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Basic study

HP0953 hypothetical protein as virulence factor from *Helicobacter pylori*, overexpresses and localization during infection to gastric epithelium cells.

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Dear editor,

The authors are very grateful for the observations and recommendations made to the manuscript by the reviewers, and each one of them was taken care of and corrected.

Again, we send the manuscript with the suggested changes, including a change in the title, one of the suggestions given by one of the reviewers seemed very pertinent to us and now the writing has as title:

HP0953 hypothetical protein as virulence factor from *Helicobacter pylori*, overexpresses and localization during infection to gastric epithelium cells

Also, editorial criteria were carefully considered, and we hope we have met them fully.

The authors are awaiting your response and once again we appreciate your contributions.

Response to reviewers

Reviewer #1:
Scientific Quality: Grade C (Good)
Language Quality: Grade B (Minor language polishing)
Conclusion: Minor revision

Specific Comments to Authors: This is a very interesting study in which the authors describe the location of the protein is determined for the first time, inside and outside the bacterium. However, the manuscript is nicely written and presented but still, there are additional points for improving the overall quality of the manuscript. Here I suggest authors should address the following points before resubmitting the manuscript.

1.The order of the results may need to be adjusted and the article more logical.

Response: Thank you for the observation, results were re-organized following the next order: *in silico* analyses, site-directed mutagenesis for *hp0953* gene, protein purification, HP0953 localization, HP0953 expression in AGS cells and finally site-directed mutagenesis for hp0953 gene

2.In fig1, should the authors determine whether the control group was infected with 0h or uninfected?

3.In the methods section the authors describe that "One bottle was used as control as its culture was not infected with the bacterial strain";

also, if Hp0953 is the genome of Hp, why the expression be detected in the AGS cell samples infected with 0h; it could not have Hp adhering to AGS cells at 0h, and if it is the Hp genome that was not eluted during the infection process, why would 0h be detected in the AGS cells? so is it appropriate to use this group as a control group?

Response: We used an uninfected bottle as control for TEM assay. For expression, the controls were the constitutive gene *glm*. Time 0 was taken as a culture of the bacterial strain without contact with AGS cells for expression assay. This was already added in materials and methods and explained in the corresponding figure.

3.as shown in Fig1 and fig5, mRNA levels are lower at 3h, why do the authors show the figure of infection at 3h not 12h in fig5, what is the authors' explanation for this?

Results: the study showed that *hp0953* expression is constitutive, however, after initial contact with AGS cells, *hp0953* mRNA undergo a diminishing period in its concentration, perhaps as a result from *H. pylori*`s adaptation to external signaling. *Hp0953* levels start to rise after 3 hours, perhaps preparing *H. pylori* to initial pathogenic process in where HP0953 is needed. It has been seen that because bacteria spend a lot of energy to synthesize their virulence factors, they first decrease the synthesis of these factors, divide themselves and then secrete their virulence factors, this they do to ensure the success of the infection and evade resistance to infection mediated by the resident microbiota (Kitamoto *et al.,* 2016)
Reference: **Kitamoto S,** Nagao-Kitamoto H, Kuffa P, Kamada N. Regulation of virulence: the rise and fall of gastrointestinal pathogens. *J Gastroenterol.* 2016;51(3):195-205. doi:10.1007/s00535-015-1141-5

Reviewer #2:
Scientific Quality: Grade C (Good)
Language Quality: Grade A (Priority publishing)
Conclusion: Minor revision
Specific Comments to Authors:

Authors investigated the manuscript titled that "Localization and expression of HP0953, a hypothetical protein from Helicobacter pylori, and a putative virulence factor". Their findings are well reported and the methods were rigorous, and pretty accurate to back their conclusions that in their manuscript.

I congratulate you for the good write up of this manuscript, however, you need to edit some points.

My comments are below; Title is reflects the main subjects.

Authors are aimed that to investigate the expression and localization of HP0953 during adhesion to an inert surface and AGS cells. Thus, abstract summarized and reflected description of work.

-Literature and references are not enough. The authors need to go through their reference list and include mostly newer references (2022 also).

Response: Literature was revised and actual references were introduced.

-Similarity rate is 27% (high). If possible, it should reduce below 20%.

Response: The manuscript was reviewed with the Viper Plagiarism Report program, and had an Overall Score of 10%.

- The manuscript should edit according to journal format.

Response: The manuscript was reviewed and edited according to the journal format.

- The limitations should be in the discussion part.

Response: Thank you for the observation, study limitations are mentioned and discussed in the corresponding section of the discussion.

- Figures are good but image of Western Blot is bright maybe contrast can be edit. Then if you use quantification program for western blot (such as Image J.) you can calculate quantify protein bands from western blot. It is important point. The quantification will reflect the relative amounts as a ratio of each protein band relative to the lane's loading control.

Response: The western blot image was edited, total protein lysates were quantified by Bradford technique, in each lane 30/50 μg of protein was loaded for SDS-PAGE protein profile.

Reviewer #3:
Scientific Quality: Grade B (Very good)
Language Quality: Grade B (Minor language polishing)
Conclusion: Minor revision
Specific Comments to Authors:

- The title was unclear, please revised it to be more informative.

Response: thank you for the observation tittle has been modified "**HP0953 hypothetical protein as virulence factor from *Helicobacter pylori*, overexpresses and localization during infection to gastric epithelium cells**"

- please describe about characteristics and profile of virulence genes of *H. pylori* strain 26695.

Response: The co-expression of other virulence genes was previously evaluated with the *fhlF* (flagellum) and *hp0015* (virB2 homolog of the type IV secretion system) genes during adherence to inert surfaces and cells(Arteaga- Resendiz *et al*., 2013). It was observed that during adherence to inert surfaces all the virulence factors expressed very little and after 3 h of incubation they began to increase. In contact

with AGS cells, only the *fhlF* gene begins to increase its expression after 3 h of incubation together with the *HP0953* gene.

- the author should be discussed about prevalence and percentage of conserved domains of HP0953 between H. pylori or close-related taxa using in silico investigation i.e. Pfam server.

Response: Preserved domains were searched on the Pfam server; however, no significant results were found. It appears that HP0953 harbors unique domains, which can only be solved by X-ray crystallography. Those analyses will be performed by groups which collaborate with our research group.

- discuss about limitation of study.

Response: Thank you for the observation, study limitations are mentioned and discussed in the corresponding section of the discussion.

the evaluation of coexpression HP0953 as well as other virulence factor could be more interesting.

Response: It was observed that the flagellum and hp0953 increased their expression with increasing incubation time during first hours of adherence, which suggested *that hp0953* was involved in the adhesion process (Arteaga- Resendiz *et al* ., 2013). This co-expression was no longer included in this study, but it would be interesting to evaluate it during infection of AGS cells.

In addition, the author could assessed pro-inflammatory response of AGS such as IL-8 when infected with HP0953 positive strain and its mutant strains.

Response: Thank you for the observation, at first intention we did not visualize immune response, however, study of immune response towards HP0953 is interesting, we would take into consideration for further research.

Directed mutagenesis was attempted by homologous recombination of the *H. pylori* 26695 strain, using pBluescript II KS as the cloning vector. A construction was made so that the gene encoding the HP0953 protein was interrupted with a chloramphenicol resistance cassette. The *H. pylori* strain 26695 was attempted to be transformed with said vector by electroporation; however, the mutant strain could not be propagated.