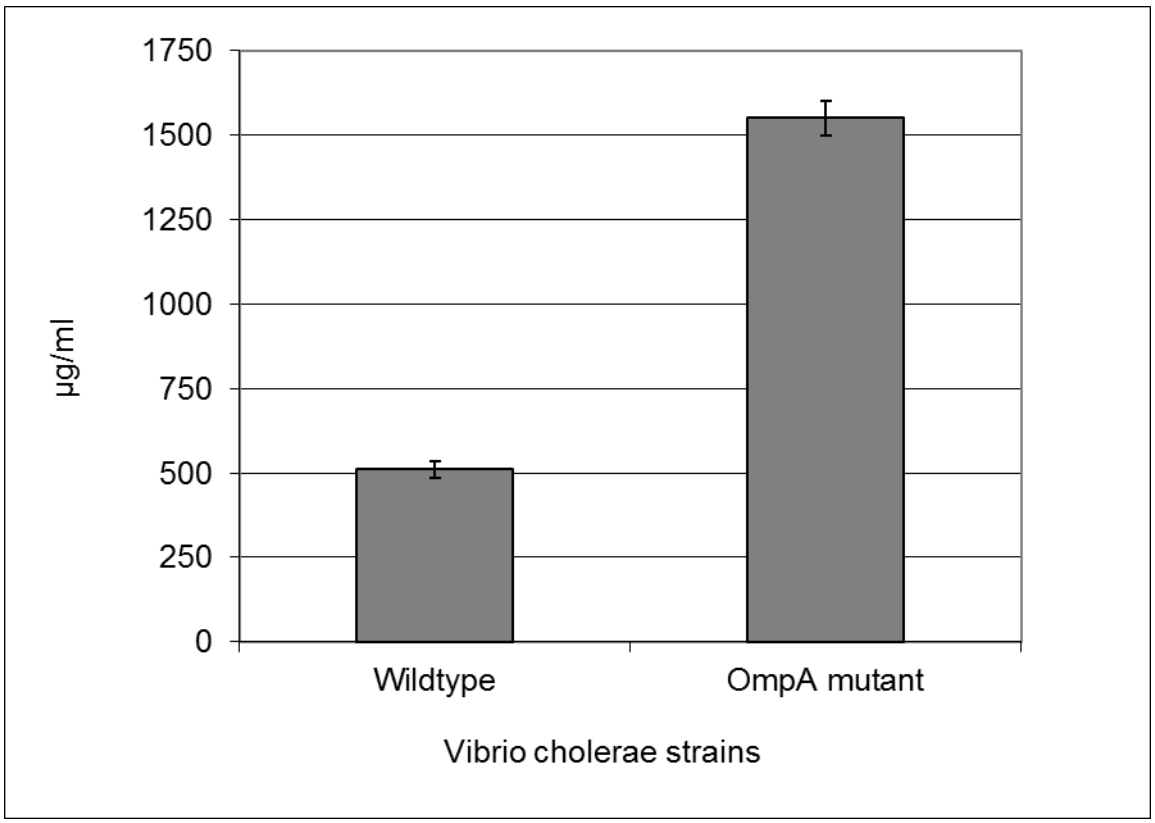


**Fig. 8.** Production of outer membrane vesicles by *V. cholerae* wild-type and *OmpA* mutant strains. Data represent mean ± SD of three different measurements.

Treatment of the amoeba with bacterial vesicles showed that the OMVs lowered the viability of the amoeba cells after 2 h of incubation. Thus the viable count of *A. castellanii* incubated with OMVs from wild-type *V. cholerae*, OMVs from the *OmpA* mutant strain and with PBS was 8.7 x $10^5$ ± 2.3 x $10^5$, 8.1 x $10^5$ ± 7.8 x $10^4$ and 1.1 x $10^6$ ± 1.3 x $10^5$ cells/mL, representing 79%, 74% and 100% viability, respectively (Fig. 9). The viable count decreased significantly compared with the PBS treatment for the amoeba treated with OMVs from *OmpA* mutant *V. cholerae* (*t*-test, $p$=0.02), but the decrease was less significant for the amoeba treated with OMVs from wild-type *V. cholerae* (*t*-test, $p$=0.05). However, this difference in amoeba viability might be due to the ability of the *OmpA* mutant of *V. cholerae* to produce more OMVs, as demonstrated here and by Song et al. (23).

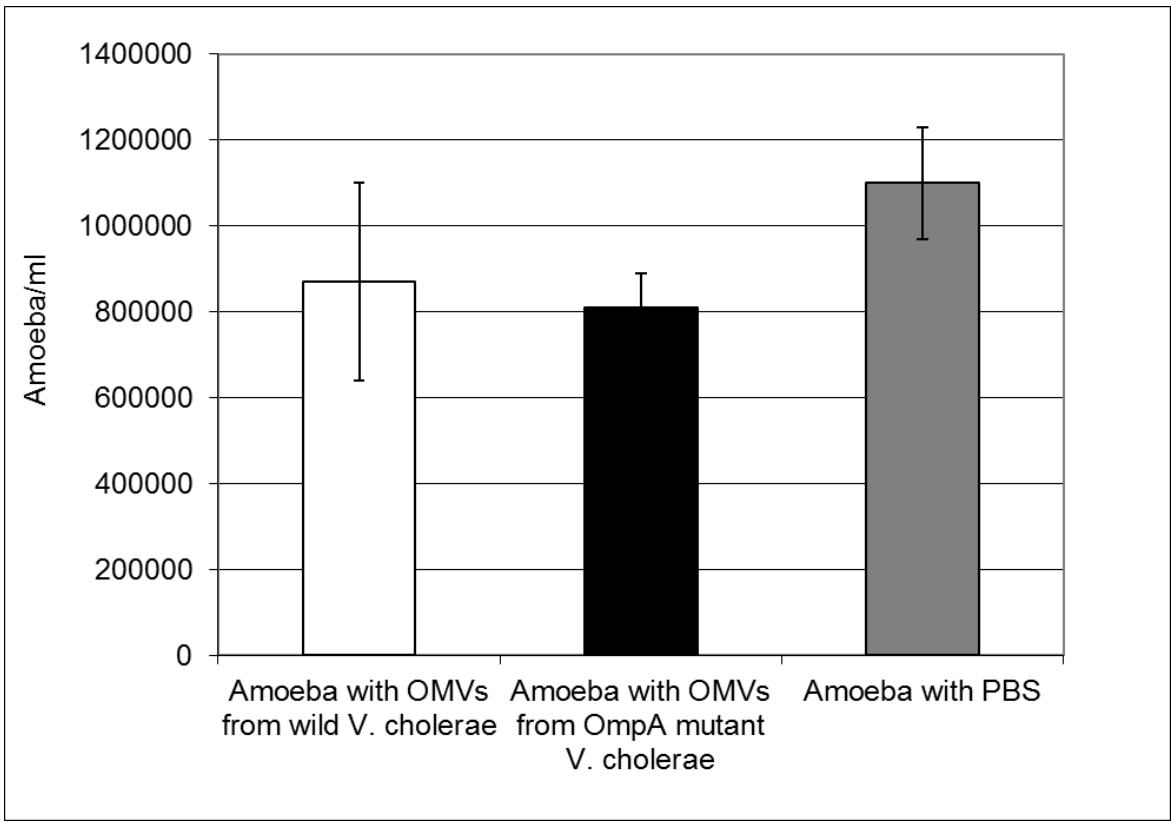


**Fig. 9.** Effect of outer membrane vesicles (OMVs) from wild-type and *OmpA* mutant strains of *V. cholerae* on viability of *A. castellanii*. Data represent mean ± SD of three different measurements.

## 4.4 SWEDISH ISOLATES OF *V. CHOLERAE* ENHANCE THEIR VIRULENCE WHEN INTERACTING INTRACELLULARLY WITH *A. CASTELLANII*

### 4.4.1 Growth of *V. cholerae* strains in the absence or presence of *A. castellanii*

The bacterial strains were cultivated in the absence and presence of *A. castellanii* to study the interaction between these microorganisms by means of viable count, as described in the Methods section. In the absence of *A. castellanii*, viable counts of *V. cholerae* O3, *V. cholerae* O4, *V. cholerae* O5, *V. cholerae* O11 and *V. cholerae* O160 increased onefold on day 1 and the bacteria showed different survival rates: *V. cholerae* O3, *V. cholerae* O4, *V. cholerae* O5, *V. cholerae* O11 and *V. cholerae* O160 survived 4 days, 15 days, 4 days, 6 days and 3 days, respectively (Fig. 10).

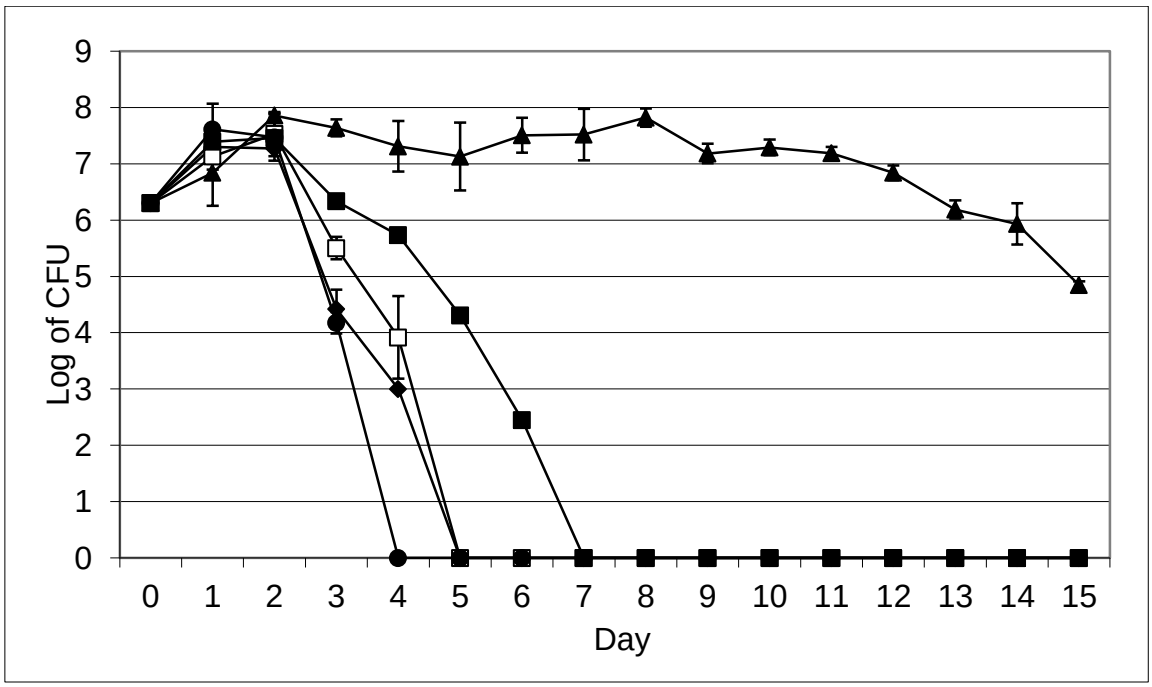


**Fig. 10.** Viable counts of *V. cholerae* in the absence of *A. castellanii*: *V. cholerae* O3 (♦), *V. cholerae* O4 (▲), *V. cholerae* O5 (●), *V. cholerae* O11 (■) and *V. cholerae* O160 (□). Data represent mean ± SD of three independent experiments.

In the presence of *A. castellanii*, the viable count of *V. cholerae* O3, *V. cholerae* O4, *V. cholerae* O5, *V. cholerae* O11 and *V. cholerae* O160 increased from 2.0 x$10^6$ cfu/mL on day 0 to 2.3×$10^7$, 2.3×$10^7$, 2.4×$10^7$, 2×$10^7$, 8.4×$10^7$ and 2.8×$10^7$ cfu/mL on day 1, and the bacteria survived 6 days, 5 days, 8 days, 5 days and 14 days, respectively (Fig. 11).

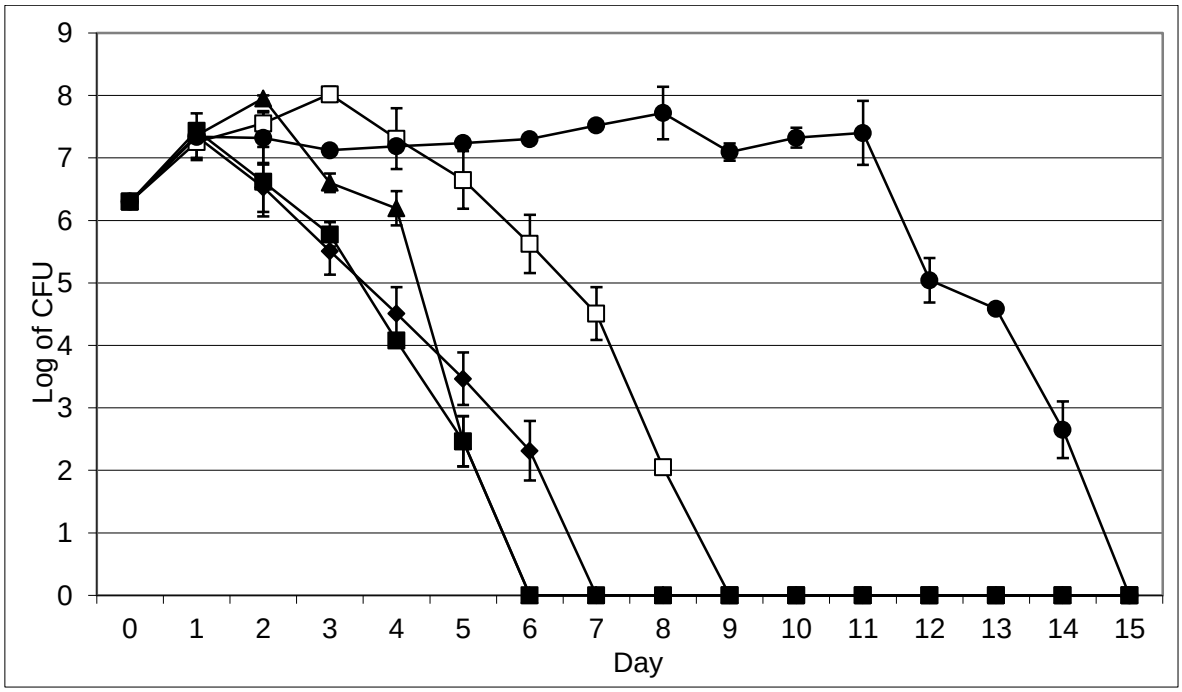

**Fig. 11.** Viable counts of *V. cholerae* in the presence of *A. castellanii*: *V. cholerae* O3 (♦), *V. cholerae* O4 (▲), *V. cholerae* O5 (□), *V. cholerae* O11 (■) and *V. cholerae* O160 (●). Data represent mean ± SD of three independent experiments.

The viable count of *V. cholerae* O3, O5 and O11 in the absence or presence of *A. castellanii* was not significant different ($p>0.05$). However, the viable count of *V. cholerae* O4 and *V. cholerae* O160 O11 in the absence or presence of *A. castellanii* was significantly different ($p\leq0.05$).

### 4.4.2 Growth of intracellular *V. cholerae* strains

Samples were taken from co-culture flasks and prepared for viable counts of intracellular growth and survival of *V. cholerae* after gentamycin killing of extracellular bacteria. Sensitivity of *V. cholerae* to gentamicin was performed by Etest. The MIC value for *V. cholerae* O3, *V. cholerae* O4, *V. cholerae* O5, *V. cholerae* O11 and *V. cholerae* O160 was 0.25, 1.0, 0.75, 1.0 and 0.75 µg/mL, respectively. These results showed that all *V. cholerae* strains examined were susceptible to gentamicin, since the susceptibility of *V. cholerae* was S≤2 µg/mL, R>4 µg/mL (Fig. 12).

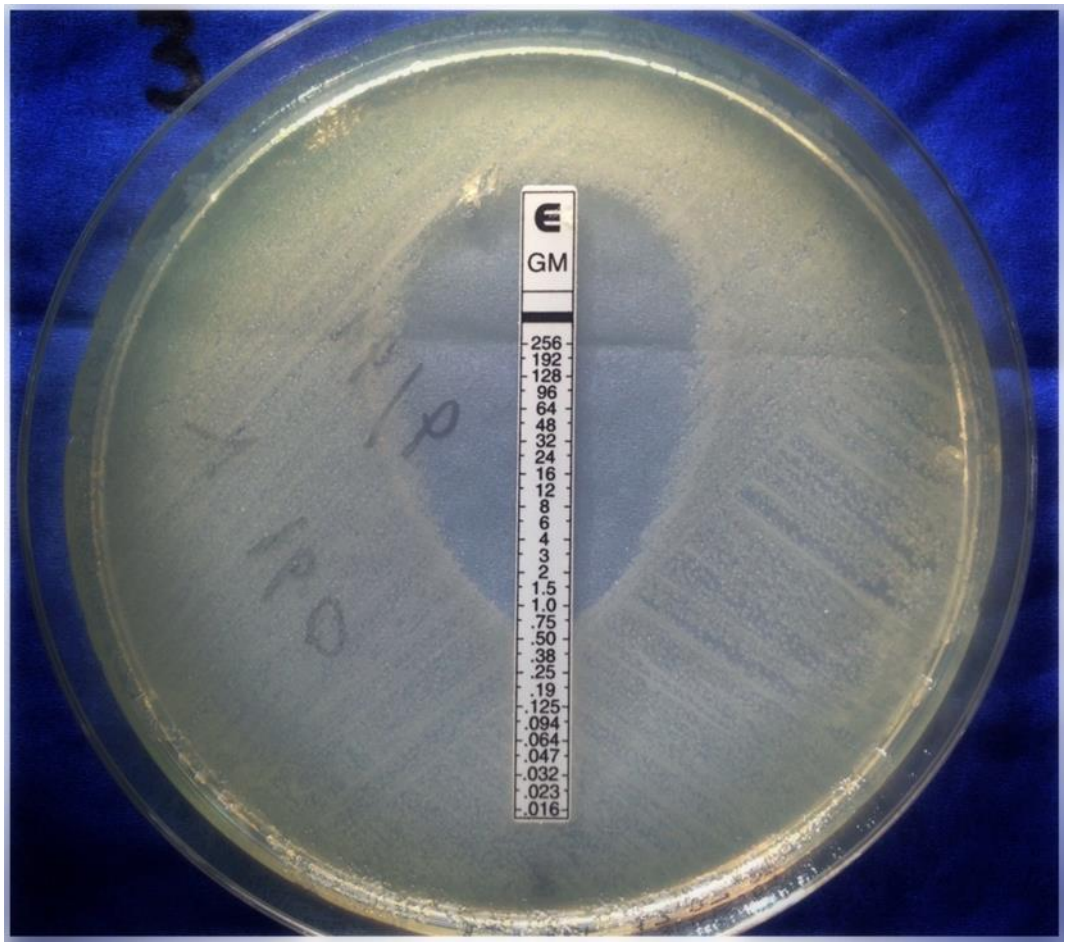


**Fig. 12.** Representative gentamicin sensitivity test of *V. cholerae* (Etest).

The intracellular assay showed that after one day, *V. cholerae* O3, *V. cholerae* O4, *V. cholerae* O5, *V. cholerae* O11 and *V. cholerae* O160 grew inside the amoeba cells to $2.7\times10^5$, $1.8\times10^5$, $5.8\times10^4$, $2.1\times10^5$ and $2.1\times10^4$ cfu/mL, respectively, and survived intracellularly for 5 days, 5 days, 6 days, 5 days and 14 days, respectively (Fig. 13).

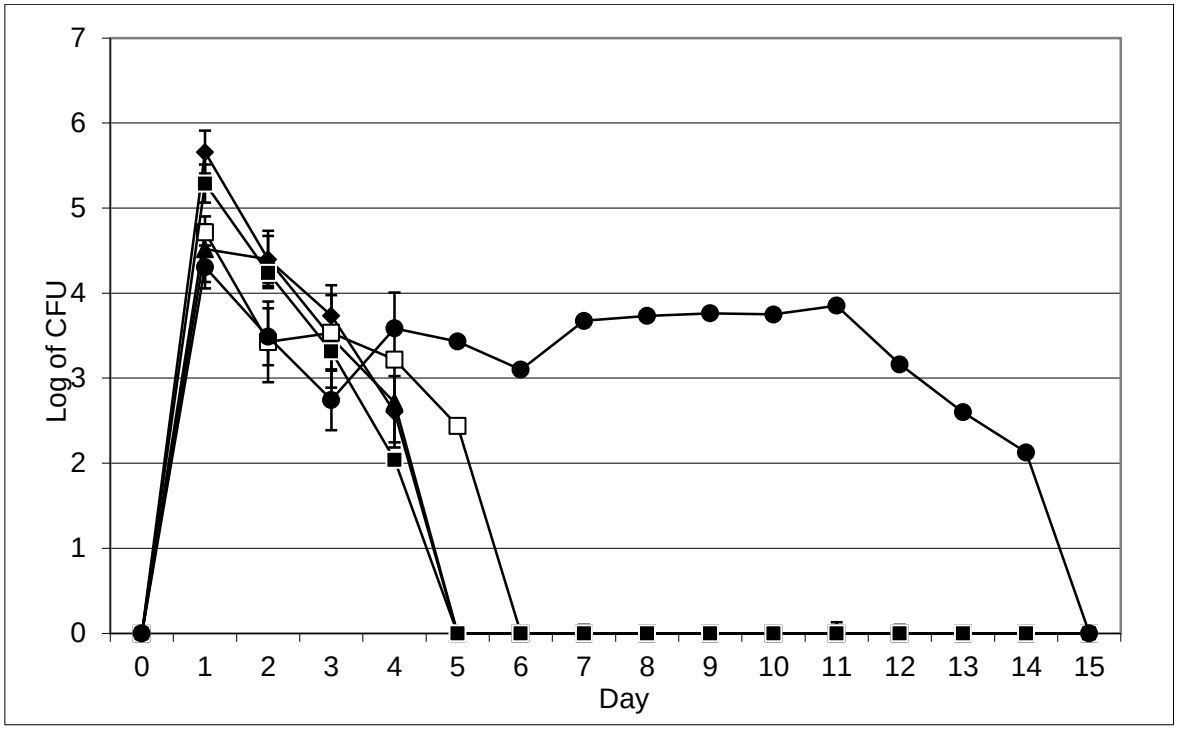


**Fig. 13.** Intracellular growth and survival of *V. cholerae*: Viable counts of intracellular *V. cholerae* O3 (♦), *V. cholerae* O4 (▲), *V. cholerae* O5 (□), *V. cholerae* O11 (■) and *V. cholerae* O160 (●). Data represent mean ± SD of three independent experiments.

### 4.4.3 Intracellular localisation of *V. cholerae*

Electron microscopy was used to confirm the intracellular localisation of *V. cholerae* in *A. castellanii*. Samples from cultures containing *A. castellanii* infected with *V. cholerae* O160 for 2 h and with *V. cholerae* O4 for 4 h were prepared separately for electron microscopy. The ultramicrography confirmed the intracellular localisation of *V. cholerae* O160 (Fig. 14) and *V. cholerae* O4 (Fig. 15) in the cytoplasmic vacuoles of trophozoites of *A. castellanii*.

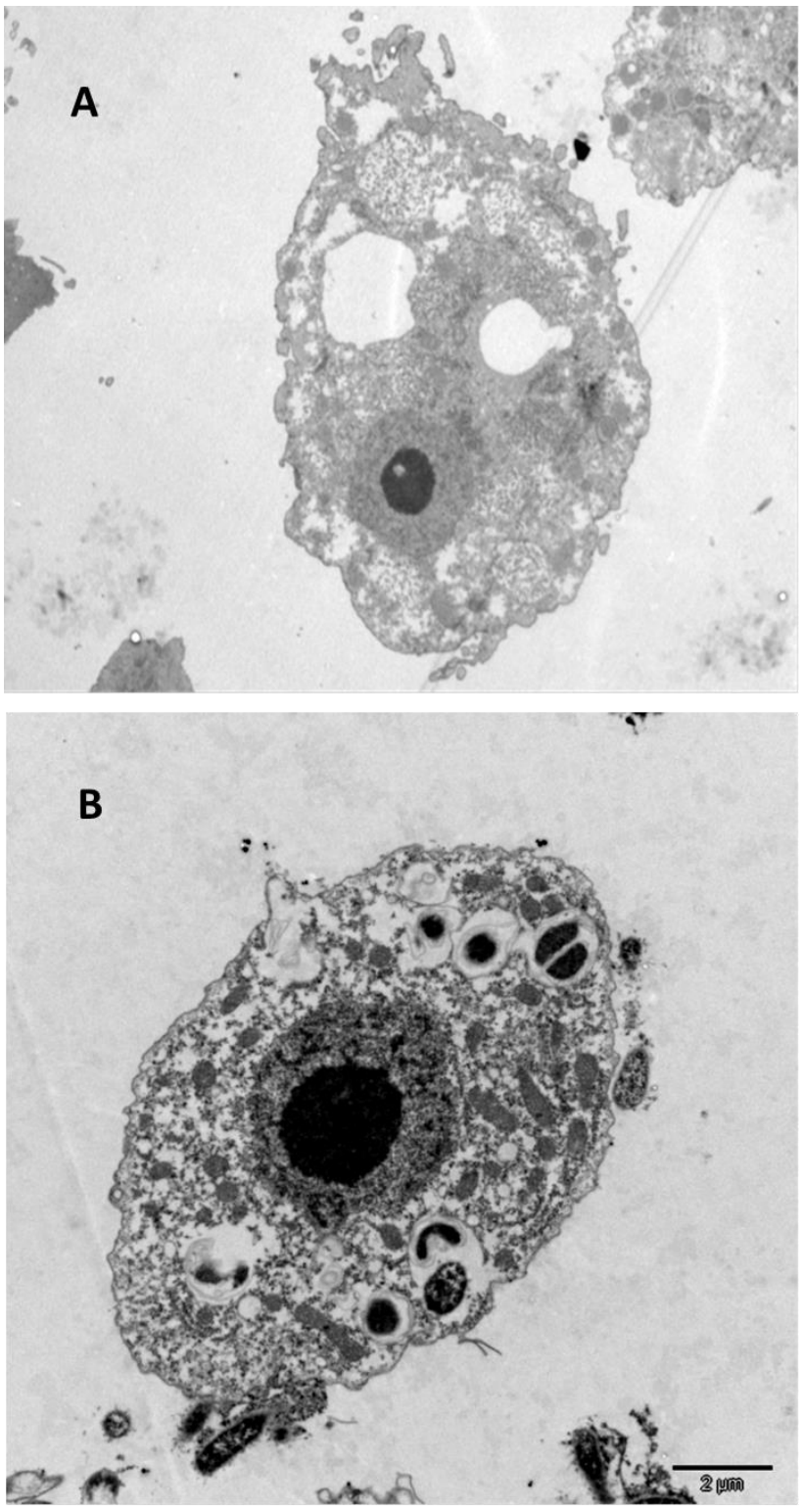


**Fig. 14.** Electron microscopy image showing the intracellular localisation of *V. cholerae* O160 in *A. castellanii*. **A**) *A. castellanii* trophozoite in absence of bacteria. B) *V. cholerae* O160 localised in cytoplasmic vacuoles of an *A. castellanii* trophozoite 2 h after co-cultivation.

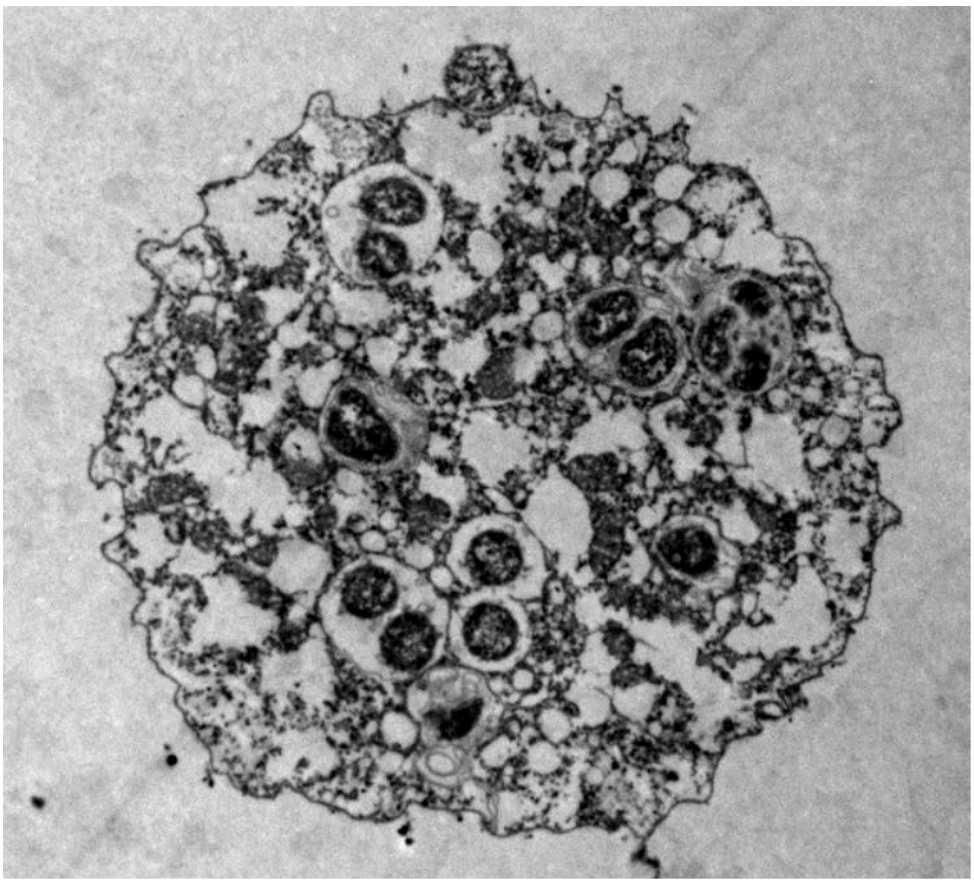

**Fig. 15.** Electron microscope image of **the** intracellular localisation of *V. cholerae* O4 in cytoplasmic vacuoles of an *A. castellanii* trophozoite 4 h after co-cultivation.

### 4.4.4 Growth of *A. castellanii* in the absence or presence of bacteria

The growth of *A. castellanii* in the absence or presence of *V. cholerae* was studied by means of viable amoeba cell counts. The viable count of the amoeba in the absence of *V. cholerae* strains increased from $2\times10^5$ cells/mL on day 0 to $1.5\times10^6$ cells/mL on day 15 (Fig. 16).

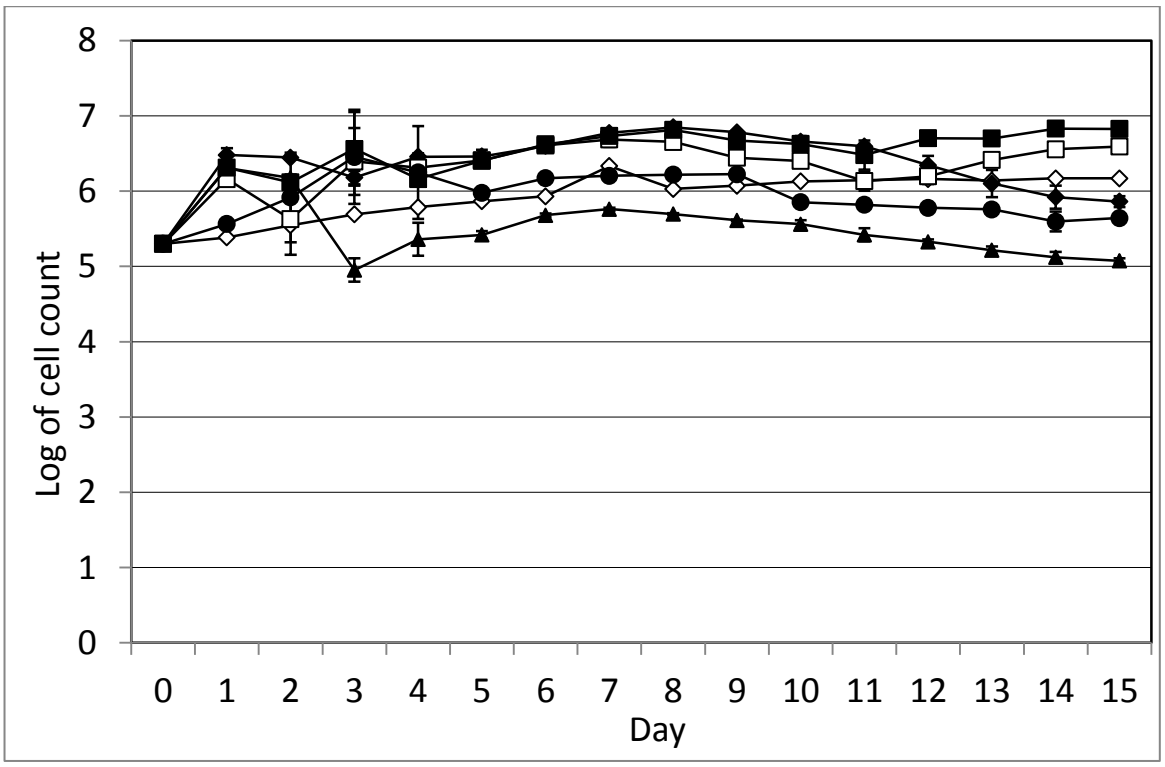


**Fig. 16.** Growth of *A. castellanii*: Viable counts of *A. castellanii* in the absence of *V. cholerae* strains (◊), and in the presence of *V. cholerae* O3 (♦), *V. cholerae* O4 (▲), *V. cholerae* O5 (□), *V. cholerae* O11 (■) and *V. cholerae* O160 (●). Data represent mean ± SD of three independent experiments.

The viable count of the amoeba in the presence of *V. cholerae* O3, *V. cholerae* O5, *V. cholerae* O11 and *V. cholerae* O160 increased from $2\times10^5$ cells/mL on day 0 to $7.3\times10^5$, $3.9\times10^5$, $6.7\times10^5$ and $4.4\times10^5$ cells/mL, respectively, on day 15 (Fig. 16). In contrast, the viable count of the amoeba in the presence of *V. cholerae* O4 decreased to $1.2\times10^5$ cells/mL on day 15 (Fig. 16). The differences in the viable count of *A. castellanii* in the absence or presence of *V. cholerae* O3, *V. cholerae* O4, *V. cholerae* O5, and *V. cholerae* O11 strains were statistically significant ($p<0.05$). In contrast, the viable count of *A. castellanii* in the absence or presence of *V. cholerae* O160 strain was not significantly different ($p>0.05$).

# 5 DISCUSSION

*Vibrio cholerae* O1 and *V. cholerae* O139 are widely distributed in aquatic environments (2), causing the diarrhoeal and waterborne disease cholera (67). *Vibrio cholerae* and *Acanthamoeba* species are present in aquatic environments, including drinking water (54-56). A number of studies report that FLA plays a role as a reservoir of pathogenic bacteria (56). However, before claiming a role for these microorganisms as an environmental reservoir of *V. cholerae*, further studies are needed, mainly on distribution and temporal occurrence of the *V. cholerae*-amoeba relationship in the environment (68). In the work presented in this thesis, water samples were collected from endemic areas in Sudan to detect the occurrence of both *V. cholerae* and *Acanthamoeba* species in the same natural water samples by PCR methodology targeting the cholera toxin gene (*toxA*) and *Acanthamoeba* 18S RNA gene. For the first time, both *V. cholerae* and *Acanthamoeba* species were detected in the same natural water samples from different cholera endemic areas in Sudan. Furthermore, 89% of detected *V. cholerae* co- existed with *Acanthamoeba* and 11% were present alone in water samples. Taken together, these and our previous findings about the intracellular growth and survival of *V. cholerae* in *Acanthamoeba* species (44, 61, 62, 64) and the endosymbiont-host relationship between these microorganisms (63) strongly indicate that *Acanthamoeba* is an environmental host, protecting and enhancing growth and survival of *V. cholerae* in the same aquatic environments. *Acanthamoeba* species are human pathogens causing different infections. For example, they can cause granulomatous amoebic encephalitis (GAE), particularly in immunocompromised or otherwise debilitated individuals, and a vision-threatening disease (keratitis) in the eyes. Moreover, they can cause infections of the lungs and skin (43, 62, 69).

*Acanthamoeba* supports bacterial growth and survival (63) and saves the bacteria from the effects of chlorination (46) and antibiotics (44, 61, 70), increasing the risk of human illness caused by the bacteria or *Acanthamoeba*. Accordingly, there is an urgent need to find an effective means of detection and elimination of both *Acanthamoeba* and their intracellular bacteria in order to reduce the risk of spread of *V. cholerae*, other bacteria, and free-living amoebae.

Unsafe water, poor sanitation and hygiene have been reported to rank third among the 20 leading risk factors for health burden in developing countries, including Sudan (71). Hafirs, tanks, zeers, lakes and streams are the main sources of drinking water in Sudan, especially

in rural areas, and not only used by humans but also by animals. Accordingly, this is the common source of contamination with protozoa and other harmful microorganisms. Although there is much scientific literature available concerning the association of protozoa with waterborne diseases in other countries, this is not the case for Sudan.

This thesis work pioneered the use of a PCR-based molecular test to search for potentially disease-causing protozoa in drinking water, resulting in the detection of four phyla of well-documented pathogenic protozoa. This is the first study in Sudan focusing on detection and identification of protozoa in drinking water. The results provide vital information regarding the protozoa found at different geographical locations, thus facilitating correlation of the identified organisms with the clinical phenotypes of infectious diseases prevalent among the population inhabiting specific geographical locations.

This thesis showed that protozoa are common in all water sources, especially in the Gadarif, Kordofan and Juba regions of Sudan, and are distributed in all drinking water sources; hafirs, zeers, lakes, tanks and streams. A total of 66 species of protozoa were identified by sequencing and they included amoebae species (28.8%), apicomplexa species (25.7%), ciliates (37.9%) and flagellates (7.6%).

In similar studies by Leiva et al. (72) and Tsvetkova et al. (73) , amoebae were found in 43% and 61% of water samples. Moreover, in a recent study in Turkey, only three species of free-living amoebae, *A. castellanii*, *A. polyphaga* and *Hartmannella vermiformis*, were identified in tap water (74). In addition, *Cryptosporidium*, *Giardia* and *Acanthamoeba* were isolated from stations in recreational lakes in Malaysia (75). However, these studies detected a limited number of amoebae, *Cryptosporidium* and *Giardia* only in water samples, whereas in this thesis 49 microorganisms comprising 16 species belonging to four different phyla of protozoa were identified. This raises concerns about the risk of water contamination, since most of these protozoa are human pathogens or zoonotic parasites.

To the best of my knowledge, there have been no previous studies performed in Sudan searching for protozoa in water sources and there are few studies about bacterial water contamination. However, Adam et al. (52) reported that *Cryptosporidium* was an important cause of diarrhoea in children in Sudan, while Hammad and Dirar (76) isolated faecal coliforms and streptococci from water in a number of zeers.

It is well known that a complex interaction between organisms is found in aquatic environments, especially amoebae and bacteria. Free-living amoebae are eukaryotic cells found in nature and include several genera such as *Acanthamoeba*, *Balamuthia*, *Naegleria* and *Sappinia.* It has been shown that *Acanthamoeba* benefits from *E. coli* and *Klebsiella*

*aerogenes* as food. In contrast, the role of *Acanthamoeba* as hosts for bacteria has been proposed for many pathogenic bacteria (77).

The interaction between bacteria and amoeba is very complex and the output of the interaction is dependent on whether the interacting bacterium is extracellular or intracellular and whether it possesses a TTSS or not, since TTSS effector proteins are observed to have a strong effect on the output of the interaction (77). Extracellular bacteria cannot multiply inside amoebal cells and TTSS-possessing extracellular bacteria such as *Pseudomonas aeruginosa* kill the amoeba. However, the extracellular bacterium *E. coli* does not possess TTSS and therefore it is ingested as food by the amoeba.

An intracellular bacterium multiplies inside an amoebal cell. The intracellular bacterium *Francisella tularensis*, which does not possess TTSS, multiplies symbiotically inside the amoeba, while the TTSS-possessing intracellular bacteria *E. coli* K1 and *Shigella flexneri* lyse the amoeba through activation of TTSS (77).

Facultative intracellular bacteria such *V. cholerae* are able to multiply in water and inside environmental phagocytic eukaryotes such as free-living amoebae. Therefore, presence of the amoeba in water will enhance presence of bacteria such as *V. cholerae* (44, 62, 64). Surprisingly, in this thesis *Acanthamoeba* species and *V. cholerae* were detected from the same water samples collected from different water sources in Sudan (77, 78) and Newsome et al. and Henke and Seidel isolated an amoeba naturally harbouring a distinctive *Legionella* species (33, 79). *Vibrio cholerae* and *Legionella pneumophila* are well known as causative agents for the waterborne diseases cholera and legionellosis.

The protozoa have a doubly infectious role in public health. They can cause infections by themselves, such as amoebiasis, cryptosporidiosis, giardiasis, amoebic encephalitis and amoebic keratitis, or they can host intracellular bacteria and thus cause multiple infections of protozoa and bacteria.

In addition to the findings on protozoa presented in this thesis, Fletcher et al. (80) reported that *Giardia intestinalis*, *Cryptosporidium* spp. and *Entamoeba* spp. are the most commonly reported protozoa associated with enteric infections and are associated mainly with food and waterborne outbreaks. These enteric protozoa are frequently isolated from diarrhoeal patients in developing regions such as Asia and sub-Saharan Africa (80). This thesis presents the first report about detection of the following protozoa: *A. castellanii, T. gondii, C. owczarzaki, B. hominis, C. muris* and *T. vaginitis* from zeers in Gadarif, Juba and Khartoum.

The overall prevalence of protozoa detected by PCR was 9.5% and the prevalence in Gadarif, Juba, Khartoum, Kordofan and Wad Madani was 14.3%, 14.0%, 9.1%, 14.0% and 2.8%, respectively. The prevalence of identified protozoa in Gadarif, Juba, Khartoum and Kordofan was not significantly different ($\chi^2$ test; $p>0.05$). However, the prevalence of identified protozoa in Wad Madani compared with Gadarif, Juba and Kordofan was highly significantly different ($p= 0.0012$, 0.0007 and 0.0003, respectively). The prevalence of identified protozoa in Wad Madani and Khartoum was not significantly different ($p=0.1210$).

These results reveal the danger posed by water in spreading infections between populations. However, this thesis presents a fast and accurate molecular detection and diagnosis of protozoa species in water.

*Vibrio cholerae* utilises several survival strategies in aquatic environments, such as biofilm formation, switching from smooth to rugose colony morphotypes and association with free-living amoebae (81). Studies have shown that *V. cholerae* has enhanced growth in association with *A. castellanii* (44, 62, 82) and both microorganisms have been detected in the same water samples from cholera endemic areas (78). This thesis investigated the role of OmpA protein and the OMVs released by *V. cholerae* on the survival and interaction of the bacterium with the eukaryotic host *A. castellanii*. The results demonstrated that in the absence of *A. castellanii*, the *OmpA* mutant *V. cholerae* survived much longer (>15 days) than wild-type *V. cholerae* (3 days). In fact, loss of all cfu from wild type *V. cholerae* cultivated in this rich medium in the absence of amoebae was observed not only in this thesis, but also previously for other bacteria such as *Shigella dysenteriae* and *Shigella sonnei* (83) and *F. tularensis* (70).

*Francisella* and *Shigella* species are facultative intracellular bacteria multiplying inside amoebal cells and remain cultivable during the experimental period, whereas in the absence of the amoeba these bacteria are non-cultivable. Moreover, studies of the interaction of the extracellular bacterium *Pseudomonas aeruginosa* with *A. castellanii* have shown that growth and survival of *P. aeruginosa* remain the same during the experimental period whether the amoeba is present or absent (84). However, in other studies *V. cholerae* O1, *V. cholerae* O139 and *V. mimicus* lost all cfu from the wild-types cultivated in the absence of the amoeba from day 4 of cultivation (44, 61, 83, 85) and it was proven that *V. cholerae* died and did not enter the viable but nonculturable (VBNC) state after the loss of all cfu from the wild type (44). The question is how the *OmpA* mutant of *V. cholerae* survived longer than the wild-type strain.

In this thesis, it was found that the *OmpA* mutant of *V. cholerae* produced significantly more OMVs than the wild-type strain, confirming previous findings by Song et al. (23) that the lack of OmpA protein leads to production of more OMVs. An interesting observation in this thesis was that significant production of OMVs might have enriched the cultivation medium and supported longer survival of the mutant strain compared with the wild-type *V. cholerae*.

To investigate the effect of OMVs on the amoeba, *A. castellanii* cells were incubated in a suspension of OMVs isolated from each *V. cholerae* strain. The viable counts demonstrated decreased viability of *A. castellanii* in both cases. This might indicate a virulence role of the OMVs towards the amoeba, in agreement with other studies (23, 86).

Interaction of *V. cholerae* strains with *A. castellanii* involves attachment of bacteria to the amoebal cells, engulfment, intracellular growth and survival inside the amoeba. The engulfment, intracellular growth and intracellular survival of the wild-type and *OmpA* mutant *V. cholerae* were not significantly different. In this context, Abd et al. (62) found that the capsule and LPS O-side chain did not affect engulfment, intracellular growth and intracellular survival of *V. cholerae* O139 when interacted with *A. castellanii*.

This thesis also showed that the presence of *A. castellanii* enhanced survival of both wild-type and *OmpA* mutant strains of *V. cholerae*. This is in agreement with previous findings that interaction of *A. castellanii* with wild-type *V. cholerae* O139, the capsule mutant strain and the capsule/LPS double mutant strain enhances survival of all these bacterial strains (62). Moreover, in spite of the fact that *V. cholerae* O1 El Tor possesses a mannose-sensitive haemagglutinin fimbria and *V. cholerae* O1 classical does not, they have enhanced survival and their intracellular growth in *A. castellanii* is not significantly different (44). All these facts may indicate that the intracellular behaviour of *V. cholerae* is a new survival strategy (44, 62).

In this thesis, the presence of the *OmpA* mutant *V. cholerae* decreased viability of *A. castellanii* significantly more than the presence of wild-type *V. cholerae*, possibly due to overproduction of OMVs by the mutant strain. The OMVs of *V. cholerae* have been suggested to promote the delivery of virulence factors to bacterial or eukaryotic cells (86). However, this thesis showed that OMVs decreased viability of the amoeba, which might indicate that vesicles are a virulence factor.

It has been observed previously that OmpA level is inversely correlated with the amount of OMVs, and that the sRNA of *V. cholerae*, which is called vibrio regulatory RNA of *OmpA* (VrrA), increases OMV production at a rate comparable to the loss of OmpA, since VrrA

positively regulates OMV release through downregulation of OmpA protein (23). However, inactivation of VrrA resulted in increased colonisation of *V. cholerae* in an infant mouse colonisation assay, indicating that OmpA protein is important for the colonisation of *V. cholerae* and that VrrA RNA may be considered a regulator that modulates the virulence of *V. cholerae* (23).

The formation of OMVs is suggested to be linked to the turgor pressure of the cell envelope during bacterial growth (87). Gram-negative bacteria have developed many strategies to enable active virulence factors to gain access to the extracellular environment, typically the tissues or bloodstream of the host organism (88). Vesicles are the means by which bacteria interact with prokaryotic and eukaryotic cells in their environment. Biochemical analysis and functional characterisation of pathogen-derived outer membrane vesicles have demonstrated that this secretory pathway has been taken by pathogens for the transport of active virulence factors into host cells (29). However, the ability of OMVs to fuse with bacterial membranes and of host cells to deliver content into the cytosol means that these vesicles may be described as bacterial 'bombs' for directed intercellular transport of particular bacterial virulence factors into host cells and tissues (29, 89-91). Further investigations are needed to reveal more about the function of the vesicles and their interaction with host cells.

Finally, this thesis showed that when both strains were cultivated alone, the *OmpA* mutant of *V. cholerae* expressed more OMVs and survived longer than the wild-type. Moreover, the amount of OMVs isolated from the *OmpA* mutant strain was sufficiently high to decrease viability of the amoeba. Co-cultivation with *A. castellanii* enhanced survival of both wild-type and *OmpA* mutant strains of *V. cholerae*.

In conclusion, OmpA might have a regulating role in survival of *V. cholerae* since it suppressed its survival, while the lack of OmpA enhanced release of OMVs. The OMVs might act as a virulence factor when they support long survival of the bacterium and decreased viability of the interacted amoeba. *Vibrio cholerae* might be adapted to survive better in association with eukaryotes.

*Vibrio cholerae* species comprise 206 serogroups (*V. cholerae* O1 to *V. cholerae* O206) (92). Only *V. cholerae* O1 and *V. cholerae* O139 produce cholera toxin and cause cholera, while the other serogroups can cause vibriosis diseases such as gastroenteritis, open wound infections and septicaemia. Recently, interaction of *V. cholerae* O1, *V. cholerae* O139 and *V. mimicus* with *Acanthamoeba* has shown that *V. cholerae* can grow and survive inside *A. castellanii* (44, 61, 62).

This thesis examined the ability of the clinical isolates *V. cholerae* O3, *V. cholerae* O4, *V. cholerae* O5, *V. cholerae* O11 and *V. cholerae* O160 to grow and survive in the absence and presence of *A. castellanii*, as well as the growth and survival of bacteria inside *A. castellanii*. Viable count assays of the bacteria and amoeba showed that in the absence of the amoeba, *V. cholerae* O3, *V. cholerae* O5, *V. cholerae* O11 and *V. cholerae* O160 died during the first week. Similar findings have been made in previous studies about growth of *V. cholerae* O1, *V. cholerae* O139 and *V. mimicus* (44, 62, 85). Surprisingly, it was found that *V. cholerae* O4 survived (>2 weeks) much longer than other serogroups mentioned above.

In the presence of the amoeba, survival of *V. cholerae* O5 and *V. cholerae* O160 was extended by up to 9 days and 14 days, respectively, compared with survival of *V. cholerae* O3, *V. cholerae* O4 and *V. cholerae* O11. The survival of *V. cholerae* O3, *V. cholerae* O4, *V. cholerae* O5 and *V. cholerae* O11 was not enhanced in the presence of the amoeba compared with the survival of *V. cholerae* O1, *V. cholerae* O139 and *V. mimicus* (44, 62, 85).

In this thesis, the intracellular growth of *V. cholerae* strains was investigated to examine their ability to grow and survive in *A. castellanii*. The results showed that *V. cholerae* O3, *V. cholerae* O4, *V. cholerae* O5, *V. cholerae* O11 and *V. cholerae* O160 were able to grow and survive inside the amoeba cells, similarly to the reported growth and survival of *V. cholerae* O1, *V. cholerae* O139 and *V. mimicus* inside *A. castellanii* (44, 62, 85).

The *V. cholerae* species from the Baltic Sea examined in this thesis and our previous studies (44, 62, 85) showed a similar growth pattern and survival to the facultative intracellular bacteria *F. tularensis*, *S. sonnei* and *S. dysenteriae*, which can grow and survive inside *A. castellanii* (70, 83).

Interaction output of *V. cholerae* O3, *V. cholerae* O4, *V. cholerae* O5, *V. cholerae* O11 and *V. cholerae* O160 with *A. castellanii* was not similar to that of the extracellular bacterium *P. aeruginosa* with *A. castellanii*. The presence or absence of the amoeba did not affect growth and survival of *P. aeruginosa*, which kills the amoeba (84).

The Swedish isolates of *V. cholerae* in this study interacted as facultative intracellular bacteria since they grew and survived in cultivation media, outside and inside the amoebal cells, which protected the intracellular bacteria from being killed by gentamicin. The role of *Acanthamoeba* in this interaction might support survival of *V. cholerae* species rather than cholera toxigenic species. The behaviour of *V. cholerae* O3, *V. cholerae* O4, *V. cholerae*

O5, *V. cholerae* O11 and *V. cholerae* O160 with *A. castellanii* identified these bacterial species as facultative intracellular bacteria.

# 6 GENERAL CONCLUSION

Our overall conclusions showed that:

- ✓ Both *V. cholerae* and Acanthamoeba species were detected in water samples, with Acanthamoeba enhancing the presence of *V. cholerae* in the samples.
- ✓ Candidate protozoal phyla were detected in the water samples and subsequently identified by molecular methods.
- ✓ Outer membrane protein A controls outer membrane vesicle (OMVs) production.
- ✓ OMVs simultaneously enhanced the survival of *V. cholerae* and inhibited the viability of amoebae.
- ✓ The Swedish isolates of *V. cholerae* interacted as facultative intracellular bacteria and the amoebae acted as an environmental host.

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