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Original article

DVC-FISH and PMA-qPCR techniques to assess the survival of *Helicobacter pylori* inside *Acanthamoeba castellanii*

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Abstract

Free-living amoebae (FLA) are ubiquitous microorganisms commonly found in water. They can act as Trojan Horses for some amoeba-resistant bacteria (ARB). *Helicobacter pylori* is a pathogenic bacteria, suggested to be transmitted through water, which could belong to the ARB group. In this work, a co-culture assay of *H. pylori* and *Acanthamoeba castellanii*, one of the most common FLA, was carried out to identify the presence and survival of viable and potentially infective forms of the bacteria internalized by the amoeba. Molecular techniques including FISH, DVC-FISH, qPCR and PMA-qPCR were used to detect the presence of internalized and viable *H. pylori*. After 24 h in co-culture and disinfection treatment to kill extra-amoebic bacteria, viable *H. pylori* cells were observed inside *A. castellanii*. When PMA-qPCR was applied to the co-culture samples, only DNA from internalized *H. pylori* cells was detected, whereas qPCR amplified total DNA from the sample. By the combined DVC-FISH method, the viability of *H. pylori* cells in *A. castellanii* was observed. Both specific techniques provided evidence, for the first time, that the pathogen is able to survive chlorination treatment in occurrence with *A. castellanii* and could be very useful methods for performing further studies in environmental samples.

Keywords: *Acanthamoeba castellanii*; *H. pylori*; Co-culture; DVC-FISH; PMA-qPCR

1. Introduction

Helicobacter pylori is a pathogenic Gram-negative bacteria considered to be the most extended infectious agent in humans, estimated to infect approximately 50% of the world's population [28]. Among all water-emerging pathogens, it is the only bacterium classified as a Class I human carcinogen by the WHO International Agency for Research on Cancer (IARC) because of its strong relationship to gastric cancer and peptic ulcer [13]. Its transmission has not yet been precisely determined, but it is strongly suggested that this bacteria is acquired by different routes, among which the fecal-oral route through water is included [10,21]. However, the relationship between illness and contaminated water is not well established, mainly due to the failure to culture the pathogen from the environment. Since *H. pylori* is sensitive to water disinfection treatments, survival mechanisms of this bacteria in water are not yet well defined.

Free-living amoebae (FLA) are ubiquitous protozoa that have been isolated from water, soil and air [23,29]. Acanthamoeba species are the most common FLA in those environments [15]. They have two developmental stages: the trophozoite, the metabolically active form, and the cyst, the dormant form, which is acquired under unfavorable conditions such as food depletion or other stress conditions. When in the trophozoite stage, Acanthamoeba spp. and other FLA feed on bacteria, some of which, instead of being phagocytosed, are able to resist or even replicate inside amoebae, being hidden

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and protected from harsh environmental conditions. Therefore, FLA could be considered "Trojan Horses" for these amoeba-resistant bacteria (ARB) [5]. In fact, there exist many studies on the role of FLA as potential transmission vehicles for several human pathogens such as Legionella, Listeria and Campylobacter [2,6,7].

Few studies have investigated the interaction between FLA and *H. pylori*. In a co-culture assay, Winiecka-Krusnell et al. [32] suggested that *H. pylori* viability could be improved by the presence of *Acanthamoeba castellanii*. Other authors [26] tried to show *H. pylori* survival and multiplication in the vacuoles of Acanthamoeba polyphaga in a co-culture assay, but without conclusive results. One study reported that co-culture of Campylobacter, a closely Helicobacter-genus-related bacteria, with *A. castellanii*, favors its survival and promotes its growth, although it is unable to replicate inside the amoeba [6]. The authors concluded that growth of this microaerobic bacterium was stimulated by a decrease in dissolved oxygen in the media due to the presence of *A. castellanii*. Therefore, the viability of *H. pylori* inside amoeba must be well established in order to clarify whether this pathogen is able to survive inside amoeba or if their co-culture simply promotes viability of the extracellular bacteria present.

Since *H. pylori* tend to acquire a viable but non-culturable (VBNC) state in the environment [12], assessing the viability of the pathogen by cultural methods is a very difficult task. Molecular methods such as fluorescent *in situ* hybridization (FISH) and quantitative polymerase chain reaction (qPCR) can be an alternative for detecting the presence of *H. pylori* inside amoeba. However, they are unable to distinguish between live and dead bacteria.

FISH in combination with direct viable count incubation (DVC-FISH) has been recently reported as a complementary technique for successfully detecting viable cells of *H. pylori* in wastewater and drinking water [17,22].

Thus, the main objective of this investigation was to apply the DVC-FISH technique to identify the presence and survival of viable and potentially infective forms of *H. pylori* inside *A. castellanii* after co-culture assay. The persistence of the pathogen inside the amoeba was also investigated by PMA-qPCR and culture.

2. Material and methods

2.1. Microorganisms and culture conditions

The reference *H. pylori* NCTC 11638 strain was obtained from the United Kingdom National Collection of Type Cultures (NCTC). The reference *A. castellanii* ATCC 30010 strain was provided by Marie-Cecile Trouilhé (Centre Scientifique et Technique du Batiment, AQUASIM, France). Both microorganisms were used in all co-culture replications.

Before each experiment, *H. pylori* was grown under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) at 37 °C for 48 h on pyruvate blood agar plates [PBA; Campylobacter selective agar (Merck, Spain) containing 10% (v/v) defibrinated horse blood (Oxoid, UK) and 0.025% (v/v) sodium pyruvate (Fisher, USA)]. Bacteria were then subcultured and incubated for 24 h in the above-mentioned conditions. An axenic *A. castellanii* culture was maintained in 10 ml of peptone-yeast extract-glucose supplemented with antibiotics [PYG + A; 20 g/l tryptose, 2 g/l yeast extract, 479 mg/l $MgSO_4$, 59 mg/l $CaCl_2$, 1 g/l $Na_3C_6H_5O_7 \cdot 2H_2O$, 25.8 mg/l $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 340 mg/l KH_2PO_4, 188 mg/l Na_2HPO_4 $7H_2O$, 18 g/l glucose, 200 µg/ml ampicillin and 200 µg/ml streptomycin] at 28 °C in 75 cm2 tissue culture flasks (Thermo Scientific, Denmark). The amoeba culture was subcultured every 7 days.

2.2. Sample preparation

H. pylori cells were resuspended in PBS 1X buffer and stained using the LIVE/DEAD® Cell Viability Kit (Invitrogen, UK) according to the manufacturer's instructions, in order to count initial inocula and assess bacteria viability during the co-culture process.

The *A. castellanii* culture was routinely observed under an inverted microscope. When most of the amoebae were in the trophozoite state, the flask was washed twice with PBS 1X buffer and Page's Amebic Saline (PAS) solution [14] was added 24 h prior co-cultivation.

2.3. Co-culture assay

Amoeba and bacteria were co-cultured in 15 ml sterile tubes. Briefly, 100 µl of a suspension of stained *H. pylori* (containing approximately 105 cells/ml) was mixed with 500 µl of a suspension of *A. castellanii* trophozoites (containing approximately 103 cells/ml) and incubated at room temperature under darkness for 1 h to allow bacterial internalization by the amoeba. This solution was then centrifuged at 500 g for 3 min to recover amoeba. The sediment was resuspended in 500 µl of PBS 1X followed by the addition of sodium hypochlorite at a final concentration of 104 ppm to kill extra-amoebic bacteria. Then, tubes were incubated at room temperature under darkness for 1 h. This solution was washed three times at 500 g for 3 min to remove sodium hypochlorite. The sediment was resuspended in PBS 1X, washed 3 times and subsequently analyzed by FISH, DVC-FISH, qPCR, PMA-qPCR and culture. In addition, bacterial fluorescence from the LIVE/DEAD® cell viability stain was evaluated *in vivo* during all co-culture assays. Three co-culture assays were carried out. A sample containing only *H. pylori* was also treated with sodium hypochlorite at the same concentration.

2.4. FISH

Aliquots of 20 µl of the buffer containing *A. castellanii* and internalized *H. pylori* were placed in gelatin-coated slides. They were air-dried, fixed in 4% paraformaldehyde for 3 h at 4 °C and washed with PBS 1X as previously described [14].

Thereafter, slides were dehydrated by successive immersions in 50%, 80% and 100% ethanol for 3 min each. Then, each well was covered with 10 µl of hybridization buffer