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

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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

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

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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, Gavle) 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 10⁶ 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 cm² cell culture flasks (Corning Incorporated Costar) filled with 50 mL of ATCC medium no. 712 containing an initial concentration of 10⁵ cells mL⁻¹ of *A. castellanii* and 10⁸ 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 gentamicin (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 10⁻⁵ 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 μg/mL 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 10⁸ 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 × 10⁵ 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 CaCl₂ 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).
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 ± 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 ≤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 ≤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).

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.
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 $2 \times 10^5$ cells/mL on day 0 to $1.5 \times 10^6$ 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 $2 \times 10^5$ cells/mL on day 0 to $7.3 \times 10^5$, $3.9 \times 10^5$, $6.7 \times 10^5$, and $4.4 \times 10^5$ cells/mL, respectively. In contrast, the viable count of the amoeba in the presence of *V. cholerae* O4 decreased to $1.2 \times 10^5$ 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 $2 \times 10^5$ cells/mL on day 0 to $7.3 \times 10^5$, $3.9 \times 10^5$, $6.7 \times 10^5$, $4.4 \times 10^5$ 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 $1.2 \times 10^5$ 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 septicemia. Recently, interaction of *V. cholerae* O1, *V. cholerae* O139, and *V. mimicus* with *Acanthamoeba* species has shown...
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.

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### Conflict of interest and funding

The authors declare that they have no conflict of interest.

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