Evaluation of a Liquid Chromatography-Tandem Mass Spectrometry Assay for Second-line Tuberculosis Drug Concentrations in Small Hair Samples

CURRENT STATUS: UNDER REVIEW

BMC Infectious Diseases  BMC Series

John Metcalfe
University of California San Francisco
✉ John.Metcalfe@ucsf.edu Corresponding Author

Peter Bacchetti
University of California San Francisco

Ali Esmail
University of Cape Town Lung Institute

Andrew Reckers
University of California San Francisco

David Aguilar
University of California San Francisco

Anita Wen
University of California San Francisco

Shu Huo
University of California San Francisco

Winnie R. Muyindike
Mbarara University of Science and Technology

Judith A. Hahn
University of California San Francisco

Keertan Dheda
University of Cape Town Lung Institute

Monica Gandhi
University of California San Francisco
Abstract
Background Treatment monitoring of multidrug-resistant (MDR) and extensively drug-resistant tuberculosis (XDR-TB) in resource-limited settings is challenging. We developed a multi-analyte assay for eleven anti-TB drugs in small hair samples as an objective metric of drug exposure.

Methods Small hair samples were collected from participants at various timepoints during directly-observed MDR/XDR-TB treatment at an inpatient tertiary referral facility in South Africa (DR-TB cohort). We assessed an LC-MS/MS index panel assay including isoniazid, ethambutol, pyrazinamide, levofloxacin, moxifloxacin, ethionamide, prothionamide, linezolid, clofazimine, pretomanid, and bedaquiline against a reference standard of inpatient treatment records. Because treatment regimens prior to hospitalization were not available, we also analyzed specificity (for all drugs except isoniazid) using an external cohort of HIV-positive patients treated for latent TB infection with daily isoniazid (HIV/LTBI cohort) in Uganda. Results Among the 57 DR-TB patients (58% with pre-XDR/XDR-TB; 70% HIV-positive) contributing analyzable hair samples, the sensitivity of the investigational assay was 94% or higher for all drugs except ethionamide (58.5%, 95% confidence interval CI, 40.7-99.9). Assay specificity was low across all tested analytes within the DR-TB cohort; conversely, assay specificity was 100% for all drugs in the HIV/LTBI cohort. Conclusions We developed an 11-drug panel assay to quantitatively ascertain drug exposure within second- and third-line DR-TB treatment regimens. Because hair concentrations reflect long-term exposure, multiple successive regimens commonly employed in DR-TB treatment may result in apparent false-positive qualitative and falsely elevated quantitative hair drug levels when prior treatment histories are not known.

Background
An estimated 1.5 million people globally have multidrug-resistant tuberculosis (MDR-TB), defined by resistance to the essential first-line agents isoniazid (INH) and rifampin (RIF). MDR-TB treatment requires prolonged treatment courses of toxic, less effective second-line drugs and is successful in only 48% of patients. Treatment of MDR-TB in the setting of HIV co-infection is further complicated by increased pill burden, overlapping drug toxicities, poor drug absorption, and high mortality. In addition, given that MDR-TB treatment is increasingly provided in community settings, better tools
are needed to assess adherence and monitor therapy to improve individual outcomes and reduce transmission.

Self-reported adherence can be limited by recall bias, poor recollection, or a desire to please the provider. In the setting of HIV treatment and prevention, this has led to exploration of alternative methods to assess adherence. Many drugs are incorporated from the systemic circulation into hair as it grows. In a manner analogous to glycosylated hemoglobin A1c (HbA1c) that provides information on average glucose levels over prolonged periods, the concentration of medication in hair reflects uptake from the systemic circulation over weeks to months. Assuming a normal scalp hair growth rate (one centimeter (cm)/month), historic drug exposure can be ascertained via segmental analysis, determining drug levels in various sections of hair with successive distance from the root. The frequency of ingestion (adherence) and pharmacokinetics of a drug are the primary determinants of hair drug concentration, and most drugs are stable in hair for months at room temperature.

Recently, we developed and validated a multi-drug assay panel for measuring eleven second-line anti-tuberculosis drug concentrations in small hair samples. Here, we report a clinical diagnostic accuracy study of this investigational assay versus treatment administration records among inpatients with DR-TB in South Africa. Because ascertainment of successive treatment regimens among patients with XDR-TB is challenging, and because carry-over of drug has been noted beyond the strict length guidelines of one centimeter/month, we validated assay specificity in an external cohort of patients receiving isoniazid alone for latent TB infection (LTBI), where ingestion of second-line drugs would be highly improbable.

Methods

Study Population and Sample Collection

The primary study cohort of adult (aged 3 18 years) patients with MDR-, pre-XDR, and XDR-TB was recruited as a convenience sample from July 12, 2016 to December 6, 2017 at Brooklyn Chest
Hospital (BCH), an inpatient referral facility for drug-resistant TB in Cape Town, Western Cape, South Africa. During the study period, pre-XDR and XDR-TB patients were treated with 24 weeks of bedaquiline within an optimized, individualized background regimen that could include levofloxacin, linezolid, and/or clofazimine. The total number of patients approached but declining to participate was not recorded. In the primary cohort, hair was collected at various times during the patient’s final treatment regimen (i.e., prior to last known treatment outcome).

A secondary study cohort of adult HIV-positive patients on ART (n=93) with LTBI was enrolled in a longitudinal cohort study at the Mbarara Regional Referral Hospital in Uganda. The participants in this study had LTBI confirmed by tuberculin skin testing (³5 mm induration) with active and prior TB and TB drug exposure ruled out; all participants were given daily self-administered 300 mg INH. This cohort was chosen for a specificity analysis of our multi-analyte assay given the low likelihood of exposure to second-line anti-TB medications but high HIV prevalence (n=19 individuals, 28 samples). In this secondary cohort, hair was collected after three and six months of INH and analyzed at both timepoints when available.

Small hair samples were collected using previously described methods. Briefly, from all participants with scalp hair and who consented for hair collection, 20–30 strands of hair were cut from the occipital region. The distal end of the hair sample was marked with a small piece of tape to denote directionality, and the hair was stored in aluminum foil at room temperature. Each participant provided written informed consent, and ethical approval was obtained from the University of Cape Town Human Research Ethics Committee (187/2016), the Mbarara University of Science and Technology Research Ethics Committee (11/10-16), and the UCSF Human Research Protection Program. All hair samples were analyzed at the University of California, San Francisco (UCSF) TB Hair Analysis Laboratory.

Investigational LC-MS/MS assay
We analyzed the samples using our validated liquid chromatography- tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantitation of eleven MDR-TB drugs in small hair samples.\textsuperscript{14} Briefly, hair strands (~20-30 cut to 3 cm and weighed to 2 mg) were pulverized and extracted with methanol. The hair extract was reconstituted to water with 1% formic acid before injection into the Agilent LC 1260 (Agilent Technologies, Sta. Clara, CA) attached to an AB Sciex API 5500 mass spectrometer (AB Sciex, Foster City, CA). The analytes were separated by gradient elution on a Phenomenex Synergi Polar RP column (2.1 x 100, 2.5 μm particle size, Phenomenex, Torrance, CA) using water with 1% formic acid as mobile phase A (MPA) and acetonitrile with 0.1% formic acid as mobile phase B (MPB). Ionization of each analyte in the mass spectrometer was achieved using electrospray ionization (ESI) in positive polarity, and mass scanning was performed via multiple reaction monitoring (MRM). Quantification of each analyte was performed by isotope dilution method using deuterated, \textsuperscript{15}N- or \textsuperscript{13}C-labeled isotopologue of each drug standard. Data analysis was done using AB Sciex Analst 1.6 and AB Sciex MultiQuant 2.1 (AB Sciex, Foster City, CA) software packages. Diagnostic specificity was also monitored during method validation using hair samples from laboratory members who have not taken any of the drugs in the panel.\textsuperscript{14}

**Statistical Analysis**

The primary objective of the study was to determine the sensitivity and specificity of the investigational assay analyzing hair concentrations of INH, pyrazinamide (PZA), ethambutol (EMB), levofloxacin (LFX), moxifloxacin (MFX), linezolid (LZD), clofazimine (CFZ), bedaquiline (BDQ), pretomanid (PTM), ethionamide (ETH), and prothionamide (PTH), against a reference standard of known drug administration, according to data abstracted from inpatient treatment records. Inpatient treatment was administered by directly observed treatment (DOT); treatment administered prior to the hospital stay was administered according to programmatic standards at each peripheral clinic, which may or may not have included DOT. In the primary analyses, the reference standards for sensitivity and specificity were considered differently. For sensitivity, the reference standard was
considered ‘positive’ if the drug was taken for at least 14 days during the hair growth window. The hair growth window was defined as the interval from 94 to five days prior to hair collection. Due to prolonged half-lives, the hair growth window was defined as an interval of 800 to four and 420 to four days prior to hair collection for BDQ and CFZ, respectively (i.e., a time period encompassing roughly five half-lives). For specificity, the reference standard was considered ‘negative’ if the drug is not taken for any days in the previous 124 days prior to and including the day of hair collection. Because of the long half-lives of BDQ and CFZ, the reference standard was ‘negative’ if there was no known history of past use of these drugs at any time. Specificity was also calculated separately using a secondary, external cohort described above. Investigational assay analysis was performed independently of reference standard treatment data. The binomial exact method was used to calculate 95% confidence intervals using Stata 14.2.

Results

Participants

A total of 111 participants with DR-TB were enrolled from July 12, 2016 to December 6, 2017 (Figure 1). Among 54 participants (49%), directionality of the hair sample (i.e., differentiation of distal and proximal hair segments) could not be reliably determined. Thus, 57 participants with MDR/XDR-TB were eligible for inclusion in the study. These participants were predominantly female (98%, n=56/57) and 70% (n=40/57) were HIV-positive. A broad spectrum of phenotypic resistance patterns was represented, and participants had been on treatment for a median of 144 days (interquartile range (IQR) 50-337 days) prior to hair sampling (Table 1). The LTBI samples (n=28, from 19 persons) were collected from 42% females; all were HIV-positive and all were on ART.

Investigational Assay versus Treatment Administration Data in Primary and Secondary Cohort

The sensitivities of the investigational assay for the detection of administered drugs were 93.9% or higher for all drugs except ETH (Table 2). Specificity, however, was high for only a few drugs, and several had upper 95% confidence bounds below 50%. In contrast, specificity as ascertained within the secondary HIV/LTBI cohort was 100% (95% CI, 88-100%) for all analytes excepting INH, for which
specificity could not be ascertained due to INH treatment among all participants. Within the primary cohort, the distributions of above-LOD drug concentrations were substantially lower among those without known treatment histories (i.e., false-positives) relative to those with confirmed treatment histories (i.e., true positives), with the first quartile among true positives higher than the third quartile among false positives for PZA, EMB, LFX, MFX, LZD, CFZ, and BDQ (Table 2).

**Discussion**

Measuring medication concentrations in small hair samples can provide a long-term metric of adherence and exposure. We assessed the diagnostic accuracy of a novel multi-analyte hair assay for eleven anti-TB drugs used in second-line and salvage regimens. In a primary cohort of patients with extensive drug resistance and multiple prior TB regimens, our assay identified drug in hair with high sensitivity but apparent poor specificity. In order to determine the etiology of the high proportion of “false-positives” in the primary cohort, we assessed specificity in a cohort of patients with a very low likelihood of exposure to anti-TB drugs other than INH. We determined that apparent false-positive assays in the primary cohort are likely due to prior undocumented treatment histories. Future clinicians utilizing hair PK assays for treatment monitoring in similar populations should be aware that apparently false-positive test results could potentially be due to actual drug exposure in the course of prior, unknown treatment histories.

Xenobiotics are thought to incorporate into hair (a pure collagen matrix in mammals) through passive diffusion into the growing hair follicle via surrounding arterial capillaries, through deposition via sweat and sebum after emergence from the scalp, and through external contamination (e.g., through physical contact). For a given absolute drug exposure and hair growth rate, incorporation of specific drugs into hair is a function of basicity, lipophilicity, and melanin content of hair, all of which increase hair drug concentrations. As an objective treatment monitoring tool, the major practical advantage of the hair biomatrix relative to blood or urine is the extended surveillance window for drug exposure over prior weeks to months, rather than hours to days. A heuristic for estimating the period of drug exposure assumes a constant 1 cm/month hair growth rate (e.g., a 2-cm segment of hair corresponds
to a two month period of drug exposure). However, a broader band of positivity from single doses of drugs have been noted in the forensic literature,\textsuperscript{17-19} occurring according to one investigator in approximately “1 case from 10 examinations.”\textsuperscript{20} This variability in the area over which incorporated drug can be distributed in the hair shaft can be due to actual rate of hair growth (range in healthy subjects of at least 0.3 to 1.8 cm/mo),\textsuperscript{19} rate of axial distribution of drug, and a number of other biologic characteristics.\textsuperscript{20}

We found a high sensitivity of our hair assay for all the DR-TB drugs except for ETH (most likely due to the poor incorporation of ETH in hair, as previously noted).\textsuperscript{14} Although patient management during the duration of our study in South Africa included inpatient treatment of MDR- or XDR-TB, second-line regimens are often started prior to final inpatient referral. In addition, diagnosis of drug-resistant TB typically takes up to two months within the National Health Service, and during this time most if not all participants in our study were treated with first-line regimens. A high or near-perfect specificity of our assay is supported by findings in our secondary cohort, as well as by the finding of substantially lower drug concentrations among individuals in our primary cohort without confirmed drug exposure in the hair growth window. Nevertheless, use of our assay in programmatic settings or in research settings involving retreatment for DR-TB will have to take account the possibility of prior successive TB treatment regimens in assessing hair drug concentrations.

An important limitation of our study is that sample collection at the beginning of the primary study failed to adequately delineate the proximal from the distal end of the hair thatch. This collection issue was corrected in the second half of the study, but approximately half of the samples could not be used as a result. Additional measures to ensure adequate labeling of the distal end of hair samples have been developed.

In conclusion, we assessed the diagnostic accuracy of an 11-drug MDR-TB panel assay to
quantitatively ascertain drug exposure within current treatment regimens. Since hair concentrations reflect long-term exposure, use of prior medications may be reflected in hair even after discontinuation. Among patients with drug-resistant TB, multiple successive regimens may result in apparent false-positive qualitative and falsely elevated quantitative hair drug levels.

**Abbreviations**

BDQ: Bedaquiline

CFZ: Clofazimine

DOT: Directly observed treatment

DR-TB: Drug resistant tuberculosis

EMB: Ethambutol

ESI: Electrospray ionization

ETH: Ethionamide

HbA1c: Hemoglobin A1c

INH: Isoniazid

LC-MS/MS: Liquid chromatography-tandem mass spectrometry

LFX: Levofloxacin

LTBI: Latent TB infection

LZD: Linezolid

MDR-TB: Multidrug-resistant tuberculosis

MFX: Moxifloxacin

MPA: Mobile phase A

MPB: Mobile phase B

MRM: Multiple reaction monitoring

PTH: Prothionamide

PTM: Pretomanid

PZA: Pyrazinamide

RIF: Rifampin
TB: Tuberculosis
XDR-TB: Extensively drug-resistant tuberculosis

Declarations

Acknowledgements

The authors would like to thank Marietjie Pretorius, clinical research coordinator, UCT. The authors further gratefully acknowledge the contributions of the participants of this study.

Authors’ contributions

Conception or design, JZM and PB; analysis and interpretation of data, JZM, PB, AR, DA, AW, SH, MG, and RG; drafting the work, JZM, PB, RG, SH, and MG; revising for important intellectual content, JZM, PB, MG, and RG; acquisition of data, JAH, AE, WRM, and KD.

Funding

This work was supported by NIH/ NIAID R01 AI123024 (P.I. Metcalfe, Gandhi) and NIH/NIAAA U01020776 (P.I. Hahn).

Availability of data and materials

The datasets generated and analyzed are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study received approval by the University of Cape Town Human Research Ethics Committee (187/2016), the Mbarara University of Science and Technology Research Ethics Committee (11/10-16), and the UCSF Human Research Protection Program. All subjects who agreed to participate in the research provided written informed consent to participate.

Consent for publication
No individual patient data is presented, therefore consent to publish was not requested.

**Competing interests**

The authors declare that they have no competing interests.

**References**

1. Global tuberculosis report 2018. Geneva: World Health Organization; 2018. Licence: CC BY-NC-SA 3.0 IGO. Accessed at https://www.who.int/tb/publications/global_report/en/.

2. O'Donnell MR, Wolf A, Werner L, Horsburgh CR, Padayatchi N. Adherence in the treatment of patients with extensively drug-resistant tuberculosis and HIV in South Africa: a prospective cohort study. *J Acquir Immune Defic Syndr.* 2014;67(1):22-29.

3. Wells CD, Cegielski JP, Nelson LJ, et al. HIV infection and multidrug-resistant tuberculosis: the perfect storm. *J Infect Dis.* 2007;196 Suppl 1:S86-107.

4. Gurumurthy P, Ramachandran G, Hemanth Kumar AK, et al. Malabsorption of rifampin and isoniazid in HIV-infected patients with and without tuberculosis. *Clin Infect Dis.* 2004;38(2):280-283.

5. Graham SM, Bell DJ, Nyirongo S, Hartkoorn R, Ward SA, Molyneux EM. Low levels of pyrazinamide and ethambutol in children with tuberculosis and impact of age, nutritional status, and human immunodeficiency virus infection. *Antimicrob Agents Chemother.* 2006;50(2):407-413.

6. Gandhi NR, Shah NS, Andrews JR, et al. HIV coinfection in multidrug- and extensively drug-resistant tuberculosis results in high early mortality. *Am J Respir Crit Care Med.* 2010;181(1):80-86.

7. Cox H, Ford N. Decentralisation of multidrug-resistant-tuberculosis care and management. *Lancet Infect Dis.* 2013;13(8):644-646.
8. Berg KM, Arnsten JH. Practical and conceptual challenges in measuring antiretroviral adherence. *Journal of Acquired Immunodeficiency Syndromes (JAIDS)*. 2006;43 Suppl 1:S79-87.

9. Kagee A, Nel A. Assessing the association between self-report items for HIV pill adherence and biological measures. *AIDS Care*. 2012;24(11):1448-1452.

10. Wertheimer BZ, Freedberg KA, Walensky RP, Yazdanapah Y, Losina E. Therapeutic drug monitoring in HIV treatment: a literature review. *HIV Clin Trials*. 2006;7(2):59-69.

11. Louissaint NA, Cao YJ, Skipper PL, et al. Single dose pharmacokinetics of oral tenofovir in plasma, peripheral blood mononuclear cells, colonic tissue, and vaginal tissue. *AIDS Res Hum Retroviruses*. 2013;29(11):1443-1450.

12. Baciu T, Borrull F, Aguilar C, Calull M. Recent trends in analytical methods and separation techniques for drugs of abuse in hair. *Anal Chim Acta*. 2015;856:1-26.

13. Metcalfe J, Bacchetti P, Gerona R, Esmail A, Dheda K, Gandhi M. Association of anti-tuberculosis drug concentrations in hair and treatment outcomes in MDR- and XDR-TB. *Eur Respir J Open*. 2019.

14. Gerona R, Wen A, Aguilar D, Shum J, Bacchetti P, Gandhi M, Metcalfe J. Simultaneous analysis of 11 medications for drug resistant TB in small hair samples to quantify adherence and exposure using a validate LC-MS/MS panel. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2019;1125:121729.

15. Wennig R. Potential problems with the interpretation of hair analysis results. *Forensic Sci Int*. 2000;107(1-3):5-12.

16. Hickey MD, Salmen CR, Tessler RA, et al. Antiretroviral concentrations in small hair samples as a feasible marker of adherence in rural Kenya. *J Acquir Immune Defic Syndr*. 2014;66(3):311-315.
17. Robertson M, Staikos V. Segmental analysis of hair in an alleged drug facilitated sexual assault—the pros and cons of segmental analysis and why results are rarely black and white. *TIAFT Bull.* 2011;41:18-19.

18. Gunther KN, Johansen SS, Wicktor P, Banner J, Linnet K. Segmental Analysis of Chlorprothixene and Desmethylchlorprothixene in Postmortem Hair. *J Anal Toxicol.* 2018;42(9):642-649.

19. Henderson GL, Harley MR, Zhou C, et al. Incorporation of isotopically labelled cocaine and metabolites into human hair: 1. Dose-response relationships. *J Anal Toxicol.* 1996;20:1-12.

20. Kintz P. Issues about axial diffusion during segmental hair analysis. *Ther Drug Monit.* 2013;35(3):408-410.

Tables

Table 1. Demographic and Clinical Characteristics of Participants at Enrollment

| Characteristic                                      | MDR/XDR-TB Cohort (primary) |
|-----------------------------------------------------|-----------------------------|
| Female sex — no./total no. (%)                      | 56/57 (98%)                 |
| Median age (range) — yr                              | 33 (22-58)                  |
| Time from most recent diagnosis to small hair sample collection (median, IQR) — days | 144 (50-337) |
| Drug-resistance status — no./total no. (%)          |                             |
| pre-XDR/XDR-TB                                      | 36/57 (63%)                 |
| MDR-TB                                              | 16/57 (28%)                 |
| Poly-resistance                                     | 1/57 (2%)                   |
| RR-TB                                                | 4/57 (7%)                   |
| Treatment Outcome — no./total no. (%)               |                             |
| Loss to follow-up                                    | 14/57 (25%)                 |
| Treatment failure                                   | 6/57 (11%)                  |
| Relapse                                              | 3/57 (5%)                   |
| Continuing Treatment                                | 18/57 (32%)                 |
| Cure                                                 | 15/57 (26%)                 |
| Complete                                             | 1/57 (2%)                   |

Table 2. Sensitivity and Specificity of the Investigational Assay, with Treatment History as the Reference Standard
| Drug          | no. detected/no. taking drugs | % (95% CI) | no. not detected/no. not taking drugs |
|--------------|------------------------------|------------|--------------------------------------|
| Isoniazid    | 31/33                        | 93.9 (79.8-99.3) | 12/20                                |
| Pyrazinamide | 54/54                        | 100 (93.3-100)  | 0/2                                  |
| Ethambutol   | 45/45                        | 100 (92.1-100)  | 0/10                                 |
| Levofloxacin | 30/30                        | 100 (88.4-100)  | 2/26                                 |
| Moxifloxacin | 41/41                        | 100 (91.4-100)  | 0/13                                 |
| Linezolid    | 22/23                        | 95.6 (78.1-99.9) | 26/32                                |
| Clofazimine  | 34/34                        | 100 (89.7-100)  | 2/22                                 |
| Bedaquiline  | 27/27                        | 100 (87.2-100)  | 7/28                                 |
| Pretomanid   | 1/1                          | 100 (2.5-100)   | 55/56                                |
| Ethionamide  | 20/34                        | 58.5 (40.7-99.9) | 20/20                                |

* For sensitivity, the reference standard was considered ‘positive’ if the drug was taken for at least 14 days during the hair growth window.

† For specificity, the reference standard was considered ‘negative’ if the drug is not taken for any days in the previous 124 days prior to and including the day of hair collection. For BDQ and CFZ, the reference standard was ‘negative’ if there was no known history of past use of the drug at any time.

‡ Not enough data points for comparison.

Figures

| Hair samples collected, n=111 | Hair samples collected, n=28 |
|-------------------------------|-------------------------------|
| Brooklyn Chest Hospital       | Mbarara Regional Referral Hospital |
| Cape Town, South Africa       | Mbarara, Uganda              |
| July 12, 2016 to December 6, 2017 | August 2017 to August 2018 |

Samples excluded:
Directionality questionable (n=54)

MDR/XDR-TB cohort, n=57
Sensitivity Assessed

HIV/LTBI cohort, n=28
Specificity Assessed

Figure 1
Participant Enrollment and Testing
