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PROTOZOOLOGY - SHORT COMMUNICATION

Vermamoeba vermiformis in hospital network: a benefit for *Aeromonas hydrophila*

Vincent Delafont[1] · Estelle Perraud[1,2] · Kévin Brunet[2,3] · Elodie Maisonneuve[4] · Sihem Kaaki[5] · Marie-Hélène Rodier[1,2]

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Abstract

Aeromonas hydrophila, considered as an emerging pathogen, is increasingly involved in opportunistic human infections. This bacterium, mainly present in aquatic environments, can therefore develop relationships with the free-living amoeba *Vermamoeba vermiformis* in hospital water networks. We showed in this study that the joint presence of *V. vermiformis* and *A. hydrophila* led to an increased bacterial growth in the first 48 h of contact and moreover to the protection of the bacteria in adverse conditions even after 28 days. These results highlight the fact that strategies should be implemented to control the development of FLA in hospital water systems.

Keywords *Vermamoeba vermiformis* · Free-living amoebae · *Aeromonas hydrophila* · Water · Environment

Introduction

Free-living amoebae (FLA) are mobile protozoa that feed on bacteria, algae, fungi, protozoa, or other organic particles. However, they may support the growth and serve as reservoirs and vehicles for a number of pathogenic microorganisms. Their life cycle comprises at least two stages: an actively feeding, dividing trophozoite form corresponding to the period of metabolic activity of the amoeba, and a dormant cyst that can resist hostile environmental conditions (Greub and Raoult 2004). *Vermamoeba vermiformis* is commonly isolated FLA from aquatic environment, thus representing an important component of the microbial community in freshwater (Scheid 2019). Besides natural aquatic environments, *V. vermiformis* is frequently found in tap water and recreational water, testifying of its ability to persist in engineered water systems, including water treatment plants, thermal water, and distribution systems (Delafont et al. 2018). This FLA is also recovered from hospital environments and was isolated more frequently than *Acanthamoeba* from samples of hospital water in Marseille, France (Pagnier et al. 2015). This study is in line with numerous others which reported the high isolation rate of *V. vermiformis* in hospital water networks (Samba-Louaka et al. 2019).

Aeromonas hydrophila, a member of the Gammaproteobacteria class, inhabits various aquatic sources, such as natural environment or community water systems, and is responsible for different types of infections both in humans and animals (Parker and Shaw 2011). This bacterium is known to cause hemorrhagic septicemia in some fish species with high mortality rate and has also been isolated from other non-aquatic animals, such as pets, invertebrates, or birds. Moreover, *Aeromonas* species can also be found in foods of animal origin. In humans, *A. hydrophila* is mainly associated with gastroenteritis or wound infections such as cellulitis or myonecrosis following an injury in an aquatic environment, consumption of contaminated food, or colonized water. *A. hydrophila* acts as an opportunistic pathogen which can cause severe deep-seated infections in immunocompromised patients (Gonçalves Pessoa et al. 2019). Thus, environmental

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✉ Vincent Delafont
vincent.delafont@univ-poitiers.fr

[1] Laboratoire Ecologie et Biologie des Interactions, UMR CNRS 7267, Université de Poitiers, Poitiers, France

[2] Laboratoire de Parasitologie et Mycologie, CHU de Poitiers, Poitiers, France

[3] Inserm U1070, Pôle Biologie Santé, 1 Rue Georges Bonnet, Poitiers, France

[4] EA BIOS 4691, Université de Reims Champagne-Ardenne, 51097 Reims, France

[5] Unité de pathologie ultrastructurale et expérimentale, Laboratoire d'anatomie et cytologie pathologiques, CHU la Milètrie, Poitiers, France

exposure of such patients is a critical aspect to monitor in hospital settings.

Vermamoeba vermiformis, a protist known to interact with a wide range of potentially pathogenic bacteria, can be found in the same hospital environment of *Aeromonas hydrophila.* This co-occurrence prompted us to investigate whether the presence of the free-living amoeba *Vermamoeba vermiformis* could impact the growth and the protection of *Aeromonas hydrophila*.

Materials and methods

Strains and growth conditions

V. vermiformis (ATCC 50256) was grown in 150-cm^2 tissue culture flasks at 27 °C in PYNFH broth (in 900 mL of distilled water: 10 g proteose peptone, 10 g yeast extract, 1 g ribonucleic acid, 15 mg acid folic, 1 mg hemin, 0.5 g $Na_2HPO_4 \cdot 2H_2O$, 0.36 g KH_2PO_4, and 100 mL fetal calf serum). When cells formed a monolayer, the trophozoites were harvested by tapping the flasks and washed three times in Page's modified Neff's amoeba saline (PAS, containing in 1 L of distilled water, 120 mg NaCl, 4 mg $MgSO_4.7H_2O$, 4 mg $CaCl_2.2H_2O$, 142 mg Na_2HPO_4, and 136 mg KH_2PO_4); then, amoebae were resuspended at a final cell concentration of 5.10^5/mL in filtered (0.22 μM) hospital water and used for the experimentations.

The strain of *A. hydrophila* used in this study was isolated from a sample of our hospital water system (Centre Hospitalier Universitaire de Poitiers, France) and identified by MALDI-TOF (Vitek MS, BioMerieux). Before experiments, this bacterium was grown on Mueller–Hinton agar slants at 37 °C for 24 h, then harvested, washed in filtered hospital water, and adjusted to a concentration of 5.10^4/mL.

Cocultivation of *A. hydrophila* with *V. vermiformis*

A hundred microliters of the suspension of *Vermamoeba* trophozoites were distributed into each well of a 96-well microplate (5.10^4 cells/100 μL) and allowed to adhere to the wells for 2 h at 27 °C. A hundred microliters containing 5.10^3 bacteria were then added in the wells to obtain a multiplicity of infection of 0.1, and incubation was carried out at 27 °C.

After 24 h and 48 h of incubation, serial dilutions of the cocultures were plated on Mueller–Hinton medium and incubated at 37 °C during 48 h to evaluate bacterial colony-forming units (CFU). Controls were carried out by incubating bacteria without amoebae in filtered hospital water. Microscopical examination of the cocultures (24 h and 48 h) using trypan blue staining was carried out in order to determine the viability of amoebae, and samples of coculture were also examined by transmission electron microscopy. All of the experiments were reproduced three times, each time in triplicate.

Incubation of *A. hydrophila* in amoeba culture supernatant

Trophozoites (5.10^5/mL) of *V. vermiformis* were incubated at 27 °C for 48 h in filtered water. The amoebae were then pelleted by gentle centrifugation (1000×*g*, 10 min) in order to prevent lysis, and *A. hydrophila* (5.10^4/mL) was incubated at 27 °C for 24 h and 48 h in the resultant filtered (0.45 μm) supernatant. After incubation, the suspension was plated on Mueller–Hinton medium and incubated at 37 °C to determine CFU counts.

Incubation of infected amoebae in encystment medium

Cocultures of *V. vermiformis* and *A. hydrophila* were first carried out in flasks at the concentrations indicated for plates. After 24 h of coincubation, the medium was replaced by encystment medium to allow cyst formation and to mimic conditions of poor nutrient availability. As a control, *A. hydrophila* alone was incubated in the same conditions. *A. hydrophila* CFU was then numbered on Mueller–Hinton agar medium after 14 days and 28 days of incubation at 27 °C. Coculture samples (28 days) were further examined by transmission electron microscopy.

Electron microscopy

In order to visualize the interactions between microorganisms, transmission electron microscopy was performed on coculture samples. Samples (approximately 5.10^6 cells) obtained in filtered tap water (24 h and 48 h) or in encystment medium (28 days) were incubated for 1 h in phosphate buffer 0.1 M, containing 4% glutaraldehyde at 4 °C. Cells were washed in PBS and post-fixed with 1% OsO_4 in phosphate buffer 0.1 M for 1 h at 4 °C. The samples were dehydrated in an acetone series and embedded in araldite resin. Sections were stained with uranyl acetate and lead nitrate before examination with a Jeol 1010 transmission electron microscope.

Results and discussion

In cocultures carried out in filtered hospital water, the presence of *V. vermiformis* induced a significant increase in *A. hydrophila* development, as compared with bacterial growth without amoebae (Kruskal–Wallis test, $\chi^2 = 46.004$, df = 2, p value = $1.024e^{-10}$; posthoc Dunn Test alone vs. coculture, Benjamini–Hochberg adjusted p value =

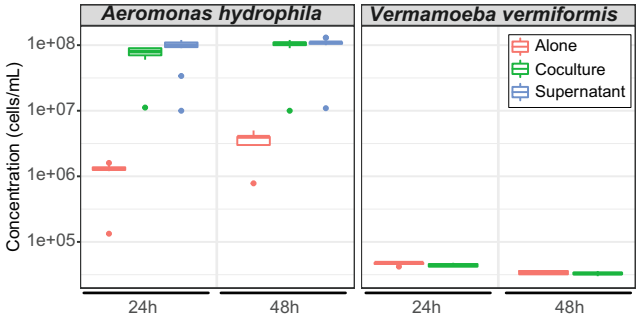

Fig. 1 Viable counts of *A. hydrophila* in tap water alone (pink), in coculture with *V. vermiformis* in tap water (green), or incubated in *V. vermiformis* supernatant (blue) during 24 h or 48 h

$1.067734e^{-06}$). The same significant results were obtained when bacteria were incubated in the presence of FLA culture supernatant (post hoc Dunn Test alone vs. supernatant, Benjamini–Hochberg adjusted p value = $2.621292e^{-10}$) (Fig. 1). These results suggested that the presence of *V. vermiformis* or of its metabolites positively influenced the bacterial growth. In addition, we have noticed that the viability of *V. vermiformis* was not different in the presence or absence of *A. hydrophila* (Fig. 1). Altogether, the presented results tend to suggest a saprophytic growth of the bacterium thanks to FLA metabolites.

So, we confirm here that the presence of *V. vermiformis* could lead to a significant bacterial development, as it has been previously shown for *Stenotrophomonas maltophilia* with the same amoeba host (Delafont et al. 2018). Using *Acanthamoeba polyphaga*, Anacarso et al. have shown that this free-living amoeba was able to interact with *A. hydrophila*, leading to an early bacterial replication within the trophozoites but ultimately to a loss of bacterial detection after 48 h. Interestingly, this behavior differs from that of *A. hydrophila* with *A. castellanii* which could have a role in the bacterial transmission. Indeed, Yousuf et al. have reported the bacterial survival within trophozoites (for at least 24 h) and cysts (for up to 10 days) of *A. castellanii* in laboratory conditions (Yousuf et al. 2013).

Transmission electron micrographs showed the presence of bacteria inside and outside of amoeba trophozoites after 24 h and 48 h of incubation (Fig. 2), mostly enclosed in vacuoles as it has been already shown in *Francisella tularensis–V. vermiformis* interactions (Santic et al. 2011) (Fig. 2A, B, D, E). We then evaluated the survival of *A. hydrophila* with and without amoebae in an oligotrophic medium and indicated that *V. vermiformis* presence led to persistent bacterial viability after 14 days ($1.47.10^8 \pm 5.77.10^6$ CFU/mL) and 28 days of incubation ($1.53.10^8 \pm 5.77.10^6$ CFU/mL), while in the same medium, bacteria without amoebae failed to grow. Interestingly, we highlighted bacteria are located in amoebal derived structures (Fig. 2C, F), devoid of typical cell organites, similar to those already described for *Stenotrophomonas maltophilia* (Cateau et al. 2014). These structures may confer to *A. hydrophila*, a higher level of protection against adverse conditions. This phenomenon could contribute to the high resistance witnessed for this bacterium in the context of artificial water networks with disinfectant residuals (Falkinham III et al. 2015). So, this opportunistic premise plumbing pathogen is very likely to benefit from the presence of free-living amoebae in the same water networks.

In conclusion, this study shows that growth and survival of *A. hydrophila* are enhanced by the presence of *V. vermiformis* in the same environment. Moreover, this amoeba seemed to confer the same mode of protection for *A. hydrophila* than for *S. maltophilia*. We confirmed that the presence of *V. vermiformis* in hospital water networks can consequently represent an increased risk of healthcare-associated infections. This study highlights the interest that should be paid to systematic detection of FLA in hospital water systems.

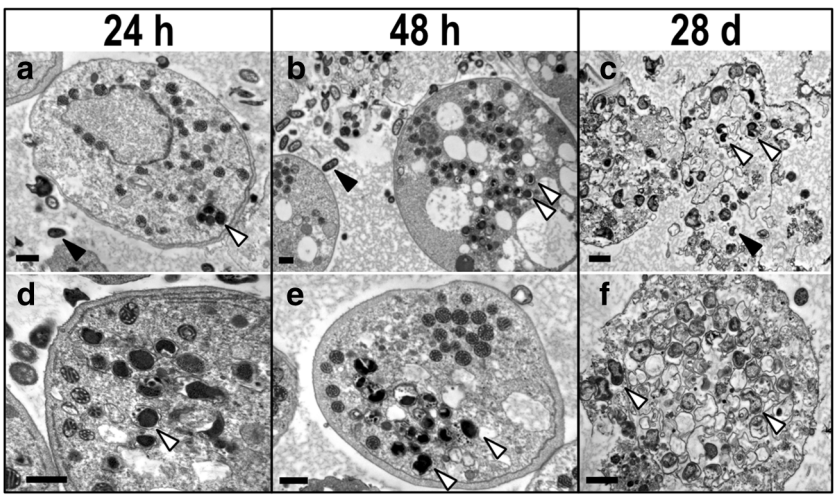

Fig. 2 Fate of *Aeromonas hydrophila* following infection of *Vermamoeba vermiformis* in tap water for 24 h (A, D) and 48 h (B, E). *Aeromonas hydrophila* can be found in amoebal derived structures after 28 days of coculture in encystment medium (C, F). White arrows highlight intra-amoebal bacteria, while black arrows indicate extracellular bacteria. Scale bar is 1 μm for all frames

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Authors' contributions MHR designed the study. MHR, EM, EP, and SK performed experiments. VD and MHR analyzed the data. MHR and VD wrote the manuscript. VD, EP, EM, KB, and MHR reviewed and edited the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

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