Author’s response to reviews

Title: Fluoroquinolone resistance and mutational profile of gyrA in pulmonary MDR tuberculosis patients

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Author’s response to reviews:

Respected Prof. Dirk Krüger
Editor-in-Chief
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Respected Prof. Dirk Krüger,

I am sending the revised manuscript “Fluoroquinolone resistance and mutational profile of gyrA gene in pulmonary MDR tuberculosis patients" for favor of its publication in your esteemed journal, BMC Pulmonary Medicine. While revising I followed the instructions of honorable reviewer and changes are mentioned in "Red color" in the manuscript.

Response to Reviewer # 1

1) Line 21-22 how many estimated cases and what is missing estimated number of cases? It is useful information in general if the context of this paper. Case finding is a major issue under current TB control
Response: A total of 562 suspected MDR-TB cases were included in this study. History of anti-tuberculosis treatment was obtained from the patients. Out of 562, 313 were the newly diagnosed cases, 97 were treatment failure cases (who completed the treatment but still positive for MTB), 59 were treatment default cases (previously taking anti-tuberculosis therapy, at least for one month, but did not complete the treatment), and for the 93 samples treatment history was unknown (Sample collection and results).
2) Lines 26-28. I suggest that authors mention how they arrived at this sample size?
Response: The samples were collected from PMDT sites (Programmatic management of drug resistant tuberculosis) of seven different districts of Punjab, Pakistan for the period of May 2018 to March 2019 (Material and Methods).

3) Lines 6-7. To which high incidence are the authors referring to? They may provide literature about the proportion of FQ resistance etc. to support their statement.
Response: The resistance to FQs occurs due to point mutations in conserved QRDR region of gyrA and gyr B gene. The mutation in QRDR change the structure of drug binding pocket (QBP) of quinolones and results in cross resistance to all FLQs. The frequency of gyr A mutations is relatively higher than gyr B (occur only rarely). In gyrA region, mostly A90V and D94G mutations are found. These A90V and D94G mutations are associated with high level of resistance to fluoroquinolone antibiotics. A90V mutation detects resistance for levofloxacin but a higher generation of FQ i.e. moxifloxacin can be used at higher dose. But if there is mutation of D94G both levofloxacin and moxifloxacin are ineffective (Mogashoa et al., 2019) (gli guideline Line probe assay) (Discussion; 5th paragraph).


Figure 2: The association of gyrA gene mutation with levofloxacin and moxifloxacin resistance.

4) Lines 34-35. Ethics statement be a stand-alone, not included under this paragraph
Response: The statement has been separated as suggested by the honorable reviewer (Page 4; lines 101-103).
The study was approved by the Research Ethics and Biosafety Committee (No.D/650/MMG) of Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan.

5) Lines 55-57. It would be good to list those districts to know geographic distribution of frequency of FQS-R proportions.
Response: The districts, from where the data was collected, were Lahore, Faisalabad, Gujranwala, Sahiwal, Sargodha, Sialkot, and Bahawalpur. The geographical map of these regions is shown below (Page 4; lines 95-96).

Figure S1: Samples were collected from Programmatic Management of Drug Resistant Tuberculosis (PMDT sites) of seven districts (Lahore, Faisalabad, Gujranwala, Sahiwal, Sargodha, Sialkot, and Bahawalpur) during May 2018 to March 2019.

6) Lines 8-10. It would be interested to read the profile of drug sensitivity/resistance to all the first line drugs with numbers and % age if space provides.
Response: GeneExpert assay was performed for the confirmation of MDR-TB. The susceptibility of first line drugs was determined against rifampcin and isoniazid by Line probe Assay. The results have shown that 430 samples were MDR (resistant to both isoniazid and rifampcin), 91 samples were mono-resistant (57 resistant only to rifampcin and 34 resistant to isoniazid only), and 41 samples were susceptible to these two drugs (Page 6; lines 130-134).
7) Lines 28-29. There is abrupt beginning to the discussion. A summary of FQ resistance and mutations followed by why resistance would occur would be more logical.

Response: Fluoroquinolones have long been widely used for several infectious diseases and easily accessible in certain region even without prescription. Such misuse of FQs has highly contributed to their efficacy in the treatment of TB and emergence of FQ-resistance [10].

In the current cross-sectional study, a presumptive multi drug resistant isolates of MTB were included. A high proportion of rifampcin (Rif) and isoniazid (INH) resistance was observed. Of the total, 92% (521/562 i.e. 430 MDR and 91 monoresistant) isolates were resistant to first line drugs (INH and Rif) either both or alone. The isolates having resistance against rifampcin and isoniazid are termed as MDR-TB and if they develop additional resistance against FLQs then they are known as Pre-XDR TB [11].

MTB develop resistance against FQs, mostly, by developing mutations against drug targeted proteins. The detection of gyrase mutations can help in predicting FQs resistance as well as estimating the levels of resistance to various fluoroquinolones [12]. GenoType MTBDRSl assay can detect mutations in the QRDR region of the gyrase gene (gyrA and gyrB ) GenoType MTBDRsl assay was used to determine the frequency of FQs resistance of our isolates. A total of 104/562 isolates were found resistant to FQs. The high level of FQs resistance was also reported in other provinces of Pakistan [10,13] and neighboring countries India [14,15], China [16,17] and Bangladesh [18,19] (Discussion; Pages 7-8; lines 173-190).

References added into the manuscript.


8) Lines 40-41. Provide the details of numbers and duration of therapy.

Response: In the current cross-sectional study, presumptive multi drug resistant isolates of MTB were included. A high proportion of rifampcin (Rif) and isoniazid (INH) resistance was observed. Of the total, 92% (521/562 i.e. 430 MDR and 91 monoresistant) isolates were resistant to first line drugs (INH and Rif) either both or alone (Discussion; Page 8; lines 176-179).

Short treatment regimens are used to reduce emergence of antimicrobial resistance in MTB. According to National guidelines for control of TB in Pakistan 2019 (adopted by WHO), the anti-TB short regimens include third or fourth generation fluoroquinolones (levofloxacin and moxifloxacin respectively) for 4 months for drug susceptible cases. They are also given in isoniazid resistant and previously treated cases in initial phase of therapy (2 months). The high proportion of FQ-R indicates the patient ineligibility for shorter regimens (Discussion; Page 8; lines 191-196). More information can be seen on the website mentioned below.


Response to Reviewer # 2
Specific Comments

Background
If the authors are seeking to determine the "potential utility" of fluoroquinolones, why is this the right population (patients who had been on therapy) and study design (identification of specific gyrA and gyrB mutations? More representative samples from Pakistan have already been surveyed for prevalence of fluoroquinolone resistance (e.g. Zignol et al Lancet Inf Dis 2016).

Reconsider how the relevance of fluoroquinolone resistance is explained. The risk of progressing to XDR is not the main reason it is important, particularly as injectable-free TB regimens are increasingly recommended and used.

Response: Convenient sampling method was used in the current study design. All the data was collected from PMDT sites where TB patients are registered for treatment. At the time when samples were collected all patients were on treatment but treatment history varies. Some patients were newly diagnosed and some had history of previous treatment including treatment failure and treatment default cases (explained in sample collection and results are mentioned).

Flouroquinolones target DNA gyrase enzyme to inhibit DNA synthesis. All type of flouroquinolones develop resistance by mutations in quinolone resistant determining region (QRDR) of gyrase gene A and B subunit (gyrA and gyrB). The resistance to FQs can be determined by detecting mutations in these genes.

In the previous study (Zignol et al Lancet Inf Dis 2016) the resistance to flouroquinolones was determined by phenotypic drug susceptibility testing. But in the current study a different approach was used by performing MTBDRsl assay and sequence analysis. In Pakistan there are some other studies using the same approach but we have included different population and broadened the geographical area by collecting samples from seven different districts of Punjab. A limited information exists regarding FQs resistance and their mutations among pulmonary TB cases in these regions. But the present study not concluded any particular variation in mutation due to differences in geographical area.

The context is changed as: During treatment of TB, MDR patients can develop resistance against flouroquinolones. The development of FQs-R in these patients is a risk factor as additional resistance to this drug can aid in transition of these patients from MDR to pre-XDR or even they can become extensively drug resistance cases with further resistance to at least one injectable second line drug.

Methods:
More detail about the patient population is needed to judge its representativeness. How were they selected, and what selection bias may have been introduced? Were the 562 samples from 562 unique patients? How much anti-tb therapy had these patients received? Enough that some had become LPA negative -- in which case this sample would biased toward those who had not responded quickly to treatment? What treatment regimens were they receiving?

How were RIF and INH susceptibility determined?

How was phenotypic susceptibility to FQs assessed?

Response: Sample collection and patient’s details have already been described. There were few patients for whom treatment history was unknown. All the patients taking anti-TB therapy including INH and RIF at least or one month was included. But due to lack of access to patient’s information we could not find out information about other TB regimens. This was the
limitation of the study as there were lack of information for all antibiotics and even treatment failure and relapse cases were unaware about their previous treatment strategy. LPA negative samples were not included in the study. Flourescent microscopy was performed before LPA testing. The smear positive samples were directly processed for LPA and smear negative samples were first cultured on MGIT® BACTEC then processed for LPA. GeneExpert assay was used for confirmation of MTB positive and rifampcin resistance. The susceptibility of first line drugs was performed by Line probe Assay. The results have shown that 430 samples were MDR (resistant to both isoniazid and rifampcin), 91 samples were mono-resistant (57 RIF-R only and 34 INH-R only) and 41 samples were susceptible to these two drugs (Results; page 6; lines 128-132). The phenotypic susceptibility was not performed. Currently, it was not the part of project but definitely we will consider this assay in future for correlation of phenotypic and genotypic drug susceptibility.

Results:

Were there any associations between patient characteristics and resistance (overall or specific mutations?)
Did each FQ resistant have only a single mutation?
What were the RIF and INH susceptibilities of those with no FQ resistance mutation identified?

Response: The patient’s characteristics and type of mutation is assessed in two ways. The one approach was to determine frequency of particular mutation according to their categorization of resistance to first line drugs and secondly by their categorization according to treatment history. In MDR and RRD cases, the FQs-R was most commonly associated with D94G mutation. This mutation shows high level resistance to all flouroquinolones even to fourth generation moxifloxacin. Monoresistance INH-R showed S91 P and D94N/Y mutation where they be either totally resistant to FQs or effective for higher dose of moxifloxacin. D94N/Y mutation was also observed in RRD. When mutation pattern of newly diagnosed TB isolates with FQs resistance was observed, it showed high proportion of A90V mutation where patients are, though, resistant to levofloxacin but moxifloxacin still remains the drug of choice at higher doses. In contrast, the mutation pattern of D94G was commonly found in treatment failure and relapse cases. The sequence analysis of gyrA gene evaluated the co-existence of S91T mutation in 95% isolates. But this type of mutation is not related with flouroquinolone resistance. However there were some other isolates (4/102) which showed co-existence of two mutations in hybridization pattern. The combination of mutation was (D94Awith D94H), (S91P with D94G), (D94G with D94N/Y) and (A90V with D94G).

As the study aimed to determine FQ resistance in MDR patients, most of the isolates (430) were resistant to INH and RIF, 57 were RIF mono-resistant and 34 INH mono-resistant and 41 were drug sensitive cases. There are the findings which suggest increase FQs-R during MDR-TB. However, the resistance of FQ in non-MDR-TB may have acquired from exposure to FQ prior to TB diagnosis (Kim et al., 2019).


Discussion:

A number of claims in the first two paragraphs lack appropriate references.
Some of the conclusions are not supported by the evidence presented. Details of the population and methods are lacking, but it appears likely that the approach taken selected for patients with FQ-R TB (because they had a history of previous treatment, and had persistent MTBDRsl positivity after some amount of treatment) and does not reflect the overall prevalence of FQ-R among clinical TB. It is also not clear what resistance would have been identified had these patients been tested at the time of initial diagnosis, as the authors recommend doing. (Perhaps less resistance would have been detected, if this is truly emergent resistance or mixed infections, or if other patients with a quicker bacteriologic response to therapy had also been included.)

Response: The study focused on MDR-TB patients and presumptive multi drug resistance isolates were collected. The patients were not tested before for susceptibility to second line drugs. We could not study emergent FQ strains which transit from FQ-sensitive to FQ resistance whilst TB therapy because of the lack of access to the patient’s information. The study represents the burden of fluoroquinolone resistance in MDR-TB patients regardless of FQ antibiotic therapy. However, relevance of genotypic and phenotypic resistance is important to accurately predict FQ-R. Even though we found FQ-R in MTB susceptible isolates but it does not reflect its true prevalence in these patients (Pages 10-11; lines 441-445). The susceptibility testing of FQ at initial time of diagnosis is recommended due to its high prevalence in MDR-TB study isolates and inadequacy for testing before starting second line drug treatment.