Author's response to reviews

Title: Analysis on Porin I genotype diversities and correlation of gene mutations and drug resistance in Neisseria gonorrhoeae isolates in Eastern China

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Author's response to reviews: see over
Editor, BMC Infect Dis

Dear Prof. Rajabi,

We would like to thank you and the reviewers for having carefully read our manuscript (No. 2890120823526510). After reading the reviewers’ comments and our original manuscript, we are sorry to find many errors, including English grammatical mistakes. As suggested by the reviewers, we reviewed many associated research papers and have done our best to rewrite the manuscript after checking the original experimental records, and Dr. IC Bruce, a native English speaker has read it. Our point-by-point replies to the reviewers’ comments are attached below.

We confirm again that the content in this revised manuscript has not been published or submitted for publication elsewhere. We also state that our experiments are ethically and legally acceptable. The authors have no conflicting financial interests.

Thank you for your consideration and we look forward to hearing from you.

With best regards,

Sincerely yours,

Prof. Jie Yan, M.D.

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Reviewer #1: Dr. Lori Snyder

In this work the authors have examined the gene sequences, and therefore predicted protein sequences, of the alleles of the gene encoding PI from a collection of clinical isolates from Eastern China. They have designed primers to amplify the whole gene and to amplify the PIA and PIB alleles of the gene in such a way that they are distinguishable on a gel. Some new mutations are described and conclusions are drawn about associations of these mutations with resistance to penicillin and tetracycline.

• Major Compulsory Revisions

1. The research question posed here and/or the hypothesis being tested is not very clear. It needs to be made clearer to the reader what the goals of this study were. This will also help the reviewer and the editor decide if these goals have been met.

Reply: We have rewritten the Introduction to make the aim of this study clearer.

2. The nomenclature used to differentiate the different porin types is possibly not the most commonly used / up to date version, i.e. PI, PII, and PIII, and therefore other terminology, like PorA and PorB, might be clearer to the reader. The authors should review the literature and use the terminology that is most commonly used by researchers in the field today.

Reply: We use porA/PorA and porB/PorB in the revised manuscript according to the reviewer’s request.

3. There have been reports of strains of N. gonorrhoeae that react to both PIA and PIB antibodies (De La Fuente and Vázquez, Sex Transm Dis, 1991; Gill et al., FEMS Microbiol Lett, 1994). This has been presumed to be due to mosaicism between porB1a and porB1b alleles of the gene. This is not mentioned in the Background and there is no discussion of mosaicism in the Results or Discussion. If none was observed, this should be mentioned. If it was observed, this should be discussed.

Reply: No mosaicism of PorB1a and PorB1b was found in this study based on the sequencing data, and we state this and cite these papers in the revised manuscript.

4. Even using a proof-reading enzyme, errors might be introduced into the sequence through the PCR amplification process. This is not insurmountable when the PCR product is then directly sequenced, since the error is likely to be visible in the base-by-base fluorescence peak analysis. In this work, however, the PCR products have been cloned and just one of the pool of PCR products has been selected within the clone – potentially freezing and preserving an error that occurred in PCR. The justification posed by the authors for cloning the PCR products was “to obtain more accurate sequencing data”. This is not the case due to the nature of PCR and the selection that occurs in cloning.

Reply: The reviewer is right. We deleted the sentence “to obtain more accurate sequencing data” and have rewritten the paragraph about sequence analysis in the revised manuscript.

5. There is no information in the Methods section on the sequencing methods used or the
analysis of the sequence data information. It is important to know if this was double-stranded sequencing (i.e. from both ends), the length of the sequence read (i.e. long enough for the whole gene), how any N’s or dye blobs were resolved, whether there were any ambiguous bases, and whether any internal primers were needed to finish the sequence.

Reply: In our study, a professional company, Invitrogen Shanghai, China, was responsible for sequencing using the dideoxy chain termination method. In the sequencing data offered by the company, there were no ambiguous bases. For the analysis of sequencing data, we used Clustalx software. All the information about the sequencing and sequence analysis are presented in the revised manuscript.

6. There are minor grammatical errors throughout that must be addressed, but are too numerous to list here. In addition, word usage should be confirmed to be correct, for example “revise” is used on page 6 when the intended word is probably “reverse”. Also there are some stray italics on page 8.

Reply: We are sorry that there are many grammatical errors including stray italics in the original manuscript. We did our best to correct the mistakes and then asked Dr. IC Bruce, a native English speaking scientist working in our college, to check the grammar of the revised manuscript.

7. The Results section of the Abstract is quite difficult to follow and seems not very dissimilar to the text on page 8 and 9. The Abstract should be re-written to be more easily understood and in a way that attracts the reader so that they will want to read the whole article.

Reply: We have rewritten the Results in Abstract.

8. Please address the following questions about the Methods. Why in the duplex PCR was 15 pmol of primer pIA/B-D-F2 used when the other 2 primers were used at 20 pmol concentrations? Also, why did the PCR reaction temperature and cycling conditions change in the duplex PCR from the normal PCR?

Reply: We are grateful to the reviewer finding the mistakes about concentration of primer pIA/B-D-F2 and annealing temperature in the duplex PCR. In fact, each concentration of the three primers used in the duplex PCR was 20 pmol and the same annealing temperature was used in the PCR and duplex PCR. In the revised manuscript, the corrected primer concentration and annealing temperature are presented.

9. It is difficult to categorically draw any conclusions about the influence of point mutations on antimicrobial resistance without the construction of isogenic mutants. The authors should be careful not to overstate discoveries of mutations in clinical isolates that also happen to be resistant to certain antibiotics before a direct association can be determined. In light of the nature of the data upon which the authors are trying to draw this conclusion, I do not believe it should be such a focus of the manuscript or that it should be so prominently in the title.

Reply: The reviewer’s opinion is important. In the revised manuscript, we changed the title and reasonably describe the possible correlation between site mutations and resistance.

10. The reader is left wondering about the nature of this isolate collection. What other information is available about these isolates from the clinical laboratories that isolated and
classified them? Was porin typing by antibodies or other methods done? Was antibiotic resistance determined? Certainly other data must be available from the hospital. Since N. meningitidis strains have been found that contain PIB (Vázquez et al., Mol Microbiol, 1995), were any tests conducted within your laboratory to confirm the classification of these isolates as N. gonorrhoeae.

Reply: More data about the sources and identification of *N. gonorrhoeae* isolates are presented in the revised manuscript. The hospitals provided the isolates that were identified using microscopy after Gram staining plus oxidase, catalase, and carbohydrate degradation tests. Our laboratory identified the isolates again using the same methods, and further determined the penicillin and tetracycline resistance by drug sensitivity tests and porin types by sequencing and sequence alignment compared to those reported in Genbank.

**Reviewer #2: Dr. Robert Nicholas**

The manuscript by Sun et al. describes the diversity of porB1a and porB1b sequences from a set of 315 gonococcal strains isolated in Eastern China between 2005 and 2008. The authors amplified and sequenced the por genes from these 315 strains, and report the MICs of penicillin and tetracycline for these strains as well. Overall, the manuscript is interesting and the data are worth publishing.

• **Major Compulsory Revisions**

1. There are many issues with the paper that need to be addressed. For example, the authors are apparently not aware that the sequence of porB1a from typical wild type strains has G120/G121, not G120/A121 (the latter sequence is found in “wild type” porB1b).

Reply: We have corrected the mistake in the revised manuscript. In the original manuscript, mutations at the 120 and 121 sites in PorA and PorB were confused.

2. There are numerous grammatical and spelling mistakes (e.g. “praline” instead of “proline”) that need to be fixed.

Reply: We are sorry to have many grammatical and spelling mistakes in the original manuscript. We did our best to correct the mistakes and then asked Dr. IC Bruce, a native English speaking scientist working in our college, to read and review the revised manuscript.

3. Lastly, and most importantly, the MICs reported are extremely high for chromosomally mediated resistant strains. For example, most chromosomally mediated penicillin-resistant strains have MICs of penicillin in the range of 2~6 µg/ml, not 4~16. Likewise, MICs of tetracycline in chromosomally mediated resistant strains are 2~4 µg/ml, not 8~32 (unless these are TetM-producing strains). This brings up a larger issue-do the authors believe that their strains are chromosomally mediated resistant strains, or could some of these be plasmid-mediated resistance? If the former, then the MICs would be strongly influenced by which resistance determinants they harbor, such as penA, mtrR, ponA, and rpsJ (the latter being involved in tetracycline resistance-- Hu M et al. Antimicrob Agents Chemother. 2005 Oct;49(10):4327-34); if the latter, it hard to know whether the por sequences contribute to resistance or are simply clonal markers. Either chromosomal or plasmid is fine, but at the very least the authors need to acknowledge these issues in the discussion. Since the MIC numbers represent a range for all of
the porB clonal strains, it is not surprising that they have a large range, but the actual numbers still seem quite high. It is also not clear how the MICs were actually obtained, which should be made clear.

Reply: We are grateful the reviewer for giving a very important opinion which makes us thinking more about further reseach. We also feel the MICs obtained in this study are relatively high. According to the reviewer’s request, we reviewed the previous literature and discuss the chromosomally-mediated resistance of penA, mtrR, ponA and rpsJ genes, and some published papers about the genes are cited in the revised manuscript. In addition, details about the method for MICs are also added.

Specific Points:
1. References to A121 in porB1a should be fixed throughout the manuscript—the actual residue is G121.
Reply: We corrected the mistake in the revised manuscript. In the original manuscript, the mutations at the 120 and 121 sites in PorA and PorB were confused.

2. On page 4, “praline” should be “proline”
Reply: This is a spelling mistake and we corrected it in the revised manuscript.

3. On pages 6 & 7, “revise” should be “reverse”
Reply: This is a spelling mistake and we corrected it in the revised manuscript.

4. On page 7, a PIB-specific primer is referred to as “PIA/B-D-F2”, but should be renamed (e.g. PIB-D-F2)
Reply: We are sorry to still use the primer name “PIA/B-D-F2” in the revised manuscript, because it difficult for us to describe clearly in table 3 that the three primers PIA/B-D-F1, PIA/B-D-F2 and PIA/B-D-R were simultaneously used in the same duplex PCR.

5. I have no idea what the “standard proportion” method for determining MICs is referring to. The authors need to be much clearer in describing how these were done.
Reply: We describe a brief protocol of the standard proportion method in the revised manuscript.

6. One very important point is that the authors describe all the mutations at a given position, e.g. when referring to mutations described in Olesky et al. (2002), they write “G120D/K/R/P or A121D/H/P”. The authors need to be clear about whether mutations at one or both positions were necessary for resistance. For example, the single G120K mutation was sufficient for full resistance, whereas both the G120D and A121D mutations had to be present to confer full resistance. For this reason, unless it is known whether the mutations have a phenotype alone, it is best to refer to the mutations in pairs (e.g. G120P/A121P).
Reply: We revised the manuscript as requested.

7. On page 11, it is written “Bash and his colleagues (2005)”. This should read “Bash and colleagues” (Margaret Bash is a she, not a he)
Reply: We thank the reviewer and corrected the mistake in the revised manuscript.
Reviewer #3: Dr. Jason Folster
The authors of this work characterized porA and porB mutations in N. gonorrhoeae and determined their role in antimicrobial resistance.

Minor essential revisions.
1. the lack of line numbers makes reviewing more difficult.
   Reply: We use line numbers in the revised manuscript.

2. the use of pIA and pIB as gene names is confusing, please change to porA and porB for the genes and PIA and PIB for the protein.
   Reply: We revised the manuscript as requested.

3. Is the duplex PCR necessary? The single PCR produces two different sized amplicons which could be used to differentiate between porA and porB.
   Reply: We think the duplex PCR is useful because there is only a 63 bp difference in size between the amplicon from the entire porA gene (981 bp) and that from the entire porB gene (1044 bp) so it is difficult to distinguish the two amplicons in agarose gel.

4. please change "strains" to "isolates"
   Reply: We changed "strains" to "isolates" in the revised manuscript.

5. page 4, line 9, PIA and PIB are
   Reply: We revised it as the requested.

6. page 4, line 10, change "anyone" to "any particular"
   Reply: We revised it as requested.

7. page 4, line 18, antimicrobials
   Reply: We revised it as the reviewer’s request.

8. page 5, line 5, "in the clinical setting"
   Reply: we revised it as requested.

9. page 5, line 20, what is well-grown? overnight culture?
   Reply: We added the incubation time in the revised manuscript.

10. page 6, line 4, "reverse" primers
    Reply: We revised it as requested.

11. page 6, line 13, remove "more accurate"
Reply: We removed "more accurate" in the revised manuscript.

12. page 6, lines 13-19, please re-word, cloning kit not used for sequencing.
Reply: We reworded the sentence in the revised manuscript.

13. page 7, line 1, remove italics on DNA concentration
Reply: We removed the italics on DNA concentration in the revised manuscript.

14. Are the primers for producing porA and porB for sequencing internal? Could the authors be missing mutations that occur within the primed sequence?
Reply: According to the all published data about mutations in the porA and porB genes, the primers we used are located in the conserved regions.

15. page 9, line 9, did the authors mean "PIA"
Reply: We are grateful to the reviewer for pointing out that PIA has been miswritten as PIB and have corrected it in the revised manuscript.

16. page 10, lines 1-3, what is the wild-type MIC value for pen and tet?
Reply: The MICs of penicillin and tetracycline for all the 98 porA+ isolates were 0.12-1 mg/L (<2 mg/L), while the 5 porB+ isolates with no G120 and A121 mutations in PIB were 0.25-0.5 mg/L (<2 mg/L). The details of MIC values are listed in table 3.

17. page 17, lines 10-13, what is considered resistant?
Reply: The MIC values considered as resistance to penicillin and tetracycline are presented under “Drug sensitivity test” in the Materials and Methods. In the Results of the revised manuscript we also describe the resistance standard in MIC detection.

18. page 18, lines 4-6, please reword Figure legends, I would use "amplicon" in place of "segments" Figure 3, the alignment is uneven and the numbers on the left are confusing. Could you use group A, B, C, etc or n=# of isolates in each group?
Reply: We use "amplicon" in place of "segments" in the figure legends of the revised manuscript. In revised Figure 3, we changed the style of calligraphy to make the alignment even.