### Title

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# 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

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**We examined factors associated with mixed-strain *Mycobacterium tuberculosis* infections among patients at high risk for drug-resistant tuberculosis in Botswana. Thirty-seven (10.0%) of 370 patients with tuberculosis had mixed *M. tuberculosis* infections, based on 24-locus mycobacterial interspersed repetitive unit–variable number of tandem repeats genotyping. In log-binomial regression analysis, age <37 years (adjusted prevalence ratio [PR], 1.92; 95% confidence interval [CI], 1.01–3.57) and prior tuberculosis treatment (adjusted PR, 2.31; 95% CI, 1.09–4.89) were associated with mixed *M. tuberculosis* infections. Among human immunodeficiency virus–infected patients, prior tuberculosis treatment (adjusted PR, 2.11; 95% CI, 1.04–4.31) and CD4$^+$ T-cell count of <100 cells/μl (adjusted PR, 10.18; 95% CI, 2.48–41.71) were associated with mixed *M. tuberculosis* infections. Clinical suspicion of mixed *M. tuberculosis* infections should be high for patients with advanced immunosuppression and a prior history of tuberculosis treatment.**

***Keywords.*** tuberculosis; mixed tuberculosis infection; HIV/AIDS; immune suppression; reinfection.

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Tuberculosis was traditionally assumed to be caused by infection with a single strain of *Mycobacterium tuberculosis* [1]. However, increasing evidence has shown that infections with multiple strains of *M. tuberculosis* (ie, mixed *M. tuberculosis* infections) occur and might be relatively common in settings of high tuberculosis endemicity [1–6]. Moreover, mixed *M. tuberculosis* infections have been associated with poor treatment outcomes, reduced diagnostic performance for detecting drug resistance, and selection and amplification of drug-resistant strains [1, 7–9].

Despite the growing recognition of the public health significance of mixed *M. tuberculosis* infections, little is known regarding risk factors for mixed *M. tuberculosis* infections. The objective of this study was to determine factors associated with mixed *M. tuberculosis* infections among human immunodeficiency virus (HIV)–infected and HIV-uninfected patients with tuberculosis in Botswana, a country hyperendemic for HIV infection and tuberculosis.

## MATERIALS AND METHODS

### Study Population

Procedures for recruitment, laboratory testing, and data collection have been reported in detail previously [8]. Briefly, we retrospectively tested sputum samples obtained from patients attending tuberculosis hospitals and clinics in Botswana who were considered to be at high risk for multidrug-resistant (MDR) tuberculosis (Supplementary Figure 1). Sputum samples were tested with Gene Xpert MTB/RIF (Xpert) and culture-based methods (ie, the Mycobacteria Growth Indicator Tube was used for *M. tuberculosis* recovery, and the proportion method with Lowenstein-Jensen medium was used for drug-susceptibility testing). Patients aged ≥18 years with a tuberculosis diagnosis confirmed by Xpert and positive culture results were included in this study.

### Data Collection and Laboratory Procedures

We abstracted clinical and demographic data from patients' medical records, laboratory records, and the national tuberculosis registry database, using standardized data collection forms. Data collected included age, sex, prior history of tuberculosis treatment, HIV test results, and CD4$^+$ T-cell counts. Sputum samples were collected and processed as previously described [8]. We performed 24-locus mycobacterial interspersed repetitive unit (MIRU) variable number of tandem repeats (VNTR) analysis on chromosomal DNA extracted from *M. tuberculosis* isolates [8]. Mixed *M. tuberculosis* infections were defined by

**Table 1. Prevalence of Mixed-Strain *Mycobacterium tuberculosis* Infections, by Demographic and Clinical Characteristics, and Adjusted Prevalence Ratios Based on Log-Binomial Regression Modeling Among Adult Patients Evaluated for Multidrug-Resistant Tuberculosis in Botswana, January 2012–March 2013**

| | Mixed-Strain Infection | | | Adjusted Prevalence Ratio (95% CI) | |
|---|---|---|---|---|---|
| Characteristic | Proportion | Prevalence, % (95% CI) | *P* Value | Model 1 (All Patients) (n = 370) | Model 2 (HIV-Infected Patients) (n = 279) |
| Overall | 37/370 | 10.0 (7.3–13.5) | | | |
| Sex | | | .726 | | |
| Male | 21/221 | 9.5 (6.3–14.1) | | 1.0 | 1.0 |
| Female | 16/149 | 10.7 (6.7–16.7) | | 1.27 (.68–2.36) | 1.52 (.83–2.79) |
| Age, y | | | .089 | | |
| <37 | 23/153 | 13.1 (10.2–21.6) | | **1.92 (1.01–3.57)** | 1.67 (.88–3.13) |
| ≥37 | 14/180 | 7.2 (4.7–12.6) | | 1.0 | 1.0 |
| Prior history of tuberculosis treatment | | | **.042** | | |
| No | 8/142 | 5.6 (2.3–8.5) | | 1.0 | 1.0 |
| Yes | 29/228 | 12.7 (9.0–17.9) | | **2.31 (1.09–4.89)** | **2.11 (1.04–4.31)** |
| HIV infection | | | .147 | | |
| No | 5/91 | 5.5 (2.4–12.2) | | 1.0 | NA |
| Yes | 32/279 | 11.5 (8.2–15.7) | | 2.32 (.94–5.73) | NA |
| $CD4^+$ T-cell count, cells/μl | | | **<.001** | | |
| <100 | 17/54 | 31.5 (20.7–44.7) | | NA | **10.18 (2.48–41.71)** |
| 100–199 | 8/74 | 10.8 (5.6–19.9) | | NA | 3.62 (.80–16.39) |
| 200–350 | 5/88 | 5.7 (2.5–12.6) | | NA | 1.76 (.36–8.75) |
| >350 | 2/63 | 3.2 (.9–10.9) | | NA | 1.0 |

Bold numbers indicate *P* values of < .05.

Abbreviations: CI, confidence interval; HIV, human immunodeficiency virus; NA, not applicable.

the presence of strains with distinct 24-loci MIRU-VNTR patterns at ≥1 loci in the same sputum sample [8].

### Statistical Analysis

We conducted statistical analyses using R, version 3.0.2 (The R Project for Statistical Computing; http://www.r-project.org). A *P* value of < .05 was determined to be statistically significant. We calculated 95% confidence intervals (CIs) for prevalence estimates, using the Score method, and compared prevalence estimates by using the $\chi^2$ test. The $\chi^2$ test for trend was used to evaluate trends in mixed *M. tuberculosis* infection prevalence across $CD4^+$ T-cell count categories.

We constructed log-binomial regression models to identify factors independently associated with mixed *M. tuberculosis* infections. Separate bivariate models were fitted for all patients and for the HIV-infected subgroup. We selected the following variables a priori for inclusion in the final models, based on their conceptual importance for reinfection and/or mixed *M. tuberculosis* infections: age (stratified dichotomously using the median), sex, prior history of tuberculosis treatment, HIV infection status (HIV infected vs HIV uninfected, for the model for all patients), and $CD4^+$ T-cell count as a categorical variable (for the model for HIV-infected patients). We also constructed a separate model for HIV-infected patients with $CD4^+$ T-cell count included as an ordinal variable to determine whether a linear relationship exists between $CD4^+$ T-cell count and mixed *M. tuberculosis* infections. Last, we evaluated whether applying the final models to subgroups of patients with and patients without MDR tuberculosis resulted in similar findings as when applied to all patients.

### Ethical Considerations

This study received approval from the University of Pennsylvania Institutional Review Board, the Botswana Ministry of Health Human Research Development Committee, and the Princess Marina Hospital Ethics Committee. We did not obtain informed consent because this study involved retrospective review of data that are routinely collected as part of tuberculosis care.

## RESULTS

Between 1 January 2011 and 30 March 2012, 370 patients met the inclusion criteria for the study. Of those, the median age was 37 years (interquartile range [IQR], 30.5–44.5 years), 149 (40.3%) were female, 228 (61.6%) had a prior history of tuberculosis treatment, 55 (14.9%) had MDR tuberculosis, and 279

(75.4%) were HIV infected. Among HIV-infected patients, the median CD4$^+$ T-cell cell count was 209 cells/μl (IQR, 111.5–330.0 cells/μl).

The overall prevalence of mixed *M. tuberculosis* infections was 10.0% (37/370; 95% CI, 7.3%–13.5%; Table 1). The prevalence of mixed *M. tuberculosis* infections was higher among patients with a prior history of tuberculosis treatment (12.7% [29/228] vs 5.6% [8/142]; $P = .042$) and higher among patients with MDR tuberculosis (20/55 [36.4%] vs 17/315 [5.4%]; $P < .001$). No statistically significant differences in mixed *M. tuberculosis* infection prevalence were observed between sex, age, and HIV infection categories. Among the 91 HIV-uninfected patients, the overall prevalence of mixed *M. tuberculosis* infections was 5.5% (5/91). All 5 mixed *M. tuberculosis* infections among HIV-uninfected patients occurred among the 63 patients who also had a prior history of tuberculosis treatment (Supplementary Figure 2).

Among HIV-infected patients, the overall prevalence of mixed *M. tuberculosis* infections was 11.5% (32/279). As with HIV-uninfected patients, the prevalence of mixed *M. tuberculosis* infections was higher among patients who also had prior history of tuberculosis treatment (14.5% [24/165] vs 7.0% [8/114] among patients with no prior history of tuberculosis treatment; Supplementary Figure). The prevalence of mixed *M. tuberculosis* infections increased from 3.2% (2/63) among patients with a CD4$^+$ T-cell count of >350 cells/μl to 31.5% (17/54) among patients with a CD4$^+$ T-cell count of <100 cells/μl ($P < .001$, by the $\chi^2$ test for trend; Table 1). When patients with a CD4$^+$ T-cell count of <100 cells/μl were stratified by their history of prior tuberculosis treatment, the prevalence of mixed *M. tuberculosis* infections was 41.4% (12/29) and 20.0% (5/25) among patients with and those without a prior history of tuberculosis treatment, respectively (Figure 1). No mixed *M. tuberculosis* infections were detected among the 55 HIV-infected patients with a CD4$^+$ T-cell count of >200 cells/μl and no prior history of tuberculosis treatment (Figure 1).

In the multivariable log-binomial model of all patients, mixed *M. tuberculosis* infections were associated with age <37 years (adjusted prevalence ratio [PR], 1.92; 95% CI, 1.01–3.57) and a prior history of tuberculosis treatment (adjusted PR, 2.31; 95% CI, 1.09–4.89; Table 1). The prevalence of mixed *M. tuberculosis* infections was higher among HIV-infected patients, but this association did not reach statistical significance (adjusted PR, 2.32; 95% CI, .94–5.73).

In the model among HIV-infected patients only, mixed *M. tuberculosis* infections were associated with a prior history of tuberculosis treatment (adjusted PR, 2.31; 95% CI, 1.09–4.89) and a CD4$^+$ T-cell count of <100 cells/μl (vs >350 cells/μl; adjusted PR, 10.18; 95% CI, 2.48–41.71; Table 1). When CD4$^+$ T-cell count was modeled as an ordinal variable, the prevalence of mixed *M. tuberculosis* infections increased with decreasing CD4$^+$ T-cell count categories, after adjustment for prior history of tuberculosis treatment (adjusted PR, 2.35 per decrease in ordinal CD4$^+$ T-cell category; 95% CI, 1.65–3.34).

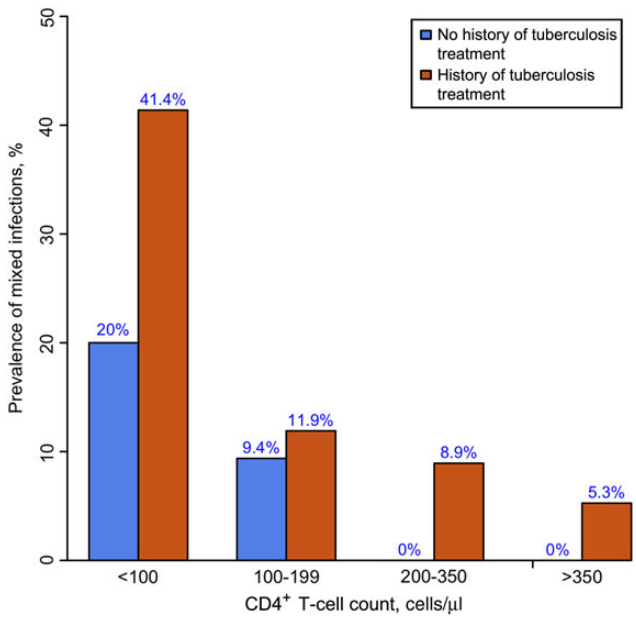


**Figure 1.** Prevalence of mixed-strain *Mycobacterium tuberculosis* infections, by CD4$^+$ T-cell count and prior history of tuberculosis treatment, among human immunodeficiency virus–infected patients evaluated for multidrug-resistant tuberculosis in Botswana, January 2012–March 2013.

We were unable to construct separate models for the subgroup of patients with MDR tuberculosis because of insufficient sample size. Models for the subgroup without MDR tuberculosis resulted in effect sizes similar to those of the models that included all patients. However, the association between previous treatment history and mixed *M. tuberculosis* infections was no longer statistically significant when restricted to the subgroup without MDR tuberculosis.

## DISCUSSION

We found a 10% prevalence of mixed *M. tuberculosis* infections among patients with pulmonary tuberculosis evaluated for MDR tuberculosis in Botswana. However, the prevalence of mixed *M. tuberculosis* infections varied substantially among patient subgroups and was as high as 41.4% among patients with a prior history of tuberculosis treatment and advanced immunosuppression (Figure 1). The prevalence of mixed *M. tuberculosis* infections found in our study population is similar to what has been reported from studies of mixed *M. tuberculosis* infections identified by MIRU-VNTR in other settings of high HIV prevalence [1, 4, 10]. However, previous studies of mixed *M. tuberculosis* infections were limited by small sample sizes of HIV-infected persons and, thus, were not able to compare the prevalence of mixed *M. tuberculosis* infection across CD4$^+$ T-cell counts.

To our knowledge, the present study is the first to describe an increasing trend in the prevalence of mixed *M. tuberculosis* infections across decreasing $CD4^+$ T-cell counts. The prevalence of mixed *M. tuberculosis* infections was 10-fold higher among HIV-infected patients with $CD4^+$ T-cell counts of <100 cells/μl, compared with that among patients with $CD4^+$ T-cell counts of >350 cells/μl. These findings are consistent with the observation that HIV-induced immunodeficiency increases the susceptibility to reinfection and the simultaneous progression to active tuberculosis [1, 2]. Alternatively, transmission events involving simultaneous infection with mixed *M. tuberculosis* strains might be possible.

Prior studies have suggested that infection with *M. tuberculosis* provides partial protection against subsequent infections, most of which remain latent without progressing to active disease [11]. On the other hand, a prior history of tuberculosis is one of the most important risk factors for incident active tuberculosis [12]. The strong association that we found between prior tuberculosis treatment and mixed *M. tuberculosis* infections suggests that the initial tuberculosis episode might increase the risk of subsequent tuberculosis caused by simultaneous infections with multiple strains, as well as the risk for relapse and reinfection.

Our findings suggest that protective immunity provided by prior *M. tuberculosis* infections might only be clinically relevant among patients with a preserved immune system in whom infection never progress to active tuberculosis. Among HIV-uninfected patients with a prior history of tuberculosis treatment, subsequent exogenous *M. tuberculosis* infection might itself suppress the immune system, causing reactivation of the prior infection if it was not completely cured. It is also possible that HIV-uninfected patients with a prior history of tuberculosis treatment in our study were more likely to have other immunosuppressive conditions, such as diabetes and malnutrition, which increase their risk for mixed *M. tuberculosis* infections. Additional research is needed to test these hypotheses.

Our findings should be interpreted with caution given that the MIRU-VNTR method does not allow for the differentiation between infection with multiple different strains (true mixed *M. tuberculosis* infection) and microevolution of a single strain [1]. Thus, it is possible that we overestimated the prevalence of mixed *M. tuberculosis* infections. The prevalence of mixed *M. tuberculosis* infections would change to 4.9% (18/370) and 2.2% (8/370) if we changed the definition to involve different patterns at ≥2 loci and ≥3 loci, respectively. However, previous studies have shown that MIRU-VNTR patterns are highly stable even in the context of microevolution [13, 14]. We therefore believe that our definition of ≥1 locus is most likely to correspond to true mixed *M. tuberculosis* infections.

Other limitations include the cross-sectional nature of our study, which limits our ability to establish causal relationships. Longitudinal cohort studies are needed to confirm whether tuberculosis treatment and advanced immune suppression increase the risk for subsequent disease caused by mixed *M. tuberculosis* infections. In addition, the MIRU-VNTR method does not allow the identification of infections with multiple strains having the same pattern. This methodological limitation might have led to underestimates of the prevalence of mixed *M. tuberculosis* infections and potentially introduced misclassification bias. Furthermore, we only tested for mixed *M. tuberculosis* infections in sputum samples taken from patients with pulmonary tuberculosis and, therefore, were unable to investigate mixed *M. tuberculosis* infections involving different strains isolated from extrapulmonary sites, which could have also led us to underestimate the true prevalence of mixed *M. tuberculosis* infections [15]. Previous history of tuberculosis treatment was determined on the basis of self-report as recorded in the medical records, which could have led to misclassification. Furthermore, we did not collect sufficient data to compare the prevalence of mixed *M. tuberculosis* infections between previously treated patients who were cured to that among patients who withdrew from or did not respond to treatment. Last, our study population consisted of retrospectively enrolled patients who were evaluated for MDR tuberculosis on the basis of predefined criteria (Supplementary Figure 1). We did not collect information on patients who did not fit these criteria, and our results might not be generalizable to the broader population of patients with tuberculosis in Botswana and elsewhere.

To our knowledge, the present study provides the strongest evidence to date of an association between HIV-related immunosuppression and mixed *M. tuberculosis* infections. Clinical suspicion of mixed *M. tuberculosis* infections should be high for patients with advanced HIV disease, particularly among those with history of a prior tuberculosis episode. While the clinical implications of mixed *M. tuberculosis* infections remain poorly understood, patients with mixed *M. tuberculosis* infections might be at increased risk for poor treatment outcomes and, thus, should be monitored with extra care [7, 8]. Future studies will need to determine the relative importance of microevolution and unrecognized tuberculosis-specific immunosuppression for acquiring mixed *M. tuberculosis* infections.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

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***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.

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