Title: Transmitted Extended-spectrum XDR Tuberculosis in Beijing with Discordant Whole-Genome Sequencing Analysis

Authors: Hao Li1,2†, Masood ur Rehman Kayani2†, Yunting Gu3†, Xiaobo Wang3, Ting Zhu2,4, Hongfei Duan5, Yifeng Ma3, Hairong Huang3‡, and Babak Javid1,4‡*

Affiliations:
1 Centre for Infectious Diseases Research, Tsinghua University School of Medicine, Beijing, CHINA, 100084
2 Tsinghua University School of Life Sciences, Beijing, CHINA, 100084
3 National Clinical Laboratory on Tuberculosis, Beijing Tuberculosis and Thoracic Tumor Institute, Beijing Chest Hospital, Capital Medical University, Beijing, CHINA, 101149
4 Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Hangzhou, CHINA, 310003
5 Department of Tuberculosis, Beijing Tuberculosis and Thoracic Tumor Institute, Beijing Chest Hospital, Capital Medical University, Beijing, CHINA, 101149

† These authors contributed equally to the work
‡ These authors contributed equally to the work
* To whom correspondence should be addressed: huanghairong@tb123.org or bjavid@gmail.com

Running title: Highly drug-resistant tuberculosis in China
Abstract:

Drug-resistance to tuberculosis remains a major public health threat. Here, we report two cases of extended-spectrum XDR (XXDR) tuberculosis, resistant to most first and second-line agents. Correlation of whole-genome sequencing (WGS) and phenotypic testing was discordant, suggesting over-reliance on WGS may miss clinically relevant resistance in extensively drug-resistant disease.

Key words: drug-resistant tuberculosis, whole genome sequencing, XDR-tuberculosis
Report of Cases

Patient EF, a 64 year old HIV negative Han Chinese male was diagnosed with sputum-culture positive tuberculosis at another hospital in 2011. Co-morbidities included type II diabetes and chronic hepatitis C infection. Initial drug susceptibility testing (DST) suggested a strain of *M. tuberculosis* that was resistant to rifampicin, isoniazid, ethambutol and streptomycin, therefore he was initiated on levofloxacin, para-amino-salicylate (PAS) and amikacin. Over the course of the next 45 days, the patient continued to lose weight (10 kgs) and the patient was transferred to the Beijing Chest Hospital where an extended DST panel was performed on a newly isolated strain (strain 8897) (Table 1). In the meantime, his regime was changed to rifapentine, high dose isoniazid, ethambutol, pyrazinamide and levofloxacin. The patient remained sputum culture positive and subsequently succumbed to newly diagnosed hepatic cancer in January 2013.

Patient ZX, a 30 year old man, was initially diagnosed with sputum smear positive tuberculosis in 2010 at another hospital after presenting with a cough and night sweats. He was treated with standard therapy (rifampicin, isoniazid, pyrazinamide and ethambutol) with initially good outcome, although his cough persisted. After 6 months, the patient remained sputum culture positive, and DST indicated that the *M. tuberculosis* was resistant to rifampicin, isoniazid, streptomycin, capreomycin, kanamycin, amikacin and ofloxacin, but remained susceptible to ethambutol, protonamide, PAS and levofloxacin. The patient’s treatment was therefore changed to pyrazinamide, ethambutol and protonamide, as well as three months of PAS. By September 2012, further DST revealed novel resistance to PAS and protonamide, but the strain was now sensitive to ethambutol, levofloxacin, moxifloxacin and capreomycin. CT imaging of the chest suggested an increase in disease burden, therefore he was transferred to the Beijing Chest
Hospital and a newly isolated tuberculosis strain (strain 11500) re-tested for drug sensitivities. The patient was not diabetic, and tested negative for HIV, HBV and HCV. His antibiotic regime was changed to pyrazinamide, ethambutol, clofazamine, moxifloxacin, linezolid and capreomycin. Due to inadequate clinical response, his regime was changed to rifapentine, levofloxacin, cycloserine, protonamide and amoxicillin-clavulanate in February 2013. His sputum converted to become culture negative by August 2014 and has remained so, therefore he was kept on this regimen for at least 20 months following sputum culture conversion.

DST on isolated strains was performed by the indirect proportion method on LJ slopes for rifampicin, rifabutin, rifapentine, isoniazid, streptomycin, ethambutol, amikacin, capreomycin, ofloxacin, levofloxacin, protonamide, PAS as the initial test and by the critical concentration method by BACTEC MGIT960 (MGIT) growth indicator tubes after reviving the strain from deep frozen storage for first and second line antibiotics according to WHO guidelines and for the following antibiotics that were available for testing: minocycline (1µg/ml), linezolid (1µg/ml), co-trimoxazole (trimethoprim 38µg/ml and sulfamethoxazole 8µg/ml). The strain derived from patient EF (8897) was resistant to all but linezolid and co-trimoxazole (Table 1). Furthermore, there was low-level resistance to ethambutol and capreomycin. There were discordant results in susceptibility to PAS and ethionamide: by the indirect proportion method the strain was resistant, but it proved susceptible when tested by MGIT. The strain from patient ZX (11500) was only sensitive to linezolid and co-trimoxazole, and was resistant to the other 17 agents tested (Table 1).

For genomic analysis, the strains were retrieved and genomic DNA extracted from logarithmically growing cells by standard methods(1). The DNA was sent for WGS on the
HiSeq2000 (Illumina) platform with an insert size of 500bp and a read length of 90bp. The mean coverage of the strains was 142x for strain 8897 and 172x for strain 11500. The raw sequencing reads were processed using a custom perl script to remove adapter contamination. Next, the low quality bases were trimmed from the reads and the high quality data used for alignment using bwa tools using the H37Rv genome (GenBank accession no.NC_000962) as a reference. Duplicates were marked and excluded from further analysis and SNPs were called using samtools. We used the software vcf tools to generate the SNP list in vcf format that was annotated using SnpEff. SNPs that were associated with antibiotic resistance (1) are reported in Table 1 (concordant phenotype-genotype are shown in **bold**). The raw sequencing reads of the two strains can be accessed on NCBI’s sequence read archive (SRA) database with accession number SRP058024.

Drug-resistance is a major threat to attempts to control tuberculosis. Multi-drug-resistant tuberculosis (MDR-TB), resistant to rifampicin and isoniazid, was first defined in the re-emergence of TB, fueled by HIV, in 1980s New York; with extensively-drug-resistant tuberculosis (XDR-TB), MDR-TB resistant to quinolones and injectables, diagnosed in South Africa in 2006. Of great concern, it is estimated that only a minority (<7%) of cases of multi-drug-resistant tuberculosis (MDR-TB) were adequately diagnosed and appropriately treated(2). In the last decade, sporadic cases of tuberculosis resistant to almost all first- and second-line agents have emerged, initially in Italy and Iran(3, 4), India(5) and most recently South Africa(6). In total, these highly resistant cases of tuberculosis number 30 cases, but this is almost certainly an under-estimate due to the resource-limiting setting in which the vast majority of tuberculosis is endemic.
Here, we report two cases of tuberculosis with strains that show phenotypic resistance to an extended panel of anti-microbials, well beyond the definitions of extensively drug-resistant (XDR) tuberculosis, which we have termed extended-spectrum extensively drug-resistant (XXDR) tuberculosis in keeping with prior reports (7-9). Phenotypically, they resemble reports of “totally drug-resistant tuberculosis” (TDR) reported in the literature recently (3-6). However, since the majority of those strains were not assessed against newer agents such as linezolid (10) and bedaquiline (11), we prefer not to use TDR, but XXDR as the descriptive term, whilst acknowledging that for now, it does not have a strict definition.

Although in one of the cases (ZX), poor compliance with taking medication was likely a factor in a successively drug-resistant strain, in the other case, EF, the first strain isolated and tested for drug-resistance was already highly resistant, raising the possibility that the patient was infected by the XXDR strain. There is some debate about whether XDR strains have a fitness cost or not, although MDR strains appear not to (12). Regardless, the relative immune paresis of the patient, with diabetes and chronic viral hepatitis and possibly an occult hepatoma will have probably made the patient more susceptible to infection, even by a strain that might have exhibited reduced fitness. For patient ZX, there were many different susceptibility results, performed at other hospitals, which we have not independently verified – and report in Table 1 only the DST performed on the first isolated strain (11500) at the Beijing Chest Hospital, and on which WGS was performed. It is possible that the patient was re-infected with the XXDR strain, on the background of an initial infection with a more susceptible strain, explaining the widely varying results. WGS on his isolated strain did not, however, suggest co-infection (1).
The use of WGS for diagnosis of drug-resistance is not yet feasible in the developing world, and has only been used in a research capacity in resource-rich nations(1) although it will likely enter routine practice in such countries within the next few years. There are several advantages to use of WGS for diagnosis of culture-positive tuberculosis. Extensive information regarding drug-susceptibility can be gained within a relatively short time span compared with phenotypic DST, which can take months, where available. Furthermore, phenotypic testing of several agents, including the front-line agent pyrazinamide is technically difficult, which makes genetic prediction of susceptibility highly attractive. Nonetheless, the genotype-phenotype correlation between many antibiotics is not 100% robust(13). It is easier to “rule-in” resistance by identification of a bona fide resistance mutation, than to rule it out(1). Moreover, there is considerable uncertainty regarding the significance of several SNPs that have been associated with resistance in clinical samples, without extensive in vitro experimental verification (e.g. with regards Rv3728 and capreomycin resistance –(14)). Not all SNPs in certain genes highly associated with resistance (e.g. pncA and pyrazinamide resistance or gyrA and quinolone resistance) are necessarily responsible for drug-resistance.

In both strains, WGS failed to identify causative mutations for the resistance to amikacin, kanamycin and capreomycin. Mutations in rrs and eis and tlyA account for most resistance to these agents, although the proportion of phenotypically resistant strains that lack mutations in these genes varies from 5-20% in studies(13, 15-17) Furthermore, in strain 8897, no genetic basis for resistance to pyrazinamide was identified, and WGS would have predicted resistance to PAS due to a mutation in thyA whereas phenotypic testing revealed a discordant result. An
advantage of WGS is the ability to predict susceptibility to agents for which no DST is locally available such as cycloserine, clofazamine, bedaquiline, and delamanid(1), to which both strains were susceptible by sequence results. Whether amoxillin-clavulanate is effective in tuberculosis is controversial, there are reports of its utility(18), and patient ZX has responded well to his regimen, despite only two of the agents (cycloserine and amoxillin-clavulanate) being potentially effective.

In summary, we report two cases of XXDR within the Beijing region of China. There is evidence of transmissibility for one of the strains, and although WGS may eventually provide a mechanism to rapidly identify drug-resistant tuberculosis to enable more effective management, further research to validate genotype-phenotype causation is required before it can fully replace phenotypic testing(1, 19).

Acknowledgements
The work was supported by the research funding from Infectious Diseases Special Project, Minister of Health of China (2012ZX10003002) to HH and from Tsinghua University to BJ. BJ is a Tsinghua-Janssen Scholar. All the type isolates and clinical isolates used in this study were obtained from the Clinical Database and Sample Bank of Tuberculosis of Beijing (D131100005313012), National Clinical Lab on Tuberculosis, Beijing Chest Hospital.

Conflicts of Interest
The authors report no conflicts of interest. BJ is a Tsinghua-Janssen Scholar, but Janssen had no role in the design, initiation or publication of the study.

**Author Contributions**

HH and BJ conceived of the project. HL, MK, YG, YM and XW performed research. HL, MK, TZ, HH and BJ analyzed data. HD assisted in patient follow-up. HL, MK and BJ wrote the paper.

**References**

extremely drug resistant (XXDR) tuberculosis strains: transmission and atomic force observation.


Table 1 Legend

Phenotype-Genotype Sensitivities and Correlations for XXDR Strains.

Phenotypic DST results from both strains, as measured by MGIT (all agents except protonamide) and indirect proportion method (all agents except those marked by *) are shown. “Fluoroquinolones” refers to testing of ofloxacin, levofloxacin and moxifloxacin – all of which gave concordant results in all assays. Agents where there is phenotype-genotype correlation for “ruled in” resistance are bolded, as are known resistance-causing mutations. SNPs of unknown significance are unbolded. †For minocycline, *M. tuberculosis* is inherently resistant to macrolides unless there is a mutation in the *whiB7* gene [3]. Mutations in *whiB7* and its promoter are also associated with resistance to other antibiotics such as aminoglycosides, and lincosamides and tetracyclines, for which phenotypic DST was not performed in this study. In strain 8897 there were discordant results in phenotypic DST by the proportion and MGIT methods for PAS and ethionamide (both were reported as resistant by proportion, but were sensitive by MGIT) – marked as R\(^\text{T}\). N refers to no mutations identified in the gene of interest.
<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Phenotypic DST</th>
<th>Gene AA change</th>
<th>Antibiotics</th>
<th>Phenotypic DST</th>
<th>Gene AA change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid</td>
<td>R</td>
<td>katG S315T</td>
<td>Isoniazid</td>
<td>R</td>
<td>katG S315T</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>R</td>
<td>rpoB S450L A1075A</td>
<td>Rifampicin</td>
<td>R</td>
<td>rpoB S450L A1075A</td>
</tr>
<tr>
<td>Rifabutin</td>
<td>R</td>
<td>rpsC W484G</td>
<td>Rifabutin</td>
<td>R</td>
<td>rpsC W484G</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>R (low level)</td>
<td>embC R027R</td>
<td>Ethambutol</td>
<td>R</td>
<td>embC R027R</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>R</td>
<td>rpsL K43R</td>
<td>Streptomycin</td>
<td>R</td>
<td>rpsL K43R</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>R</td>
<td>pncA N</td>
<td>Pyrazinamide</td>
<td>R</td>
<td>pncA G102A</td>
</tr>
<tr>
<td>Amikacin</td>
<td>R</td>
<td>rpsA R212R</td>
<td>Amikacin</td>
<td>R</td>
<td>rpsA R212R</td>
</tr>
<tr>
<td>Capreomycin</td>
<td>R (low level)</td>
<td>rts L111</td>
<td>Capreomycin</td>
<td>R</td>
<td>rts L111</td>
</tr>
<tr>
<td>PAS</td>
<td>R</td>
<td>thyA L111</td>
<td>PAS</td>
<td>R</td>
<td>thyA L111</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>R</td>
<td>gyrA S125Y</td>
<td>Fluoroquinolones</td>
<td>R</td>
<td>gyrA A09Y, E21Q, S95T, G668D</td>
</tr>
<tr>
<td>Ethionamide</td>
<td>R</td>
<td>ethA L15P</td>
<td>Ethionamide</td>
<td>R</td>
<td>ethA S266R R261W</td>
</tr>
<tr>
<td>Minocycline*</td>
<td>R</td>
<td>whiB7† N</td>
<td>Minocycline*</td>
<td>R</td>
<td>whiB7† N</td>
</tr>
<tr>
<td>Linezolid *</td>
<td>S</td>
<td>rrl N</td>
<td>Linezolid *</td>
<td>S</td>
<td>rrl N</td>
</tr>
<tr>
<td>TMP-SMX *</td>
<td>S</td>
<td>dfrA N</td>
<td>TMP-SMX *</td>
<td>S</td>
<td>dfrA N</td>
</tr>
</tbody>
</table>

* Denotes resistance to high levels, ** indicates resistance to low levels.