Journal of Microbiological Methods

A QUICK FLOW CYTOMETRY PROTOCOL TO ASSESS HELICOBACTER PYLORI VIABILITY

--Manuscript Draft--

Manuscript Number:	MIMET_2020_318R1
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Keywords:	Helicobacter pylori; viability; flow cytometry; Syto 9; propidium iodide.
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Abstract:	Here we present an easy flow cytometry protocol to study the viability of Helicobacter pylori which also enables the detection of even low live bacteria densities. This protocol has potential utility for a fast and accurate assessment of experimental eradication methods against H. pylori .
Suggested Reviewers:	Jose Machado josem@ipatimup.pt He is an expert on H. pylori research Colm O'Moráin profcolmomorain@imeddoc.net He is an expert on H. pylori research Alfredo Lucendo ajlucendo@hotmail.com He is an expert on H. pylori research
Response to Reviewers:	Dear editor and reviewers, Thank you for considering our work for publication in Journal of Microbiological Methods. Below, we provide an answer to all your comments point by point: Editor and Reviewer Comments: -Editor Please address the following points before publication can be considered:

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-Reviewer 1

- Accept - no comments

We thank the reviewer.

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This study provides data on the applicability of flow cytometry to characterize bacterial cells of the species *Helicobacter pylori* grown in pure cultures under experimental conditions. Flow cytometric results were compared with colony counts upon calibration with reference beads. Viability tests by Syto9/PI double staining were also used to assess the efficacy of inactivation treatments.

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	<p>this regard new methods are in a developmental stage as is the case of photodynamic inactivation assays. We believe this flow cytometry method to assess HP viability is suitable for the in vitro fast evaluation of the efficiency of this kind of novel approaches, where a large set of different photosensitizers can be easily tested for their efficacy thus allowing a faster optimization of the chemical structures of the photosensitizing dyes.</p> <p>Therefore, we agree there should be some experimentation in this regard before its application in translational/clinical research. Unfortunately, we were unable to do it. The COVID-19 pandemic has strongly hit Madrid (city where we are working), and since middle-march we have not permission to obtain biological samples due to the potential infective risks. As we have also converted the manuscript into a short communication (as suggested by the editor), there is little room to properly discuss this point, Nevertheless, we have included a brief discussion highlighting the need of validation of the technique in clinical practice (paragraph starting in line 84).</p> <p>2.The use of flow cytometry for characterizing bacteria in liquid suspensions of various origins is very well established, with a consistent literature focused on the advantages of this technique to complement cultivation-based methods. It is hard to see any novel aspect in this study and the overall impression is to have read just one more scholastic application of flow cytometry to a microbial target.</p> <p>We agree that there have been many advances in the usage of flow cytometry in many bacteria species, but not H. pylori and in this point relies the novelty of this protocol. It is estimated that H. pylori infection affects half of the world's population, and it is strongly related to the development of gastric cancer. So, from a global health point of view, advances in the way we can investigate this bacterium are needed. This protocol demonstrates that H. pylori viability can be easily tested by flow cytometry, what could be translated in a faster method to investigate inactivation treatments against it. We apologize that we had not explained that in depth so we now talk about it in the paragraphs starting in lines 19 and 84.</p> <p>3.The study rationale appears weak (see L76-80). Although the consistent literature in the field, only few reference studies are cited herein.</p> <p>We have now included some additional references (line 24).</p> <p>4.Several methodological details are erroneously reported in the results section. This make difficult to follow the entire protocol and the rationale behind the performed tests.</p> <p>We have adapted the manuscript into a short communication format (as suggested by the editor). We hope this new-shortened version will be clearer.</p> <p>5.The presented dataset is rather limited and generally not sufficient to justify a full-research article on this journal.</p> <p>We agree that a full-length article maybe is not the best option for this work. Therefore, we have adapted the manuscript into a short communication format.</p> <p>Finally, we want to thank the editor and reviewers for considering the publication of our work in the Journal of Microbiological Methods. Of course, we will be pleased to provide further answers or amendments if they were needed or required.</p> <p>Best regards,</p> <p>Alicia C. Marin On behalf of the study group.</p>
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Funding Information:	
Manuscript Classifications:	Human pathogens; Microbial infections; Cell viability; Flow cytometry; Viability assays; Helicobacter

Declaration of interests

X The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

A QUICK FLOW CYTOMETRY PROTOCOL TO ASSESS HELICOBACTER PYLORI VIABILITY

SHORT TITLE

FACS and *H. pylori* viability.

AUTHORS

Claudio Alba^{1,†}, Alicia C. Marin^{2,3,†,*}, Adrian G. McNicholl^{2,3}, Ana Montalban-Arques^{2,3}, Irene Mora-Gutierrez², Antonio José Sánchez-Arroyo⁴, Tamara Soler¹, David García-Fresnadillo⁴, Javier P. Gisbert^{2,3}, Teresa Alarcón^{1,‡}, David Bernardo^{2,3,5,‡}.

¹ Microbiology Unit, Hospital Universitario de La Princesa and Instituto de Investigación Sanitaria Princesa (IP), Madrid, Spain.

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[†] Both authors had equal contribution as main authors.

[‡] Both authors had equal contribution as senior authors.

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AUTHOR'S CONTRIBUTION: AGM, DGF, JPG and DB had the original concept and idea for the study. DB and TA designed the protocols and the experimental approaches. CA, ACM, AMA, IMG, TS and AJSA performed the experiments. CA and ACM analyzed the data and wrote a first draft of the manuscript. All authors reviewed and approved the final version of the manuscript.

CONFLICT OF INTEREST: none

ABBREVIATIONS

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CRediT author statement

Claudio Alba: Investigation, formal analysis, writing – original draft, writing – reviewing and editing.

Alicia C. Marín: Investigation, formal analysis, visualization, writing – original draft, writing – reviewing and editing.

Adrian G. McNicholl: Conceptualization, writing – reviewing and editing.

Ana Montalban-Arques: Investigation, writing – reviewing and editing.

Irene Mora-Gutierrez: Investigation, writing – reviewing and editing.

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Tamara Soler: Investigation, writing – reviewing and editing.

David García-Fresnadillo: Conceptualization, supervision, writing – reviewing and editing.

Javier P. Gisbert: Conceptualization, resources, supervision, writing – reviewing and editing.

Teresa Alarcón: Methodology, resources, supervision, writing – reviewing and editing.

David Bernardo: Conceptualization, methodology, supervision, visualization, supervision, writing – reviewing and editing.

Response to the editor and reviewers

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Best regards,

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Alicia C. Marin
On behalf of the study group.

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SUMMARY

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KEYWORDS

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Helicobacter pylori infection is the main cause of gastritis, peptic ulcer disease and gastric cancer. Culture methods are the most accepted techniques to investigate the effect of eradication assays (mainly antibiotic-based) on *H. pylori*, but they are material and time consuming. Nevertheless, flow cytometry (FC) is a rapid, easy, accurate and reproducible method to study bacteria (Ambriz-Aviña et al., 2014). However, few studies have attempted to use FC to assess *H. pylori* viability (Faghri et al., 2014; Poursina et al., 2013; Sarafnejad et al., 2007). Given the importance of *H. pylori* infection from a healthcare point of view, it is imperative to design novel procedures to study *H. pylori* taking the advantage of FC. Hence, we aimed to design a novel FC-based protocol to study *H. pylori* viability.

Strain TIGR-26695 was cultured in Columbia Agar medium supplemented with blood (COS) in a microaerobic atmosphere at 36°C. A three day-old culture (stationary growth phase) was resuspended in 0.9% NaCl solution (NCS; Fresenius Kabi, Spain) and adjusted to a turbidity of 3 using McFarland standards. Three aliquots of 1 mL were centrifuged (2 minutes; 10,000 g) and bacteria pellets were resuspended and treated in different conditions: (a) NCS and heating (100°C, 3 minutes); (b) 70% isopropanol (25°C, 45 minutes); (c) NCS (25°C, 45 minutes). Aliquots were washed twice and resuspended in 1 mL of NCS. Colony-counting was performed by 10-fold dilutions in COS (5-days in a CO₂ incubator, 36°C). For FC viability determination, live (condition c) and dead bacteria (conditions a and b) were mixed in 10% intervals ranging from 0% to 100% live.

FC was performed using Syto 9, propidium iodide (PI) and microsphere beads (MB), obtained from the Live/Dead BacLight™ Kit (Invitrogen, L34856). Dyes were titrated, being their optimum dose selected based on their stain index. Ten µL of each bacteria suspension were stained with 1.5 µL of Syto 9 and 0.2 µL of PI in a final volume of 990 µL of NCS (room conditions -RC-, 15 minutes, dark). Ten µL of MB were subsequently added, in order to determine the absolute number of live bacteria per mL. Samples were kept in dark and acquired within 1 hour on a FACSCanto II flow cytometer (BD Bioscience). No “bleaching effect” on Syto 9 (Stiefel et al., 2015) was observed using this protocol.

MB and bacteria were identified based on Syto 9 (FITC-channel) and side scatter (SSC-A; Figure 1). Next, live/dead cells were determined within the bacteria gating using Syto 9 and PI (PE-channel). Bacteria with intact membranes are not permeable to PI, rendering only bacteria with compromised membranes as PI⁺ (Figure

1A). On the contrary, Syto 9 diffuses both in live and dead cells, but since the nucleus-binding affinity of PI is higher than that of Syto 9, live bacteria have higher fluorescence signal from Syto 9 (Stiefel et al., 2015). Thus, mixed live/dead bacteria can be identified as shown in Figure 1B.

Noteworthy, the analysis of a suspension of growing bacteria (representing 100% live cells) revealed a small proportion of cells within the dead gate (Figure 1C). *H. pylori* is a microaerophile and capnophile microorganism, being important for its *in vitro* growing not only the oxygen and carbon dioxide tension, but also the bacterial density and the type of culture media (Bury-Moné et al., 2006). As staining was performed at RC (21% oxygen), we tested whether those dead cells (Figure 1C) were caused during staining. Bacterial samples were analysed by FC and colony-counting, before and after keeping them at RC for 2 hours, and no significant changes in viability were observed. This result is probably reflecting that not all the bacteria from the cultures were viable, maybe associated with the culture conditions.

Therefore, an adjustment was done to refer the data to the real number of viable cells added in each tube. To that end, mixes ranging 0-100% live bacteria were analysed as shown in Figure 1; in the tube 100% live, the number of events gated as viable was used as the real number of viable bacteria we were using in the experiment. Hence, an adjustment was performed to obtain the real percentage of viable bacteria in a certain mix by multiplying by 100 the ratio between the percentage of viable bacteria in the problem solution and the 100 % live solution:

$$\% \text{ viable bacteria in a certain mix} = (\text{number of events gated as viable in the mix} / \text{number of events gated as viable in the 100\% live suspension}) * 100$$

Following the adjustment, there was a correlation between the obtained results and the expected ones (Figures 2 and 3), being the technique more accurate at lower percentages. Given that an acceptable treatment should be effective in >90% of the cases (Gisbert et al., 2016), viable bacteria after treatment are expected to be <10%, a percentage easy to identify by this FC-protocol.

Bacteria counts obtained with the MB were also compared to those obtained from the culture-counts, showing a direct correlation between both techniques (data not shown), with a lower detection limit of 10^4 bacteria/ml.

In summary, this FC-protocol could be an alternative to culture methods for determining *H. pylori*'s viability, with the advantage of being faster and sensitive enough for the detection of even low live bacteria densities. By combining two

87 fluorophores with opposed profiles for both viable/dead bacteria, their discrimination is
88 more accurate and objective compared to methods including only one dye (Faghri et al.,
89 2014; Poursina et al., 2013; Sarafnejad et al., 2007). This protocol could be
90 implemented for the research of new non antibiotic-based methods, as the
91 photodynamic inactivation assays (Calvino-Fernández et al., 2013). Further research
92 should focus on its application in cultures obtained from biological samples (i.e., gastric
93 biopsies or stomach lavage), requiring its validation in this scenario.

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Figure 1. Gating strategy. Microsphere beads and *H. pylori* (Hp) were gated based on their FITC and side scatter (SSC-A) properties. Debris (down on the left) was ungated and, therefore, excluded from the analysis. Hp region was further analysed as live or dead bacteria based on the expression of Syto 9 and PI. Representative flow cytometry dot plots of **A)** 100% dead heat-killed Hp, **B)** 50% live-dead mixed suspension or **C)** growing bacteria directly obtained from the culture plates are shown. Results are representative from several independent experiments performed with similar results.

Figure 2. Heat-killed bacteria correlation between expected and obtained. Correlation between the percentage in which the live and dead suspensions were mixed (called “expected percentage”) and the adjusted percentages of live bacteria for the heat-killed model of death. The adjusted calculations were referred to the number of live events on the tube 100% live *H. pylori* (Hp). Results are representative from two independent experiments performed with similar results.

Figure 3. Isopropanol-killed bacteria correlation between expected and obtained.

Correlation between the percentage in which the live and dead suspensions were mixed (called “expected percentage”) and the adjusted percentages of live bacteria for the isopropanol-killed model of death. The adjusted calculations were referred to the number of live events on the tube 100% live *H. pylori* (Hp). Two outliers were removed to adjust the trend-line (corresponding to 80% and 90% theoretical values). Results are representative from two independent experiments performed with similar results.

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