OPEN PEER REVIEW

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Advantages of "Open Peer Review" process

- More transparency, constructiveness and tactful comments of the peer review process: leads to an increase in the quality of reviews
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- Authors' satisfactions from the review process: Increases honesty between authors and reviewers
- Education of both authors and new students
- Prevents reviewers from following their individual agendas and leading to the detection of reviewers' conflicts of interests

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Jundishapur Journal of Microbiology
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Peer Review Report for "Prevalence and Molecular Genotyping of *Cryptosporidium* Spp. in Diarrheic Patients from Bandar Abbas City, Southern Iran"

Author(s): Majid Najafi-Asl, Habibollah Faraji, Saeed Hosseini Teshnizi, Khojasteh Sharifi-Sarasiabi

Review Timeline:

Submit Date: 16 Mar 2020
Revised Date: 7 Jun 2020
Accepted Date: 8 Jun 2020

Revision (0)

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

Hossein Hooshyar: Reviewer | Revision (0)

9 Apr 2020

Dear AE,
Comments for the authors:
Title:
- prevalence and genotypes of Cryptosporidium spp. in Diarrheal patients of Bandar Abbas, Southern Iran.
Abstract:
1- Cryptosporidium species are recognized as important gastrointestinal pathogens change to: as one of the most important
2- The level of significance is not needed in the abstract.
Background:
1- This sentence is not clear: A low dose of oocyst can be caused by disease, so it has the major importance of public health
Methods:
1- The descriptive checklist included items that are not needed, all of them can be written as demographic aspects.
2- Statistical analysis must be located at the end of the methods section.
3- The PCR condition (thermal condition and no of cycles) is not clear.

Discussion:

1- Please explain the major differences between the result of this study and the prevalence of cryptosporidium in Bandar abbas in the previous study.

2- Are there any animal studies of prevalence and genotype of cryptosporidium in Hormzgan for compared with the result of this study?

Thanks.

REFEREE: Reviewer | **Revision** (0) 12 Apr 2020

Dear AE,

The following points should be noticed and considered by the authors:

- Title: Prevalence and molecular genotyping of Cryptosporidium sp. in diarrheic patients from Bandar Abbas City, Southern Iran

ABSTRACT section:

Objectives

- This study aimed to determine the prevalence and subgenotypes of Cryptosporidium sp. among diarrheic patients in Bandar Abbas City, Iran.

Methods

- Diarrheic fecal samples were collected from 170 patients in three hospitals of Bandar Abbas, Iran, from December 2018 to June 2019.
- Initial parasitological identification of Cryptosporidium sp. was performed ….
- The level of significance was < 0.05.

Results

- Of the 170 diarrheic patients, 98 (57.6%) were male and 72 (42.4%) were female.
- Prevalence of Cryptosporidium sp. by parasitological examination was ….
- Sequence analysis of gp60 gene ….
- Two distinct nucleotide sequences obtained from this study were deposited in GenBank under the accession numbers MN820453 and MN820454.

Conclusion

- The predominance of C. parvum (subtype family IId) in this study emphasizes the importance of zoonotic Cryptosporidium transmission in Bandar Abbas, Southern Iran.

Keywords: Genotypes, Subtypes, Cryptosporidium, gp60 gene, Diarrhea, Iran

Background section:

Objectives: This study aimed to determine the prevalence and subgenotypes of Cryptosporidium sp. among patients with diarrhea in Bandar Abbas City, southern Iran.

Methods section:

These sections must be explained separately:

1- Study area.

2- Sample collection and processing.

- How Cryptosporidium oocysts positive samples were preserved and kept?

3- Microscopic examination

4- DNA extraction and Nested-PCR

- The primers used and amplification procedure should be explained.
- The PCR method mentioned in the text was first applied and described by:
- Abe N, Matsubayashi M, Kimata I, Iseki M. Subgenotype analysis of Cryptosporidium parvum isolates from humans and animals in Japan using the 60-kDa glycoprotein gene

sequences. Parasitol Res. 2006; 99(3): 303-305.
6- Please add a 400-bp band PCR gel electrophoresis image for the gp60 gene.
7- Sequence analysis
8- Statistical analysis
- How the degree of agreement between microscopy and PCR was determined?
Results section:
1- Prevalence of Cryptosporidium sp. infection by modified ZN staining was ….
2- It has been stated that: The level of agreement between the two methods was above average (Kappa = 64%).
Note: Cohen's k-index definitions: poor agreement (k < 0.20), fair agreement (k = 0.21-0.40), moderate agreement (k = 0.41-0.60), substantial agreement (k = 0.61-0.80) and perfect agreement (k = 0.81-1.00).
3- Table 1. Patient 4. genotype IIdA15 (or IIdA15G1, which one is correct?).
4It has been stated that:
- In order to recognize isolate subtypes, trinucleotide repeats TCA (red ink) and TCG (green ink) in the variable region located in the 5′ region of gp60 gene were enumerated.
Note: The gp60 gene has a number of tandem repeats at the 5' end of the gene consisting of TCA, TCG, or TCT, and also further variation in the non-repeat 3' end of the gene, enabling classification of C. hominis and C. parvum into subtype families on the basis of the number and type of trinucleotide repeat.
Discussion section:
1- Reference no. 16 is located after references 17, 18, and 19.
References section:
1- Reference 21: please include the publication yea.
Thanks.

REFEREE: Associate Editor | **Revision** (0) 12 Apr 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.

Authors' Response to the Reviewers' Comments

Journal: *Jundishapur Journal of Microbiology*
Manuscript #: *ID: 102706*
Title of Paper: *Cryptosporidiosis: Prevalence, Genotype Analysis in Diarrheal patients of Bandar Abbas, Southern Iran.*
Authors: *Majid Najafi-Asl, Habibollah Faraji, Saeed Hosseini Teshnizi, Khojasteh Sharifi-Sarasiabi*

Dear Prof. Ali Zarei Mahmoudabadi,

Please find our revised manuscript entitled '*Cryptosporidiosis: Prevalence, Genotype Analysis in Diarrheal patients of Bandar Abbas, Southern Iran*'. We would like to appreciate the reviewers for their valuable and constructive comments which helped us to improve our manuscript. We have carefully taken their comments into consideration in preparing the revision. The point-to-point reply to the reviewers is enclosed and, in the article, highlighted.

All authors are aware of and agree to the content of the manuscript. We very much hope that the present version of the manuscript satisfactorily addresses all the observations made by you and the reviewers and meets the quality standard for publication in the *Jundishapur Journal of Microbiology*

Thank you very much for your kind consideration.

Yours sincerely,

Khojasteh Sharifi-Sarasiabi (PhD)

Associate Professor of Medical Parasitology, Infectious and Tropical Diseases Research Center, Hormozgan Health Institute, Hormozgan University of Medical Sciences, Bandar Abbas, Iran

E-mail: sharifisarasiabi@gmail.com.

COMMENTS FROM THE EDITOR:

Comment 1*: Thank you for submitting your valuable manuscript to our journal. We would like to inform you that the peer review process of your manuscript is complete and requires "**Major Revision**". Although it has several useful points, it needs **Major Revision** to be reviewed again. Below you can find the abstracts of the peer review decision made by our editorial board members.*

Response: We appreciate for such a kind consideration to our manuscript. We agree with the reviewers and in order to address respective concern, we answer and cover reviewer's insightful comments in revised manuscript.

Reviewers' Comments:

Reviewer #1:

Authors' Response to the Reviewers' Comments

The following points should be noticed and considered:

Comment 1: *Title: Prevalence and molecular genotyping of Cryptosporidium sp. in diarrheic patients from Bandar Abbas City, Southern Iran*

Response: Thanks for such an insightful comment. We agree with the reviewer, hence, title of manuscript is modified as wanted.

Comment 2: *ABSTRACT section:*

Objectives

- This study aimed to determine the prevalence and subgenotypes of Cryptosporidium sp. among diarrheic patients in Bandar Abbas City, Iran.

Response: We agree with the reviewer, therefore, sentence "The aim of this study was to determine the prevalence and genotypes of Cryptosporidium spp. in Bandar Abbas diarrheal patients." is corrected.

Comment 3: Methods

- Diarrheic fecal samples were collected from 170 patients in three hospitals of Bandar Abbas, Iran, from December 2018 to June 2019.

Response: We appreciate for the comment mentioned. It is corrected as well.

Comment 4: *Initial parasitological identification of Cryptosporidium sp. was performed ….*

Response: The word of parasitological is added to the sentence according to reviewer comment.

Comment 5: *The level of significance was < 0.05.*

Response: With regard to reviewer 2 comment, this sentence is removed.

Comment 6: Results

- Of the 170 diarrheic patients, 98 (57.6%) were male and 72 (42.4%) were female.

Response: Thanks for such an insightful comment, therefore, the sentence was paraphrased according to reviewer comment. In other word, the sentence "One hundred seventy individuals with diarrheic stool were recruited, 98 (57.6%) were males and the rest (42.4%) female." change to shortly one "Of the 170 diarrheic patients, 98 (57.6%) were male and 72 (42.4%) were female.

Comment 7: *Prevalence of Cryptosporidium sp. by parasitological examination was ….*

Response: We agree with reviewer comment. Therefore, it is corrected.

Comment 8: *Sequence analysis of gp60 gene ….*

Response: It is done. Plural word "Sequences" change to single form.

Comment 9: *Two distinct nucleotide sequences obtained from this study were deposited in GenBank under the accession numbers MN820453 and MN820454.*

Response: Thanks for such a constructive comment. Therefore, it is corrected with removing the common part of assigned accession numbers.

Comment 10: Conclusion

- The predominance of C. parvum (subtype family IId) in this study emphasizes the importance of zoonotic Cryptosporidium transmission in Bandar Abbas, Southern Iran.

***Response*:** Thanks for creating such a strongly phrase. Therefore, it is paraphrased according to reviewer comment.

Comment 11: Keywords*: Genotypes, Subtypes, Cryptosporidium, gp60 gene, Diarrhea, Iran*

Response: We agree with reviewer to add another important keyword related to article as "Subtypes".

Comment 12: Background section*: Objectives: This study aimed to determine the prevalence and subgenotypes of Cryptosporidium sp. among patients with diarrhea in Bandar Abbas City, southern Iran.*

Response: Thanks for the comment. Hence, the sentence "the present study was performed in order to find prevalence and genotypes of Cryptosporidium spp. among patients with diarrhea in Bandar Abbas city, Southern Iran." is modified in accordance with reviewer comment.

Comment 13: Methods section:

These sections must be explaining separately:

Response: We appreciate such a precise consideration to the manuscript for improving its quality. In order to address reviewer concern, we revise thoroughly Methods section. Subtitles is created in accordance with reviewer comment and deleted previously ones. It composes of Study area, Sample collection and processing, Microscopic examination, DNA extraction and Nested-PCR, Sequence analysis and Statistical analysis, respectively. Then, we explain reviewer queries and comments related to each section.

Comment 13-1: 1- Study area.

Response: We add a paragraph in its own section.

"In this descriptive cross-sectional study, specimens were collected from patients in 3 hospitals of Bandar Abbas, Iran, from October 2018 to May 2019. This city is located in southern Iran; a tropical region, attached to the Persian Gulf with high humidity (20%-100%) and warm climate (9)."

Comment 13-2: *2-* ***Sample collection and processing.***

Response: After obtaining the written consent, the researcher administered a comprehensive questionnaire to each patient in the period of time mentioned above. Recipients of anti-parasitic drugs and diarrhetic patients by *Shigella* spp. were excluded. The checklist included items on patient demographic aspects. Subsequently, a single fecal specimen was collected from 170 diarrheic patients.

Comment 13-3 *How Cryptosporidium oocysts positive samples were preserved and kept?*

Response: Thanks for the valuable comments. The positive specimens determined by the staining method, and 47 suspected ones of *Cryptosporidium* spp, were stored in 2.5% potassium dichromate (K2Cr2O7) and stored at 4˚C for DNA extraction (Reference).

Comment 13-4 *4- Microscopic examination*

Response: To identify oocysts of *Cryptosporidium* spp., a permanent slide was prepared for each sample after the formalin-ether concentration method Slides stained with the modified ZN- Staining were viewed under a light microscope at a final magnification of 1,000 to observe *Cryptosporidium* oocysts (Reference). We insert a paragraph in related section.

Comment 13-5 *5- DNA extraction and Nested-PCR*

Response: We insert a paragraph in related section (Line 131-152). "Three positive specimens determined by the staining method, and 47 suspected ones of Cryptosporidium spp, were further evaluated by genetic analysis of gp60 gene. Approximately 200µL of fecal suspension was washed three times in distilled water before extraction. Genomic DNA was then extracted using the FavorPrep Stool DNA Isolation Mini Kit (FAVORGEN, Taiwan,) according to the manufacturer's instruction. For subtype analysis of Cryptosporidium, a gp60 gene fragment (400bp) was targeted using the nested PCR method as previously described (10). Briefly, in both reactions, total volume was 20 µL containing 3 µL of MgCl2 solution (25 mM), 2 µL of 10 × reaction buffer, 1.5 µL of 10 mM dNTPs mix, 2 µL of Primer mix (10 pm/µL), ~4 ng of DNA template and 0.25 µL of Taq DNA polymerase (5 U/µL) (all from Parstous, Mashhad, Iran). Two PCR cycle were as follow: an initial denaturation at 94 °C for 5 min, followed by 35 cycle of 94 °C for 45 s, 55/58 °C for 1 min/45 s, and 72 °C for 60 s, then final extension at 72 °C for 7 min. 1Kb DNA Ladder RTU (SinaClon, Iran) was used to compare the DNA fragment sizes. The amplified PCR fragment was separated by electrophoresis in a 1.5% agarose gel, stained with green viewer (Parstous) and visualized under a UV light and photographed with an UVidoc GEL Documentation System (UVitec, UK). For the first reaction, outer primers GP60 forward1 (5′- ATAGTCTCCGCTGTATTC -3′) and GP60 reverse 1 (5′--GCAGAGGAACCAGCATC -3′) were used at annealing temperature 55°C, with a product size of 980–1,000 bp. For the second reaction, inner primers GP60 forward 2 (5′-TCCGCTGTATTCTCAGCC -3′) and GP60 reverse 2 (5′-GAGATATATCTTGGTGCG -3′) were used at annealing temperature 58°C".

Comment 13-6*- The primers used and amplification procedure should be explained.*

Response: Thanks for the insightful comments. Two PCR cycle were as follow: Two PCR cycle were as follow: an initial denaturation at 94 °C for 5 min, followed by 35 cycle of 94 °C for 45 s, 55/58 °C for 1 min/45 s, and 72 °C for 60 s, then final extension at 72 °C for 7 min. For the first reaction, outer primers GP60 forward1 (5′-ATAGTCTCCGCTGTATTC -3′) and GP60 reverse1 (5′-GCAGAGGAACCAGCATC -3′) were used at annealing temperature 55°C, with a product size of 980–1,000 bp. For the second reaction, inner primers GP60 forward 2 (5′TCCGCTGTATTCTCAGCC -3′) and GP60 reverse 2 (5′-GAGATATATCTTGGTGCG -3′) were used at annealing temperature 58 °C with a product

size of nearly 400 bp. It is added to 'DNA extraction and Nested-PCR' section.

Comment 13-7*- The PCR method mentioned in the text was first applied and described by: - Abe N, Matsubayashi M, Kimata I, Iseki M. Subgenotype analysis of Cryptosporidium parvum isolates from humans and animals in Japan using the 60-kDa glycoprotein gene sequences. Parasitol Res. 2006; 99(3): 303-305.*
Response: We appreciate reviewer comment, accordingly, we cite above paper.

Comment 13-8*- Please add 400-bp band PCR gel electrophoresis image for gp60 gene.*
Response: Thanks for such a constructive comment. Accordingly, we insert shortly sentence in result section. Also, 400-bp band PCR gel electrophoresis image for gp60 gene and its legend is added as Figure 1. Sequentially, Figure 1 change to Figure 2.

Comment 13-9*- Sequence analysis*
Response: We insert the paragraph in related section.

Comment 13-10*- Statistical analysis*
Response: Statistical analysis section locate after "Sequence analysis" section.

Comment 13-11*-How the degree of agreement between microscopy and PCR was determined?*
Response: We appreciate the reviewer's insightful question. To assess the degree of agreement between PCR and ZN staining for detecting of *Cryptosporidium* spp. *Cohen's kappa-index: poor agreement (k < 0.20), fair agreement (k = 0.21-0.40), moderate agreement (k = 0.41-0.60), substantial agreement (k = 0.61-0.80) and perfect agreement (k = 0.81-1.00)* was applied (Reference). It is inserted in related section.

Comment 14 **Results section:**
1- Prevalence of Cryptosporidium sp. infection by modified ZN staining was ….
Response: It is corrected changing the word "cryptosporidiosis" to "*Cryptosporidium* spp. infection".

Comment 15 *It has been stated that: The level of agreement between the two methods was above average (Kappa = 64%).*
Note: Cohen's k-index definitions: poor agreement (k < 0.20), fair agreement (k = 0.21-0.40), moderate agreement (k = 0.41-0.60), substantial agreement (k = 0.61-0.80) and perfect agreement (k = 0.81-1.00).
Response: We appreciate for the comment mentioned together with its useful information. We modified related sentence.

Comment 16 Table 1. Patient 4. genotype IIdA15 (or IIdA15G1, which one is correct?).
Response: We apologize for typing error made. *Cryptosporidium* spp. positive patient 4 has genotype IIdA15 with 15TCA repeats without TCG trinucleotide in variable region of gp60 gene which is mentioned in table 1. Also, apart from one, the rest of IIdA15G1 in text is

changed to IIdA15.

***Comment* 17** It has been stated that:
- In order to recognize isolates subtypes, trinucleotide repeats TCA (red ink) and TCG (green ink) in variable region located in the 5′region of gp60 gene were enumerated.
Note: The gp60 gene has a number of tandem repeats at the 5' end of the gene consisting of TCA, TCG, or TCT, and also further variation in the non-repeat 3' end of the gene, enabling classification of *C. hominis* and *C. parvum* into subtype families on the basis of the number and type of trinucleotide repeat.
Response: Thanks for such a valuable question. With respect to the query raised by the reviewer, elaboration on the details can be found in the following review paper:
Chalmers RM, Robinson G, Elwin K, Elson R. Analysis of the *Cryptosporidium* spp. and gp60 subtypes linked to human outbreaks of cryptosporidiosis in England and Wales, 2009 to 2017. Parasit Vectors. 2019; 12(1): 95. doi: 10.1186/s13071-019-3354-6, PMCID: PMC6417012.
For enabling classification of *C. hominis* and *C. parvum*, as described by Chalmers and colleagues (ref.), firstly, an allelic family is identified from the sequence of a 3′conserved region (e.g IId) of gp60 gene. Variation in a 5′trinucleotide repeat region of the gene then identifies subtypes within each family, (e.g A15G1). Finally, in some gp60 families, number of contiguous copies of a short repeat sequence, *Rn, located in a region between two gene ends can also* contribute to subtype identification (e.g R1). In this study, in order to recognize isolates subtypes, trinucleotide repeats TCA (red ink) and TCG (green ink) in variable region located in the 5′region of gp60 gene were enumerated (Figure 2).

***Comment* 18** Discussion section:
1- Reference no. 16 is located after references 17, 18, and 19.
Response: Thanks. It is corrected.
Comment 19 References section:
1- Reference 21: please include the publication year
***Response*:** Publication year is 2009 added to the reference.

Reviewer #2: Title*:*
Comment 1 *prevalence and genotypes of Cryptosporidium spp. in Diarrheal Patients of Bandar Abbas, Southern Iran.*
Response: Thanks for such an insightful comment. We agree with both reviewer that title of manuscript must change.

Comment 2: Abstract*:*
1-Cryptosporidium species are recognized as important gastrointestinal pathogens change to: as one of the most important
Response: Thanks. It is corrected in the revised manuscript.

Comment 3: The level of significance is not needed in the abstract.

Response: We agree with reviewer, according it is removed.

Comment 4: **Background**:
1- This sentence is not clear: A low dose of oocyst can be caused by disease, so it has the major importance of public health
Response: Thanks for precise consideration to the manuscript for improving its quality. Due to the ambiguity in the sentence, we change it to 'Exposure to low doses of *Cryptosporidium* oocysts can be caused of disease, so it has the major importance public health.
Comment 5: **Methods**:
1-The descriptive of checklist included items are not needed, all of them can be written as demographic aspects.
Response: Thanks for such a valuable comment. Accordingly, we mention all variables as demographic aspects.

Comment 6 Statistical analysis must be located at the end of the methods section.
Response: Thanks for the constructive comments. It is done.

Comment 7 The PCR condition (thermal condition and no of cycles) is not clear.
Response: With respect to previous comment mentioned by reviewer 1 to add amplification procedure, we explain PCR condition in detail.

Comment 8 **Discussion**:
Please explain the major differences between the result of this study and the prevalence of *cryptosporidium* in Bandar Abbas in the previous study.
Response: We appreciate reviewer for such an insightful question. There are differences between our study and previously one done in Bandar Abbas. This study had less prevalence since our subjects included all individuals with diarrhea whereas previously study performed in children with diarrhea. As a result, the latter had higher prevalence (7%). Other differences contained molecular genotyping. Also, additional sentence "In the previous study that was performed in children with diarrhea in Bandar Abbas, prevalence of *Cryptosporidium* spp. was 7%" is deleted.

Comment 9 -Are there any animal study of prevalence and genotype of *cryptosporidium* in Hormozgan for compared with the result of this study?
Response: Thanks for the comment. Unfortunally, there was no animal study of prevalence and genotype of *cryptosporidium* in Hormozgan to be associated with the results of our study. It is added to discussion section.

Associate Editor's Comments:
Comments
Response: All comments is mentioned before by two reviewers. We response them.

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)** 8 Jun 2020

Dear Authors,
According to the associated editor report, your manuscript is accepted for publishing in JJM and sent to the publisher for the next process.
Note:
Please add ORCID number into you and your collages account, then automatically appear in your published papers in JJM both in HTML and pdf.
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