Association of *Vibrio cholerae* with fresh water amoebae

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**Summary.** An investigation was undertaken to determine whether *Acanthamoeba polyphaga* SHI and *Naegleria gruberi* 1518/le could affect the survival of various strains of *Vibrio cholerae* in laboratory microcosms. In microcosms pre-inoculated with trophozoites of amoebae, all six strains of *V. cholerae* tested survived and multiplied during 24 h. In control microcosms without trophozoites of amoebae, survival of the *V. cholerae* strains was much decreased. Two strains of *V. cholerae* were used to determine whether *V. cholerae* might survive ingestion within amoebae and subsequent encystment. Strain 152 was re-isolated from excysting *N. gruberi* 1518/le but not from *A. polyphaga* SHI. Strain 9112 could not be isolated from cysts of either species of amoebae.

**Introduction**

Evidence for an aquatic reservoir of *Vibrio cholerae* is derived from laboratory based investigations of the effects of physico-chemical stresses on the survival of *V. cholerae* and from field studies in which *V. cholerae* was isolated from water without any evident association with cases of clinical cholera. Furthermore, it has been shown that *V. cholerae* can survive long periods of low nutrient stress as coccoid ultra-microbacteria, a possible resting stage formed by reductive division and size reduction. Despite the isolation of *V. cholerae* from multiple environmental sites and living organisms, including plants, plankton, shellfish and fish, the precise identity of the ecological niche favouring its survival in an aquatic environment remains obscure. However, other bacteria are known to survive in association with amoebae and symbiotic relationships between infecting bacteria and host amoebae have been demonstrated.

*L. pneumophila* has been shown to persist within various species of amoebae. The fastidious nature of *L. pneumophila* in laboratory culture combined with evidence of legionellae infecting macrophages in man led some to postulate that it is not a free-living aquatic organism and to examine whether it might be associated with free-living amoebae. Subsequently, it was shown that *L. pneumophila* could infect and multiply within amoebae of *Acanthamoeba* and *Naegleria* spp. This suggests that amoebae may be the primary reservoir of *L. pneumophila* in the environment.

However, there has been no similar investigation of a potential association between *V. cholerae* in the aquatic environment and free-living amoebae. If *V. cholerae* could survive ingestion by amoebae, the controlled local micro-environment would afford protection from external fluctuations. Furthermore, amoebae encyst in response to environmental stress. The cyst form protects the amoebae from desiccation and food deprivation and it might also protect the vibrio. Here the potential for survival of *V. cholerae* in association with amoebic trophozoites and within amoebic cysts was examined.

**Materials and methods**

*Bacteria and amoebae*

Suspensions of *V. cholerae* strains 17 (clinical isolate, Tanzania), 102 (Tanzania), 152 (water, Dacca), 164 (Bangladesh), 9112 (clinical isolate, Australia) and 9351 (water, Australia) were prepared in Page's saline containing NaCl 0.1% (MPS), from overnight cultures incubated at 25°C or 37°C on Trypticase Soy Agar (TSA; Oxoid). The suspensions were adjusted to an OD _600_ of 0.04 and 3 ml were inoculated on to pure adherent cultures of *N. gruberi* 1518/le and *A. polyphaga* SHI trophozoites seeded at 10⁵/ml in plastic flat-sided tissue-culture tubes (Nunc Ltd). The amoebae had been grown as axenic cultures at 30°C in SCGYEM medium as modified by Auy et al. Tubes inoculated with bacteria alone served as controls and were taken through the whole experimental procedure. The amoebae culture tubes and controls were incubated at 25°, 30° and 37°C for 1 h in the trophozoite survival experiment and for 1 or 3 h in the encystment survival experiment. All experiments were performed in duplicate.

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Survival of V. cholerae in trophozoites

Amoebae were exposed to V. cholerae as outlined above. After incubation for 1 h, unattached bacteria were decanted and the tubes were rinsed with MPS. Tubes were placed in an ice-bath to detach the amoebae which were then resuspended in MPS. Suspended amoebae (0.1 ml) were inoculated on to non-nutrient agar (NNA) overlaid with heat inactivated Escherichia coli and incubated at 30°C for up to 7 days to confirm the presence of viable trophozoites.

Ten-fold serial dilutions were prepared from the suspensions of amoebae, and from suspensions that had been freeze-thawed once, and 0.1-ml portions spread on to TSA at pH 8-6. Plates were incubated overnight at 37°C and colonies were counted.

In a second series of experiments, following the rinse after incubation for 1 h, the tubes were refilled with MPS and re-incubated for 24 h before further processing as above.

Survival of V. cholerae in cysts

After challenge of amoebae with V. cholerae, unattached bacteria were decanted and the tubes were rinsed in MPS. Encystment medium19 was added to each tube and the tubes were incubated at room temperature for 18 h. After centrifugation at 890 g for 2 min, the supernate was discarded, the tubes were washed twice in MPS containing HCl 0.5% v/v and centrifuged at 890 g for 2 min; the pellet was resuspended in fresh MPS containing HCl 0.5% v/v and incubated at room temperature for 18 h. The centrifugation and washing procedures were then repeated but with MPS as the wash medium. Finally, the cysts were resuspended in MPS containing heat-inactivated E. coli and incubated for up to 96 h at room temperature. Samples (0.1 ml) of the suspension were plated on to NNA overlaid with E. coli, TSA and Thiosulphate Citrate Bile Salts Agar (TCBS; Oxoid) plates. The suspensions remaining were freeze-thawed once and additional samples were spread on to TSA and TCBS plates. All the plates were incubated overnight at 37°C and colonies were counted.

Results and discussion

All six strains of V. cholerae survived in association with trophozoites of both species of amoebae for 24 h. In most co-cultures with amoebae, the viable count of vibrios at the end of the 24-h period was greater than in the original inoculum, whereas control cultures of V. cholerae incubated in the absence of amoebae showed a decrease in viable count. Typical results are presented in figs. 1–4. Of the two strains of V. cholerae tested for survival in amoebic cysts, strain 152 was recovered from cysts of N. gruberi 1518/1e (fig. 5) but not from cysts of A. polyphaga SHI, whereas strain 17 could not be isolated from either species of amoeba following encystation. Longer exposure of amoebae to the inoculating dose of V. cholerae resulted in a greater recovery of viable bacteria from amoebic cysts. No viable V. cholerae was isolated from the control cultures incubated without amoebic cysts (fig. 5).

V. cholerae has not previously been associated with

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Fig. 2. Survival of V. cholerae strain 17, from inoculum grown at (a) 25°C or (b) 37°C, with N. gruberi 1518/1e trophozoites (10⁵/ml) over 24 h. Amoeba co-cultures (X, ○, △) and V. cholerae control suspensions (■, ●, △) were incubated at 25°C (X, ■), 30°C (○, ●) or 37°C (△, △).

Fig. 3. Survival of V. cholerae strain 9112, from an inoculum grown at (a) 25°C or (b) 37°C, with A. polyphaga SHI trophozoites (10⁵/ml) over 24 h. Amoeba co-cultures (X, ○, △) and V. cholerae control suspensions (■, ●, △) were incubated at 25°C (X, ■), 30°C (○, ●) or 37°C (△, △).
polyphaga of sources not only survived, but multiplied over 24 h in microcosms containing amoebic trophozoites. Control control microcosms lacking amoebae. Different strains of other enteric pathogens of man, such as shigellae, of survival within amoebae. The presence of A. cholerae an intracellular mode of existence. Unlike a number survival of enterocolitica2'. Additionally, it was shown that V. cholerae isolated from environmental and clinical isolations and a means of dispersal. An alteration in conditions and a means of dispersal. An alteration in temperature ('C) incubation temperature ('C) (total expts)

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<tr>
<th>Vibrio growth temperature ('C)</th>
<th>Experimental incubation temperature ('C)</th>
<th>Number of isolations (total expts)</th>
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<tr>
<td>25</td>
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V. cholerae and Water Amoebae

an intracellular mode of existence. Unlike a number of other enteric pathogens of man, such as shigellae, entero-invasive E. coli, salmonellae and Yersinia enterocolitica20 the pathogenesis of V. cholerae does not involve cellular invasion. The results of this investigation have shown that V. cholerae is capable of survival within amoebae. The presence of A. polyphaga or N. gruberi trophozoites increased the survival of V. cholerae when compared to that in control microcosms lacking amoebae. Different strains of V. cholerae isolated from environmental and clinical sources not only survived, but multiplied over 24 h in microcosms containing amoebic trophozoites. Control cultures of V. cholerae incubated alone declined in viability over the same period.

Furthermore, it was shown that V. cholerae could survive encystment within N. gruberi cysts. The introduction of an acid wash following encystment ensured that all extra-amoebal V. cholerae and remaining trophozoites were killed. All control cultures of V. cholerae were sterile after this treatment. The re-isolation of viable V. cholerae after excysting of amoebic cultures demonstrated that the vibrio was ingested by the trophozoites and that it could survive within amoebic cysts (table).

No single test condition exclusively favoured survival of V. cholerae within amoebae. It is interesting to note that of the two strains of V. cholerae tested for survival in cysts, it was the environmental isolate and not the clinical isolate that survived ingestion and subsequent encystment by the environmental species of amoebae (N. gruberi). However, as only two species of amoebae and two strains of V. cholerae were used in the encystment experiments, no firm conclusions on inter-strain variations can yet be drawn.

The results of this study are compatible with those of King et al.21 who investigated the susceptibility of coliform bacteria and bacterial pathogens to free chlorine residues in the presence or absence of amoebae and ciliate protozoa. Their results led to the proposal that resistance to digestion by predatory protozoa was an evolutionary precursor of bacterial pathogenicity and a survival mechanism for bacteria in aquatic environments.

In the context of this hypothesis,21 a study of the phylogenetic relationship between Chlamydia (obligate intracellular parasites) and other bacteria gave interesting results in that ribosomal RNA from Chlamydia hybridised preferentially with DNA from V. cholerae.22

The combination of these results and those from the current study indicate that V. cholerae may have an intra-cellular/amoebal habitat. An intra-amoebal habitat would not necessitate an invasive capacity since internalisation of the vibrio would be effected by the amoebae. That no single combination of conditions tested particularly favoured an association with amoebae does not detract from the significance of the association. The survival of V. cholerae within cysts of certain species of amoebae for long periods would provide a protected niche under unfavourable conditions and a means of dispersal. An alteration in conditions could result in the emergence of the trophozoite from the cyst, the intra-amoebal multiplication of the vibrio, and its subsequent release into the environment.

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References