Moreno Mesonero, L.; Moreno Trigos, MY.; Alonso Molina, JL.; Ferrús Pérez, MA. (2016). DVC-FISH and PMA-qPCR techniques to assess the survival of Helicobacter pylori inside Acanthamoeba castellanii. Research in Microbiology. 167(1):29-34. doi:10.1016/j.resmic.2015.08.002.

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

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Running title: Viable *H. pylori* in co-culture with *A. castellanii*

Keywords: *A. castellanii*, *H. pylori*, co-culture, DVC-FISH, PMA-qPCR

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, such as FISH, DVC-FISH, qPCR and PMA-qPCR were used to detect the presence of internalized and viable *H. pylori*. After 24 hour in co-culture and a 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 method DVC-FISH, viability of *H. pylori* cells into *A. castellanii* was observed. Both specific techniques provided evidence for the first time that the pathogen is able to survive a chlorination treatment in occurrence with *A. castellanii* and could be very useful methods for performing further studies in environmental samples.

1. Introduction

Helicobacter pylori is a pathogenic gram-negative bacteria considered to be the most extended infectious agent in humans, thus being estimated to infect approximately 50% of the world's population [26]. Among all the 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 relation to gastric cancer and peptic ulcer [11]. Its transmission has not been exactly determined yet, but it is strongly suggested that this bacteria could be acquired by different routes, among which the fecal-oral route through water is included [8, 19]. 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, the survival mechanisms of this bacteria in water are not well defined yet.

Free-living amoebae (FLA) are ubiquitous protozoa which have been isolated from water, soil and air [21, 27]. *Acanthamoeba* species are the most common FLA in those environments [13]. 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 phagocyted, are able to resist or even replicate inside amoebae, being hidden and protected from harsh environmental conditions. Therefore, FLA could be considered to be "Trojan Horses" for these amoeba-resistant bacteria (ARB) [4]. In fact, there are many studies about the role of FLA as potential transmission vehicles for several human pathogens such as *Legionella*, *Listeria* and *Campylobacter* [2, 5, 6].

There are few studies investigating the interaction between FLA and *H. pylori*. In a co-culture assay, Winiecka-Krusnell et al. [29] suggested that *H. pylori* viability could be improved by the presence of *Acanthamoeba castellanii*. Other authors, [24] tried to show *H. pylori* survival and multiplication in the vacuoles of *Acanthamoeba polyphaga* in a co-culture assay but without conclusive results. A study reported that the 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 [5]. The authors concluded that the growth of this microaerobic bacterium was stimulated by the decrease of the dissolved oxygen in the media due to the presence of *A. castellanii*. Therefore, the viability of *H. pylori* inside amoeba has to be well established in order to clarify whether this pathogen is able to survive inside amoeba or if their co-culture only promotes the viability of the extracellular bacteria present.

Since *H. pylori* tend to acquire a viable but non culturable (VBNC) state in the envir onment [10], assessing the viability of the pathogen by cultural methods is a very difficu lt task. Molecular methods such as Fluorescent *in Situ* Hybridization (FISH) and quantit ative Polymerase Chain Reaction (qPCR) can be an alternative to detect 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 rec ently reported as a complementary technique to successfully detect viable cells of *H. pyl ori* in wastewater and drinking water [15, 20].

Thus, the main objective of this investigation was to apply the DVC-FISH technique in order to identify the presence and survival of viable and potentially infective forms of

H. pylori inside *A. castellanii* after a co-culture assay. The persistence of the pathogen i nside 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 3001 0 strain was provided by Marie-Cecile Trouilhé (Centre Scientifique et Technique du Ba timent, AQUASIM, France). Both microorganisms were used in all co-culture replicatio ns.

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 hours on Pyruvate Blood Agar plates [PBA; *Campylobacter* selective agar (Merck, Spain) containing 10% (v/v) defibrinated horse b lood (Oxoid, UK) and 0.025% (v/v) sodium pyruvate (Fisher, USA)]. Bacteria were the n subcultured and incubated for 24 hours in the above mentioned conditions. An axenic *A. castellanii* culture was maintained in 10 ml of Peptone-Yeast Extract-Glucose supple mented 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 stre ptomycin] at 28°C in 75 cm^2 tissue culture flasks (Thermo Scientific, Denmark). The a moeba culture was subcultured every 7 days.

2.2. Sample preparation

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

The *A. castellanii* culture was routinely observed under an inverted microscope. Whe n most of the amoebae were in the trophozoite state, the flask was washed twice with P BS 1X buffer and Page's Amebic Saline (PAS) solution [12] was added 24 hours prior c o-cultivation.

2.3. Co-culture assay

Amoeba and bacteria were co-cultured in 15 ml sterile tubes. Briefly, 100 µl of a su spension of stained *H. pylori* (containing approximately 10^5 cells/ml) was mixed with 5 00 µl of a suspension of *A. castellanii* trophozoites (containing approximately 10^3 cells/ ml) and incubated at room temperature under darkness for 1 h to allow bacterial internal ization 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 o f 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 w as 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 analysed by FISH, DVC -FISH, qPCR, PMA-qPCR and culture. Additionally, bacterial fluorescence from the LI VE/DEAD® Cell Viability stain was evaluated *in vivo* along all co-culture assays. Three co-culture assays were carried out. In a previous study, a sample containing only *H. pyl*

ori 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* we re placed in gelatine-coated slides. They were air-dried, fixed in 4% paraformaldehyde f or 3 h at 4 °C and washed with PBS 1X as previously described [12].

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 buff er (0.9 M NaCl, 20 mM HCl-Tris, 0.01% SDS and 30% formamide, pH 7.5), which con tained 50 ng of each probe. The reaction was carried out under darkness at 46°C for 1.5 h. A combination of three EUB338 probes complementary to a region of the *Eubacteria* domain 16S rRNA was used as a positive control. For the specific detection of *H. pylori* , a previously designed probe [16] with LNA modifications to increase its specificity [2 0] was used; LNA-HPY: 5'- CTG GAG AGA C^{+}TA AGC CC^{+}T CC-3'.

Subsequently, slides were washed under darkness at 48 °C for 15 min in 50 ml of wa shing solution (0.10 M NaCl, 0.02 HCl-Tris, 0.01% SDS and 0.005 M EDTA). Finally, they were washed with distilled water and air-dried under darkness. Slides were mounte d with FluoroGuard Antifade Reagent (Bio-Rad, Spain) between the coverslip and the sl ides. They were visualized using an Olympus BX 50 fluorescence microscope with the f ilters U-MWB, U-MWIB and U-MWIG. Photographs were taken with an Olympus DP-12 camera. A pure culture of fixed *H. pylori* cells was used as a positive control of the F ISH reaction.

2.5. DVC-FISH

The DVC procedure was carried out by adding 1 ml of the co-culture sample in 9 ml of DVC broth (BBL™ Brucella Broth supplemented with 5% Fetal Bovine Serum (FBS) and 0.5 mg/l Novobiocin) followed by 24 hours of incubation under *H. pylori* optimal conditions [20].

Once incubated, DVC tubes were centrifuged at 5000 g for 8 min and resuspended in 1 ml of PBS 1X. Afterwards, samples were fixed for FISH analysis and dehydrated, hyb ridized and analyzed as previously described by Moreno et al., (2003) [16]. A pure cultu re of fixed *H. pylori* cells were used as a positive control of the FISH reaction.

2.6. qPCR and PMA-qPCR

The mammalian tissue protocol from the GeneJet™ Genomic DNA Purification kit (ThermoScientific, Germany) was used to isolate DNA from 500 µl of the co-culture sa mple, following the manufacturer's instructions, with the exception of the incubation ti me at 56 °C, which was increased from 10 min to 30 min. Afterwards, specific *H. pylori* Real Time PCR based on SYBR Green I fluorescence was carried out using VacA prim ers to amplify a 372 bp fragment [18] in LightCycler® 2.0 Instrument (Roche Applied S cience, Spain). The final reaction volume was 20 µl, which contained: 2 µl of LightCycl er® FastStart DNA SYBR Green I (Roche Applied Science, Spain), 1.6 µl of $MgCl_2$ (50 mM), 0.5 µl of each primer (20 µM) and 2 µl of DNA template. The amplification consi sted of an initial DNA denaturalization at 95 °C for 10 min, followed by 40 cycles of: 9 5 °C for 10 s, 62 °C for 5 s and 72 °C for 16 s. Finally, one cycle at 72 °C for 15 s and o

ne at 40 °C for 30 s [20]. Amplifications were made by triplicate. A positive control wit h *H. pylori* DNA and a control of external contamination, qPCR mix without DNA, wer e added to the qPCR analysis. PCR products were also visualized in 1% agarose gel elec trophoresis prepared with 0.01% GelRed (Biotium, USA).

Prior to the performance of the qPCR, another 500 µl of the co-culture samples were treated with Propidium Monoazide (PMA) (GenIUL, Spain) which was dissolved in 20 % dimethylsulfoxide (DMSO) according to the manufacturer´s instructions. Samples we re incubated under darkness for 10 minutes with a final concentration of the reagent of 5 0 µM with occasional mixing to allow better reagent penetration. Then, they were expos ed to light for 15 min in a photo activation system (PhAST Blue, GenIUL, Spain). Ther eafter, samples were centrifuged at 15300 g for 5 min and resuspended in 200 µl of PBS 1X [1].

2.7. Culture

One hundred µl of the co-culture sample was incubated on PBA for 3 days under *H. pylori* specific conditions above described. Plates were daily monitorized to observe cha racteristic *H. pylori* colonies.

3. Results

3.1. Co-culture fluorescence evaluation

Before the co-culture assay, we checked by means of LIVE/DEAD® Cell Viability stain that most of the *H. pylori* cells were in their helical viable form, stained in green, and only some of them were observed in red, being non-viable. The number of viable cells in the inoculum was adjusted to be 10^5 cells/ml. After 1 h in co-culture, viable and non-viable *H. pylori* cells were observed inside *A. castellanii* (Figure 1). After the addition of sodium hypochlorite at a final concentration of 104 ppm, *H. pylori* cells kept their viability inside amoeba, while outside them, bacterial cells were dead and thus observed with red fluorescence. The staining of the sample containing only *H. pylori* showed that all cells were dead. Moreover, even 24 hours after the sodium hypochlorite treatment, viable *H. pylori* cells were observed inside *A. castellanii*.

3.2. H. pylori detection by FISH and DVC-FISH

By direct FISH analysis, the specific *H. pylori* hybridization showed *H. pylori* cells in coccoid and helical morphologies, both outside and inside (Figure 2) *A. castellanii* in all co-culture samples. Only a portion of *H. pylori* cells was internalized.

After removing the chlorine by washing the co-culture suspension, during the DVC incubation (24 h for *H. pylori*), only the active (live) *H. pylori* excreted cells, protected from treatment, became longer and/or fatter and stopped their replication because of the Novobiocin action. Dead cells present in the broth kept their original size. After FISH analysis combined with the previuos DVC incubation, we were able to distinguish between elongated *H. pylori* cells with strong fluorescence and non-elongated cells outside amoebae (Figure 3). According to other authors, this type of cells present a bigger size than the original one (control) and also show a stronger fluorescence, being considered as viable cells [20].

3.3. H. pylori detection by qPCR and PMA-qPCR

Specific amplification product was obtained both by qPCR and by PMA-qPCR. By means of qPCR, Ct (cycle threshold) mean value was 26.34. However, when PMA-qPCR was carried out, Ct mean value was 28.01. To confirm amplicon size, qPCR and PMA-qPCR products were visualized in 1% agarose gel. All amplicons were 372 bp (Figure 4).

3.4. H. pylori detection by culture

After incubation of co-culture samples under *H. pylori* conditions, no presumptive colonies were observed.

4. **Discussion**

It is known that several human waterborne pathogens such as *Campylobacter*, *Salmonella*, *Legionella* or *Listeria* [2, 5, 6, 9] are able to colonize and survive into FLA, like *Acanthamoeba*. The resistance of those intracellular pathogens to digestion by protozoa may increase their potential of infectivity in eukaryotic cells. Since FLA are resistant to several adverse conditions, such as disinfection, they could act as reservoirs, protecting and improving the survival of hosting bacteria [28] and even promoting their multiplication [7]. Several authors have proposed that *H. pylori* could belong to the ARB group, since it has been reported to be phagocyted by FLA [29].

In this study, internalization of viable and non-viable *H. pylori* cells by *A. castellanii* was observed along the co-culture assay. Co-cultures were exposed to an agressive sodium hypochlorite treatment able to kill *H. pylori* based on previous works [17, 22]. After this treatment, external non-viable *H. pylori* cells, stained in red, were observed, while viable *H. pylori* cells were seen inside amoeba, showing green fluorescence by the LIVE/DEAD® Cell Viability staining. Therefore, in agreement with other authors [24, 29], it was checked how *H. pylori* was internalized by *A. castellanii*. Even 24 hours after the sodium hypochlorite treatment, green (live) stained *H. pylori* cells were observed inside amoeba suggesting the survival of the pathogen. Most ARB are able to enter into the VBNC state, losing the ability to grow on synthetic agar media, and, therefore, this could lead to the underestimation of the presence of viable bacteria when samples are analyzed by culture. Identification by molecular techniques could be an alternative and, in fact, they have been previously applied to monitor the presence of *H. pylori* in co-culture with *Acanthamoeba* [24, 29].

FISH with the specific probe showed the presence of *H. pylori*. However, this technique cannot prove that bacteria remain alive into amoeba, even though some authors consider FISH to be directly related to viability [29]. When the combined method DVC-FISH was applied, viability of *H. pylori* cells was evidenced [20]. In this work we have observed that when a portion of the co-culture was incubated into DVC medium, a fraction of *H. pylori* cells were excreted by *A. castellanii* during the incubation period. This fact was also observed in a previous study of an *in vitro* predation assay of *Salmonella*, in which FLA excrete endogenous bacteria every 2-4 hours after ingestion [9]. Since external cells were killed by the sodium hypochlorite treatment, after DVC incubation, viable cells able to increase their size and fluorescent hybridization signal were only possible to come from inside *A. castellanii*, through their excretion. Although there are some studies in which *H. pylori* internalization by amoebae has been studied *in vitro* [24, 29], none of them have proved its survival after disinfection, being this the first demonstration that *H. pylori* is able to survive a chlorination treatment in occurrence with *A. castellanii.* Because of these results and the fact that *H. pylori* has been detected in different water systems, including rivers,

wastewater and drinking water [15, 20, 25], our study supports the hypothesis that this pathogen could belong to the ARB group and could survive inside FLA, which would act as "Trojan horses", protecting this bacteria and becoming a transmission vehicle [4].

When qPCR was applied to detect *H. pylori* in co-culture samples, we obtained amplification of the total DNA present in the sample: from dead external cells, external DNA and DNA from *H. pylori* cells from inside amoeba. The PMA treatment prior to the DNA isolation was used to remove exogenous DNA and DNA from non-internalized *H. pylori* dead cells after the sodium hypochlorite treatment, allowing the detection of *H. pylori* DNA only from the inside of *A. castellanii.* The fact that the Ct mean value from qPCR analyzed samples was lower than the Ct mean value from PMA-qPCR analyzed ones supports that PMA eliminates DNA from dead cells and exogenous DNA [3], yielding more accurate results of the presence of bacteria inside amoeba but without distinguishing between viable and dead internal cells.

Although it has been shown that the growth of *H. pylori* in co-culture with *Acanthamoeba* is encouraged [29], no report has concluded that internal amoeba conditions are optimal for the growth of *H. pylori*. Thus, not being able to isolate *H. pylori* by culture suggests that these bacteria could acquire the VBNC state, since its viability after the co-culture assay has been shown by DVC-FISH in this report. In this study *A. castellani* was not disintegrated prior to cultivation under *H. pylori* specific conditions. Many ARB are not able to self-release, although according to Marciano-Cabral and Cabral [14], *H. pylori* can be liberated after 4 days from *Acanthamoeba astronyxis*, and thus the incubation period of 3 days carried out in this study may not be longer enough for *H. pylori* to release from *A. castellanii.* Besides, we hypothesize that it could also be possible that certain FLA species are capable of promoting the growth and maintenance of viable *H. pylori* cells to a greater extent, for longer periods of time or in better conditions than others. Therefore, one of the most important challenges following this study would be to assess whether *H. pylori* is able to replicate inside amoeba or the bacteria only survives in a VBNC state until a more suitable environment or host is found. However, in this work survival of *H. pylori* cells phagocyted by *A. castellanii* was demonstrated using molecular methods for the first time, since, up to now, no report had identified viable *H. pylori* cells coming only from inside FLA.

Acknowledgements

This study was partially supported by a grant of the "Recovery and joint Resources from I+D+i of VLC/CAMPUS program" funded by the MECD (International Excellence Campus Program). The authors thank the Generalitat Valenciana for FEDER financial support 2007-2013 (T0104000).

Conflict of interest statement

The authors declare no conflict of interest.

References

[1] Agustí, G., Codony, F., Fitipaldi, M., Adrados, B., Morató, J. (2010). Viability determination of *Helicobacter pylori* using propidium monoazide quantitative PCR. Helicobacter **15:** 472-476.

[2] Akya, A., Pointon, A., Thomas, C. (2009). Viability of *Listeria monocytogenes* in co-culture with *Acanthamoeba* spp. FEMS Microbiol Ecol **70:** 20-29.

[3] Amman, R.I., Ludwig, W., Schleifer, K.H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev **59:** 143-169.

[4] Barker, J., Brown, M.R.W. (1994). Trojan horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment. Microbiol **140:** 1253-1259.

[5] Bui, X.T., Winding, A., Qvortrup, K., Wolff, A., Bang, D.D., Creuzenet, C. (2012). Survival of *Campylobacter jejuni* in co-culture with *Acanthamoeba castellanii*: role of amoeba-mediated depletion of dissolved oxygen. Environ Microbiol **14:** 2034-2047.

[6] Conza, L., Casati, S., Gaia, V. (2013). Detection limits of *Legionella pneumophila* in environmental samples after co-culture with *Acanthamoeba polyphaga*. BMC Microbiol **13:** 1-6

[7] Dupuy, M., Mazoua, S., Berne, F., Bodet, C., Garrec, N., Herbelin, P., Ménard-Szczebara, F., Oberti, S., Rodier, M.H., Soreau, S., Wallet, F., Héchard, Y. (2011). Efficiency of water disinfectants against *Legionella pneumophila* and *Acanthamoeba.* Water Res **45:** 1087-1094.

[8] Engstrand, L. (2001). *Helicobacter* in water and waterborne routes of transmission. J. Appl. Microbiol **90*:*** 80-84.

[9] Gaze, W.H., Burroughs, N., Gallagher, M.P., Wellington, E.M. (2003). Interactions between *Salmonella typhimurium* and *Acanthamoeba polyphaga,* and observation of a new mode of intracellular growth within contractile vacuoles. Microb Ecol **46:** 358-369.

[10] Hulten., K., Enroth, H., Nystrom, T., Engstrand, L. (1998). Presence of *Helicobacter pylori* in surface water in the United States. J Appl Microbiol **85:** 282-286.

[11] IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. (1994). IARC monographs on the evaluation of carcinogenic risks to humans. Vol 61. Schistosomes*,* liver flukes and *Helicobacter pylori*. International Agency for Research on Cancer, Lyon, France, pp. 177-240.

[12] Ioveno, A., Ledee, D.R., Miller, D., Alfonso, E.C. (2010). Detection of bacterial ensodymbionts in clinical *Acanthamoeba* isolates. Ophtalmology **117:** 445-452.

[13] Khan, N.A. (2006). *Acanthamoeba*: biology and increasing importance in human health. FEMS Microbiol Rev **30:** 564-595.

[14] Marciano-Cabral, F., Cabral, G. (2003). *Acanthamoeba* spp. as agents of disease in

humans. Clin Microbiol Rev **16:** 273-307.

[15] Moreno, Y., Ferrús, M.A. (2012). Specific detection of cultivable *Helicobacter pylori* cells from wastewater treatment plants. Helicobacter **17:** 327-332.

[16] Moreno, Y., Ferrús, M.A., Alonso, J.L., Jiménez, A., Hernández, J. (2003). Use of fluorescent *in situ* hybridization to evidence the presence of *Helicobacter pylori* in water. Water Res **37:** 2251-2256.

[17] Moreno, Y., Piqueres, P., Alonso, J.L., Jiménez, A., González, A., Ferrús, M.A. (2007). Survival and viability of *Helicobacter pylori* after inoculation into chlorinated drinking water. Water Res. **41:** 3490-3496.

[18] Nilsson, H., Blom, J., Al-Soud, W., Ljungh, A., Andersen, L., Eadström, T. (2002) Effect of cold starvation, acid stress and nutrients on metabolic activity of *Helicobacter pylori*. Appl. Environ. Microbiol **68:** 11-19.

[19] Percival, S.L., Thomas, J.G. (2009). Transmission of *Helicobacter pylori* and the role of water and biofilms. J Water Health **7:** 469-477.

[20] Piqueres, P., Moreno, Y., Alonso, J.L., Ferrús, M.A. (2006). A combination of direct viable count and fluorescent *in situ* hybridization for estimating *Helicobacter pylori* cell viability. Res Microbiol **157:** 345-349.

[21] Rodríguez-Zaragoza, S. (1994). Ecology of free-living amoebae. Crit Rev Microbiol **20:** 225-241.

[22] Rutala, W., Weber, D., Committee HICPA. (2008). Guidelines for disinfection and sterilization in healthcare facilities, 2008. Atlanta, GA: Centers for Disease Control and Prevention http://www.cdc.gov/ncidod/dhap/pdf/guideslines/Disinfection_nov_2008.pdf. (10-6-15)

[23] Santiago, P., Moreno, Y., Ferrús, M.A. (2015). Identification of viable *Helicobacter pylori* in drinking water supplies by cultural and molecular techniques. Helicobacter. *doi: 10.1111/hel.12205*

[24] Smith, C.D., Ashbolt, N.J. (2012). The fate of *Helicobacter pylori* phagocytised by *Acanthamoeba polyphaga* demonstrated by fluorescent in situ hybridization and quantitative polymerization chain reaction tests. Curr Microbiol **65:** 805-812.

[25] Tirodimos, I., Bobos, M., Kazakos, E., Haidich, A.B., Dardavessis, T., Kostopoulos, I., Arvanitidou, M. (2014). Molecular detection of *Helicobacter pylori* in a large Mediterranean river, by direct viable count fluorescent *in situ* hybridization (DVC-FISH). J Water Health **12:** 868-873.

[26] Torres, J., Pérez-Pérez, G., Goodman, K.J., Atherton, J., C., Gold, B.D., Harris, P.R., Madrazo-de-la-Garza, A., Guarner, J., Muñoz, O. (2000). A comprehensive review of the natural history of *Helicobacter pylori* infection in children. Arch Med Res **31:** 431-469.

[27] Trabelsi, H., Dendana, F., Sellami, A., Sellami, H., Cheikhrouhou, F., Neji, S., Makni, F., Ayadi, A. (2012). Pathogenic free-living amoebae: Epidemiology and clinical review. Pathol Biol (Paris) **6:** 399-405.

[28] Wingender, J., Flemming, H.C. (2011). Biofilms in drinking water and their role as reservoir for pathogens. Int J Hyg Environ **214:** 417-423.

[29] Winiecka-Krusnell, J., Wreiber, K., Euler, A., Engstrand, L., Linder, E. (2002) Free-living amoebae promote growth and survival of *Helicobacter pylori*. Scand J Infect Dis **34:** 253-256.

Figure legends

Fig.1 LIVE/DEAD® Cell Viability staining of *H. pylori* cells inside *A. castellanii* after 1 hour in co-culture and prior to the addition of sodium hypochlorite. (A) live cells stained with SYTO9; (B) dead cells stained with propidium iodide; (C) white light. X100 magnification (Olympus, Japan)

Fig.2 Identification by means of FISH of *H. pylori* inside *A. castellanii* after the sodium hypochlorite treatment of the co-culture: (A) Hybridization with HPY-LNA probe. (B) Hybridization with EUB-338 probe. (C) Trophozoite morphology visualized under a fluorescence microscope with white light. X100 magnification (Olympus, Japan)

Fig.3 Elongated cells of *H. pylori* after the chlorine treatment of the co-culture identified by DVC-FISH. X100 magnification (Olympus, Japan)

Fig.4 Gel electrophoresis showing the specific VacA amplified fragment from *H. pylori*. Lane 1: qPCR of the co-culture. Lane 2: PMA-qPCR of the co-culture. Lane 3: 100 bp ladder. Lane 4: negative control. Lane 5: positive control

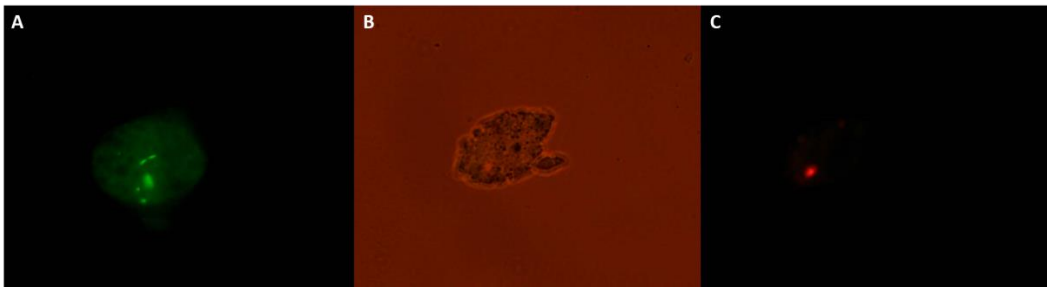

Figure 1.

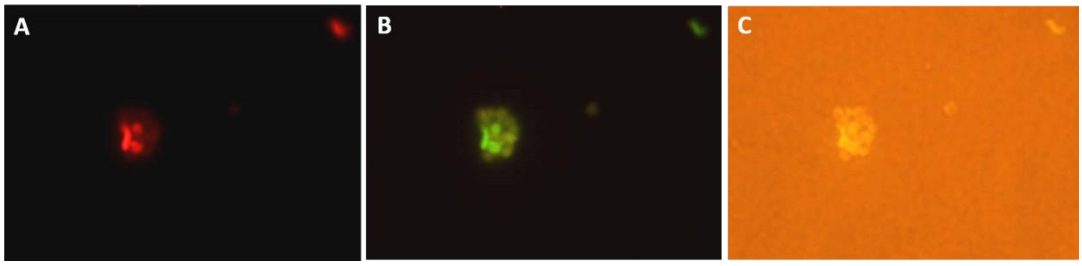

Figure 2.

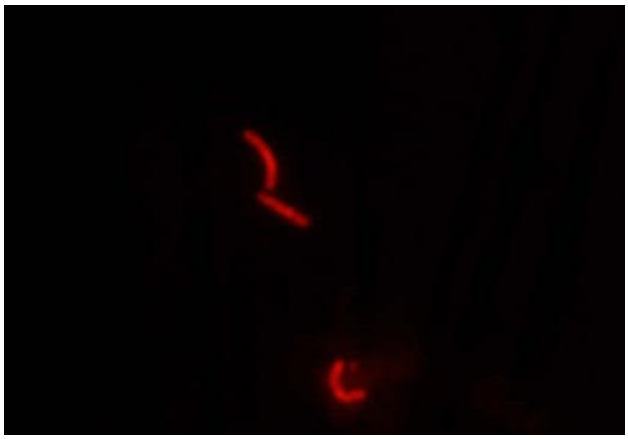

Figure 3.

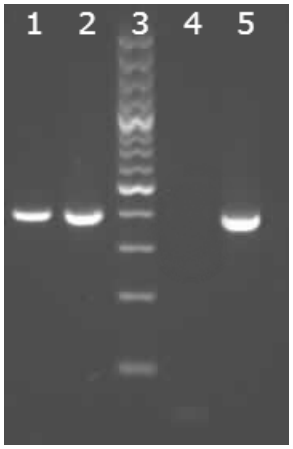

Figure 4.

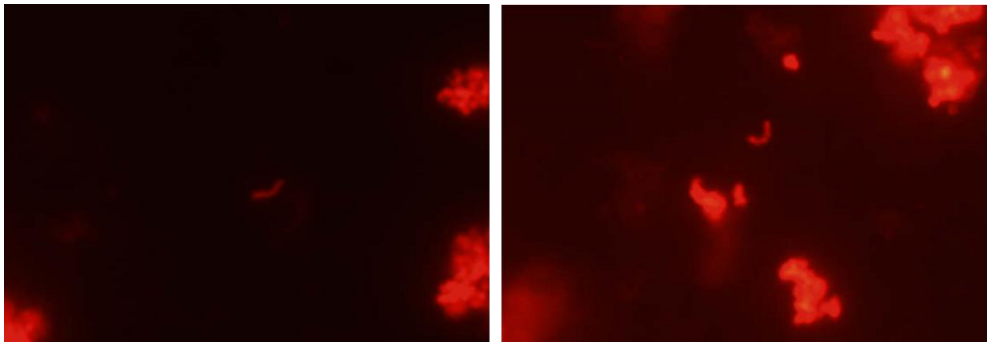

Figure 5.