The Journal of Infectious Diseases

BRIEF REPORT

Within-Host Heterogeneity of *Mycobacterium tuberculosis* Infection Is Associated With Poor Early Treatment Response: A Prospective Cohort Study

Ted Cohen,[1] Leonid Chindelevitch,[1] Reshma Misra,[4] Maria E. Kempner,[2] Jerome Galea,[3] Prashini Moodley,[4] and Douglas Wilson[5]

[1]Department of Epidemiology of Microbial Diseases, Yale School of Public Health, New Haven, Connecticut; [2]Brigham and Women's Hospital, and [3]Partners in Health, Boston, Massachusetts; [4]Infection Prevention and Control, University of KwaZulu-Natal, Durban, and [5]Department of Internal Medicine, Edendale Hospital, University of KwaZulu-Natal, Pietermaritzburg, South Africa

The clinical management of tuberculosis is a major challenge in southern Africa. The prevalence of within-host genetically heterogeneous *Mycobacterium tuberculosis* infection and its effect on treatment response are not well understood. We enrolled 500 patients with tuberculosis in KwaZulu-Natal and followed them through 2 months of treatment. Using mycobacterial interspersed repetitive units–variable number of tandem repeats genotyping to identify mycobacterial heterogeneity, we report the prevalence and evaluate the association of heterogeneity with treatment response. Upon initiation of treatment, 21.1% of participants harbored a heterogeneous *M. tuberculosis* infection; such heterogeneity was independently associated with a nearly 2-fold higher odds of persistent culture positivity after 2 months of treatment (adjusted odds ratio, 1.90; 95% confidence interval, 1.03–3.50).

Keywords. within-host heterogeneity; mixed infection; coinfection; tuberculosis; HIV/TB.

Previous reports suggest that within-host genetic diversity of *Mycobacterium tuberculosis* infection, especially when comprising isolates that differ in terms of drug susceptibility, can compromise the performance of diagnostic tools [1], reduce treatment effectiveness [2–4], and alter the expected population-level effects of interventions [5]. We describe a prospective study of 500 patients with pulmonary tuberculosis from an area of high tuberculosis incidence and human immunodeficiency virus (HIV) prevalence. We aimed to estimate the prevalence of within-host diversity (ie, complex infection), identify factors associated with complex infection, and determine whether complexity affects early treatment response.

METHODS

Study Setting and Design

Our study was based in the Edendale Hospital catchment area, in Pietermaritzburg, KwaZulu-Natal (KZN), the South African province with the highest rates of incident tuberculosis in the country. The tuberculosis notification rate in KZN was 889 cases/100 000 individuals in 2012, and approximately 60% of individuals with new tuberculosis cases are coinfected with HIV [6].

Between June 2011 and November 2012, we enrolled 500 patients with tuberculosis from 5 primary healthcare clinics. All smear-positive adults (age, ≥18 years) who had not initiated treatment for the current episode of tuberculosis were eligible. Study nurses approached potential participants on site when smear microscopy results were communicated.

We collected 2 pretreatment sputum specimens from each study participant in spot-spot collection on the day of enrollment. For participants unable to produce a sufficient volume of sputum, study nurses used saline nebulizers for sputum induction. We administered a questionnaire that ascertained demographic characteristics (eg, sex, age, and race) and medical information (eg, previous tuberculosis treatment, HIV infection status, and use of antiretroviral drugs). HIV testing was recommended to all participants regardless of reports of previous test results. Treatment charts were reviewed for the latest $CD4^+$ T-cell count among individuals reporting positive results of previous HIV tests. Immediately after enrollment, treatment was started with rifampicin, isoniazid, ethambutol, and pyrazinamide combination tablets in accordance with current South African Department of Health guidelines. Streptomycin was discontinued in the retreatment regimen during mid-2012.

We followed each participant for 2 months and recorded the number of doses taken. At this follow-up visit, we collected 1 additional sputum specimen; induction with saline nebulizers was done as needed. Study data were collected on paper forms and double entered into REDCap (Research Electronic Data Capture) [7]. The biomedical research ethics committee at the University of KwaZulu-Natal and the KwaZulu-Natal Department of Health, as well as the Partners Human Research Committee (Boston, Massachusetts), approved the protocol (2009-P-001938) for this study.

Laboratory Testing

Sputum samples were refrigerated immediately and transported to the clinical laboratory at the University of KwaZulu-Natal (UKZN; Durban, South Africa) within 24 hours. To minimize

Received 23 December 2015; accepted 6 January 2016; published online 14 January 2016.
Correspondence: T. Cohen, Yale School of Public Health, 60 College St, New Haven, CT 06510 (theodore.cohen@yale.edu).

The Journal of Infectious Diseases® 2016;213:1796–9

DOI: 10.1093/infdis/jiw014

elimination of minority subpopulations of mycobacteria, we adopted a slightly gentler sputum decontamination procedure, using a 4% NALC-NaOH solution, compared with a 5% NALC-NaOH solution that is typically used in this laboratory.

All laboratory tests (with the exception of mycobacterial interspersed repetitive units–variable number of tandem repeats [MIRU-VNTR] typing) were conducted at UKZN. Sputum specimens from individual patients were pooled, and microscopy was performed on the decontaminated specimens, using Ziehl-Neelsen staining. To improve the sensitivity for detecting minority subpopulations [8], we cultured specimens on 3 solid plates (Middlebrook 7H11 agar) and in liquid medium (Mycobacteria Growth Indicator Tube [MGIT] 960). Direct susceptibility tests were also performed on the decontaminated specimen, using the 1% agar proportion method, and cultures were read in a double-blinded fashion after 3 weeks. For all cultures that yielded resistant *M. tuberculosis* or were contaminated, repeat samples were obtained for analysis by either MGIT or solid cultures.

Mycobacterial colonies were swept from half of each of the 3 plates and suspended in 200 μL of sterile distilled water. From MGIT cultures, 1 mL of liquid culture was aspirated into 2-mL Eppendorf tubes and centrifuged at 15 000 *g* for 15 minutes. The supernatant was discarded, and mycobacterial pellets were resuspended in 200 μL of sterile distilled water, incubated at 95°C for 30 minutes, and centrifuged at 15 000 *g* for 1 minute. The supernatant was harvested, and 100 μL of the lysate was inoculated into 96-well plates and shipped for 24-loci MIRU-VNTR typing at Genoscreen (Institute Pasteur, Lille, France) [9]. The lysates were stored and transported at −20°C.

Classification of Heterogeneous Infections

We defined MIRU-VNTR patterns that included at least 1 locus with >1 copy number as heterogeneous infections [10]; all other patterns were designated as simple infections.

Statistical Analysis

We used multivariable logistic regression to assess (1) whether any factors were associated with the presence of a heterogeneous infection at the time of treatment initiation and (2) the independent association of baseline factors with culture conversion after 2 months of treatment. For our multivariable models, we included all variables and report unadjusted and adjusted estimates in Tables 1 and 2. Statistical analyses were performed in StataSE, version 13.1 (College Station, Texas).

RESULTS

A total of 442 of 500 recruited participants (88.8%) had a positive *M. tuberculosis* culture. As our analyses depend on the availability of mycobacterial isolates available for genotyping by MIRU-VNTR, results are limited to participants with positive baseline cultures (Supplementary Figure).

Of the 442 participants with positive baseline cultures, 308 (71.1%) had HIV infection by self-report, medical record review, and testing after enrollment. Only a fraction of the HIV-positive participants had $CD4^+$ T-cell counts recorded in their medical records; among the 161 participants with such records, the median $CD4^+$ T-cell count was 205 cells/mm^3 (interquartile range, 96–358 cells/mm^3; Supplementary Table).

Table 1. Baseline Factors Associated With Complex Infection at Enrollment

Factor	OR (95% CI)	aOR[a] (95% CI)
Age (per 1-year increase)	0.97 (.95–1.00)	0.98 (.96–1.00)
Male sex	1.45 (.91–2.31)	1.19 (.71–1.99)
Smear positivity	1.35 (.85–2.13)	1.05 (.63–1.74)
Previous treatment	0.51 (.26–1.00)	0.57 (.28–1.17)
HIV infected	0.71 (.44–1.17)	0.85 (.50–1.45)
MDR disease at baseline	0.77 (.21–2.72)	0.94 (.25–3.47)

Abbreviations: aOR, adjusted odds ratio; CI, confidence interval; HIV, human immunodeficiency virus; MDR, multidrug resistant; OR, odds ratio.

[a] All listed variables were included in a multivariable model.

Findings at Baseline

Although participants were recruited on the basis of a smear-positive diagnostic sputum result and had not yet initiated treatment, upon repeated sampling at enrollment only 53.8% of participants had a smear-positive specimen. MIRU-VNTR typing results were available from the isolates of specimens collected in 436 participants at enrollment. Of these individuals, 344 (78.9%) had simple infections and 92 (21.1%) had heterogeneous infections. In univariate analyses, previous treatment and younger age were associated with a reduced odds of heterogeneous infection, but after adjustment for other measured covariates, these associations did not remain statistically significant predictors of heterogeneity (Table 1).

Findings After 2 Months of Treatment

A total of 444 participants (88.8%) were successfully followed through the 2-month visit. Reasons for noncompletion of the study included death (2.0% of participants), withdrawal of consent (1.2%), loss to follow-up (4.2%), moving out of the study area (3.0%), and other unspecified reasons (0.8%). Of the 444

Table 2. Baseline Factors Associated With 2-Month Culture Positivity

Factor	OR (95% CI)	aOR[a] (95% CI)
Any baseline complex infection	1.72 (.98–3.00)	1.90 (1.03–3.50)
Age (per 1-year increase)	1.03 (1.01–1.05)	1.03 (1.01–1.06)
Male sex	1.71 (1.05–2.81)	1.74 (.98–3.10)
Smear positivity	2.56 (1.53–4.28)	1.64 (.93,2.92)
Previous treatment	1.15 (.64–2.05)	0.96 (.49–1.88)
HIV infected	0.54 (.32–0.91)	0.63 (.36–1.11)
MDR disease at baseline	3.04 (.95–9.70)	3.84 (1.11–13.27)

Abbreviations: aOR, adjusted odds ratio; CI, confidence interval; HIV, human immunodeficiency virus; MDR, multidrug resistant; OR, odds ratio.

[a] All listed variables were included in a multivariable model.

participants successfully followed through 2 months, we obtained positive culture results for 87 of 399 (21.8%) who were able to produce sputum specimens that were of adequate volume and were uncontaminated in the laboratory (Supplementary Figure).

MIRU-VNTR typing results were obtained for isolates from 77 participants after 2 months of treatment. Among these participants, 64 (83.1%) had simple infections, whereas 13 (16.9%) had evidence of heterogeneous infections.

The presence of a complex infection was independently associated with a nearly 2-fold increased odds of persistent culture positivity (adjusted odds ratio [aOR], 1.90; 95% confidence interval [CI], 1.03–3.50; $P = .04$). Other variables independently associated with persistent culture positivity included multidrug-resistant disease present at baseline (aOR, 3.79; 95% CI, 1.12–12.84; $P = .03$) and older age at treatment initiation (aOR, 1.03 for every additional year of age; 95% CI, 1.01–1.06; $P = .01$; Table 2).

DISCUSSION

We found that approximately 1 in 5 patients with smear-positive tuberculosis had evidence of a complex infection at the time of treatment initiation. No measured factors were predictive of complex infection at the time of treatment initiation. Although the association did not reach statistical significance, previous receipt of tuberculosis treatment trended toward a reduced odds of heterogeneous infection, suggesting that the selective pressure of previous treatment may serve as an evolutionary bottleneck or limit the window of time in which a participant can be reinfected. In a previous post mortem analysis conducted in this same setting, we also found a trend toward a negative association between previous treatment and complex infection (OR, 0.27; 95% CI, .06–1.20) [11]. This finding stands in contrast to a recent report that found previous treatment to be associated with a higher risk of polyclonal infections in a study of HIV-infected individuals in Botswana [12]. This may reflect differences in the levels of immunosuppression between study populations, differences in strain distribution and transmission intensity, or differences in the timing between episodes and effectiveness of prior treatment between study settings.

We did not find that the HIV infection status of participants was associated with complex *M. tuberculosis* infection. While Shin et al [12] found polyclonal infections to be most common amongst HIV-infected participants with the lowest $CD4^+$ T-cell counts, we were not able to comprehensively assess the association between complex infection and immunosuppression among HIV-infected individuals because we did not have sufficiently complete $CD4^+$ T-cell count data. However, in a subanalysis limited to the 198 HIV-coinfected participants with recorded $CD4^+$ T-cell counts, we found an inverse association between $CD4^+$ T-cell count and baseline risk of a complex infection for those infected with HIV (OR, 0.996 per cell/mm^3; 95% CI, .993–.999), which is consistent with these previous findings.

Our estimated prevalence of complex infection is within the range reported by other studies in southern Africa that have used different designs and laboratory approaches for identifying within-host complexity [5]. We had anticipated that, by culturing multiple specimens in parallel, we might increase the opportunity to identify rare variants, but this design did not lead us to find a higher prevalence of complex infections than has been previously reported. Our ability to detect these rare variants is still limited by several factors: (1) failure to harvest all existing variants within the participant in the spot sputum samples; (2) loss of rare variants during sample transport, decontamination, and culture; (3) outcompetition of variants in culture; and (4) limited sensitivity of genotyping by MIRU-VNTR for detecting complexity. Higher-resolution sequencing methods that analyze sputum samples directly will address some of these limitations [13, 14].

Despite this limitation, we found that the detection of complex infection was independently associated with an increased odds of persistent culture-positive tuberculosis. In our study setting, this deleterious effect of within-host diversity of infection on early treatment response appears to be independent of a relationship with drug resistance. Previous reports have demonstrated that infections with 2 strains that differ in drug susceptibility or infections with differing susceptibilities (ie, heteroresistance) can compromise treatment outcomes [2–4]. Our result suggests that the presence of a heterogeneous mycobacterial population itself may predict poorer treatment response, but we are unable to determine whether this association is due to a causal link between heterogeneity and treatment response (eg, a genetically diverse mycobacterial population presents special challenges for drug treatment or for host immune clearance) or because it serves as a marker of increased host vulnerability (eg, greater immunosuppression and possibly greater mycobacterial burden). Because our follow-up ended at 2 months, we were unable to examine the relationship between complex infections and final treatment outcome.

Our study is consistent with previous reports suggesting that the within-host heterogeneity of *M. tuberculosis* infection is not a rare event in tuberculosis-hyperendemic areas and is likely underestimated by use of current approaches [15]. We found significant effects of within-host diversity on 2-month culture conversion, even in the absence of a relationship between complexity and drug resistance. If transmitted drug resistance increases, we expect the association between heterogeneity and poor treatment response to strengthen.

While our study has strengths including a large study size and a prospective design with good follow-up, further work is needed to describe the impact of within-host heterogeneity on final treatment outcomes. Although these findings cannot be generalized to settings of lower HIV prevalence, the observation that heterogeneous infections compromise early treatment response is important and should be reexamined. Higher-resolution genomic typing of strains, such as whole-genome sequencing or

metagenomic analysis, will facilitate improved understanding of the within-host complexity of infections and the dynamic response of these mycobacterial populations under the selective pressure of treatment.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

Acknowledgments. We thank our study nurses, Patricia Bartman and Lungile Maziya, for their commitment to this study; the KwaZulu-Natal Department of Health: and nursing staff and management at the Umgungundlovu District primary healthcare clinics.

Disclaimer. The content is solely the responsibility of the authors and does not necessarily represent the official views of the Office of the Director, the National Institute of General Medical Science, or the National Institutes of Health (NIH).

Financial support. This work was supported by the NIH (award DP2 OD006663 from the Office of the Director and award U54 GM088558 from the National Institute of General Medical Sciences).

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Zetola NM, Shin SS, Tumedi KA, et al. Mixed *Mycobacterium tuberculosis* complex infections and false-negative results for rifampin resistance by GeneXpert MTB/RIF are associated with poor clinical outcomes. J Clin Microbiol **2014**; 52:2422–9.
2. Zetola NM, Modongo C, Moonan PK, et al. Clinical outcomes among persons with pulmonary tuberculosis caused by *Mycobacterium tuberculosis* isolates with phenotypic heterogeneity in results of drug-susceptibility tests. J Infect Dis **2014**; 209:1754–63.
3. van Rie A, Victor TC, Richardson M, et al. Reinfection and mixed infection cause changing *Mycobacterium tuberculosis* drug-resistance patterns. Am J Respir Crit Care Med **2005**; 172:636–42.
4. Liu Q, Via LE, Luo T, et al. Within patient microevolution of *Mycobacterium tuberculosis* correlates with heterogeneous responses to treatment. Sci Rep **2015**; 5:17507.
5. Cohen T, van Helden PD, Wilson D, et al. Mixed-strain *Mycobacterium tuberculosis* infections and the implications for tuberculosis treatment and control. Clin Microbiol Rev **2012**; 25:708–19.
6. KwaZulu-Natal Department of Health Annual Report 2013:[54 p.]. http://www.kznhealth.gov.za/AnnualReport/2013_2014.pdf. Accessed 20 September 2015.
7. Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap)–a metadata-driven methodology and workflow process for providing translational research informatics support. J Biomed Inform **2009**; 42:377–81.
8. Hanekom M, Streicher EM, Van de Berg D, et al. Population structure of mixed *Mycobacterium tuberculosis* infection is strain genotype and culture medium dependent. PLoS One **2013**; 8:e70178.
9. Oelemann MC, Diel R, Vatin V, et al. Assessment of an optimized mycobacterial interspersed repetitive- unit-variable-number tandem-repeat typing system combined with spoligotyping for population-based molecular epidemiology studies of tuberculosis. J Clin Microbiol **2007**; 45:691–7.
10. Garcia de Viedma D, Alonso Rodriguez N, Andres S, Ruiz Serrano MJ, Bouza E. Characterization of clonal complexity in tuberculosis by mycobacterial interspersed repetitive unit-variable-number tandem repeat typing. J Clin Microbiol **2005**; 43:5660–4.
11. Cohen T, Wilson D, Wallengren K, Samuel EY, Murray M. Mixed-strain *Mycobacterium tuberculosis* infections among patients dying in a hospital in KwaZulu-Natal, South Africa. J Clin Microbiol **2011**; 49:385–8.
12. Shin SS, Modongo C, Ncube R, Sepako E, Klausner JD, Zetola NM. Advanced immune suppression is associated with increased prevalence of mixed-strain *Mycobacterium tuberculosis* infections among persons at high risk for drug-resistant tuberculosis in Botswana. J Infect Dis **2015**; 211:347–51.
13. Colman RE, Schupp JM, Hicks ND, et al. Detection of low-level mixed-population drug resistance in *Mycobacterium tuberculosis* using high fidelity amplicon sequencing. PLoS One **2015**; 10:e0126626.
14. Brown AC, Bryant JM, Einer-Jensen K, et al. Rapid whole-genome sequencing of *Mycobacterium tuberculosis* directly from clinical samples. J Clin Microbiol **2015**; 53:2230–7.
15. Perez-Lago L, Palacios JJ, Herranz M, Ruiz Serrano MJ, Bouza E, Garcia-de-Viedma D. Revealing hidden clonal complexity in *Mycobacterium tuberculosis* infection by qualitative and quantitative improvement of sampling. Clin Microbiol Infect **2015**; 21:147.e1–7.