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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*

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Summary

The ability of free living and waterborne amoebae to feed on bacteria in the surroundings, as well as to host several human bacteria suggests that both amoebae and bacteria are involved in complex interactions. The extracellular bacterium, *Vibrio cholerae* requires 10^8 to 10^9 cells to cause cholera, and accordingly it needs an environmental host to grow to such high numbers to be able to cause the infection in humans. The current review discusses the properties of *V. cholerae* to be able to grow inside the environmental protozoa *Acanthamoeba* species, findings of our field study applied molecular detection of both microorganisms in the same natural water samples from cholera endemic area, and role of *Acanthamoeba* as a protozoal host to *V. cholerae* in nature beside human.

Keywords: Cholera; Natural water; *Acanthamoeba*; Second host; *V. cholerae*; Intracellular survival

Introduction

Vibrio cholerae is a gram-negative bacterium and it known to cause cholera. Cholera is an acute diarrhoeal disease that can kill within hours if left untreated. The actual global disease burden is estimated to be 3–5 million cases and 100 000–130 000 deaths per year [1]. *V. cholerae* and free-living amoebae (FLA) are present in aquatic environments, including drinking water [2,3,4].

Acanthamoeba is a genus of FLA, which are environmental eukaryotic cells distributed worldwide in nature [5,6] and they are found to support bacterial growth and survival [5]. Recently, studies have shown that *Pseudomonas aeruginosa* kills *A. castellanii* [7] and *Aeromonas hydrophila* inhibits growth of *A. castellanii* [8]. Interestingly, *V. cholerae* O1, O139 and *V. mimicus* have grown and survived inside *Acanthamoeba* species [9-14].

Since ability of different bacteria to interact differently with FLA needs to disclose mechanisms of the interactions, which are important to the medical- and environmental microbiology, the current review discusses findings about growth and survival of *V. cholerae* inside *Acanthamoeba castellanii* and *A. polyphaga*, detection of both microorganisms in the same natural water samples from cholera endemic area, and role of *Acanthamoeba* as an environmental host to *V. cholerae*.

Biology and ecology of *V. cholerae*

Vibrio is a genus of Gram-negative bacteria that comprise nearly 70 species [15] such as *V. cholerae, V. parahaemolyticus* and *V. mimicus*. These bacteria found in water and they can be carried by sea living animals, such as shellfishes. The prevalence rate of infections caused by *Vibrio* species appears to be increasing globally. The combination of increased water temperature and salinity may contribute to increased association rates of the bacteria with sea living animals or protozoa. In the event of a natural disaster, the disturbance to the environment may increase the risk of infectious diseases such as *Vibrio* infections. Vibrio species can produce multiple extracellular cytotoxins and enzymes that are associated with extensive tissue damage and that may play a major role in the development of sepsis [16].

V. cholerae includes more than 200 serogroups based on the O antigenic structures [17]. Only serogroup O1 and O139 possess cholera toxin gene and cause epidemic and pandemic cholera that affects many millions annually creating a worldwide health problem [18-21]. Non-O1/O139 *V. cholerae* are serologically diverse strains, which are abundant in estuarine environments. They are sporadically involved in cholera-like diarrheal disease [22-26], but rarely in outbreaks. *V. cholerae* O141 and *V. cholerae* O75 possess cholera toxin gene but not produce cholera toxin [27,28].

Vibrio mimicus shares similar properties with *V. cholerae* such as existence of virulence associated genes, namely cholera toxin as well as toxin co-regulated pilus genes [29] and both species possess LuxO protein that regulates protease activity [30].

V. cholerae species are straight or curved rods widely distributed in aquatic environments [31]. Evidence suggests that *V. cholerae* is a component of the autochthonous flora of brackish water, estuaries, and salt marshes of coastal areas of the temperate zone, posing an ongoing hazard to public health [32,33]. Various *V. cholerae* O1 strains have become endemic in many regions in the world, including Australia and the Gulf Coast region of the US [20,34].

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 [23,33,35,36,37,38].

Food supplies may be contaminated by the use of human faeces as fertilizer or by freshening vegetables for market with contaminated water [17,35,39,40,36]. 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.

V. cholerae harbors naturally in the zooplankton of fresh, brackish, and salt water, attached primarily to their chitinous exoskeleton [41]. 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 [42].

Despite amoebae are significant predators of bacteria in soil and aquatic environments [43], many biological factors affect the survival of *V. cholerae* in aquatic environments such as loss to predators [44] and regulating the level of viable cells of *V. cholerae* [45] to be able to infect humans. The dose of *V. cholerae* needed to cause cholera is approximately 10^8 cells [21]. Therefore, the bacteria need a biological reservoir as 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 host and reservoir for *V. cholerae* outside its aquatic environment [46] but according to the need of high numbers of bacteria to cause infection it seems likely that the bacterium has an environmental host beside man and that such a host may support growth and survival of the bacterium in nature. Finding of aquatic reservoirs of *V. cholerae* is an important factor in the epidemiology of cholera.

Amoebae predators or hosts for bacteria but special bacteria kill the amoebae

FLA are eukaryotic cells found in nature [5] and including several genera such as *Acanthamoeba*, Balamuthia, *Naegleria*[6] *Sappinia* [47] and *Dictyostelium* [48]. Association of *Dictyostelium* was studied with a number of pathogenic bacteria for more than hundred years ago since the role of the accompanying bacteria rightfully occupied the center of attention [49]. Interestingly, Raper and Smith (1939) found an excellent growth of *D. discoideum* with *Escherichia coli* and a suppressed growth with *P. aeruginosa* compared to good growth occurred in association with *Shigella dysenteriae, Salmonella enteritis* and *S. paratyphi* [49]. Moreover, Thom et al., 1992 showed that *V. cholerae* could grow and survive during 24 h in microcosms pre-inoculated with trophozoites of freshwater amoebae [50].

In this context, it has been shown that *Acanthamoeba* benefit from *E. coli* and *Klebsiella aerogenes* as food [51]. In contrast, the role of *Acanthamoeba* as hosts for bacteria, has been proposed for many pathogenic bacteria, for example *Campylobacter jejuni* [52], *Coxiella burnetii* [53], *E. coli* O157 [54], *E. coli* K1 [55], *Francisella tularensis* [56], *Helicobacter pylori* [57], *Legionella pneumophila* [58], *Listeria monocytogenes* [59], *Mycobacterium avium* [26], *S. dysenteriae* [25], *S. sonnei* [60, 61, 25] and *S. typhimurium* [62]. The *Acanthamoeba* support bacterial growth and survival and save the bacteria from chlorination [63] increasing the risk of human illness caused by bacteria or *Acanthamoeba*. However, Fritsche et al. [64] has found that 25% of environmental and clinical *Acanthamoeba* species isolates contain obligate bacterial endosymbionts.

Why some bacteria are utilised as food by the amoebae and other bacteria utilised the amoebae as hosts, and why a number of special bacteria kill the amoeba!

Generally, bacteria can be divided into intracellular and extracellular bacteria according to avoid phagocytosis or not and to multiply inside or outside the host cells [65]. Many bacteria possess type three secretion system (TTSS) other bacteria do not. These differences between bacteria may affect their interaction with the amoebae.

TTSS is found in many gram-negative pathogenic bacteria, such as *Yersinia* spp., *P. aeruginosa*, *Salmonella* spp., *Shigella* spp., enteropathogenic, enterohemorrhagic and neuropathogenic *E. coli* strains. TTSS consists of structural-, effectors proteins and chaperones. The structural proteins build the apparatus consisting of base, inner rod and needle. The needle translocates effector proteins into the plasma membranes to inject the effector proteins into the cytoplasm of eukaryotic cells to promote infection in target cells. The chaperones bind the effectors in the bacterial cytoplasm, protect them from aggregation and degradation and direct them towards the needle complex [84]. TTSS plays a key role in the bacterial pathogenicity and infection; therefore, output of extracellular and intracellular bacteria that possess or lack TTSS, on interaction with amoebae was studied by the researchers.

It has been found that the extracellular bacterium *P. aeruginosa* possessing TTSS injects four different effector proteins (exoenzymes) described as ExoS, ExoT, ExoU, and ExoY. These effector proteins are able to inhibit DNA synthesis as well as phagocytosis in the host cell and to induce necrosis and cell lysis associated with cell injury damage and dissemination of *P. aeruginosa* within infected hosts [7].

Abd et al., studied the interaction between *A. castellanii* and *P. aeruginosa* and the result has shown that neither the presence nor absence of *A. castellanii* has any effect on growth of *P. aeruginosa*, since large numbers of bacteria remain outside the amoebae during the interaction, revealing *P. aeruginosa* to be strictly extracellular bacteria growing better outside than inside eukaryotic cells. However, the encystation of *A. castellanii* can be stimulated by either wild type *P. aeruginosa* possessing different TTSS effector proteins or TTSS-deficient strains. Interestingly, wild type *P. aeruginosa* strains kill *A. castellanii*, while TTSS deficient strains inhibit growth of the amoebae. Thus, stimulation of encystation and inhibition of amoebic growth might be caused by the extracellular virulence factors such as extracellular factors produced by the Rhl quorum sensing system. *P. aeruginosa* wild type or deficient in TTSS effector proteins can cause necrosis and apoptosis in amoebae, but TTSS effector proteins of the wild type are also capable of lysing more amoeba cells, resulting in decreased numbers of necrotic, apoptotic and normal amoeba cells. The difference between the effect of strains possessing TTSS effector proteins and strains deficient in these proteins on *A. castellanii* is clearly the difference between killing and inhibition [7]. In this context, It has been reviewed that *P. aeruginosa* utilises TTSS effector proteins to kill macrophages, epithelial cells and the amoeba D. discoideum, as is also the case with A. *castellanii* [7]. Thus, TTSS is a potent virulence factor for the extracellular bacterium *P. aeruginosa* and *A. castellanii* represents a novel model to study wild type bacteria and parental mutants in TTSS.

In comparison, it was found that the extracellular and non-pathogenic *E. coli* K12 lacking TTSS had no effect on *A. castellanii* viability and the bacterium failed to grow inside the amoeba cells [55]. However, co-cultivation of the facultative intracellular neuropathogenic *E. coli* K1 and *E. coli* K12 associated with *A. castellanii* showed that *E. coli* K1 lysed the amoebae and grew exponentially, whereas *E. coli* K-12 exhibited minimal growth [85]. Surprisingly, Siddiqui et al., has demonstrated recently that the neuropathogenic *E. coli* K1 strains

possesses TTSS and the deletion mutant of TTSS in *E. coli* exhibits defects in the invasion and intracellular survival in *A. castellanii* [85].

F. tularensis is a gram-negative bacterium causing tularemia in humans and animals. Tularemia is a zoonotic disease, which results in fever, rash and swollen lymph nodes. The transmission of the bacterium occurs by several modes such as bites by infected ticks, flies or mosquitoes, or intake of contaminated water, food or soil as well as inhalation of aerosol containing the bacteria and other direct contact means such as handling of tissues or fluids from infected animals. Ecology of the bacterium has shown that it has been isolated from more than 250 animal species such as hares, rabbits and rodents and it can be recovered from contaminated water and soil but its principal natural reservoir is unknown. It is suggested that the bacterium can persist in water courses, possibly in association with amoebae [56].

F. tularensis is facultative intracellular bacterium lacking TTSS [86]. It has potential ability to grow and survive intracellularly in *A. castellanii*. *F. tularensis* cells infect trophozoites of the amoeba, replicate and grow in membrane bounded vacuoles inside the trophozoites. Cell organelles of the amoeba such as mitochondria and endoplasmic reticulum are recruited to the vacuoles containing bacteria. Infected trophozoites are found both as intact viable cells filled with vacuoles containing *F. tularensis* and, in the process of cytolysis, excreting vesicles containing *F. tularensis*. Some infected trophozoites are also seen undergoing encystations and *F. tularensis* cells can be found in precysts and in mature cysts. The infection process of *F. tularensis* in *A. castellanii* seems to display many features in common with *Legionella* infection in *A. castellanii* and in macrophages [56].

The ability of *F. tularensis* to survive in trophozoites of *A. castellanii* and its cysts may have implications for the mode of transmission of the microorganism. The close connection of tularaemia with water and the isolation of the bacterium from water samples used for domestic purposes, as well as from natural water systems, support the hypothesis that amoebae play a role in the natural transmission of the facultative intracellular/lacking TTSS *F. tularensis*. The model of non-mammalian cells and waterborne bacteria can be useful in the search for biological methods to inhibit transmission of pathogens from water to humans.

The interaction of TTSS possessing or lacking extracellular bacteria (*P. aeruginosa* or *E. coli* K12) and that of TTSS possessing or lacking intracellular bacteria (*E. coli* K1 or *F. tularensis*) is reviewed in this paper. Next, the reviewed outputs of the extracellular and intracellular bacteria that possess or lack TTSS, on interaction with amoebae will be compared to that of *V. cholerae* O1 and O139 lacking TTSS [84]. *V. cholerae* is a free-living cell in aquatic environment [66] and it is held to be an extracellular bacterium [4, 67]. What will happen if *V. cholerae* is cultivated with the environmental phagocyte *Acanthamoeba castellanii* or *A. polyphaga* in a liquid microcosm? Theoretically, it is expected that *V. cholerae* multiply in the culture liquid and not inside *Acanthamoeba* cells because it is an extracellular bacterium. But how does the experiment answer this question?

Enhanced growth, viability and survival of *V. cholerae* and *V. mimics* by co-cultivation with *Acanthamoeba* species

The interaction of *A. castellanii* or *A. polyphaga* with *V. cholerae* O1, *V. cholerae* O139 and *V. mimicus* was studied by cultivation of each microorganism alone and by cultivation of each bacterial species or serogroup with the amoebae for two weeks. Growth of the microorganisms was estimated by viable counts and the bacterial localisation in amoebae was disclosed by microscopy.

It was found that the number of alone- and co-cultivated amoeba increased tenfold after one week. Irrespective of *V. cholerae* species or serogroup present the growth of the amoebae was not inhibited [9-14]. The growth of co-cultivated *V. cholerae* O1 classical and El Tor biogroups, *V. cholerae* O139 and *V. mimicus* with amoebae was enhanced and they remained viable under the performed experimental time. The presence of the amoebae enhanced survival of co-cultivated Vibrio species during two weeks, while all these species in the absence of amoebae died within few days and did not enter viable and non-culturable state [9-14].

It was shown that growth of wild type *V. cholerae* O139 MO10, capsule mutant strain, and capsule + lipopolysaccharide (LPS) double mutant strain were enhanced in the presence of *A. castellanii* [10]. Previous studies have shown that both capsule and LPS O side chain of *V. cholerae* MO10 enhance adherence to the human intestinal mucosa [68,69] and the capsule may contribute to partially resist phagocytosis [70].

In comparison to macrophages, *Acanthamoeba* utilise different mechanisms to capture bacteria by means of specific and non-specific adherence as well as food cup formation. It is well known that *A. castellanii* takes up bacteria by pseudopodia to form food vacuoles in which phagocytosis and digestion occur within phagolysosomes [24] or by food cup formation and ingestion of particulate matter [71]. Adherence of bacteria to eukaryotes is the first step in interaction between bacteria and host cell [72]. Bacterial adherence to various surfaces includes several manners, including hydrophobic and ionic bonds and also lectin-like interactions between bacterial ligands and complementary molecules of the substrate or receptors on eukaryotes [73,74]. Lock et al., 1987 found that bacteria possessing mannose-sensitive fimbriae were able to adhere to *Acanthamoeba* and to leucocytes and that addition of mannose inhibited completely bacteria adherence to leucocytes, whereas, it inhibited only partly adherence to *Acanthamoeba* [73].

It is known that mannose-sensitive haemagglutinin fimbria of *V. cholerae* O139 contributed to its attachment to plankton in aquatic habitats [75]. In this context, Abd et al., 2009 showed that the addition of mannose neither affected adherence nor uptake and intracellular growth of *V. cholerae* MO10 in *A. castellanii* [10].This supports the previous finding by Lock et al., 1987 [73] and may indicate that specific adherence mediated by other factors such as outer membrane protein, toxin co-regulated pilus, beside non-specific adherence participate in the interaction between *V. cholerae* and *A. castellanii*.

A. castellanii and *V. cholerae* lived together in the microcosm. The co-existence of these microorganisms did not inhibit growth of amoebae but enhanced growth, viability and survival of the bacteria instead. How did the enhanced bacterial growth, viability and survival occur?

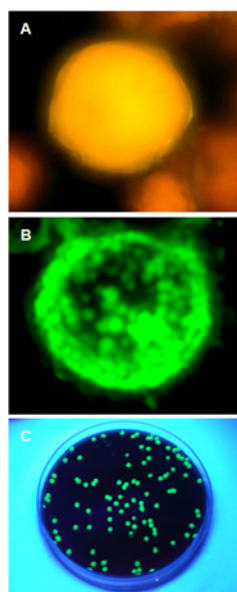## Intracellular behaviour of *V. cholerae* and *V. mimicus* as a new property

To answer the previous question, the ability of *V. cholerae* and V. mimics to grow and survive inside *A. castellanii* or *A. polyphaga* was investigated by gentamicin treatment to kill extracellular bacteria from co-culture and by sodium deoxycholate solution treatment, which permeabilities amoebae to release intracellular bacteria. The outcome of the treatments was detected by viable counts and visualized by fluorescence microscopy and the localization of intracellular occurring bacteria was visualized by electron microscopy.
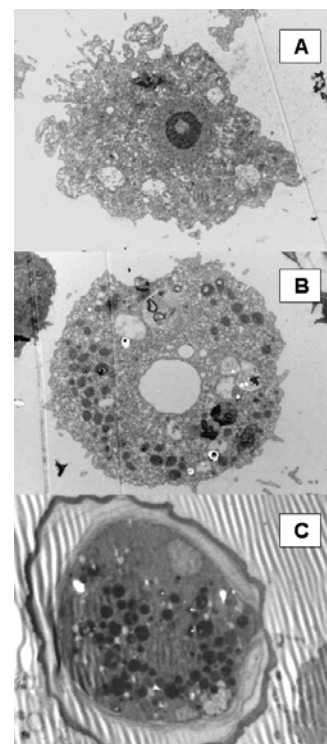
Surprisingly intracellular growth of *V. cholerae* and *V. mimicus* occurred and the viable counts of the intracellular growing bacteria showed an increase in the number from non-detectable levels on day 0 to 10^3 and 10^5 CFU/ml on day 1 and 14, respectively [9-14]. The intracellular localisation of *V. cholerae* strains in *A. castellanii* was confirmed by fluorescence microscopy and electron microscopy. The fluorescence microscopic analysis confirmed viability of intracellular *V. cholerae* O1 tagged green fluorescent protein (GFP), which emits green fluorescence (Figure 1B), compared to *A. castellanii* cells, which emits red auto fluorescence (Figure 1A). To know whether the fluorescent bacteria entered viable but non-culturable state or not, the viable count assay confirmed that the bacteria are viable and culturable because they grew on agar plates, and the growing colonies emitted green fluorescence also when exposed to ultraviolet light (Figure 1C). Electron microscopy confirmed the intracellular localisation of *V. cholerae* cells in the trophozoites (Figure 2B) as well as in the cysts of *A. castellanii* (Figure 2C) compared to the trophozoite that did not contain bacteria (Figure 2A).

Trophozoites of *A. castellanii* undergo encystation as a vital, protective, and reversible process to face adverse conditions such as changes in pH, temperature, and food deprivation [76]. However, our studies showed that encystation of *A. castellanii* in presence or absence of *V. cholerae* did not affected by intracellular internalisation of the bacteria [12] in contrast to interaction of *A. castellanii* with the extracellular bacterium *P. aeruginosa*, which killed the amoebae [7]. Thus, output of different interactions between *V. cholerae, P. aeruginosa* and *A. castellanii* indicated adaption of *V. cholerae* to the intracellular milieus and symbiotical relation between *V. cholerae* and *A. castellanii*.
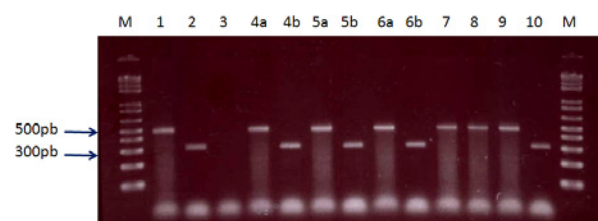
Despite *V. cholerae* is considered as an extracellular bacterium [4] our studies show that *V. cholerae* has interacted with the environmental phagocyte *A. castellanii* as a facultative intracellular bacterium, similar to *F. tularensis* [56].

Figure 2: Localisation of *V. cholerae* in *A. castellanii* with a magnification of 3700x.
A. alone cultivated *A. castellanii* trophozoite as negative control, characterized by nucleus, vacuoles and pseudopodia. B. Many empty vacuoles, pseudopodia and more than 50 cells of *V. cholerae* O139 inside *A. castellanii* trophozoite on day 6 of co-cultivation. C. More than 40 cells of *V. cholerae* O139 inside *A. castellanii* cyst characterized by double wall called ecto-and endocyst, on day 7 of co-cultivation.

Figure 1: Viability and cultivability of the intracellular *V. cholerae* O1 tagged green fluorescent protein (GFP). A. Alone cultivated *Acanthamoeba castellanii* cyst emitting autoflourescence. B. Intracellular *V. cholerae* emitting green fluorescence. C. The intracellular *V. cholerae* grew on blood agar plate in presence of ampicillin. The colonies emitted green fluorescent when exposed to UV light.

Figure 3: 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 bacterial positive control (308bp), 3 negative control, 4 to 6 samples contain both *Acanthamoeba* (a) and *V. cholerae* (b), 7 to 9 samples contain amoebae only, 10 sample containing *V. cholerae* only.

It is well known that extracellular bacteria, such as *Corynebacterium diphtheriae, Pseudomonas* species [46], and *Bacillus anthracis* [77], are sensitive to phagocytosis; therefore, they may benefit from killing phagocytes before they are ingested [38]. We have found that the extracellular bacterium *P. aeruginosa* induced both apoptosis and necrosis in *A. castellanii* [7], which is in contrast to the facultative intracellular bacterium, *L. pneumophila* that does not induce apoptosis in another species of *Acanthamoeba*, *A. polyphaga* [78].

The growth pattern of *P. aeruginosa* PA103 in the absence or presence of amoebae [7] indicates that the behaviour of this extracellular bacterium differs from the behaviour of facultative

intracellular bacteria, such as *F. tularensis* [56] and from *V. cholerae* that has shown a facultative intracellular behaviour since the growth of *V. cholerae* is enhanced in the presence and inhibited in the absence of amoebae [9-14].

Behaviour of *V. cholerae* differs also from other strict extracellular bacteria, such as *E. coli* and *K. aerogenes*, which provide excellent nourishment for *A. castellanii* and *A. polyphaga* [55, 51].

Growth pattern of *V. cholerae* O1 strains showed that numbers both of amoebae and intracellular bacteria increased over the experimental time, and the statistical analysis showed that the growth of intracellular bacteria is dependent on the growth of amoebae in a strong positive linear relation; R2 was 0.87 [12]. The behaviour of *V. cholerae* O1 and *V. cholerae* O139 in our studies [10,12-14] resembles the behaviour of the facultative intracellular bacterium *F. tularensis* [56] when it interacts with *A. castellanii* but differs from the behaviour of the extracellular bacterium *P. aeruginosa*. Recent study showed that *P. aeruginosa* grew to 10^9CFU/ml on day 1, in spite of different multiplicities of infection (MOIs) when this bacterium was cultured alone or with amoebae, and that both alone and co-cultivated *P. aeruginosa* survived at the same level during the experimental time, which was 12 days [7]. In comparison, growth and survival of *F. tularensis* [56] as well as *V. cholerae* [10, 12-14] were enhanced in the presence and inhibited in the absence of amoebae.

The common property of toxigenic *V. cholerae* serogroups is the presence of toxin-co-regulated pilus (TCP), which is a colonization factor to the intestine of humans [31]. *V. cholerae* O1 El Tor and O139 possess mannose-sensitive haemagglutinin (MSHA), which is required for colonization to zooplankton [75]. However, it is well documented that *V. cholerae* O139 possesses a capsule whereas O1 strains do not [79,80].

Although there are differences between toxigenic *V. cholerae* serogroups and biotypes, our studies [10,12-14] showed that *V. cholerae* O1 and O139 grow and survive intracellularly in *A. castellanii*. These findings confirm a facultative intracellular behavior of *V. cholerae* strains as a new common property, which is previously unknown.

The new behaviour of *V. cholerae* to behaveas intracellular bacteria with *A. castellanii* did enhance growth and viability of the co-cultivated bacteria and protected the intracellularly *V. cholerae* from antibiotics [10,12-14]. Moreover, growth and viability of the amoebae did not affected, indicating that free-living amoeba acts as host to *V. cholerae* in nature in addition to man. In this context, do the amoebae hosting intracellular *V. cholerae* play a role as biological factor in the epidemiology of cholera?

Enhanced growth of *V. cholerae* to higher number than needed to cause cholera by re-cultivation of *A. castellanii* hosting intracellular *V. cholerae*

To examine the role of amoebae harbouring intracellular *V. cholerae* as biological factor in the epidemiology of cholera, *A. castellanii* was cultivated with *V. cholerae* for 1 week. After gentamicin killing and washing of extracellular *V. cholerae*, the number of viable intracellular bacteria as well as viable amoebae was estimated by viable count of bacteria and viability of amoeba cells by using erythrosine staining.

It was found that 4 x10^5 cell/ml *A. castellanii* harbouring 2 x10^5 cell/ml intracellular *V. cholerae* was re-cultivated in 50 ml ATCC medium 712 for 2 weeks. The result showed an enhanced growth of *A. castellanii* to 2.3×10^6cell/ml and *V. cholerae* to 2 x10^{11} CFU/ ml and both

microorganisms survived for more than 2 weeks. In this experiment the amoeba works as a biological incubator where intracellular growth occurred under 4 days to increase number of the bacteria one million fold and maintains overall viability of *V. cholerae* during 2 weeks [81].

V. cholerae shows the facultative intracellular bacterial behaviour, because it is found intracellularly as well as extracellularly in the culture medium, which increases the number of the bacteria over the infection dose stipulated for humans. Thus, the ability of *V. cholerae* to grow and survive intracellularly in *A. castellanii* presented in our studies disclosed one of the biological factors that could enhance the survival of *V. cholerae* in aquatic environments. The free-living amoeba is a possible biological factor, which enhances growth of the bacteria in water and may thereby increase the probability to infect human with cholera [12,81].

Detection of both *V. cholerae* and *Acanthamoeba* in same natural water samples from cholera endemic area

V. cholerae and *Acanthamoeba* species are present in aquatic environments, including drinking water [2-4]. A number of studies report that FLA support survival of pathogenic bacteria [5] and more studies are still needed on distribution of *V. cholerae* and FLA in nature [82]

A molecular method was developed to detect *V. cholerae* and *A. castellanii* if found together in the same environmental water samples, by using primers targeting cholera toxin gene (ctxA) and *Acanthamoeba* 18S rDNA gene [14]. Recently this method was utilised to detect presence of *V. cholerae* and *Acanthamoeba* species in same natural water samples collected from endemic areas in Sudan. It was found that eight samples contained both *V. cholerae* and *Acanthamoeba* species. Furthermore, it was found that one sample contained just *V. cholerae* compared to 13 samples which contained *Acanthamoeba* only. Surprisingly, the detected number of together- and alone-identified amoebae and bacteria was significantly differed (pvalue of $\chi 2$ was < 0.05). *V. cholerae* needs to be found with other microorganisms such as *Acanthamoeba* a finding disclosed by this study since 89% of detected *V. cholerae* was found with *Acanthamoeba*e compared to 11% *V. cholerae*, which was found alone [83]. For the first time this study showed that both *V. cholerae* and Acanthamoeba species can be detected in the same natural water samples collected from different cholera endemic areas in Sudan. Taken together role of *Acanthamoeba* species in survival of *V. cholerae* [10,12-14] may strongly disclose *Acanthamoeba* as a biological factor enhancing survival of *V. cholerae* in nature.

Conclusions

Output of the interaction between bacteria and amoeba is depending on whether the interacted bacterium is an extracellular or intracellular and if it possesses TTSS or not since TTSS effector proteins are observed to affect strongly output of the interaction.

Extracellular bacteria cannot multiply inside amoeba cell. TTSS possessing extracellular bacteria such as *P. aeruginosa* kill the amoeba compared to *E. coli* that does not possess TTSS is ingested as food by the amoeba.

Intracellular bacteria multiply inside amoeba cell. The intracellular bacterium that does not possess TTSS *F. tularensis* multiplies symbiotically inside the amoebae, while TTSS possessing intracellular bacterium *E. coli* K1 lyses the amoebae according to activation of TTSS.

The ability of *V. cholerae* to grow and survive inside *A. castellanii*

confirms that *V. cholerae* strains have a facultative intracellular behaviour as a new property, previously unknown.

The intracellular growth as well as survival of the bacteria does not inhibit growth and viability of amoeba cells indicating a symbiotic relationship between *V. cholerae* and *A. castellanii*.

A. castellanii enhances growth and survival of *V. cholerae* and protects the intracellular bacteria from antibiotic effects, in addition to detection both microorganisms from same natural water samples, supporting the role of amoebae as environmental hosts for *V. cholerae*.

A. castellanii is a suitable model in research to differentiate between extracellular and intracellular bacteria, since the intracellular bacteria are able to multiply inside the amoeba while the extracellular bacteria do not.

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