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Additional Information

1 **Optimization of pre- treatments with Propidium Monoazide and PEMAX™ before**

2 **real-time quantitative PCR for detection and quantification of viable *Helicobacter***

3 ***pylori* cells.**

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11 **ABSTRACT**

12 Accurate detection of *H. pylori* in different environmental and clinical samples is

13 essential for public health studies. Now, a big effort is being made to design PCR

14 methodologies that allow for the detection of viable and viable but non-culturable

15 (VBNC) *H. pylori* cells, by achieving complete exclusion of dead cells amplification

16 signals. The use of DNA intercalating dyes has been proposed. However, its efficacy is

17 still not well determined.

18 In this study, we aimed to test the suitability of PMA and PEMAX™ dyes used prior to

19 qPCR for only detecting viable cells of *H. pylori*. Their efficiency was evaluated with cells

20 submitted to different disinfection treatments and confirmed by the absence of growth

21 on culture media and by LIVE/DEAD counts. Our results indicated that an incubation

22 period of 5 min for both, PMA and PEMAX™, did not affect viable cells. Our study also

23 demonstrated that results obtained by using intercalating dyes may vary depending on

24 the cell stress conditions. In all dead cell's samples, both PMA and PEMAX^{TM} pre-qPCR
25 treatments decreased the amplification signal (>10^3 Genomic Units (GU)), although none
26 of them allowed for its disappearance confirming that intercalating dyes, although
27 useful for screening purposes, cannot be considered as universal viability markers. To
28 investigate the applicability of the method specifically to detect *H. pylori* cells in
29 environmental samples, PMA-qPCR was performed on samples containing the different
30 morphological and viability states that *H. pylori* can acquire in environment. The
31 optimized PMA-qPCR methodology showed to be useful to detect mostly (but not only)
32 viable forms, regardless the morphological state of the cell.

33 *Keywords: Helicobacter pylori, PMA-q PCR, PEMAX^{TM}-qPCR, Viability, Disinfection treatment,*
34 *Morphological states.*

35

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41

42 INTRODUCTION

43 *Helicobacter pylori* is the major causative agent of chronic gastroenteritis, duodenal,
44 non-cardia gastric cancer and gastric Mucosa-Associated Lymphoid Tissue (MALT)
45 lymphoma (Hosseini *et al*., 2012; Calvet *et al*., 2013). For this reason, *H. pylori* has been

46 classified as human carcinogen Type I by the World Health Organization and US Food
47 and Drug Administration included it in the list of microorganisms that pose a serious
48 threat to public health (FDA, 2014).

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

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

60 Depending on the environmental conditions the microorganism adopts different vital
61 states, associated with morphological changes: *H. pylori*, usually presents spiral shape
62 but, when exposed to stressful conditions such as increased oxygen concentration,
63 changes in pH (alkaline), increased temperature, absence of nutrients, prolonged
64 incubation periods, treatment with antimicrobial agents or exposure to visible light and
65 UV irradiation, the organism can change from spiral to coccoid form (Del Campo *et al*.,
66 2009; Cunningham *et al*., 2009; Andersen and Rasmussen, 2009). This transformation of
67 rod shape to the coccoid form can occur through intermediate forms (“V” and “U”),
68 corresponding to viable but non-cultivable spiral cells (Bai *et al.,* 2010). Some authors

69 had proposed that acquiring coccoid form indicates cellular degeneration and
70 subsequently death (Kusters *et al*., 1997). However, some works strongly support that
71 some of these forms are viable, although they cannot be cultured (Viable but non-
72 culturable, VBNC), as they preserve metabolic activity; express virulence genes as *ure*A,
73 *ure*B, *hpa*A, *Bab*A, *vac*A and *cag*A; keep the urease activity and continue synthesizing
74 proteins and small amounts of DNA (Oliver, 2005; Azevedo *et al*., 2007). Thus, some
75 authors have differentiated coccoid forms into two types, with different morphological
76 and functional characteristics: Type A is irregular, with rough surface and is considered
77 a dead cell. Type B is smoother, with strictly membranous structure and is considered a
78 viable but not cultivable form (Sarem and Corti, 2016; Flores-Encarnacion *et al.,* 2015).
79 On some occasions, spiral non-viable cells have been also described (Orta de Velásquez
80 et al., 2016)

81 Taking all this into account, vital states of *H. pylori* cells could be classified as: viable
82 spiral form (V), non-viable spiral form (NV), viable but non-cultivable (VBNC) coccoid or
83 spiral form and non-viable degenerative coccoid form (NVC) (Saito *et al.,* 2003). The
84 stage in which *H. pylori* can be present in a sample is of great epidemiological interest,
85 because VBNC forms seem to play a crucial role in the process of transmission through
86 water or in the relapse of the infection after antimicrobial treatment (Dworkin, 2010;
87 Flores-Encarnacion *et al*., 2015).

88 VBNC forms cannot be detected by culture techniques, only by PCR (Codony *et al.,*
89 2015). However, PCR cannot differentiate between viable and nonviable cells because
90 DNA of live and dead cells, as well as extracellular DNA, is amplified (Pathak *et al*., 2012).
91 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

115 treatment protocol with PEMAX that allows for neutralizing DNA signals from up to 5.0

116 x 10^7 dead cells from a pure culture of *Salmonella spp*. However, this methodology has

117 never been applied to *Helicobacter spp.*

118 The purpose of the present study was, firstly, the optimization of PMA and PEMAX™

119 protocols to achieve the total extinction of the qPCR signal from dead bacteria.

120 Concentration of dyes and incubation periods were assayed in accordance with previous

121 studies about the effects of different PMA concentrations on viable *H. pylori* cells (Zeng

122 *et al*., 2016). We also studied the efficacy of these dyes for detecting viable cells in

123 samples submitted to different disinfection treatments. Finally, we evaluated the

124 performance of PMA for detecting the different morphological and viability states

125 adopted for *H. pylori* under stress conditions. Our objective was to determine the

126 suitability of a pre-qPCR treatment with PMA or PEMAX™ for detection and

127 quantification of DNA from *H. pylori* viable cells in environmental samples.

128

129 **MATERIALS AND METHODS**

130 **Bacteria strain and growth conditions**

131 *H. pylori* NCTC 11637 strain (National Collection of Type Cultures, UK) was cultured in

132 Blood Agar Medium as previously described (Hortelano *et al.,* 2020), The cultures were

133 incubated under micro-aerobic conditions (5% oxygen, 10% carbon dioxide, and 85%

134 nitrogen) and 90-95% humidity, by using CampyGen™ 3-5L Atmosphere Generation

135 Systems (Oxoid, UK) in anaerobic jars (Oxoid, UK) at 37 °C for 48 h-14 days.

136 For the different assays, the initial inoculum was prepared by suspending a portion of a
137 48 h growth agar culture in 6 mL of PBS buffer (phosphate-buffered saline, pH 7.5) and
138 adjusted by the LIVE/DEAD method as below described, to reach a concentration of 10^6
139 viable cell/mL. For assessing LIVE/DEAD counts results, CFU counts were also performed,
140 by culturing 10-fold serial dilutions of the inoculum in Blood Agar Medium as described
141 above.

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

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

146 Briefly, an aliquot of 125 µL of each PBS cell suspension (inoculum, controls, and treated
147 samples) was mixed with 0.4 µL of a mixture of SYTO9 and Propidium Iodide (1:1), re-
148 suspended and incubated under dark conditions for 5 min at room temperature. A 5 µL
149 aliquot was spotted on a poly-L-lysine (Polysine® slides, Menzel-Glaser, Thermo
150 Scientific, Germany) coated slide. The count of viable (green) and dead (red)
151 microorganisms was performed by using an Olympus epifluorescence microscope
152 (BX50) with U-MWB filter. Count was determined as the mean value obtained from 20
153 microscopic fields from each of two different slides. When observed, information about
154 morphology of *H. pylori* cells present in each sample was also collected.

155

156 For testing cultivability of cells, 100 µL of each sample were spread onto blood agar
157 plates, incubated at 37 °C, as above described, and observed daily after 3 days for the
158 following 11 days.

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

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

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

169 In order to determine the best concentration of each dye for only amplifying living cells,
170 suspension of a 48 h *H. pylori* culture was adjusted by LIVE/DEAD test to a final
171 concentration of 10^6 cells/mL viable cells. Three mL from this initial stock were used as
172 positive controls, and another 3 mL were incubated at 85 °C for 30 min, killing all the
173 cells. Viability (LIVE/DEAD test) and culture analysis were carried out before and after
174 heat treatment to verify the state of the cells. Then, 1.5 mL aliquots of viable and heat-
175 killed cells suspensions were treated with PMA or PEMAX^{TM} prior to qPCR, at a final
176 concentration of 25 µM and 50 µM, in both cases.

177 To test if pre-PCR treatment with dyes affected cells viability, two different suspensions
178 (A and B) from the same inoculum containing viable *H. pylori* cell were analyzed
179 independently in triplicate and submitted to qPCR before and after pre-treatment with
180 PMA and PEMAX™, at incubation times of 5 and 10 min. Additionally, a 1.5 mL aliquot
181 of each sample was processed with the PEMAX™ GenIUL Reaction Buffer. Briefly, the
182 inoculum samples (10^6 cells/mL viable cells *H. pylori* cell suspension) were centrifuged
183 at 10000 rpm for 10 min, the supernatant was removed, and 1.5 mL amount of Reaction
184 Buffer was added before PEMAX™ staining, followed by an incubation period of 5 min
185 and 10 min. Samples were then stained with PEMAX™, as described above.

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

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

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

200 PEMAX^{TM} Reaction Buffer), as described above, and incubated for 5 and 10 min prior to
201 qPCR analysis.

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

204 To obtain different viability states of *H. pylori* during its morphological transformation
205 from bacillary to coccoid form, an initial bacterial suspension in PBS was prepared from
206 a 48 h *H. pylori* NCTC 11637 pure culture and adjusted with LIVE/DEAD method to a final
207 concentration of 10^8 viable (green) cells/mL, as described before. A 3 mL aliquot from
208 the initial inoculum was taken as control (viable spiral shape). Other four aliquots of 1.5
209 mL were exposed to different environmental conditions, according to previous studies:
210 100 ppm sodium hypochlorite, 5% of hydrogen peroxide, at 25 °C and 4 °C.

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

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

220 When the desired morphology of cells was reached, samples were centrifuged at 10000
221 rpm for 5 min, pellets were re-suspended in 1.5 mL of PBS and treated with PMA at a

222 final concentration of 50 µM. Samples were incubated at room temperature in dark
223 conditions for 5 min.

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

227 **DNA extraction**

228 DNA was extracted from all the samples by using the GeneJet™ Genomic DNA
229 Purification Kit (ThermoScientific, Germany), according to Moreno-Mesonero *et al*.
230 (2016). Isolated DNA was stored at -20 °C until use.

231 **Real-time quantitative PCR assay**

232 A SYBR®Green I qPCR assay was performed to amplify a 372 bp fragment of *H. pylori*
233 VacA gene (Vesga *et al.,* 2018) in a final volume of 18 µl, containing 2 µL LightCycler®
234 FastStart DNA Master SYBR Green I (Roche Applied Science, Spain), 1.6 µL $MgCl_2$ (50
235 mM), 0.5 µL of each primer (20 mM) and 2 µL of DNA template. Amplification protocol
236 consisted in an initial DNA denaturation step at 95 °C for 10 min, followed by 40 cycles
237 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
238 and another at 40 °C for 30 s. Amplification was carried out in a LightCycler® 2.0 (Roche
239 Applied Science, Spain) and LightCycler® 4.1 Software (Roche Applied Science, Spain)
240 was used to obtained automatically the Cp values, which marked the cycle when the
241 fluorescence of a given sample significantly exceeded the baseline signal, and
242 quantification of DNA using the "Abs. Quant Analysis" and the "Automated Second
243 Derivative" method. It offers automated data calculation without any user influence

244 (except for the selection/deselection of standards) and provides the advantage of high
245 reproducibility. The quantification of samples was obtained according to an external
246 standard curve as previously developed by Santiago *et al.* (2015) (Figure 2.S). Briefly, six
247 log of *H. pylori* NCTC 11637 DNA concentration, in the range 2.16 x 10^1 to 1.79 x 10^6
248 genomic units, corresponding to cycle threshold (Cp) media values ranged from 34 to
249 15.62 (Cp = -3.7333 · Log10 (GU) + 38.976; $R^2 = 1$), was used to elaborate the standard
250 curve. GU were calculated considering the existence of an only *Vac*A gene copy in each
251 cell and following the next: Genomic Units (GU)= DNA concentration/atomic mass (Linke
252 *et al*., 2010).

253 **Statistical analysis**

254 All experiments were performed in triplicate and repeated at least once in an
255 independent experiment. Mean values and standard deviation for the different
256 experiments performed in triplicate, used to evaluate the relation of intercalating dyes
257 treatment and viability *H. pylori* states, were calculated using Microsoft Excel.v.10.

258 The effect of PMA, PEMAX^{TM} with and without PEMAX^{TM} Reaction Buffer on viable and
259 killed *H. pylori* cells by different disinfectant treatment were determined by calculating
260 log10 reduction of GU (genomic units) using qPCR C_p values. The following equation was
261 used to calculate log10 in detectable genome copies:

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

263 Where:

264 ΔGU_C = GU value from control samples calculated with *H. pylori* standard curve.

265 ΔGU_{TS} = GU value from PMA, PEMAX^{TM} with and without PEMAX^{TM} Reaction Buffer
266 treated samples, calculated with *H. pylori* standard curve. Amplification efficiency (E)
267 was estimated by using the slope of the standard curve and the formula E¼ [10(
268 −1/slope)] −1.

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

274 **RESULTS**

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

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

279 Two different dye concentrations, 50 and 25 μM, were tested. As showed in Table 1,
280 qPCR results for the dead cells sample without pre-treatment showed a reduction in
281 counts of only 0,95 log GU relative to the control (live cells sample). The use of
282 concentrations of 50μM for both intercalating dyes, PMA and PEMAX^{TM}, yielded near
283 statistically significant (P=0.06) reductions in DNA counts from dead cells samples,
284 compared with the viable cells control sample, showing average signal decreases of 3.01
285 and 2.5 log GU, respectively. Pre-treatment with PMA and PEMAX^{TM} at concentrations
286 of 25μM showed less efficacy, resulting in 1.67 and 2.29 log GU reduction.

287 In addition, the potential toxic action of intercalating dyes on viable cells was also
288 examined by treating independently two different samples from the same inoculum of
289 viable *H. pylori* cells with PMA and PEMAX™ (with and without using PEMAX™ Reaction
290 Buffer) (Table 2). No effect on the qPCR could be observed when using PMA at both
291 incubation times, since their average signal reduction (ΔCp) from the two different
292 viable *H. pylori* cells samples were not significantly different from the untreated
293 controls. Nevertheless, Sample B, incubated for 10 minutes with PMA caused a slightly
294 higher average reduction of 0.30 log GU (probably due to the physiological
295 heterogeneity of the cells) compared with the control, suggesting more toxic effect in
296 viable cells than a shorter 5 min incubation period.

297 Regarding PEMAX™, pre-treatment at both incubation times rendered similar results
298 for the two samples analyzed. Sample A showed a decreased in q-PCR signal by 4.000
299 log and 3.97 log GU reduction; and Sample B, demonstrated a reduction of 0.75 log and
300 1.12 log GU reduction, after incubation for 5 and 10 min, respectively. Difference
301 between samples with and without PEMAX™ treatment were statistically difference
302 (p=0.00001 (A); p=0.0065 (B)).

303 The use of PEMAX™ GenIUL Reaction Buffer previously to add PEMAX™ showed the
304 most significant effect on qPCR results, since the average of genomic units from the two
305 viable *H. pylori* cell samples. Sample A, resulted in a decrease of q-PCR signal of 1.13 and
306 1.97 log GU reduction, for 5 and 10 min incubation periods, respectively. Regarding with
307 sample B, led to a decline of q-PCR signal of 1.97 and 2.82 log GU reduction, for 5 and
308 10 min incubation periods, respectively.

309 **Evaluation of PMA, PEMAX^{TM} and PEMAX^{TM}-Buffer efficacy after different disinfection**

310 **treatments.**

311 Samples were subjected to pre-PCR treatment with 50 µM PMA and 25 µM PEMAX^{TM} at

312 two incubation times, 5 and 10 min, in order to compare the results. The effectiveness

313 of the PEMAX^{TM} GenIUL Reaction Buffer was also tested (Table 3).

314 Exposure of *H. pylori* viable cells, to 70% of ethanol led to a complete loss of cultivability

315 and LIVE/DEAD test showed non-viable *H. pylori* forms. PMA pre-treatment induced a

316 significant reduction of qPCR signal ($p=0.0057$) on ethanol-killed *H. pylori* cells: signals

317 decreased 2.46 and 2.84 log GU related to untreated inactivated *H. pylori* cells, after

318 incubation for 5 and 10 min, respectively. No significant difference could be observed

319 depending on the incubation period.

320 Similar results were obtained for PEMAX^{TM}: intercalating dye pretreatment reduced the

321 signal by 2.83 and 2.87 log GU after incubation for 5 and 10 min, respectively. When

322 PEMAX^{TM} was used in combination with enhancer Buffer PEMAX^{TM}-qPCR signal was

323 reduced by 3.14 and 3.06 log GU.

324 After treating a pure culture of *H. pylori* (viable cells), with 100 ppm sodium

325 hypochlorite, any colony grew on culture plates and LIVE/DEAD test showed only non-

326 viable *H. pylori* forms. The Cp values observed after pre-treatment with PMA presented

327 significant differences when compared to non-treated inactivated *H. pylori* cells (29,1

328 vs. 17,15 and 17,78 after incubation for 5 and 10 min respectively). Similar results were

329 obtained with PEMAX^{TM} (29,1 vs. 17,6 and 20,38 after incubation for 5 and 10 min

330 respectively). PEMAX^{TM} used with standard Buffer reduced qPCR signal by 0.31 and 1.04

331 log, as compared to non-treated inactivated *H. pylori* cells, results that were not
332 different from those obtained by using PEMAX^{TM} without enhancer Buffer.

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

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

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

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

348 The study was carried out with previously established optimal conditions for PMA
349 treatment (50 µM and 5 min incubation). Morphologies, viability, and cultivability of
350 cells were confirmed by viability LIVE/DEAD test (Figure 1) and the presence/absence of
351 colonies in culture plates.

352 As expected, no effect of PMA pretreatment was observed on viable morphological
353 states of *H. pylori,* since their average counts were not significantly different (Table 4).
354 On the contrary, PMA pretreatment induced a significant reduction of the qPCR signal
355 in non-viable spiral shape (p= 0.03) and non-viable coccoid forms (p= 0.01) samples,
356 when compared with untreated *H. pylori* control.

357 **DISCUSSION**

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

370 As previously reported by Nam *et al.* (2011), we found that PMA concentration had no
371 significant effect on the reduction of qPCR signal from viable *H. pylori* cells. This can be
372 attributed to the low cytotoxicity of PMA and its affinity to penetrate only cells with
373 strongly damaged membranes (Nocker and Camper *et al.*, 2006). The small logarithmic
374 reduction in genomic units on viable cells after PMA treatment may be due to the death

375 of cells because osmotic stress when pure culture was spiked in PBS (Delgado-Viscogliosi
376 *et al*., 2009). Underestimation of viable cell populations has been reported for other
377 bacterial species (Yáñez *et al.,* 2011; Liu and Mustapha, 2014; Barbau-Piednoir *et al*.,
378 2014) when using high concentrations of intercalating dye or lower number of targets
379 (Yasunaga *et al.,* 2013). Concerning PEMAX^{TM}, we found a significant reduction on qPCR
380 signal from live cells for both concentrations tested, what seems to suggest an unspecific
381 toxic effect in viable cells treated with PEMAX^{TM} or DNA neutralization in live cells
382 presenting non-lethal damage membrane. An even larger effect was noticed when
383 PEMAX^{TM} was used in combination with commercial Buffer. PEMAX^{TM} has been
384 proposed to be more accurate than PMA, detecting only cells with intact membrane
385 structure and active metabolism (Agustí *et al.,* 2017), what may explain that some viable
386 non-lethally damaged cells are not detected. To our knowledge, there is no reference in
387 the literature mentioning similar results for *H. pylori*.

388 Regarding the assay with dead cells, we chose heating at 85 °C during 30 min because
389 heat induces effusion of intracellular substances (Hurst *et al.,* 1974) and loss of
390 membrane lipopolysaccharides (Tsuchido *et al.,* 1985). Results confirmed that heat
391 altered membrane permeability, allowing PMA/PEMAX^{TM} to enter the cell, but did not
392 result in the complete loss of qPCR signal. This is in accordance with several studies that
393 describe incomplete PMA-qPCR signal inhibition, resulting in strong overestimation of
394 viable cells (LØvdal *et al*., 2011). Previous studies have proposed that the presence of
395 high number of dead cells can exceed the intercalating dyes capacity, what may result
396 in insufficient binding to DNA in damaged cell (Yáñez *et al*., 2007). Other researchers

397 have suggested that short amplicon size in qPCR cannot be completely suppressed by
398 PMA pretreatment (Luo *et al*., 2010).

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

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

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

413 The highest and most significant signal reduction without intercalating dyes
414 pretreatment occurred after exposition to 100 ppm of sodium hypochlorite during 45
415 min. Sodium hypochlorite effects include the destruction of key metabolic enzymes
416 (Wyss *et al.,* 1961) and the disruption of protein synthesis (Agranoff, 1967). Our data
417 showed that this disinfectant treatment reduced the qPCR signal by 3.47 log GU,
418 showing no significant differences among results obtained before and after the use of

419 both intercalating dyes and standard buffer. This can be explained because hypochlorite
420 at high concentrations affects both, cellular membrane, and nucleic acids, hindering the
421 covalent joint between DNA and intercalating dye (Delgado-Viscogliosi *et al.,* 2009; Lee
422 *et al*., 2015).

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

428 Regarding hydrogen peroxide, it generates oxidative stress and causes lipid
429 peroxidation, resulting in a decrease in membrane fluidity and its inactivation (Nebe-
430 von-Caron *et al*., 2000). Our data showed no significant difference between counts
431 obtained for live and dead control samples, what can be explained because hydrogen
432 peroxide leads to loss of membrane integrity but does not damage DNA (Krüger *et al*.,
433 2014).

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

438 We also aimed to investigate if PMA-qPCR results could be affected by *H. pylori*
439 morphological state, as this pathogen is frequently present in the environment in a
440 coccoid shape, which presents some different structural and functional properties

441 (Krüger *et al.,* 2014). As expected, no effect of PMA was observed on all viable
442 morphological states of *H. pylori,* since their average counts were not significantly
443 different, confirming that this intercalating dye is excluded from viable cells and, thus, it
444 does not reduce the qPCR signal from viable and viable but non-cultivable states of *H.*
445 *pylori*, either spiral or coccoid. Our research also showed that the use of PMA reduced
446 the signal of non-viable coccoid and spiral morphologies. However, again this assay did
447 not totally avoid amplification of DNA from non-viable cells (LØvdal et *al*, 2011).

448 **CONCLUSIONS**

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

456

457

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648

Figure 1: LIVE/DEAD method used to determinate 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 (VBNC) spiral form (4 °C). (D) Viable but Non-Cultivable (VBNC) Coccoid form (25 °C). (E) Non-Viable Coccoid form (100 ppm sodium hypochlorite). Bar: 10 µm. Observation 100X.Viable but Non-Cultivable (VBNC) coccoid form (25 °C). (E) Non-Viable Coccoid form (100 ppm sodium hypochlorite). Bar: 10 µm. Observation 100X.

	V Spiral (A)	**NV Spiral (B)**	**VBNC Spiral (C)**	**VBNC Coccoid (D)**	**NV Coccoid (C)**
H. pylori					

657 **Table 1:** Effects of PMA and PEMAX^{TM} concentrations in qPCR results for heat-killed *H. pylori* cells.

Sample	Concentration (µM)	Cp[b]	ΔCp[c]	GU[d]	LogGU[e]
Viable cells Inoculum control[a]		15.64		6.3	
Dead cells Inoculum control		19.01	3.37	5.35	-0.95
PMA pre-treatment	50[F]	26.71 ± 0.353	11.07	3.29 ± 0.095	-3.01
PEMAX pre-treatment		24.78 ± 0.184	9.14	3.8 ± 0.049	-2.5
PMA pre-treatment	25[G]	21.72 ± 0.233	6.08	4.63 ± 0.062	-1.67
PEMAX pre-treatment		23.67 ± 0.113	8.03	4.09 ± 0.080	-2.29

658 [a]Inoculum control: without PMA and PEMAX treatment.
659 [b]Cp: Crossing Point. Values are mean Cp values (n=3) ± SD.
660 [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%).
661 [c]ΔCp: $\Delta Cp = \Delta Cp_C - \Delta Cp_{TS.}$
662 [e] LogGU: ΔGU: $\Delta GU = \Delta GU_C - \Delta GU_{TS}$
663 ΔCp_C = Cp value from inoculum *H. pylori* control.
664 ΔCp_{TS} = Cp value from PMA, PEMAXTM treated samples.
665 ΔGU_C = GU value from inoculum *H. pylori* control samples calculated with *H. pylori* standard curve.
666 ΔGU_{TS} = GU value from PMA, PEMAX^{TM} treated samples, calculated with *H. pylori* standard curve.
667 [F]*p*-valor (50 µM): 0.06.
668 [G]*p*-valor (50 µM): 0.01.
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671 **Table 2:** Effect of PMA PEMAX^{TM} and PEMAX^{TM}-Buffer in viable *H. pylori* cells. Two assays (A and B) were performed independently in triplicate.

		SAMPLE A				SAMPLE B			
Intercalating dye	**Incubation time (min)**	**Cp[b]**	**ΔCp[c]**	**GU[d]**	**LogGU[e]**	**Cp[b]**	**ΔCp[c]**	**GU[d]**	**LogGU[e]**
Inoculum Control[a]		16.1		6.12		13		6.96	
PMA[H]	5	16.48 ± 0.056	0.38	6.02 ± 0.015	-0.10	13.75 ± 0.035	0.75	6.77 ± 0.009	-0.19
	10	16.38 ± 0.052	0.28	6.03 ± 0.011	-0.09	14.12 ± 0.021	1.12	6.66 ± 0.005	-0.3
PEMAX[I]	5	20.1 ± 0.140	4.00	5.05 ± 0.038	-1.07	18.98 ± 0.055	5.98	5.41 ± 0.015	-1.55
	10	20.07 ± 0.120	3.97	5.06 ± 0.032	-1.06	20.04 ± 0.095	7.04	5.08 ± 0.025	-1.88
PEMAX+Buffer[J]	5	20.29 ± 0.026	4.19	4.99 ± 0.007	-1.13	20.34 ± 0.080	7.34	4.99 ± 0.021	-1.97
	10	23.47 ± 0.032	7.37	4.15 ± 0.008	-1.97	23.52 ± 0.076	10.52	4.14 ± 0.024	-2.82

672 [a]Inoculum control: without PMA and PEMAX treatment.
673 [b]Cp: Crossing Point. Values are mean Cp values (n=3) ± SD
674 [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%).
675 [c]ΔCp: $\Delta Cp = \Delta Cp_C - \Delta Cp_{TS.}$
676 [e] LogGU: ΔGU: $\Delta GU = \Delta GU_C - \Delta GU_{TS}$
677 ΔCp_C = Cp value from inoculum *H. pylori* control.
678 ΔCp_{TS} = Cp value from PMA, PEMAXTM with and without PEMAXTM Reaction Buffer treated samples.
679 ΔGU_C = GU value from inoculum *H. pylori* control samples calculated with *H. pylori* standard curve.
680 ΔGU_{TS} = GU value from PMA, PEMAX^{TM} with and without PEMAX^{TM} Reaction Buffer treated samples, calculated with *H. pylori* standard curve.
681 [H]*p*-valor (sample A): 0.85; *p*-valor (sample B): 0.81.
682 [I]*p*-valor (sample A): 0.00001; *p*-valor (sample B): 0.006.
683 [J]*p*-valor (sample A): 0.068; *p*-valor (sample B): 0.030.

684 **Table 3:** Reduction in qPCR counts from a suspension of non-viable *H. pylori* cells, killed by different disinfectant treatments.

Sample	Incubation time (min)*	Ethanol 70%				Sodium Hypochlorite 100ppm				Hydrogen Peroxide 5%			
		Cp[b]	ΔCp[c]	GU[d]	LogGU[e]	Cp[b]	ΔCp[c]	GU[d]	LogGU[e]	Cp[b]	ΔCp[c]	GU[d]	LogGU[e]
Viable inoculum	0	14.45		6.56		14.45		6.56		14.45		6.56	
Treated inoculum	0	19.16		5.31		17.1		5.86		16.3		6.79	
[K]PMA pre-treatment	5	23.62 ± 0.031	9.17	4.1 ± 0.008	-2.46	17.15 ± 0.025	2.7	5.83 ± 0.007	-0.73	22.28 ± 0.181	7.83	3.74 ± 0.048	-2.82
	10	25.13 ± 0.015	10.68	3.72 ± 0.040	-2.84	17.78 ± 0.026	3.33	5.67 ± 0.007	-0.89	21.21± 0.061	6.76	4.02 ± 0.016	-2.54
[L]PEMAX pre-treatment	5	24.99 ± 0.026	10.54	3.73 ± 0.007	-2.83	17.6 ± 0.055	3.15	5.71 ± 0.015	-0.84	21.64 ± 0.127	7.19	4.63 ± 0.034	-1.93
	10	25.17 ± 0.059	10.72	3.69 ± 0.016	-2.87	20.38 ± 0.025	5.93	4.98 ± 0.007	-1.58	24.4 ± 0.100	9.95	3.89 ± 0.027	-2.67
[M]PEMAX + Buffer pre-treatment	5	26.15 ± 0.025	11.7	3.42 ± 0.007	-3.14	18.34 ± 0.030	3.89	6.25 ± 0.008	-0.31	25.95 ± 0.061	11.5	3.48 ± 0.016	-3.08
	10	25.87 ± 0.055	11.42	3.5 ± 0.015	-3.06	20.38 ± 0.097	5.93	5.52 ± 0.025	-1.04	25.67 ± 0.113	10.22	3.82 ± 0.03	-2.74

685 [a]Inoculum control: without PMA and PEMAX treatment.
686 [b]Cp: Crossing Point. Values are mean Cp values (n=3) ± SD
687 [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%).
688 [c]ΔCp: ΔCp= $\Delta Cp_C - \Delta Cp_{TS}$.
689 [e] LogGU: ΔGU: ΔGU= $\Delta GU_C - \Delta GU_{TS}$
690 ΔCp_C = Cp value from inoculum *H. pylori* control.
691 ΔCp_{TS} = Cp value from PMA, PEMAXTM with and without PEMAXTM Reaction Buffer treated samples.

692 ΔGU_C = GU value from inoculum *H. pylori* control samples, calculated with *H. pylori* standard curve.
693 ΔGU_{TS} = GU value from PMA, PEMAX™ with and without PEMAX™ Reaction Buffer treated samples, calculated with *H. pylori* standard curve.
694 [K]*p*-valor (Ethanol 70%): 0.005; *p*-valor (Sodium Hypochlorite 100ppm): 0.89; *p*-valor (Hydrogen Peroxide 5%): 0.053.
695 [L]*p*-valor (Ethanol 70%):0.001; *p*-valor (Sodium Hypochlorite 100ppm): 0.082; *p*-valor (Hydrogen Peroxide 5%): 0.025.
696 [M]*p*-valor (Ethanol 70%): 0.0053; *p*-valor (Sodium Hypochlorite 100ppm): 0.025; *p*-valor (Hydrogen Peroxide 5%): 0.00345.
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698 **Table 4**: Effects of pre-treatment with PMA in qPCR counts of *H. pylori* samples considering different morphological and viability states.

Morphologic state	**V Spiral**				**VBNC Spiral**				**NV Spiral**				**VBNC Coccoid**				**NV Cocoid**			
	Cp[b]	**ΔCp[c]**	**GU[d]**	**LogGU[e]**	**Cp[b]**	**ΔCp[c]**	**GU[d]**	**LogGU[e]**	**Cp[b]**	**ΔCp[c]**	**GU[d]**	**LogGU[e]**	**Cp[b]**	**ΔCp[c]**	**GU[d]**	**ΔGU[e]**	**Cp[b]**	**ΔCp[c]**	**GU[d]**	**ΔGU[e]**
Inoculum Control	12.69		7.04		12.69		7.04		14.30		6.61		14.75		6.49		13.40		6.85	
Sample without PMA pre-treatment	12.69 ± 0.305		7.04 ± 0.082		13.48 ± 0.327		6.83 ± 0.088		16.31 ± 0.321		6.07 ± 0.086		16.87 ± 0.286		5.92 ± 0.077		21.20 ± 0.207		4.76 ± 0.055	
[N]Sample with PMA pre-treatment	12.21 ± 0.300	0.48	7.17 ± 0.101	0.13	14.11 ± 0.142	0.63	6.66 ± 0.038	-0.17	22.36 ± 0.112	6.05	4.45 ± 0.029	-1.34	18.29 ± 0.311	3.64	5.54 ± 0.083	-0.38	26.17 ± 0.166	12.77	3.43 ± 0.044	-1.08

699 [b]Cp: Crossing Point. Values are mean Cp values (n=3) ± SD
700 [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%).
701 [e]ΔGU: $\Delta GU = \Delta GU_C - \Delta GU_{TS}$
702 ΔCp_C = Cp value from inoculum *H. pylori* control.
703 ΔCp_{TS} = Cp value from PMA, PEMAXTM with and without PEMAXTM Reaction Buffer treated samples.
704 ΔGU_C = GU value from inoculum *H. pylori* control samples calculated with *H. pylori* standard curve.
705 ΔGU_{TS} = GU value from PMA, PEMAX™ with and without PEMAX™ Reaction Buffer treated samples, calculated with *H. pylori* standard curve.
706 VC: Viable and culturable
707 VBNC: Viable but non-culturable
708 NV: Non-viable
709 [N]*p*-valor (VC Spiral): 0.423; *p*-valor (VBNC Spiral): 0.072; *p*-valor (NV Spiral): 0.003; *p*-valor (VBNC Coccoid): 0.24; *p*-valor (NV Coccoid): 0.01
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