Author's response to reviews

Title: Antimicrobial susceptibility and genetic characteristics of Neisseria gonorrhoeae isolates from India, Pakistan and Bhutan in 2007-2011

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Version: 2 Date: 29 December 2012

Author's response to reviews:

Dr. Philippa Harris and Dr. Sepehr Tabrizi
Editors, BMC Infectious Diseases

Dear Editor Dr. Harris and Dr. Tabrizi,

Re: MS: 7692293398390200 (Antimicrobial susceptibility and genetic characteristics of Neisseria gonorrhoeae isolates from India, Pakistan and Bhutan - 2007-2011)

Comments from the Editors:

“We would be grateful if you could address the comments in a revised manuscript and provide a cover letter giving a point-by-point response to the concerns.

* In addition we would like to request that you clarify, within your methods section, that only bacterial isolates taken as part of standard care were isolated and stored and as a result no ethical approval was required.”

Thank you for your positive response concerning our manuscript. We have carefully considered all the comments from the reviewers, and in response we have made substantial changes to the manuscript and point-by-point response to the concerns are outlined below. As requested, we have also included one sentence in the methods section that clarifies that no ethical approval was required because only bacterial isolates taken as part of the routine diagnostics were isolated and stored.

The manuscript represents original work and no paper resembling the enclosed article has been or will be published except in BMC Infectious Diseases. All authors have read and agreed to the revised version of the manuscript and none of the authors have any conflict of interest.
Reviewer comments

Reviewer #1

Comment #1

“The paper title and introduction indicated the authors want to find out the prevalence and characteristics of N. gonorrhoeae in South Asia. However, the strains were mainly from three countries. It is more appropriate to indicate three countries or indicate the authors definition of South Asia.”

In the revised paper, we have in most places specified the name of these three countries. In some places where we discuss resistance levels in also other countries in the region, we have referred to South-east Asia, where all the countries we are referring to are located.

Comment #2

“L82-84. Disk Diffusion if done properly should be able to classify susceptibility of N. gonorrhoeae. However, intermediate strains should be confirmed with MIC determination. The advantage of MIC determination is the ability to track decreasing susceptibility. Quality assurance and quality control should be performed for both disk diffusion and MIC methods. Therefore, it would be better to reword these two sentences for readers. Do the authors want to say that previous data in this region are invalid due to lack of quality assurance and quality control. It is also important to methods are validated internationally for global surveillance. Therefore, MIC tests become method of choice if affordable.”

We agree with the reviewer that appropriately performed, optimized and fully quality assured disc diffusion method may reflect and classify susceptibility and resistance. However, in N. gonorrhoeae the zone sizes suboptimally reflect the MICs for most/all antimicrobials. This is very clear particularly for the extended-spectrum cephalosporins that currently are the most important to appropriately monitor in detail, i.e. when you at current time try to even monitor the creep of MICs (of the MIC distribution of the gonococcal population) up on the MIC scale. This is not possible to adequately monitor using disc diffusion methods. Accordingly, we truly consider, and have evidence-basis for expressing, that there is no really appropriate disc diffusion method for N. gonorrhoeae, i.e. that ideally reflects the exact MIC over the entire MIC range. We totally agree that all methods, independent on if it is agar dilution, Etest or disc diffusion, need to be strictly quality assured and quality controlled. In the revised manuscript, we have also mentioned that we used the 2008 WHO N. gonorrhoeae reference strains as quality controls in all antimicrobial susceptibility testing. Furthermore, as requested we have slightly rephrased the sentences the reviewer mention.

Comment #3

“A reference for genetic determination of ciprofloxacin resistance in strains from this region should be included.”
Revised as requested.

Comment #4
“L 92. Insert reference of now out-dated DNA-based method. DNA sequencing is one of the DNA based method.”

The referred sentence did not add anything to the manuscript and, accordingly, has been excluded in the revised manuscript.

Comment #5
“Azithromycin resistance could be caused by ribosomal RNA gene mutation besides efflux. This paper did not sequence the ribosomal RNA genes of resistant isolates. Is that based on the level of resistance or other reasons? 11 of the 15 isolates has increased efflux, what are the mechanisms of the other four azithromycin resistant or reduced susceptible strains (L 182-185). In discussion L245, 13 has Mtr promoter mutations.”

The focus regarding resistance mechanisms was on the extended-spectrum cephalosporins, and ciprofloxacin. Accordingly, to comprehensively elucidate the resistance mechanisms to all examined antimicrobials, including azithromycin, was no aim of the present paper. In regard to azithromycin the main reason that we did not completely elucidate the resistance mechanisms was the level of resistance, i.e. no isolates had higher MIC of azithromycin than 4 mg/L (one single isolate). If we would have found some strains with high-level resistance to azithromycin, we would have sequenced all the four alleles of the 23S rRNA gene. It is also described in the revised manuscript that 11 isolates showed the A-deletion and two showed a T-insertion in the mtrR promoter (i.e. 13 isolates had MtrR promoter mutations), which all most probably increase the efflux through the MtrCDE efflux pump and increase the MICs of azithromycin. For the remaining two isolates with intermediate susceptibility/resistance to azithromycin (with relatively low MICs of azithromycin), we do not know the resistance mechanisms. However, for example 2611 mutation in some (but not all) of the four alleles of the 23S rRNA gene could result in this elevated azithromycin MIC.

Comment #6
“L280-281 Do the authors collect epidemiological data of patients so typing could be linked to modes of transmission?”

Unfortunately, basically no useful epidemiological data were available for the present study. Accordingly, in this region this is the first study investigating the antimicrobial resistance using quality assured MIC-based methods and genetic methods for antimicrobial resistance and molecular epidemiology. However, we had to use basically all viable isolates available independent of epidemiological and in general metadata. You have to start somewhere and hopefully in the future also epidemiological data may be available for isolates from this region of the world, which has also been mentioned in the revised version.

Comment #7
“Are ST types in this region different from those in other parts of the world?”

This is the first study examining the NG-MAST STs in these countries. Only among the relatively few examined isolates, 43 new STs (not described previously worldwide) were found. All this is mentioned in the revised manuscript. However, this probably mainly reflects that it was the first study in the region and many locally evolved STs were found. This has been observed also in other regions, i.e. when the first study in the specific region was performed.

Comment #8

“L87-91. It is a good idea to have a population based study. However, the sample size in particular in Bhutan is rather small and has little power for interpretation for comparison of different communities etc. At the most, the sample is adequate for comparison at country level. Each year the number of isolates was small. It is important for the reader to know the incidence of N. gonorrhoeae and population size in these countries to understand the representativeness of the selection of strains or clinically specimen and relevance to an epidemiological study.”

We totally agree with the reviewer and, unfortunately, the sample size was very small, particularly in Bhutan. Due to the lack of culture-based diagnostics as well as lack of deep freezers or equivalent for storage of gonococcal isolates very few isolates are available from these countries. In fact, we shipped more than 300 strains to Sweden, but the majority was not viable when they arrived. Nevertheless, this is the first study investigating the antimicrobial resistance using quality assured, internationally validated MIC-based methods and genetic methods for antimicrobial resistance and molecular epidemiology in the region. Accordingly, you have to start somewhere and these countries need to be strongly supported for enhancing their surveillance. However, due to the low number of isolates all the results need of course to be interpreted with caution which is also mentioned in the discussion section of the revised manuscript. Unfortunately, due to use of suboptimal diagnostics, case reporting and epidemiological surveillance no at all reliable incidence figure for gonorrhoea is available from the investigated countries. In fact, gonorrhoea is not even mandatorily reported in all these countries.

Comment #9

“L 94-102. To have high discriminatory power, N. gonorrhoeae often is based on multiple typing methods and not a single method. Of course the most discriminatory is whole genome sequencing. If the authors indicate NG-MAST or por sequencing could be used only, it require good evidence. In Line 107-108 the authors used two molecular typing methods (L 94-95 indicate only one method is needed).”

We totally agree with the reviewer that genome sequencing is the most discriminatory typing method. However, for many epidemiological considerations, such as more long-term investigations, genome sequencing may be even too discriminatory and hard to interpret. Accordingly, in a specific study the clinical,
epidemiological or scientific questions should guide the selection of the most suitable typing method (or methods when required). We consider there are good evidence that NG-MAST or porB gene sequencing can be recommended for many epidemiological considerations, with possible exception of macroepidemiology such as very long-term epidemiology (many years-decades) or global epidemiology over time. This as well as the international recommendation to use NG-MAST or porB sequencing were described and comprehensively discussed in reference 39 of the revised manuscript. This comprehensive review also includes many references providing the evidence-basis for this statement. We consider that in most situations only one of these two methods are needed and in the revised manuscript we have excluded all information regarding full-length porB gene sequencing. For further information, see comment #6, reviewer #2.

Comment #10

“In the discussion, different rates of resistance were reported from different studies. Does this study has less biased sampling? What is the confidence level of the rates of in this study in comparison of other studies? Could the difference be explained by methodology only as author suggested in the paper or the difference in sample bias? Does the scenario in L 267-275 affects the results of this study?”

Due to the low number of isolates and biased selection of the isolates (i.e. few isolates but all viable isolates from several years) examined in the present as well as previous studies, appropriate and reliable confidence levels cannot be calculated. Furthermore, as indicated by the reviewer also the high number of gonococcal infections remaining undiagnosed using laboratory testing in the included countries further biases the results and interpretations of the present study. Accordingly, the largest bias in the current and previous studies from this region is the low number of isolates examined, which also introduce a bias in regard to examined isolates (i.e. selection of isolates). Of course, also different methodology for antimicrobial resistance testing affects the comparisons of the present and previous studies. The present study is the first study investigating the antimicrobial resistance using quality assured, internationally validated MIC-based methods that should at least minimize the biases introduced by the used methodology. In the revised manuscript, we have mentioned all these possible biases and that all the results need to interpreted with caution due to these biases.

Reviewer #2

Comment #1

“The paper could be edited and checked for syntax.”

The revised manuscript has been carefully edited and checked for syntax.

Comment #2

“Further, the sections on mechanisms of resistance in the results are a mix of
introductory comments, results and discussion. There is almost no background on mechanisms of resistance in the Introduction.”

In the revised manuscript, this has been clarified and some descriptions in the results section have been moved into the introduction and/or discussion. Accordingly, some of that replaced information is used as background information regarding mechanisms of resistance in the introduction section of the revised manuscript.

Comment #3
“Data on genetic mechanisms of resistance should be summarized in a Table.”

Revised as suggested!

Comment #4
“The authors state that most of the isolates tested had intermediate susceptibility to an amoxicillin/clavulanate combination. Then (abstract) they state that this antibiotic combination should not be used. The authors should explain why this no-longer recommended combination was tested. Further, some (younger) readers might forget that #lactamase acts against amoxicillin and that, with 52% of the isolates producing #lactamase, this combination cannot be recommended despite seemingly intermediate MICs. This needs to be discussed more thoroughly. I would not recommend reporting these results as they are misleading.”

We are very sorry but we are not completely clear about these comments from the reviewer. Accordingly, the amoxicillin/clavulanate combination was tested for all isolates because this combination might rarely be used for treatment of gonorrhoea in India. Furthermore, we totally agree with the reviewer that beta-lactamase that was produced by 52% of the isolates acts also against amoxicillin, however, it is not effective against amoxicillin if this antimicrobial is combined with clavulanate that inhibits the beta-lactamase enzyme activity. Anyway, we totally agree with the reviewer that it may be dangerous to report this combination in the paper, because some readers may consider it appropriate for use based on the reported results. Accordingly, as recommended in the revised manuscript we have excluded all the results for the amoxicillin/clavulanate combination.

Comment #5
“Many results, for example the very high number of NG-MAST strain types, might be explained because the selection of isolates was random and occurred over several years from several countries. Given that scenario, it is not surprising that so many NG-MAST types were observed and there is a great deal of literature (uncited) to support such an observation. Furthermore, data on the STs are not provided, even though the abstract claims that 43 STs were never before reported.”

We agree with the reviewer that the high number of NG-MAST sequence types
may be partly explained by the fact that the selection of isolates was random (basically all viable isolates were examined but many isolates obtained were not viable) and occurred over several years. However, the high diversity probably also reflects that it was the first study examining the NG-MAST STs in the region and many locally evolved STs were found. This has been observed also in other regions, i.e. when the first study in the specific region was performed. These explanations as well as additional explanations have been mentioned in the revised manuscript. In the revised manuscript, the NG-MAST STs identified in the present study are provided as supplemental material.

Comment #6
“The authors also conducted full porB sequencing analysis. That is also a valid typing method. Were the results compared to the NG-MAST results?”

We totally agree with reviewer that full-length porB sequencing is also a valid and most useful molecular epidemiological typing method. During double-checking of the porB sequences we have identified that several of our porB sequences are not reliable sequences for the full-length gene. Accordingly, in the revised manuscript we have excluded the information regarding full-length porB sequencing.

Comment #7
“The collection, identification and testing and storage of strains need to be described. Where and how were strains collected? Where were the isolates initially cultured, identified and stored. Was all testing done at the same site, or were strains or DNA shipped to various sites? What was the basis of isolate collection – all isolates collected in one year at a given hospital? Other? Such information might elucidate some of the data obtained (such as NG-MAST).”

In the revised manuscript, we have clarified most of these issues, i.e. by including all our available data.

Comment #8

High cost for the individuals and society due to gonococcal infections and their associated severe sequelae, particularly calculated as disability-adjusted life years, social trauma and/or stigma to have gonorrhea in many communities, etc. Many different factors are described in the references included in the revised manuscript.

Comment #9
“Line 71. The statement on dual antimicrobial therapy should be explained. Is dual therapy being used in the countries covered in this study? What is the recommended therapy in these countries? Such an explanation would make the discussion more relevant.”

In the referred sentence in the general background, we only expressed that it is
higher threat of untreatable gonorrhoea in settings where dual antimicrobial therapy may not be feasible or affordable. Dual antimicrobial therapy has to our knowledge only been introduced in the USA and Europe, and not in any of the countries covered in the present paper. Due to this reason we have excluded the referred information in the revised manuscript. In the countries covered in this study, basically ceftriaxone is recommended for treatment. However, most importantly in practice a variety of other antimicrobials are also used in the treatment of gonorrhoea, particularly in the private sector. Accordingly, ceftriaxone but also penicillins, fluoroquinolones, macrolides, tetracycline and spectinomycin remain in use for treatment of gonorrhoea in this region. This has also been mentioned in the revised manuscript.

Comment #10
“Line 91. What methods are outdated? A reference is needed for that statement.

Line 101-102. Methods of interpretation of ribotyping, Opa-typing and other methods which require discrimination of bands are described as being “relatively subjective”. Do the authors mean that these methods cannot be compare results between laboratories because there is no standardized interpretive data base? The sentence reads as if the method itself is subjective. The method(s) is/are very reproducible within laboratories.”

The sentence regarding outdated typing methods has been excluded in the revised manuscript. Regarding interpretation of the typing methods that the reviewer mentions, we meant that these gel-based methods are read subjectively (mostly visually) and that it can be hard to compare results between laboratories (compared to when you are using sequencing-based typing methods), due to this fact and especially due to the lack of a standardized interpretive data base. We totally agree with the reviewer and have further rephrased the sentence for clarification.

Comment #11
“Line 103. “selected” genetics…. determinants. What was the basis of this selection?”

We focused on studying resistance determinants for extended-spectrum cephalosporins and ciprofloxacin (the current and previous first-line antimicrobials for treatment of gonorrhoea). This has been further clarified also in the aims of the revised manuscript. The reasons for focusing on resistance determinants for those antimicrobials were that i) extended-spectrum cephalosporins are the recommended first-line antimicrobials in most countries globally and ii) most worryingly ciprofloxacin remains relatively frequently used for treatment in the countries covered in the present study.

Comment #12
“Materials and methods:
The medium used for MIC testing should be stated.”
Revised as requested.

Comment #13
“Table 1 should include results for #lactamase testing.”

Revised as requested, i.e. the results for the beta-lactamase testing are now mentioned in a footnote for Table 1.

Comment #14
“A Table should be developed describing the various mechanisms of resistance.”

Revised as requested.

Comment #15
“Statements such as this –Line 184. “penB alterations causing a decreased efflux…” are unreferenced and un-described. Another such statement occurs in line 165 – ‘which explains the high level of intermediate susceptibility”. There is no reference or explanation subsequently.”

In the revised manuscript, we have included some background information regarding genetic resistance mechanisms to extended-spectrum cephalosporins and ciprofloxacin. Furthermore, all the statements referred above are referenced in the revised manuscript.

Comment #16
“The discussion is very general and should compare and contrast more relevant publications on mechanisms of resistance.”

We have focused the discussion mostly on the phenotypic antimicrobial resistance. The reasons for this were that we did not find any decreased susceptibility or resistance to extended-spectrum cephalosporins, or any isolates containing the main resistance determinants for those antimicrobials (penA mosaic allele or A501-altered allele of PBP2). Accordingly, for the most important antimicrobials (currently recommended first-line for empiric treatment of gonorrhoea in most countries) we did not have any really interesting novel findings that we could discuss and compare with previous publications. Nevertheless, in the revised manuscript we have included some background information regarding genetic resistance determinants to extended-spectrum cephalosporins and fluoroquinolones in the introduction, included slightly more descriptions in the discussion and all this is appropriately referenced. Three additional references regarding genetic resistance mechanisms to extended-spectrum cephalosporins and fluoroquinolones have also been included in the revised manuscript (Reference 26, 27 and 38).

Comment #17
“The paragraph starting on line 77 should be split into several, referenced sentences.”
Revised as requested.

Comment #18
“The sentence starting on Line96 (In south Asia) should be split.”

Revised as requested.

Comment #19
“There are other grammatical errors throughout –the addition of the, a etc. The text should be carefully reviewed.”

As requested, the text of the revised manuscript has been carefully reviewed and corrected by a native English speaking professional.

Best regards,
Magnus Unemo