OPEN PEER REVIEW

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# Peer Review Report for "Association of *cagA*, *cagC*, *virB2*, and *vacA* Subtypes of *Helicobacter pylori* with Adenocarcinoma Development in Iranian Patients"

**Author(s):** Pouya Khodadadi, Mohammad Kargar, Mahdi Bijanzadeh , Abbas Doosti, Shapoor Aghaei

## Review Timeline:

Submit Date: 25 Jan 2020
Revised Date: 9 Jul 2020
Accepted Date: 24 Jul 2020

Revision (0)

Here, you can see the **Reviewers**, **Associate Editors** and **EICs'** comments from the beginning to the end of the revision process.

**Farhad Safarpoor Dehkordi**: Associate Editor | Revision (0) — 13 May 2020

Dear EIC,
The manuscript is studied, and there are some comments which should be addressed by the author. According to the comments, the document needs major revision.
Kind regards,

**REFEREE**: EIC | Revision (0) — 16 May 2020

Dear Authors,
Thank you for submitting your manuscript to JJM. I have completed the review of your manuscript and a summary is appended below (Pleases find attached files). The reviewers recommend the reconsideration of your paper following major revision. I invite you to resubmit your manuscript after addressing all reviewer comments. Please answer point by point to reviewer's comments on a separate sheet under WORD format and write your modifications in the revised version (WORD format) in colored or highlighted letters. Your

answers to reviewers' queries are not only for the Editorial Board but for the readers and should be included, when appropriate, in the manuscript for improvement and a better understanding.

When resubmitting your manuscript, please carefully consider all issues mentioned in the reviewers' comments, outline every change made a point by point, and provide suitable rebuttals for any comments not addressed. In addition, JJM INSTRUCTION attached to the mail, Pleases find it.

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Thanks.

OPEN PEER REVIEW

Revision (1)

## Reply to Reviewers

Ideally, the reviewing process can significantly improve the submitted manuscripts by allowing the authors to take into account the advice of reviewers. Author(s) must reply to all reviewers' comments in a separate Word file, point by point. A "**Reply to Reviewers**" document is submitted along with revised manuscript during submission of revised files, summarizing the changes that the authors made in response to the reviewers' comments. The responses to reviewers' comments specifies how the authors addressed each comment the reviewers made.

You can read the authors' responses to the reviewers' comments in the next page.

**June 14, 2020**

**Manuscript number***: 101275*

**Manuscript Full Title***: Association of cagA, cagC, virB2 and vacA subtypes of Helicobacter pylori with adenocarcinoma development in Iranian patients*

**Journal***: Jundishapur Journal of Microbiology*

**Dear Prof. Ali Zarei Mahmoudabadi (Editor-in-Chief)**

Thanks for the valuable comments on the manuscript. We tried to improve the draft based on these suggestions. The revised section has been highlighted as yellow. Thanks for considering our revision letter.

**Corresponding author: Prof. Mohammad Kargar**
**Associate Editor's Comments:**

*1- Text is full of grammatical and typographical mistakes. Please use it from a native English speaker person who is an expert in microbiology to revise your text.*

**Author Response:** Thanks for the comment. Edited.

*2- The Abstract is not written in a good way. Please confirm your claim with a scientific base.*

**Author Response:** Thanks for the comment. Edited.

*3- Keywords are not exactly related to your topic and your claim.*

**Author Response:** Thanks for the comment. Edited.

*4- cagA, cagC, virB2 and vacA subtypes or genotypes?*

**Author Response:** Thanks for the comment.

The subtype term is referring to *vacA* gene. Mosaicism in *vacA* alleles with two distinct families of *vacA* signal sequences (*s1* and *s2*), two distinct families of middle region alleles (*m1* and *m2*) and two intermediate regions (*i1* and *i2*) were found. Due to combination of different distinct regions of *vacA s/m/i* resulted in eight possible combinations (subtypes). (All of them are genotypes, because of mosaicism in *vacA*, the term subtype also can be used.)

Mentioned in the background, paragraph 4

*5- Please identify gastroduodenal symptoms.*

**Author Response:** Edited.

Some of clinical symptoms in patient with gastrodeudenal diseases are include vomiting, gastrointestinal bleeding, or weight loss. Also some people suffering from dysphagia, difficulty swallowing, stomach ulcers, reflux, indigestion, and atrophy.

Mentioned in the background, paragraph two

*6- Some parts of the text need additional gaps and some parts of the text need additional space between letters and words of the text.*

**Author Response:** Thanks for the comment. Edited.

*7- The name of bacteria and genes should be italic.*

**Author Response:** Thanks for the comment. Edited.

*8- The scientific names should start with capital letters and then small letters. For example Helicobacter pylori.*

**Author Response:** Thanks for the comment. Edited.

*9- Authors mentioned: DNA was isolated from biopsy samples using DNA extraction kit (Qiagen DNA Mini Kit, Iran)…… Then, they also mentioned DNA was extracted from each clinical isolate using a Genomic DNA Purification Kit (Fermentas, Germany). So I think that two steps of DNA extraction were done? Please identify them one by one. Also please add the reason for two steps DNA extraction. Please note that the name of fermentas company has been changed to another name. Please changed it.*

**Author Response:** Thanks for the comment. Edited.

DNA was isolated from biopsy samples using DNA extraction kit (Qiagen DNA Mini Kit, Iran) according to manufacturer's instructions. It is also corrected that only one extraction DNA step was performed.

Mentioned in the methods, part 3.2. Extraction of genomic DNA from *Helicobacter pylori*

*10- table(1) should change to table 1.*

**Author Response:** Thanks for the comment. Edited.

*11- Statistical analysis should add to another part of the method.*

**Author Response:** Thanks for the comment. Edited.

**3.4. Statistical analysis**

Statistical analysis was done using the SPSS 20 statistical software (SPSS Inc., Chicago, IL, USA). Chi-square test and Fisher's exact 2-tailed tests were used to assess any significant relationship between prevalence of *H. pylori* strains and their genotypes. P value < 0.05 was considered as statistical significant level.

Mentioned at the end of method, part 3.4. statistical analysis

*12- Please add the positive and negative control of the PCR reactions. Specific positive control for each gene should add to the method.*

**Author Response:** Thanks for the comment.

*H. pylori* strain ATCC 43504 was used as a positive control. A *cagA* negative *H. pylori* strain was obtained from the Department of Microbiology, Jundishapur University of medical sciences.

Mentioned at the end of method, part 3.3**.** Amplification of *vacA (s1, s2, m1, m2, i1, i2) and cag A* genes

*13- If authors designed the primers, they should add the full procedure used for their designation. If not, please add their references.*

**Author Response:** Thanks for the comment.

The sequence of the *cagA*, *cagC*, *virB2*, and *vacA* genes was deposited in the GenBank database. Finally, *Gene Runner 3.5* was employed to design specific forward and reverse primers to the amplified genes.

Mentioned in method, 3.2. Extraction of genomic DNA from *Helicobacter pylori*

*14- I think that the error bars of figure 2 has been designate according to percent. Authors should add their own error bars in figures.*

**Author Response:** Thanks for the comment. Edited and done.

*15- Similar to the above comment for fig 3 and 4.*

**Author Response:** Thanks for the comment. Edited.

*16- In my opinion, authors cannot confirm their results about the association of studied genes on the development of adenocarcinoma. It is better to a person who has high experiences in the statistic to take a glance at the data.*

**Author Response:** Thanks for the comment. Mentioned in manuscript

CagA is also a surface protein with high immunization potential, which can result in deformation in the spindle-shaped cells after entrance into the stomach epithelial cells. Entering the CagA protein into the target cells and cell signaling interference increases the pathogenicity power and expresses *cagPAI* related genes. In addition, *cagA* expression in the strains can lead to several morphologic changes, including bacterial generation and apoptosis in the host cells. It was found that *cagA* positive strains have a high potent to nesting, erosion, and inflammation (18). Furthermore, *vir* genes are a part of *cagPIA*, which have a similar structure to T4SS called *cagPAI* and ComB before and after *dupA* locus, and thus have been considered as the 3$^{rd}$ T4SS (19). *vacA* gene includes three main regions of the signal zone that are characterized by *s1* and *s2*, middle region (*m1,m2*), and intermediate region (*i1, i2, i3*). These polymorphic zones exhibit different combinations, some of which have the highest activity to create human gastric diseases ( 20, 21). Due to mosaicism, a combination of different distinct regions of *vacA s/m/i* resulted in eight possible combinations (subtypes) (11). Because of *vacA* mosaicism, different *H. pylori* strains may show different pathogenicity capacities (22(Dabiri, 2017 #64)(Dabiri, 2017 #1) (Dabiri, 2017 #64)). However, it is clear that the *i1* allele has more activity than *i2*. One of the studies in the field showed the relationship of a combination of strain, *i1* allele, and *s1m1* alleles with more cytotoxic activity and peptic ulceration. Finally, the *s1i1* genotype induces severe vacoulating activity of *vacA* and thus can lead to metaplasia and inflammation compared with *s1i2* or *s2i2* (23).

Mentioned in the background, paragraph 3 and 4.

*17- Please add the figure of gel electrophoresis.*

**Author Response:** Thanks for the comment. Edited and done.

*18- How authors detect the Helicobacter pylori? With PCR? With culture? Which one of the*

*primers was used for the detection of the Helicobacter pylori? Please identify it in detail. The method section has some defects.*

**Author Response:** Thanks for the comment.

Two samples were taken from each patient. These samples were selected based on the endoscopic observations and histological examinations. DNA samples derived from gastric biopsy were *H. pylori* positive according to the RUT and Gimsa staining. Also, all of the isolates were confirmed by the *ureC* gene PCR assay.

Mentioned in method, 3.1. Collection of the patients' samples

*19- Some parts of the text need additional gaps and some parts of the text need additional space between letters and words of the text.*

**Author Response:** Thanks for the comment. Edited.

*20- Please add the exact names of positive and negative controls.*

**Author Response:** Thanks for the comment. Edited.

*Helicobacter pylori* strain ATCC 43504 was exploited as a positive control. A *cagA* negative strain was obtained from the Department of Microbiology, Ahwaz Jundishapur University of medical sciences.

Mentioned in method, 3.3. Amplification of *vacA (s1, s2, m1, m2, i1, i2) and cag A* genes

*21- (Figure ...) and (Table ...) should change to (figure ...) and (table ...). Make it throughout the text.*

**Author Response:** Thanks for the comment. Edited.

*22- The discussion section has some defects. Please complete the introduction part using the explanation about the characters of the topic. Additionally, add some explanation about the fact that why other surveys cannot solve the issue which caused that you and your colleague performed this research. Additionally, the novelty of work and also application of the results obtained from this work. Some of your references were old. You should replace them with some novel references. Are there any surveys conducted on this field in Iran and some neighboring countries?*

**Author Response:** Thanks for the comment. Edited.

Old references replace with the novel one: number 3, 5, 6, 8, 18, 20, 22, 26, 30, 31, 32, 33.

*Helicobacter pylori* and related diseases are common all over the world and while no treatment and improved could significantly increase the risk of adenocarcinoma. Therefore, it is necessary to study it in all countries. Many studies based on genotyping and survey on important virulence genes in *H. pylori* were done in Iran and another neighboring countries such as Iraq, Turkey, Pakistan and etc. Identification of individuals at high risk for gastric cancer that may enter a surveillance program and intervention during the precancerous process is the most suitable strategy for decreasing mortality due to this malignancy. In addition, the determination of the genotype of *H. pylori* isolates from this population may allow us to further understand the relationship between putative virulence genes and clinical disease. In most studies were done in Iran *i* region *vacA* genotyping has receive less attention. In this research we considered all three *vacA* gene regions. Our results interestingly implicated that *m1* and *i1* alleles had significant relation with gastritis and adenocarcinoma cases. Also both genotypes *s1m1i1* and *s2m1i1* relevant with gastritis and adenocarcinoma had got *i1* allele. These data supports the idea that the strain with *vacAi1* and *vacAm1* alleles secret the toxin more efficiently. Also chronic infection enhanced gastric mucosal inflammation and increased risk of atrophy, intestinal metaplasia and some malignancies.

Mentioned in conclusion and the first paragraph of the discussion

Old references replace with the novel one: number 3, 5, 6, 8, 18, 20, 22, 26, 30, 31, 32, 33.

*23- Please complete the conclusion with some more relevant information about the exact finding of your survey and also their applications.*

**Author Response:** Thanks for the comment.

Data analysis confirm this fact that all desired genes related to adenocarcinoma outcome significantly. *vacA s1m1i1* genotype plays an important role in gastric malignancies. Although in most studied the *i* region *vacA* genotyping has receive less attention, current results interestingly showed that *i1* allele had significant relation with gastritis and adenocarcinoma cases. the determination of the genotype of *H. pylori* isolates from this population may allow us to further understand the relationship between putative virulence genes and clinical disease.

Mentioned in abstract and conclusion

*24- What are the main applications of your research in the field of microbiology based on the clinical view.*

**Author Response:** Thanks for the comment.

Due to the fact that *Helicobacter pylori* is the main cause of many gastrointestinal diseases such as gastritis, duodenal ulcer, gastric ulcer MALT lymphoma and especially gastric cancer. Also according to epidemiological research, the prevalence of this bacterium is high so that half of the human infected by *H. pylori*. Thereafter primarily recognize the patient by clinical features and then samples were collected and infected samples identified by biochemical and microbial tests and finally *H. pylori* genotyping was done by molecular test (PCR). Clinical symptoms such as vomiting, difficult swallowing, and abdominal pain, it is possible to detect bacterial infection in the early stage. On the other hand, clinical manifestations are a useful way to bacterial surveillance. However, because of the increased *H. pylori* antibiotic resistance, bacterial diagnosis in the early stages may be the best way to eradicate the disease.

Mentioned in the background, paragraph 3

*25- Please also unique the style of references throughout the reference lists.*

**Author Response:** Thanks for the comment. Edited.

Added references are highlighted

We really appreciate your great and pertinent comments about the manuscript. We have tried to do our best to respond to the points.

**OPEN PEER REVIEW**

**Revision (1)**

Here, you can see the **Reviewers**, **Associate Editors** and **EICs'** comments from the beginning to the end of the revision process.

**REFEREE**: EIC | **Revision (1)** 24 Jul 2020

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