INFECTION ECOLOGY & EPIDEMIOLOGY
THE ONE HEALTH JOURNAL

co-action PUBLISHING

ORIGINAL RESEARCH ARTICLE

Swedish isolates of *Vibrio cholerae* enhance their survival when interacted intracellularly with *Acanthamoeba castellanii*

Salah Shanan, PhD[1]*, Magdi Bayoumi, MD, MSc, Assistant Professor[1], Amir Saeed, PhD, Assistant Professor[2], Gunnar Sandström, PhD, Professor[2] and Hadi Abd, PhD, Researcher[2]

[1]Faculty of Medical Laboratory Sciences, University of Medical Sciences and Technology, Khartoum, Sudan; [2]Division of Clinical Microbiology, Department of Laboratory Medicine, Karolinska Institute, Karolinska University Hospital, Stockholm, Sweden

Vibrio cholerae is a Gram-negative bacterium that occurs naturally in aquatic environment. Only *V. cholerae* O1 and *V. cholerae* O139 produce cholera toxin and cause cholera, other serogroups can cause gastroenteritis, open wounds infection, and septicaemia. *V. cholerae* O1 and *V. cholerae* O139 grow and survive inside *Acanthamoeba castellanii*. The aim of this study is to 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*. The interaction between *A. castellanii* and *V. cholerae* strains was studied by means of amoeba cell counts, viable counts of the bacteria in the absence or presence of amoebae, and of the intracellularly growing bacteria, visualised by electron microscopy. These results show that all *V. cholerae* can grow and survive outside and inside the amoebae, disclosing that *V. cholerae* O3, *V. cholerae* O4, *V. cholerae* O5, *V. cholerae* O11, and *V. cholerae* O160 all can be considered as facultative intracellular bacteria.

Keywords: V. cholerae; *Swedish isolates*; *intracellular*; A. castellanii; *gentamicin protection*

Responsible Editor: Tanja Strand, Uppsala University, Sweden.

*Correspondence to: Salah Shanan, University of Medical Sciences and Technology, P.O. Box 12810, Khartoum, Sudan, Email: salahshan@yahoo.co.uk

Received: 20 January 2016; Revised: 23 February 2016; Accepted: 23 February 2016; Published: 25 April 2016

Vibrio is a genus of Gram-negative bacteria found in water. The genus comprises nearly 70 species such as *Vibrio parahaemolyticus*, *V. vulnificus*, and *V. cholerae* (1).

V. cholerae species are comprised of serogroups depending on antigen O (O1–O206) (2), 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 (3).

V. cholerae and free-living amoebae (FLA) are present in aquatic environments, including drinking water (4–6). FLA are unicellular eukaryotic protozoa found in all soil and aquatic environments (7). The FLA belong to the genera *Balamuthia*, *Naegleria*, and *Acanthamoeba* and are responsible for opportunistic and non-opportunistic infections in mammals (8). *Acanthamoeba* causes three main types of diseases: keratitis, encephalitis and disseminated infection (8–10).

Sea living animals may carry the vibrio, and the combination of increased water temperature and salinity may contribute to increased association rates of the bacteria with sea-living animals or protozoa (11). The ability of FLA to act as reservoirs for many bacteria has been studied (reviewed in (12)). 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 (12). 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 (12). *V. cholerae* O1, *V. cholerae* O139, and *V. mimicus* have been found to be able to grow inside *A. castellanii* (13–15). In 2004, more than 50 cases of vibriosis were reported in Sweden after

Citation: Infection Ecology and Epidemiology 2016, **6**: 31060 - http://dx.doi.org/10.3402/iee.v6.31060
(page number not for citation purpose)

exposure to water from the Baltic Sea or swimming outdoors in summer (16–19).

The aim of this study is to 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*.

Materials and methods

Microorganisms

A. castellanii ATCC 30234 was obtained from the American Type Culture Collection, Manassas, VA. The clinical isolated strains: *V. cholerae* 03 (wound infection, 2006, Ronneby), *V. cholerae* 04 (sepsis, 2006, Karlskrona), *V. cholerae* 05 (blood, 2006, Stockholm), *V. cholerae* 011 (ear infection, 2004, Karlskrona), and *V. cholerae* 0160 (blood, 2006, Gävle) were obtained from the Public Health Agency of Sweden.

Culture media, growth conditions and analyses

V. cholerae strains were grown on blood agar plates overnight at 37°C. *A. castellanii* was grown at 30°C to a final concentration of 10^6 cells ml^{-1} in ATCC medium no. 712 (Karolinska Institutet, Stockholm, Sweden). In order to infect the amoeba, *V. cholerae* were grown in Luria–Bertani (LB) broth to an absorbance of 0.6 at 600 nm. Co-cultures of each bacterial strain and *A. castellanii* were incubated in 75 cm^2 cell culture flasks (Corning Incorporated Costar) 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 viable counts.

Gentamycin susceptibility test

Sensitivity of *V. cholerae* strains to gentamicin was determined by E-test. The test measured the minimal inhibitory concentration of gentamycin (MIC) utilising a plastic strip according to the Swedish Reference Group for Antibiotics (SRGA) (20).

Growth of V. cholerae *strains in the absence or presence of amoebae*

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 amoebae 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.

Growth and survival of V. cholerae *strains inside* A. castellanii

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 minimize extracellular *V. cholerae* contamination. The pellets were resuspended in 1 mL of PBS and incubated with 500 $\mu g/ml^{-1}$ 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 two-fold with 0.1% sodium deoxycholate. Series of 10-fold dilution 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.

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

A. 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 2×10^5 cells mL^{-1}.

Microscopy analysis

A. castellanii cells, in the absence and presence of bacteria were counted in a Bürker chamber (Merck Eurolab, Sweden) under a light microscope (Carl Zeiss, 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 post-fixed 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).

Citation: Infection Ecology and Epidemiology 2016, **6**: 31060 - http://dx.doi.org/10.3402/iee.v6.31060

Statistical analysis

Student's *t*-test was used for comparison between viable counts of alone and co-cultivated microorganisms. A p value of ≤ 0.05 was considered statistically significant. Data represent mean $\pm$SD of three independent experiments over the whole course of the experiment (including every day).

Results

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 one log on day one and the bacteria showed different survival rates. *V. cholerae* O3, O4, O5, O11, and O160 survived 4, 15, 4, 6, and 3 days, respectively (Fig. 1).

In the presence of *A. castellanii* the viable counts of *V. cholerae* O3, O4, O5, O11, and O160 increased one log on day 1 and the bacteria survived 6, 5, 8, 5, and 14 days, respectively (Fig. 2).

To compare between the viable counts of the bacterial strains in the absence or presence of *A. castellanii.* The Student's *t*-test was used. Viable count of *V. cholerae* O3, O5, and O11 in the absence or presence of *A. castellanii* was not significant (p values were >0.05). However, viable count of *V. cholerae* O4 and *V. cholerae* O160 in the absence or presence of *A. castellanii* was significant ($p \leq 0.05$).

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 gentamycin was performed by E-test. The MIC value for *V. cholerae* O3, O4, O5, *V. cholerae* O11, O160 was 0.25, 1.0, 0.75, 1.0, and 0.75 μg/mL, respectively. These results showed that all *V. cholerae* examined strains were susceptible to gentamycin since the susceptibility of *V. cholerae* was (S $\leq$2 μg/mL, R >4 μg/mL).

The intracellular assay showed that after 1 day *V. cholerae* O3, O4, O5, O11, and O160 grew inside the amoeba cells to 2.7×10^5, 1.8×10^5, 5.8×10^4, 2.1×10^5, and 2.1×10^4 cfu/mL, respectively, and survived intracellularly for 5, 5, 6, 5, and 14 days, respectively (Fig. 3).

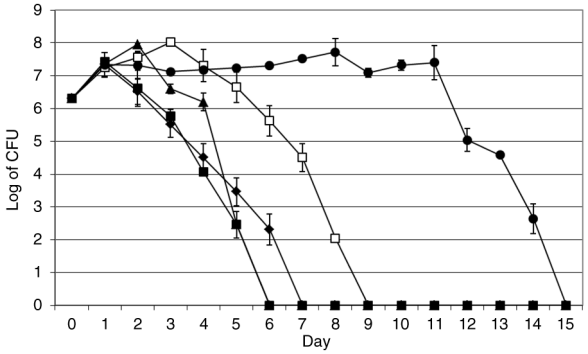

Fig. 2. Viable counts of *V. cholerae* in the presence of *A. castellanii*. *V. cholerae* O3 (◆), O4 (▲), O5 (□), O11 (■), and O160 (●). Data represent means $\pm$SD of three independent experiments.

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 and *V. cholerae* O4 (Fig. 4) in the cytoplasmic vacuoles of trophozoites of *A. castellanii*.

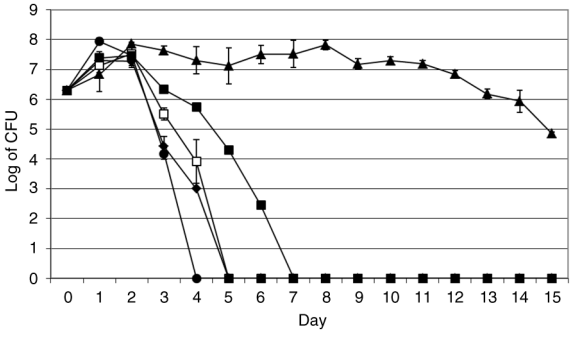

Fig. 1. Viable counts of *V. cholerae* in the absence of *A. castellanii*. *V. cholerae* O3 (◆), O4 (▲), O5 (□), O11 (■) and O160 (●). Data represent means $\pm$SD of three independent experiments.

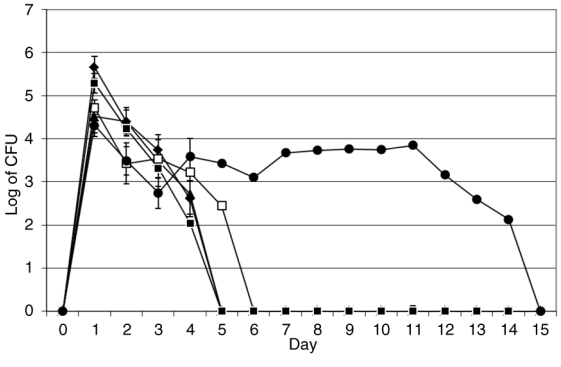

Fig. 3. Intracellular growth and survival of *V. cholerae*. Viable counts of intracellular *V. cholerae* O3 (◆), O4 (▲), O5 (□), O11 (■), and O160 (●). Data represent mean $\pm$SD of three independent experiments.

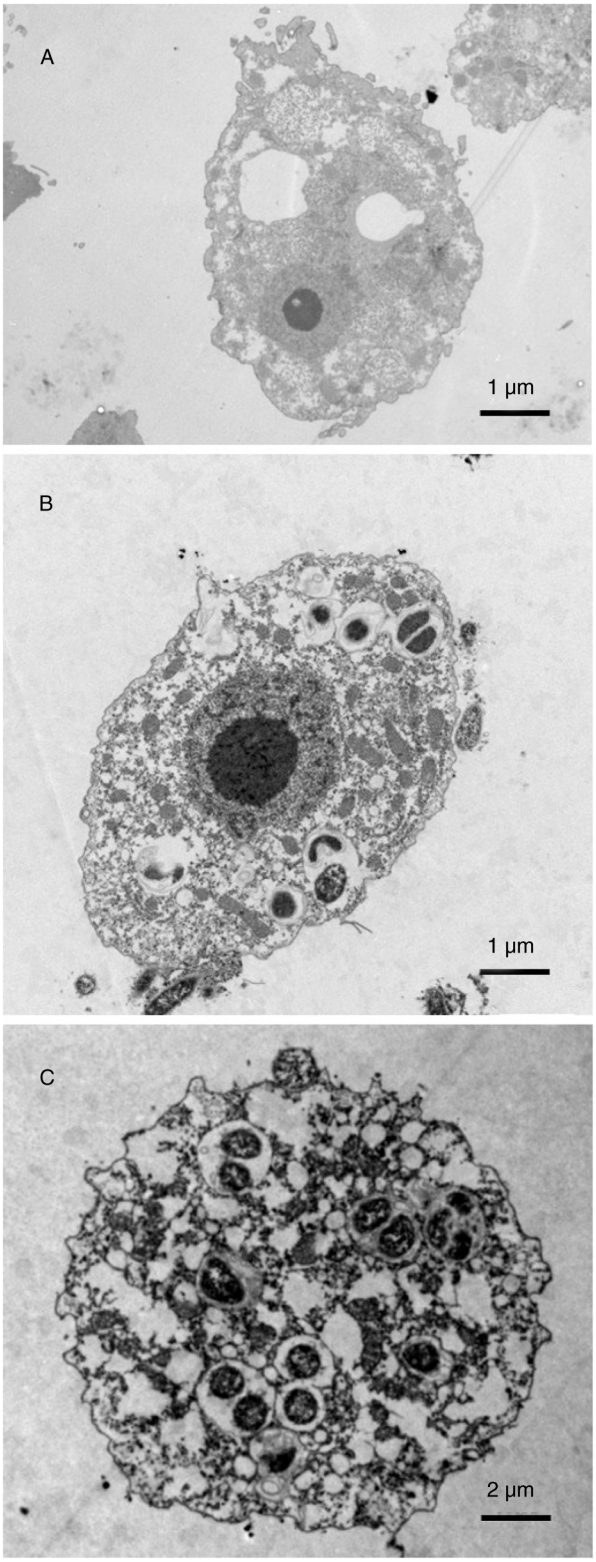

Fig. 4. Electron microscope image showing the intracellular localisation of *V. cholerae* in *A. castellanii*. **A.** *A. castellanii* trophozoite in the absence of bacteria. **B**. *V. cholerae* O160 localised in cytoplasmic vacuoles of *A. castellanii* trophozoite, 2 h after co-cultivation. **C**. *V. cholerae* O4 in cytoplasmic vacuoles of *A. castellanii* trophozoite, 4 h after co-cultivation.

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

The growth of *A. castellanii* in the absence or in the 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 2×10^5 cells/mL on day 0 to 1.5×10^6 cells/mL on day 15 (Fig. 5).

The viable count of *A. castellanii* in the presence of *V. cholerae* O3, O5, O11, and O160 increased from 2×10^5 cells/mL on day 0 to 7.3×10^5, 3.9×10^5, 6.7×10^5, and 4.4×10^5 cells/mL, respectively. In contrast, the viable count of the amoeba in the presence of *V. cholerae* O4 decreased to 1.2×10^5 cells/mL on day 15 (Fig. 5).

The viable count of *A. castellanii* in the presence of *V. cholerae* O3, O5, O11, and *V. cholerae* O160 increased from 2×10^5 cells/mL on day 0 to 7.3×10^5, 3.9×10^5, 6.7×10^5, 4.4×10^5 cells/mL, respectively, on day 15 (Fig. 5). However, the viable count of *A. castellanii* in the presence of *V. cholerae* O3, O5, O11, O160 and presuming also *V. cholerae* O4, shows values comparable to those on day 15, already on day 1, that is, the increase was observed much earlier than on day 15. In contrast, the viable count of the amoeba in the presence of *V. cholerae* O4 decreased to 1.2×10^5 cells/mL on day 15 (Fig. 5).

The differences in the viable count of *A. castellanii* in the absence or presence of *V. cholerae* O3, O4, O5, and O11 strains were statistically significant by student t-test ($p < 0.05$). In contrast, the viable count of *A. castellanii* in the absence or presence of *V. cholerae* O160 strain was not significantly different ($p > 0.05$).

Discussion

More than 200 serogroups of *V. cholerae* are human pathogens causing cholera and vibriosis such as gastroenteritis, open wounds infection, and septicaemia. Recently, interaction of *V. cholerae* O1, *V. cholerae* O139, and *V. mimicus* with *Acanthamoeba* species has shown

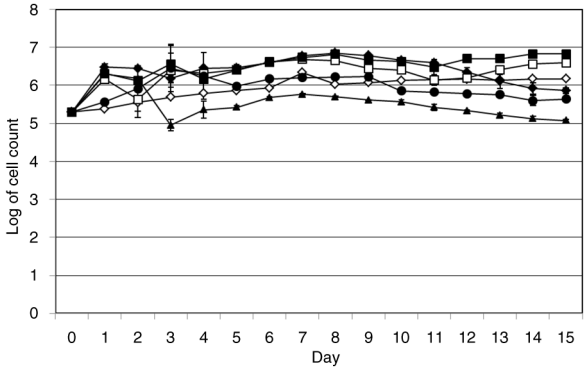

Fig. 5. Growth of *A. castellanii*. Viable counts of *A. castellanii* in the absence of *V. cholerae* strains (◇), and in the presence of *V. cholerae* O3 (◆), O4 (▲), O5 (□), O11 (■), and O160 (●). Data represent mean ±SD of three independent experiments.

Citation: Infection Ecology and Epidemiology 2016, **6**: 31060 - http://dx.doi.org/10.3402/iee.v6.31060

that *V. cholerae* can grow and survive inside *A. castellanii* (13, 14, 21).

The current study examined the ability of the Swedish clinical isolates *V. cholerae* O3, O4, O5, O11, and 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* to highlight the interaction between the Swedish isolates of *V. cholerae* with amoebae.

Viable count of the bacteria in the absence of amoeba showed that *V. cholerae* O3, O5, O11, and O160 died during the first week. This finding is similar to that of previous studies on growth of *V. cholerae* O1, *V. cholerae* O139, and *V. mimicus* (13–15). Surprisingly, it was found that *V. cholerae* O4 survived (>2 weeks) much longer than did the other serogroups.

In the presence of the amoebae, survival of *V. cholerae* O3, O5, and *V. cholerae* O160 was enhanced up to 6, 8, and 14 days compared with suppressed survival of *V. cholerae* O4 and *V. cholerae* O11. The survival of *V. cholerae* O3, O4, O5, and O11 was not enhanced in the presence of the amoebae compared to previous studies on the survival of *V. cholerae* O1, *V. cholerae* O139, and *V. mimicus* that enhanced from days to weeks (13–15).

Furthermore, 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, O4, O5, O11, and O160 could grow and survive inside the amoeba cells similar to what previously has been shown on the growth and survival of *V. cholerae* O1, *V. cholerae* O139, and *V. mimicus* inside *A. castellanii* (13–15).

Surprisingly, *V. cholerae* O4 and *V. cholerae* O160 cells were seen in the cytoplasmic vacuoles of trophozoites of *A. castellanii* only. The cysts of *A. castellanii* containing *V. cholerae* were not found indicating that intracellular *V. cholerae* might not suppress the trophozoites to undergo encystation. However, *V. cholerae* O160 showed more adaption, than other strains in this study, to survive in presence of the amoeba and intracellularly as well (Figs. 2 and 3).

The *V. cholerae* species from the Baltic Sea examined in this study and our previous studies (13–15) showed a similar growth pattern and survival to the facultative intracellular bacteria *Francisella tularensis*, *Shigella sonnei*, and *S. dysenteriae*, which can grow and survive inside *A. castellanii* (22, 23). Huws and Smith 2006 showed that *Staphylococcus aureus* could grow and survive intracellularly as well as extracellularly in amoebae. The numbers of viable amoebae in the presence or absence of *S. aureus* were not found to be significantly different (24).

Interaction output of *V. cholerae* O3, O4, O5, O11, and O160 with *A. castellanii* was found to be different from that of the extracellular bacteria such as *P. aeruginosa* and *Aeromonas* species during interaction with *A. castellanii*. The presence or absence of the amoebae did not affect growth and survival of *P. aeruginosa* which instead kills the amoebae (25). Moreover, *Aeromonas hydrophila* and *A. veronii* has been shown to inhibit growth of *A. castellanii* (26). The Swedish isolates of *V. cholerae* in this study interacted as facultative intracellular bacteria since they grew and survived in cultivation medium, outside and inside the amoebae cells.

A. castellanii in this interaction supported survival of *V. cholerae* species rather than cholera toxigenic species. The behaviour of *V. cholerae* O3, O4, O5, O11, and O160 with *A. castellanii* highlighted the role of the FLA and their intracellular pathogenic microorganisms as risks for water quality.

In summary, interesting differences can be observed regarding the interaction between these strains and *A. castellanii*, where the amoebae dramatically prolong the survival of strain *V. cholerae* O160 and in contrast, dramatically reduce the survival of strain O4. This warrants future studies of the mechanisms behind bacterial defence to amoeba predation in these organisms.

Acknowledgements

The authors gratefully acknowledge Karolinska Institute for supporting this project.

Conflict of interest and funding

The authors declare that they have no conflict of interest.

References

1. Euzeby JP. List of bacterial names with standing in nomenclature: a folder available on the Internet. Int J Syst Bacteriol 1997; 47: 590–2.
2. Aydanian A, Tang L, Morris JG, Johnson JA, Stine OC. Genetic diversity of O-antigen biosynthesis regions in *Vibrio cholerae*. Appl Environ Microbiol 2011; 77: 2247–53.
3. Centers for Disease Control and Prevention (CDC). Summary of notifiable diseases – United States, 2010. MMWR Morb Mortal Wkly Rep 2012; 59: 1–111.
4. Brown MR, Barker J. Unexplored reservoirs of pathogenic bacteria: protozoa and biofilms. Trends Microbiol 1999; 7: 46–50.
5. Backer H. Water disinfection for international and wilderness travelers. Clin Infect Dis 2002; 34: 355–64.
6. Greub G, Raoult D. Microorganisms resistant to free-living amoebae. Clin Microbiol Rev 2004; 17: 413–33.
7. Page FC. A new key to freshwater and soil Gymnamoebae. Ambleside, Cumbria, UK: Freshwater Biological Association; 1988.
8. Visvesvara GS, Moura H, Schuster FL. Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. FEMS Immunol Med Microbiol 2007; 50: 1–26.
9. Marciano-Cabral F, Cabral G. Acanthamoeba spp. as agents of disease in humans. Clin Microbiol Rev 2003; 16: 273.
10. Siddiqui R, Khan NA. Biology and pathogenesis of *Acanthamoeba*. Parasit Vectors 2012; 5: 6.
11. Huq A, West PA, Small EB, Huq MI, Colwell RR. Influence of water temperature, salinity, and pH on survival and growth of

toxigenic *Vibrio cholerae* serovar 01 associated with live copepods in laboratory microcosms. Appl Environ Microbiol 1984; 48: 420–4.
12. Abd H, Shanan S, Saeed A, Sandström G. Survival of Vibrio cholerae inside *Acanthamoeba* and detection of both micro-organisms from natural water samples may point out the amoeba as a Protozoal Host for *V. cholerae*. J Bacteriol Parasitol 2011: 1–3.
13. Abd H, Saeed A, Weintraub A, Nair GB, Sandstrom G. *Vibrio cholerae* O1 strains are facultative intracellular bacteria, able to survive and multiply symbiotically inside the aquatic free-living amoeba *Acanthamoeba castellanii*. FEMS Microbiol Ecol 2007; 60: 33–9.
14. Abd H, Saeed A, Weintraub A, Sandstrom G. *Vibrio cholerae* O139 requires neither capsule nor LPS O side chain to grow inside *Acanthamoeba castellanii*. J Med Microbiol 2009; 58: 125–31.
15. Abd H, Valeru SP, Sami SM, Saeed A, Raychaudhuri S, Sandstrom G. Interaction between *Vibrio mimicus* and *Acanthamoeba castellanii*. Env Microbiol Rep 2010; 2: 166–71.
16. Smittskyddsinstitutet. Epidemiologisk årsrapport 2004. Solna; 2005. Available from: http://www.smittskyddsinstitutet.se/upload/Publikationer/Epi-arsrapport-050623.pdf [cited 16 November 2015].
17. Steen A. Farliga bakterier i träbadkaret. Smittskydd. Contract No.: 5. Folkhälsomyndigheten; 2004.
18. Collin B, Rehnstam-Holm AS. Occurrence and potential pathogenesis of *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* on the South Coast of Sweden. FEMS Microbiol Ecol 2011; 78: 306–13.
19. Rehnstam-Holm AS, Collin B. [Vibrio species in the waters of Southern Sweden caused bath-wound fever. Increased bacteria frequency according to studies on clams]. Lakartidningen 2009; 106: 435–8.
20. Antibiotics SRGf. Web-page of the Swedish Reference Group for Antibiotics (SRGA). Available from: www.srga.org/RAFMETOD/etest.htm [cited 11 April 2008].
21. Abd H, Weintraub A, Sandstrom G. Intracellular survival and replication of *Vibrio cholerae* O139 in aquatic free-living amoebae. Environ Microbiol 2005; 7: 1003–8.
22. Abd H, Johansson T, Golovliov I, Sandstrom G, Forsman M. Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii*. Appl Environ Microbiol 2003; 69: 600–6.
23. Saeed A, Abd H, Edvinsson B, Sandstrom G. *Acanthamoeba castellanii* an environmental host for *Shigella dysenteriae* and *Shigella sonnei*. Arch Microbiol 2009; 191: 83–8.
24. Huws SA, Smith AW, Enright MC, Wood PJ, Brown MR. Amoebae promote persistence of epidemic strains of MRSA. Environ Microbiol 2006; 8: 1130–3.
25. Abd H, Wretlind B, Saeed A, Idsund E, Hultenby K, Sandstrom G. *Pseudomonas aeruginosa* utilises its type III secretion system to kill the free-living amoeba *Acanthamoeba castellanii*. J Eukaryot Microbiol 2008; 55: 235–43.
26. Rahman M, Abd H, Romling U, Sandstrom G, Mollby R. *Aeromonas-Acanthamoeba* interaction and early shift to a viable but nonculturable state of *Aeromonas* by *Acanthamoeba*. J Appl Microbiol 2008; 104: 1449–57.