Optimization of pre-treatments with Propidium Monoazide and PEMAX™ before real-time quantitative PCR for detection and quantification of viable Helicobacter pylori cells.

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ABSTRACT

Accurate detection of H. pylori in different environmental and clinical samples is essential for public health studies. Now, a big effort is being made to design PCR methodologies that allow for the detection of viable and viable but non-culturable (VBNC) H. pylori cells, by achieving complete exclusion of dead cells amplification signals. The use of DNA intercalating dyes has been proposed. However, its efficacy is still not well determined.

In this study, we aimed to test the suitability of PMA and PEMAX™ dyes used prior to qPCR for only detecting viable cells of H. pylori. Their efficiency was evaluated with cells submitted to different disinfection treatments and confirmed by the absence of growth on culture media and by LIVE/DEAD counts. Our results indicated that an incubation period of 5 min for both, PMA and PEMAX™, did not affect viable cells. Our study also demonstrated that results obtained by using intercalating dyes may vary depending on
In all dead cell's samples, both PMA and PEMAX™ pre-qPCR treatments decreased the amplification signal (>10^3 Genomic Units (GU)), although none of them allowed for its disappearance confirming that intercalating dyes, although useful for screening purposes, cannot be considered as universal viability markers. To investigate the applicability of the method specifically to detect *H. pylori* cells in environmental samples, PMA-qPCR was performed on samples containing the different morphological and viability states that *H. pylori* can acquire in environment. The optimized PMA-qPCR methodology showed to be useful to detect mostly (but not only) viable forms, regardless the morphological state of the cell.

**Keywords:** Helicobacter pylori, PMA-qPCR, PEMAX™-qPCR, Viability, Disinfection treatment, Morphological states.

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**INTRODUCTION**

*Helicobacter pylori* is the major causative agent of chronic gastroenteritis, duodenal, non-cardia gastric cancer and gastric Mucosa-Associated Lymphoid Tissue (MALT) lymphoma (Hosseini *et al.*, 2012; Calvet *et al.*, 2013). For this reason, *H. pylori* has been
classified as human carcinogen Type I by the World Health Organization and US Food and Drug Administration included it in the list of microorganisms that pose a serious threat to public health (FDA, 2014).

This pathogen affects 50% of the world population. Its prevalence is higher in countries with economic and social underdevelopment, associated to hygiene deficit, unhealthy diet and use of non-potable water. In developed countries 0.5% of the population is affected by *H. pylori* (Vale and Vítor, 2010; Eusebi *et al*., 2014).

Currently, it is widely accepted that transmission occurs most probably through fecal-oral, gastro-oral and oral-oral routes (Cellini *et al*., 2001, Azevedo *et al*., 2007). It has been demonstrated that *H. pylori* can survive in aquatic environments (Fernández-Delgado *et al*., 2016), tap water (Vesga *et al*., 2018) and vegetables (Atapoor *et al*., 2014; Zamani *et al*., 2017; Moreno-Mesonero *et al*., 2020). According to that, some authors have proposed the existence of indirect transmission via contaminated water and food (Ramy *et al*., 2013; Atapoor *et al*., 2014).

Depending on the environmental conditions the microorganism adopts different vital states, associated with morphological changes: *H. pylori*, usually presents spiral shape but, when exposed to stressful conditions such as increased oxygen concentration, changes in pH (alkaline), increased temperature, absence of nutrients, prolonged incubation periods, treatment with antimicrobial agents or exposure to visible light and UV irradiation, the organism can change from spiral to coccoid form (Del Campo *et al*., 2009; Cunningham *et al*., 2009; Andersen and Rasmussen, 2009). This transformation of rod shape to the coccoid form can occur through intermediate forms (“V” and “U”), corresponding to viable but non-cultivable spiral cells (Bai *et al*., 2010). Some authors
had proposed that acquiring coccoid form indicates cellular degeneration and subsequently death (Kusters et al., 1997). However, some works strongly support that some of these forms are viable, although they cannot be cultured (Viable but non-culturable, VBNC), as they preserve metabolic activity; express virulence genes as ureA, ureB, hpaA, BabA, vacA and cagA; keep the urease activity and continue synthesizing proteins and small amounts of DNA (Oliver, 2005; Azevedo et al., 2007). Thus, some authors have differentiated coccoid forms into two types, with different morphological and functional characteristics: Type A is irregular, with rough surface and is considered a dead cell. Type B is smoother, with strictly membranous structure and is considered a viable but not cultivable form (Sarem and Corti, 2016; Flores-Encarnacion et al., 2015).

On some occasions, spiral non-viable cells have been also described (Orta de Velásquez et al., 2016). Taking all this into account, vital states of H. pylori cells could be classified as: viable spiral form (V), non-viable spiral form (NV), viable but non-cultivable (VBNC) coccoid or spiral form and non-viable degenerative coccoid form (NVC) (Saito et al., 2003). The stage in which H. pylori can be present in a sample is of great epidemiological interest, because VBNC forms seem to play a crucial role in the process of transmission through water or in the relapse of the infection after antimicrobial treatment (Dworkin, 2010; Flores-Encarnacion et al., 2015).

VBNC forms cannot be detected by culture techniques, only by PCR (Codony et al., 2015). However, PCR cannot differentiate between viable and nonviable cells because DNA of live and dead cells, as well as extracellular DNA, is amplified (Pathak et al., 2012). To avoid this drawback, many studies have proposed the use of DNA-intercalating dyes,
such as Ethidium Monoazide Bromide (EMA) or Propidium Monoazide Iodide (PMA), for viability PCR assays. The technique has been successfully used to detect viable bacteria, viruses, and protozoa (Dabrowska et al., 2014; Gyawali et al., 2017). EMA and PMA, derived from ethidium bromide and propidium iodide respectively, are membrane-impermeant dyes that can penetrate only compromised membranes of non-viable cells. Once inside the cells, EMA or PMA bind with DNA and form stable covalent bonds when exposed to bright light (Randazzo et al., 2018). This DNA will be unable to be amplified by PCR reaction. Hence, when a sample is treated with EMA or PMA prior to PCR, only amplification of DNA from viable cells (with intact membrane) will occur (Kibbee and Örmeci, 2007).

Recently, the use of PEMAX™ (GenIUL, S.L, Barcelona, Spain), a double photoactivable dye methodology, was proposed as a new alternative. PEMAX™ is the result of the adequate combination of EMA (<10µM) and PMA (>20µM) (Codony, 2014; 2015; Agustí et al., 2017), which present different size and charge. The smaller molecule, EMA, can cross cell membranes, but the process can be reverted by efflux pumps, which could lead to false positive results. The second dye, PMA, is necessary to improve the process when high amounts of dead cells are present in the sample (Codony et al., 2015). Some authors have also proposed the use of a specific buffer, PEMAX™ GenIUL Reaction Buffer, which helps to preserve the homeostasis of living cells, increasing the efficiency of viability PCR procedures (Lizana et al., 2017; Agustí et al., 2017).

Pre-PCR treatment with PEMAX™ has been used for monitoring some bacterial pathogens in environment. Lizana et al. (2017) confirmed the presence of Legionella spp in 32.1% of 116 analyzed water samples. Thanh et al. (2017) developed a sample
treatment protocol with PEMAX that allows for neutralizing DNA signals from up to 5.0 x 10^7 dead cells from a pure culture of Salmonella spp. However, this methodology has never been applied to Helicobacter spp.

The purpose of the present study was, firstly, the optimization of PMA and PEMAX™ protocols to achieve the total extinction of the qPCR signal from dead bacteria. Concentration of dyes and incubation periods were assayed in accordance with previous studies about the effects of different PMA concentrations on viable H. pylori cells (Zeng et al., 2016). We also studied the efficacy of these dyes for detecting viable cells in samples submitted to different disinfection treatments. Finally, we evaluated the performance of PMA for detecting the different morphological and viability states adopted for H. pylori under stress conditions. Our objective was to determine the suitability of a pre-qPCR treatment with PMA or PEMAX™ for detection and quantification of DNA from H. pylori viable cells in environmental samples.

MATERIALS AND METHODS

Bacteria strain and growth conditions

H. pylori NCTC 11637 strain (National Collection of Type Cultures, UK) was cultured in Blood Agar Medium as previously described (Hortelano et al., 2020). The cultures were incubated under micro-aerobic conditions (5% oxygen, 10% carbon dioxide, and 85% nitrogen) and 90-95% humidity, by using CampyGen™ 3-5L Atmosphere Generation Systems (Oxoid, UK) in anaerobic jars (Oxoid, UK) at 37 °C for 48 h-14 days.
For the different assays, the initial inoculum was prepared by suspending a portion of a 48 h growth agar culture in 6 mL of PBS buffer (phosphate-buffered saline, pH 7.5) and adjusted by the LIVE/DEAD method as below described, to reach a concentration of $10^6$ viable cell/mL. For assessing LIVE/DEAD counts results, CFU counts were also performed, by culturing 10-fold serial dilutions of the inoculum in Blood Agar Medium as described above.

**Viability analysis, cell count and morphological determination**

Counts of viable and dead bacteria were carried out before and after every assay, by using the Film Tracer™ LIVE/DEAD™ Viability Kit (Molecular Probes, USA), according to manufacturer’s instructions.

Briefly, an aliquot of 125 µL of each PBS cell suspension (inoculum, controls, and treated samples) was mixed with 0.4 µL of a mixture of SYTO9 and Propidium Iodide (1:1), re-suspended and incubated under dark conditions for 5 min at room temperature. A 5 µL aliquot was spotted on a poly-L-lysine (Polysine® slides, Menzel-Glaser, Thermo Scientific, Germany) coated slide. The count of viable (green) and dead (red) microorganisms was performed by using an Olympus epifluorescence microscope (BX50) with U-MWB filter. Count was determined as the mean value obtained from 20 microscopic fields from each of two different slides. When observed, information about morphology of *H. pylori* cells present in each sample was also collected.
For testing cultivability of cells, 100 µL of each sample were spread onto blood agar plates, incubated at 37 °C, as above described, and observed daily after 3 days for the following 11 days.

**Optimization of PMA and PEMAX™ pre-PCR treatments protocol**

PMA (Biotium, Hayward, CA, USA) and PEMAX™ (GenIUL, Barcelona, Spain) were dissolved each in sterile distilled water to obtain a final stock solution of 2.5 mM and stored at -20 °C in the dark.

For all the assays, prior to DNA extraction PMA and PMAX™ were added to the samples at different concentrations and incubated in dark at room temperature for 5 or 10 min shaking, to promote penetration of DNA intercalating dyes (Agustí et al., 2010). After photo-induced cross-linking for 15 minutes using the high-power LED equipment PhAST Blue (GenIUL, Spain), samples were centrifuged at 10000 rpm for 5 min. The pellet was re-suspended in 200 µL of PBS and placed at -20 °C until DNA extraction and qPCR assays.

In order to determine the best concentration of each dye for only amplifying living cells, suspension of a 48 h *H. pylori* culture was adjusted by LIVE/DEAD test to a final concentration of 10^6 cells/mL viable cells. Three mL from this initial stock were used as positive controls, and another 3 mL were incubated at 85 °C for 30 min, killing all the cells. Viability (LIVE/DEAD test) and culture analysis were carried out before and after heat treatment to verify the state of the cells. Then, 1.5 mL aliquots of viable and heat-killed cells suspensions were treated with PMA or PEMAX™ prior to qPCR, at a final concentration of 25 µM and 50 µM, in both cases.
To test if pre-PCR treatment with dyes affect
ted cells viability, two different suspensions
(A and B) from the same inoculum containing viable H. pylori cell were analyzed
independently in triplicate and submitted to qPCR before and after pre-treatment with
PMA and PEMAX™, at incubation times of 5 and 10 min. Additionally, a 1.5 mL aliquot
of each sample was processed with the PEMAX™ GenIUL Reaction Buffer. Briefly, the
inoculum samples (10⁶ cells/mL viable cells H. pylori cell suspension) were centrifuged
at 10000 rpm for 10 min, the supernatant was removed, and 1.5 mL amount of Reaction
Buffer was added before PEMAX™ staining, followed by an incubation period of 5 min
and 10 min. Samples were then stained with PEMAX™, as described above.

Evaluation of PMA, PEMAX™ and PEMAX™-Buffer efficacy after different disinfection
treatments.

To evaluate if the efficacy of DNA intercalating dyes varied depending on the conditions
causing death of the cells, H. pylori cells were exposed to various disinfectant
treatments: Ethanol 70% for 10 min; 100 ppm of sodium hypochlorite for 45 min and
hydrogen peroxide 5% for 45 min.

An initial stock of 6 mL of viable cell suspension in PBS was prepared from a 48 h fresh
culture of H. pylori NCTC 11637 and adjusted to a final concentration of 10⁶ viable
cells/mL by using LIVE/DEAD test. An aliquot of 1.5 mL was taken as positive control and
aliquots with the same volume were subjected to three different disinfection processes.
After killing process, the supernatant was carefully removed after a centrifugation step
of 10000 rpm for 5 min and cells were re-suspended in 1.5 mL of PBS. Loss of viability of
H. pylori cells was determined by LIVE/DEAD test and culture. Then, processed bacterial
suspensions were treated with 50 µM PMA, 25 µM PEMAX™ (with and without
PEMAX™ Reaction Buffer), as described above, and incubated for 5 and 10 min prior to qPCR analysis.

Study of PMA efficacy for detecting *H. pylori* at different morphology and viability states.

To obtain different viability states of *H. pylori* during its morphological transformation from bacillary to coccoid form, an initial bacterial suspension in PBS was prepared from a 48 h *H. pylori* NCTC 11637 pure culture and adjusted with LIVE/DEAD method to a final concentration of $10^8$ viable (green) cells/mL, as described before. A 3 mL aliquot from the initial inoculum was taken as control (viable spiral shape). Other four aliquots of 1.5 mL were exposed to different environmental conditions, according to previous studies:

- 100 ppm sodium hypochlorite, 5% of hydrogen peroxide, at 25 °C and 4 °C.

To obtain non-viable (NV) coccoid and bacilli *H. pylori* forms, two samples were treated with 100 ppm sodium hypochlorite and 5% of hydrogen peroxide for 45 min (Orta de Velásquez *et al.*, 2016), respectively. Samples were tested every 15 min with LIVE/DEAD test until at least 95% of the cells in the sample presented the desired morphology.

To induce the morphological changes to viable but non-cultivable (VBNC) coccoid and spiral forms, other 1.5 mL samples ($10^8$ viable cells/mL of bacterial suspension in PBS) were incubated at 25 °C and 4 °C (Zamani *et al.*, 2017) respectively, in a humid chamber for two months, inside a sterile 10 mL tube, and examined every day with LIVE/DEAD method until at least 95% of the cells in the sample presented the desired morphology.

When the desired morphology of cells was reached, samples were centrifuged at 10000 rpm for 5 min, pellets were re-suspended in 1.5 mL of PBS and treated with PMA at a
final concentration of 50 µM. Samples were incubated at room temperature in dark conditions for 5 min.

All assays were made in triplicate and repeat at least once in other independent experiment. For all of them, loss of viability of *H. pylori* cells was determined by LIVE/DEAD test and culture.

**DNA extraction**

DNA was extracted from all the samples by using the GeneJet™ Genomic DNA Purification Kit (ThermoScientific, Germany), according to Moreno-Mesonero *et al.* (2016). Isolated DNA was stored at -20 °C until use.

**Real-time quantitative PCR assay**

A SYBR®Green I qPCR assay was performed to amplify a 372 bp fragment of *H. pylori* VacA gene (Vesga *et al.*, 2018) in a final volume of 18 µl, containing 2 µL LightCycler® FastStart DNA Master SYBR Green I (Roche Applied Science, Spain), 1.6 µL MgCl₂ (50 mM), 0.5 µL of each primer (20 mM) and 2 µL of DNA template. Amplification protocol consisted in an initial DNA denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 62 °C for 5 s and 72 °C for 16 s; and finally, one cycle at 72 °C for 15 s and another at 40 °C for 30 s. Amplification was carried out in a LightCycler® 2.0 (Roche Applied Science, Spain) and LightCycler® 4.1 Software (Roche Applied Science, Spain) was used to obtained automatically the Cp values, which marked the cycle when the fluorescence of a given sample significantly exceeded the baseline signal, and quantification of DNA using the “Abs. Quant Analysis” and the “Automated Second Derivative” method. It offers automated data calculation without any user influence
(except for the selection/deselection of standards) and provides the advantage of high reproducibility. The quantification of samples was obtained according to an external standard curve as previously developed by Santiago et al. (2015) (Figure 2.S). Briefly, six log of *H. pylori* NCTC 11637 DNA concentration, in the range $2.16 \times 10^1$ to $1.79 \times 10^6$ genomic units, corresponding to cycle threshold (Cp) media values ranged from 34 to 15.62 ($Cp = -3.7333 \cdot \log_{10} (GU) + 38.976; R^2 = 1$), was used to elaborate the standard curve. GU were calculated considering the existence of an only *VacA* gene copy in each cell and following the next: Genomic Units (GU) = DNA concentration/atomic mass (Linke et al., 2010).

**Statistical analysis**

All experiments were performed in triplicate and repeated at least once in an independent experiment. Mean values and standard deviation for the different experiments performed in triplicate, used to evaluate the relation of intercalating dyes treatment and viability *H. pylori* states, were calculated using Microsoft Excel v.10. The effect of PMA, PEMAX™ with and without PEMAX™ Reaction Buffer on viable and killed *H. pylori* cells by different disinfectant treatment were determined by calculating log10 reduction of GU (genomic units) using qPCR $C_p$ values. The following equation was used to calculate log10 in detectable genome copies:

$$\Delta GU = \text{Difference in GU values} = \Delta GU_C - \Delta GU_{TS}$$

Where:

$$\Delta GU_C = \text{GU value from control samples calculated with } H. pylori \text{ standard curve.}$$
$\Delta GU_{TS} = GU$ value from PMA, PEMAX$^{TM}$ with and without PEMAX$^{TM}$ Reaction Buffer treated samples, calculated with H. pylori standard curve. Amplification efficiency (E) was estimated by using the slope of the standard curve and the formula $E = \frac{10}{[1 - 1/slope]} - 1$.

The statistical differences in GU values calculated from Cp values and H. pylori standard curve, from different experiments, was evaluated by analysis of variances (ANOVA), with Microsoft Excel v.10, to examine the differences between treatments. In all cases, differences and correlations were considered statistically significance at p values less than 0.05.

RESULTS

Optimization of PMA and PEMAX$^{TM}$ pre-PCR treatments protocol

The bacteria present in the samples were killed by incubation at 85 °C for 30 min. All the cells in the heat-killed bacterial suspension appeared red when inspected by fluorescent microscope by LIVE/DEAD test (Figure 1. S), and no colony grew on culture plates.

Two different dye concentrations, 50 and 25 μM, were tested. As showed in Table 1, qPCR results for the dead cells sample without pre-treatment showed a reduction in counts of only 0.95 log GU relative to the control (live cells sample). The use of concentrations of 50μM for both intercalating dyes, PMA and PEMAX$^{TM}$, yielded near statistically significant (P=0.06) reductions in DNA counts from dead cells samples, compared with the viable cells control sample, showing average signal decreases of 3.01 and 2.5 log GU, respectively. Pre-treatment with PMA and PEMAX$^{TM}$ at concentrations of 25μM showed less efficacy, resulting in 1.67 and 2.29 log GU reduction.
In addition, the potential toxic action of intercalating dyes on viable cells was also examined by treating independently two different samples from the same inoculum of viable *H. pylori* cells with PMA and PEMAX™ (with and without using PEMAX™ Reaction Buffer) (Table 2). No effect on the qPCR could be observed when using PMA at both incubation times, since their average signal reduction (ΔCp) from the two different viable *H. pylori* cells samples were not significantly different from the untreated controls. Nevertheless, Sample B, incubated for 10 minutes with PMA caused a slightly higher average reduction of 0.30 log GU (probably due to the physiological heterogeneity of the cells) compared with the control, suggesting more toxic effect in viable cells than a shorter 5 min incubation period.

Regarding PEMAX™, pre-treatment at both incubation times rendered similar results for the two samples analyzed. Sample A showed a decreased in q-PCR signal by 4.000 log and 3.97 log GU reduction; and Sample B, demonstrated a reduction of 0.75 log and 1.12 log GU reduction, after incubation for 5 and 10 min, respectively. Difference between samples with and without PEMAX™ treatment were statistically difference (p=0.00001 (A); p=0.0065 (B)).

The use of PEMAX™ GenIUL Reaction Buffer previously to add PEMAX™ showed the most significant effect on qPCR results, since the average of genomic units from the two viable *H. pylori* cell samples. Sample A, resulted in a decrease of q-PCR signal of 1.13 and 1.97 log GU reduction, for 5 and 10 min incubation periods, respectively. Regarding with sample B, led to a decline of q-PCR signal of 1.97 and 2.82 log GU reduction, for 5 and 10 min incubation periods, respectively.
Evaluation of PMA, PEMAX™ and PEMAX™-Buffer efficacy after different disinfection treatments.

Samples were subjected to pre-PCR treatment with 50 μM PMA and 25 μM PEMAX™ at two incubation times, 5 and 10 min, in order to compare the results. The effectiveness of the PEMAX™ GenIUL Reaction Buffer was also tested (Table 3).

Exposure of H. pylori viable cells, to 70% of ethanol led to a complete loss of cultivability and LIVE/DEAD test showed non-viable H. pylori forms. PMA pre-treatment induced a significant reduction of qPCR signal (p=0.0057) on ethanol-killed H. pylori cells: signals decreased 2.46 and 2.84 log GU related to untreated inactivated H. pylori cells, after incubation for 5 and 10 min, respectively. No significant difference could be observed depending on the incubation period.

Similar results were obtained for PEMAX™: intercalating dye pretreatment reduced the signal by 2.83 and 2.87 log GU after incubation for 5 and 10 min, respectively. When PEMAX™ was used in combination with enhancer Buffer PEMAX™-qPCR signal was reduced by 3.14 and 3.06 log GU.

After treating a pure culture of H. pylori (viable cells), with 100 ppm sodium hypochlorite, any colony grew on culture plates and LIVE/DEAD test showed only non-viable H. pylori forms. The Cp values observed after pre-treatment with PMA presented significant differences when compared to non-treated inactivated H. pylori cells (29,1 vs. 17,15 and 17,78 after incubation for 5 and 10 min respectively). Similar results were obtained with PEMAX™ (29,1 vs. 17,6 and 20,38 after incubation for 5 and 10 min respectively). PEMAX™ used with standard Buffer reduced qPCR signal by 0.31 and 1.04
log, as compared to non-treated inactivated *H. pylori* cells, results that were not
different from those obtained by using PEMAX™ without enhancer Buffer.

Treatment pure culture of *H. pylori* with 5% hydrogen peroxide also killed all the cells,
as checked by culture plates and by LIVE/DEAD test.

Pre-PCR PMA treatment reduced the amplification signal by 2.82 and 2.54 log GU as
compared to the non-treated inactivated *H. pylori* cells, after incubation for 5 and 10
minutes, respectively. PEMAX™ pretreatment yielded similar results, since qPCR counts
were reduced by 1.93 and 2.67 log GU. Additionally, enhancer Buffer reduced qPCR
counts by 3.08 and 2.74 log GU for both incubation times, compared with non-treated
inactivated *H. pylori* cells.

Overall, all pre-qPCR treatments used yielded statistically significant (*P*=0.0053)
reductions in DNA counts after PCR, compared with results obtained for untreated
inactivated control sample, regardless of incubation time. When results from both
incubation times were compared, no significant difference was observed for any pre-qPCR dye treatment.

**Study of PMA treatment effects on amplification of DNA from different viability and**
**morphological states of *Helicobacter pylori***.

The study was carried out with previously established optimal conditions for PMA
treatment (50 µM and 5 min incubation). Morphologies, viability, and cultivability of
cells were confirmed by viability LIVE/DEAD test (Figure 1) and the presence/absence of
colonies in culture plates.
As expected, no effect of PMA pretreatment was observed on viable morphological states of *H. pylori*, since their average counts were not significantly different (Table 4).

On the contrary, PMA pretreatment induced a significant reduction of the qPCR signal in non-viable spiral shape (p= 0.03) and non-viable coccoid forms (p= 0.01) samples, when compared with untreated *H. pylori* control.

**DISCUSSION**

In previous works, many authors have observed the presence of *H. pylori* in aquatic environments, demonstrating that disinfectant treatments may be inefficient enough for inactivating this pathogen (Castillo et al., 2019; Sakudo et al., 2018; Orta de Velásquez et al., 2016). However, most of these studies rely on molecular techniques, such as PCR, that cannot differentiate between DNA from viable or dead cells. This point is crucial from a Public health point of view. For these reason, we have evaluated PMA-qPCR and PEMAX™ pre-q PCR methods for its applicability in the detection of viable *H. pylori* cells in environmental samples. Different parameters, such as the concentration of dyes, use of enhancer buffer and dark incubation time, were considered. Moreover, for the first time we studied the performance of this technology when applied to samples previously submitted to different lethal procedures which are commonly used during wastewater disinfection, food processing or disinfection.

As previously reported by Nam et al. (2011), we found that PMA concentration had no significant effect on the reduction of qPCR signal from viable *H. pylori* cells. This can be attributed to the low cytotoxicity of PMA and its affinity to penetrate only cells with strongly damaged membranes (Nocker and Camper et al., 2006). The small logarithmic reduction in genomic units on viable cells after PMA treatment may be due to the death
of cells because osmotic stress when pure culture was spiked in PBS (Delgado-Viscogliosi et al., 2009). Underestimation of viable cell populations has been reported for other bacterial species (Yáñez et al., 2011; Liu and Mustapha, 2014; Barbau-Piednoir et al., 2014) when using high concentrations of intercalating dye or lower number of targets (Yasunaga et al., 2013). Concerning PEMAX™, we found a significant reduction on qPCR signal from live cells for both concentrations tested, what seems to suggest an unspecific toxic effect in viable cells treated with PEMAX™ or DNA neutralization in live cells presenting non-lethal damage membrane. An even larger effect was noticed when PEMAX™ was used in combination with commercial Buffer. PEMAX™ has been proposed to be more accurate than PMA, detecting only cells with intact membrane structure and active metabolism (Agustí et al., 2017), what may explain that some viable non-lethally damaged cells are not detected. To our knowledge, there is no reference in the literature mentioning similar results for H. pylori.

Regarding the assay with dead cells, we chose heating at 85 °C during 30 min because heat induces effusion of intracellular substances (Hurst et al., 1974) and loss of membrane lipopolysaccharides (Tsuchido et al., 1985). Results confirmed that heat altered membrane permeability, allowing PMA/PEMAX™ to enter the cell, but did not result in the complete loss of qPCR signal. This is in accordance with several studies that describe incomplete PMA-qPCR signal inhibition, resulting in strong overestimation of viable cells (LØvdal et al., 2011). Previous studies have proposed that the presence of high number of dead cells can exceed the intercalating dyes capacity, what may result in insufficient binding to DNA in damaged cell (Yáñez et al., 2007). Other researchers
have suggested that short amplicon size in qPCR cannot be completely suppressed by PMA pretreatment (Luo et al., 2010).

Trying to better characterize the usefulness of this viability-PCR methodologies, we compared their efficiency to discriminate between viable and non-viable H. pylori cells killed by different disinfection treatments. Three different procedures were selected considering physical and chemical treatments that develop cell stress conditions in H. pylori cells for promote their adaptive response (Dinh et al., 2017). Moreover, sodium hypochlorite and hydrogen peroxide are usually applied to environmental disinfection, mainly wastewater or drinking water.

Other authors (Lee et al., 2015; Cho et al., 2010) reported that the effectiveness of PMA-qPCR detection varies according to the disinfectant pretreatment used. Therefore, we considered that viability assays by using intercalating dyes should be optimized depending on the different cell stresses.

All treatments decreased the qPCR signal of dead cells, although none of them allowed for its total disappearance, confirming that the use of intercalating dyes, although useful for screening purposes, does not yield entirely consistent viable cells quantifications.

The highest and most significant signal reduction without intercalating dyes pretreatment occurred after exposition to 100 ppm of sodium hypochlorite during 45 min. Sodium hypochlorite effects include the destruction of key metabolic enzymes (Wyss et al., 1961) and the disruption of protein synthesis (Agranoff, 1967). Our data showed that this disinfectant treatment reduced the qPCR signal by 3.47 log GU, showing no significant differences among results obtained before and after the use of
both intercalating dyes and standard buffer. This can be explained because hypochlorite at high concentrations affects both, cellular membrane, and nucleic acids, hindering the covalent joint between DNA and intercalating dye (Delgado-Viscogliosi et al., 2009; Lee et al., 2015).

Ethanol treatment dehydrates bacteria, injures cell wall, and causes coagulation of proteins (Huffer, et al., 2011). Our results showed a reduction of qPCR signal about 2.46 logs genomic units when compared with viable H. pylori control. There were not significant different between pretreatments. However, our results indicated a higher significant reduction when PEMAX™ was used with standard Buffer.

Regarding hydrogen peroxide, it generates oxidative stress and causes lipid peroxidation, resulting in a decrease in membrane fluidity and its inactivation (Nebe-von-Caron et al., 2000). Our data showed no significant difference between counts obtained for live and dead control samples, what can be explained because hydrogen peroxide leads to loss of membrane integrity but does not damage DNA (Krüger et al., 2014).

After all these assays, we selected 50 μM PMA incubated for 5 min as the optimum pre-qPCR treatment methodology, because results indicated significant reduction of qPCR signal from dead cells and less qPCR signal reduction (less cytotoxic effects) from viable cells.

We also aimed to investigate if PMA-qPCR results could be affected by H. pylori morphological state, as this pathogen is frequently present in the environment in a coccoid shape, which presents some different structural and functional properties
(Krüger et al., 2014). As expected, no effect of PMA was observed on all viable morphological states of *H. pylori*, since their average counts were not significantly different, confirming that this intercalating dye is excluded from viable cells and, thus, it does not reduce the qPCR signal from viable and viable but non-cultivable states of *H. pylori*, either spiral or coccoid. Our research also showed that the use of PMA reduced the signal of non-viable coccoid and spiral morphologies. However, again this assay did not totally avoid amplification of DNA from non-viable cells (Løvdal et al, 2011).

**CONCLUSIONS**

In conclusion, PMA- and PEMAX™-qPCR techniques can significantly reduce the DNA amplification signal from dead cells but are still unable to totally discriminate between viable and non-viable cells present in a sample. Although PMA-qPCR is the most direct method to minimize false positive results in the detection of dead cells, and provides rapid results compared with culture methods (Goh and Gin, 2015), this approach cannot be considered as a universal viability marker, and other factors, such as bacterial species, origin of the sample and disinfection treatment should be considered.
References:


Figure 1: LIVE/DEAD method used to determine the different *H. pylori* morphological states after different treatment conditions. (A) Viable Spiral (Control Inoculum). (B) Non-Viable Spiral Form (treated with 5% of hydrogen peroxide for 45 min). (C) Viable but Non-cultivable (VBN C) spiral form (4 °C). (D) Viable but Non-Cultivable (VBN C) coccoid form (25 °C). (E) Non-Viable Coccoid form (100 ppm sodium hypochlorite). Bar: 10 µm. Observation 100X.

<table>
<thead>
<tr>
<th>H. pylori</th>
<th>V Spiral (A)</th>
<th>NV Spiral (B)</th>
<th>VBN C Spiral (C)</th>
<th>VBN C Coccoid (D)</th>
<th>NV Coccoid (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image A" /></td>
<td><img src="image2.png" alt="Image B" /></td>
<td><img src="image3.png" alt="Image C" /></td>
<td><img src="image4.png" alt="Image D" /></td>
<td><img src="image5.png" alt="Image E" /></td>
<td></td>
</tr>
</tbody>
</table>
Table 1: Effects of PMA and PEMAX™ concentrations in qPCR results for heat-killed *H. pylori* cells.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µM)</th>
<th>Cp(^b)</th>
<th>ΔCp(^c)</th>
<th>GU(^d)</th>
<th>LogGU(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable cells inoculum control(^a)</td>
<td>15.64</td>
<td>6.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dead cells Inoculum control</td>
<td>19.01</td>
<td>3.37</td>
<td>5.35</td>
<td>-0.95</td>
<td></td>
</tr>
<tr>
<td><strong>PMA pre-treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50(^f)</td>
<td>26.71 ± 0.353</td>
<td>11.07</td>
<td>3.29 ± 0.095</td>
<td>-3.01</td>
<td></td>
</tr>
<tr>
<td><strong>PEMAX pre-treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24.78 ± 0.184</td>
<td>9.14</td>
<td>3.8 ± 0.049</td>
<td>-2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PMA pre-treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21.72 ± 0.233</td>
<td>6.08</td>
<td>4.63 ± 0.062</td>
<td>-1.67</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PEMAX pre-treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23.67 ± 0.113</td>
<td>8.03</td>
<td>4.09 ± 0.080</td>
<td>-2.29</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Inoculum control: without PMA and PEMAX treatment.

\(^b\)Cp: Crossing Point. Values are mean Cp values (n=3) ± SD.

\(^c\)ΔCp: ΔCp = ΔCp\(_C\) – ΔCp\(_TS\).

\(^d\)GU: Genomic Unit. Values are mean GU values (n=3) ± SD; calculated with *H. pylori* standard curve (correlation coefficient =1; \(y=-3.7333x + 38.976\); PCR efficiency= 99.8%).

\(^e\)LogGU: ΔGU: ΔGU = ΔGU\(_C\) – ΔGU\(_TS\)

\(^f\)ΔCp\(_C\) = Cp value from inoculum *H. pylori* control.

\(^g\)ΔCp\(_TS\) = Cp value from PMA, PEMAX™ treated samples.

\(^h\)ΔGU\(_C\) = GU value from inoculum *H. pylori* control samples calculated with *H. pylori* standard curve.

\(^i\)ΔGU\(_TS\) = GU value from PMA, PEMAX™ treated samples, calculated with *H. pylori* standard curve.

\(^j\)p-value (50 µM): 0.06.

\(^k\)p-value (50 µM): 0.01.
Table 2: Effect of PMA PEMAX™ and PEMAX™-Buffer in viable *H. pylori* cells. Two assays (A and B) were performed independently in triplicate.

<table>
<thead>
<tr>
<th>Intercalating dye</th>
<th>Incubation time (min)</th>
<th>Cp&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ΔCp&lt;sup&gt;c&lt;/sup&gt;</th>
<th>GU&lt;sup&gt;d&lt;/sup&gt;</th>
<th>LogGU&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Cp&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ΔCp&lt;sup&gt;c&lt;/sup&gt;</th>
<th>GU&lt;sup&gt;d&lt;/sup&gt;</th>
<th>LogGU&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inoculum Control&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>16.1</td>
<td>6.12</td>
<td>-0.10</td>
<td>13.75 ± 0.035</td>
<td>0.75</td>
<td>0.38</td>
<td>6.02 ± 0.015</td>
<td>6.77 ± 0.009</td>
</tr>
<tr>
<td>PMA&lt;sup&gt;H&lt;/sup&gt;</td>
<td>5</td>
<td>16.48 ± 0.056</td>
<td>0.38</td>
<td>6.02 ± 0.015</td>
<td>-0.10</td>
<td>13.75 ± 0.035</td>
<td>0.75</td>
<td>0.38</td>
<td>6.02 ± 0.015</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>16.38 ± 0.052</td>
<td>0.28</td>
<td>6.03 ± 0.011</td>
<td>-0.09</td>
<td>14.12 ± 0.021</td>
<td>1.12</td>
<td>6.66 ± 0.005</td>
</tr>
<tr>
<td>PEMAX&lt;sup&gt;I&lt;/sup&gt;</td>
<td>5</td>
<td>20.1 ± 0.140</td>
<td>4.00</td>
<td>5.05 ± 0.038</td>
<td>-1.07</td>
<td>18.98 ± 0.055</td>
<td>5.98</td>
<td>5.41 ± 0.015</td>
<td>-1.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>20.07 ± 0.120</td>
<td>3.97</td>
<td>5.06 ± 0.032</td>
<td>-1.06</td>
<td>20.04 ± 0.095</td>
<td>7.04</td>
<td>5.08 ± 0.025</td>
</tr>
<tr>
<td>PEMAX+Buffer&lt;sup&gt;J&lt;/sup&gt;</td>
<td>5</td>
<td>20.29 ± 0.026</td>
<td>4.19</td>
<td>4.99 ± 0.007</td>
<td>-1.13</td>
<td>20.34 ± 0.080</td>
<td>7.34</td>
<td>4.99 ± 0.021</td>
<td>-1.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>23.47 ± 0.032</td>
<td>7.37</td>
<td>4.15 ± 0.008</td>
<td>-1.97</td>
<td>23.52 ± 0.076</td>
<td>10.52</td>
<td>4.14 ± 0.024</td>
</tr>
</tbody>
</table>

<sup>a</sup>Inoculum control: without PMA and PEMAX treatment.  
<sup>b</sup>Cp: Crossing Point. Values are mean Cp values (n=3) ± SD.  
<sup>c</sup>ΔCp: ΔCp = ΔCp<sub>TS</sub> - ΔCp<sub>IC</sub>.  
<sup>d</sup>GU: Genomic Unit. Values are mean GU values (n=3) ± SD; calculated with *H. pylori* standard curve (correlation coefficient =1; y=-3,7333x + 38,976; PCR efficiency= 99.8%).  
<sup>e</sup>LogGU: LogGU = LogGU<sub>IC</sub> - LogGU<sub>TS</sub>.  
<sup>H</sup>ΔCp<sub>IC</sub> = Cp value from inoculum *H. pylori* control.  
<sup>I</sup>ΔCp<sub>TS</sub> = Cp value from inoculum *H. pylori* control samples calculated with *H. pylori* standard curve.  
<sup>J</sup>ΔGU<sub>IC</sub> = GU value from inoculum *H. pylori* control samples.  
<sup>K</sup>ΔGU<sub>TS</sub> = GU value from PMA, PEMAX™ with and without PEMAX™ Reaction Buffer treated samples calculated with *H. pylori* standard curve.  
<sup>l</sup>p-value (sample A): 0.85; p-value (sample B): 0.81.  
<sup>m</sup>p-value (sample A): 0.00001; p-value (sample B): 0.006.  
<sup>n</sup>p-value (sample A): 0.068; p-value (sample B): 0.030.
Table 3: Reduction in qPCR counts from a suspension of non-viable *H. pylori* cells, killed by different disinfectant treatments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Incubation time (min)*</th>
<th>Cp</th>
<th>ΔCp</th>
<th>GU</th>
<th>LogGU*</th>
<th>Cp</th>
<th>ΔCp</th>
<th>GU</th>
<th>LogGU*</th>
<th>Cp</th>
<th>ΔCp</th>
<th>GU</th>
<th>LogGU*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable inoculum</td>
<td>0</td>
<td>14.45</td>
<td></td>
<td>6.56</td>
<td></td>
<td>14.45</td>
<td></td>
<td>6.56</td>
<td></td>
<td>14.45</td>
<td></td>
<td>6.56</td>
<td></td>
</tr>
<tr>
<td>Treated inoculum</td>
<td>0</td>
<td>19.16</td>
<td></td>
<td>5.31</td>
<td></td>
<td>17.1</td>
<td></td>
<td>5.86</td>
<td></td>
<td>16.3</td>
<td></td>
<td>6.79</td>
<td></td>
</tr>
<tr>
<td>1PMA pre-treatment</td>
<td>5</td>
<td>23.62 ± 0.031</td>
<td>9.17</td>
<td>4.1 ± 0.008</td>
<td>-2.46</td>
<td>17.15 ± 0.025</td>
<td>2.7</td>
<td>5.83 ± 0.007</td>
<td>-0.73</td>
<td>22.28 ± 0.181</td>
<td>7.83</td>
<td>3.74 ± 0.048</td>
<td>-2.82</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>25.13 ± 0.015</td>
<td>10.68</td>
<td>3.72 ± 0.040</td>
<td>-2.84</td>
<td>17.78 ± 0.026</td>
<td>3.33</td>
<td>5.67 ± 0.007</td>
<td>-0.89</td>
<td>21.21 ± 0.061</td>
<td>6.76</td>
<td>4.02 ± 0.016</td>
<td>-2.54</td>
</tr>
<tr>
<td>1PEMAX pre-treatment</td>
<td>5</td>
<td>24.99 ± 0.026</td>
<td>10.54</td>
<td>3.73 ± 0.007</td>
<td>-2.83</td>
<td>17.6 ± 0.055</td>
<td>3.15</td>
<td>5.71 ± 0.015</td>
<td>-0.84</td>
<td>21.64 ± 0.127</td>
<td>7.19</td>
<td>4.63 ± 0.034</td>
<td>-1.93</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>25.17 ± 0.059</td>
<td>10.72</td>
<td>3.69 ± 0.016</td>
<td>-2.87</td>
<td>20.38 ± 0.025</td>
<td>5.93</td>
<td>4.98 ± 0.007</td>
<td>-1.58</td>
<td>24.4 ± 0.100</td>
<td>9.95</td>
<td>3.89 ± 0.027</td>
<td>-2.67</td>
</tr>
<tr>
<td>MPEMAX + Buffer pre-treatment</td>
<td>5</td>
<td>26.15 ± 0.025</td>
<td>11.7</td>
<td>3.42 ± 0.007</td>
<td>-3.14</td>
<td>18.34 ± 0.030</td>
<td>3.89</td>
<td>6.25 ± 0.008</td>
<td>-0.31</td>
<td>25.95 ± 0.061</td>
<td>11.5</td>
<td>3.48 ± 0.016</td>
<td>-3.08</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>25.87 ± 0.055</td>
<td>11.42</td>
<td>3.5 ± 0.015</td>
<td>-3.06</td>
<td>20.38 ± 0.097</td>
<td>5.93</td>
<td>5.52 ± 0.025</td>
<td>-1.04</td>
<td>25.67 ± 0.113</td>
<td>10.22</td>
<td>3.82 ± 0.03</td>
<td>-2.74</td>
</tr>
</tbody>
</table>

* Inoculum control: without PMA and PEMAX treatment.

* Cp: Crossing Point. Values are mean Cp values (n=3) ± SD.

* GU: Genomic Unit. Values are mean GU values (n=3) ± SD; calculated with *H. pylori* standard curve (correlation coefficient =1; y=-3.7333x + 38.976; PCR efficiency = 99.8%).

* ΔCp: ΔCp = ΔCpTS.

* LogGU: LogGU = ΔGU - ΔGU0.

ΔCpTS = Cp value from inoculum *H. pylori* control.

ΔCp = Cp value from PMA, PEMAXTM with and without PEMAXTM Reaction Buffer treated samples.
ΔGU = GU value from inoculum *H. pylori* control samples, calculated with *H. pylori* standard curve.

ΔGU = GU value from PMA, PEMAX™ with and without PEMAX™ Reaction Buffer treated samples, calculated with *H. pylori* standard curve.

*p*-value (Ethanol 70%): 0.005; *p*-value (Sodium Hypochlorite 100ppm): 0.89; *p*-value (Hydrogen Peroxide 5%): 0.053.

*p*-value (Ethanol 70%): 0.001; *p*-value (Sodium Hypochlorite 100ppm): 0.082; *p*-value (Hydrogen Peroxide 5%): 0.025.

*p*-value (Ethanol 70%): 0.0053; *p*-value (Sodium Hypochlorite 100ppm): 0.025; *p*-value (Hydrogen Peroxide 5%): 0.00345.

**Table 4**: Effects of pre-treatment with PMA in qPCR counts of *H. pylori* samples considering different morphological and viability states.

<table>
<thead>
<tr>
<th>Morphologic state</th>
<th>V Spiral</th>
<th>VBNC Spiral</th>
<th>NV Spiral</th>
<th>VBN Coccoid</th>
<th>NV Coccoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cp</td>
<td>ΔCp</td>
<td>GU</td>
<td>Cp</td>
<td>ΔCp</td>
<td>GU</td>
</tr>
<tr>
<td>Sample without PMA pre-treatment</td>
<td>12.69 ± 0.305</td>
<td>7.04 ± 0.082</td>
<td>13.48 ± 0.327</td>
<td>6.83 ± 0.088</td>
<td>16.31 ± 0.321</td>
</tr>
<tr>
<td>Sample with PMA pre-treatment</td>
<td>12.21 ± 0.300</td>
<td>7.17 ± 0.101</td>
<td>0.13</td>
<td>14.11 ± 0.142</td>
<td>0.63</td>
</tr>
</tbody>
</table>

*Cp*: Crossing Point. Values are mean Cp values (n=3) ± SD.

*GU*: Genomic Unit. Values are mean GU values (n=3) ± SD; calculated with *H. pylori* standard curve (correlation coefficient =1; y=−3.7333x + 38.976; PCR efficiency= 99.8%).

*ΔGU*: ΔGU = ΔGU<sub>C</sub> − ΔGU<sub>TS</sub>.

*VC*: Viable and culturable.

*VBNC*: Viable but non-culturable.

*NV*: Non-viable.

*p*-value (VC Spiral): 0.423; *p*-value (VBNC Spiral): 0.072; *p*-value (NV Spiral): 0.003; *p*-value (VBNC Coccoid): 0.24; *p*-value (NV Coccoid): 0.01.