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Mixed-Strain *Mycobacterium tuberculosis* Infections among Patients Dying in a Hospital in KwaZulu-Natal, South Africa▽

Ted Cohen,[1,2]* Douglas Wilson,[3] Kristina Wallengren,[2] Elizabeth Y. Samuel,[4] and Megan Murray[1,2,5]

Division of Global Health Equity, Brigham and Women's Hospital, Boston, Massachusetts[1]; Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts[2]; Department of Medicine, Edendale Hospital, University of KwaZulu-Natal, Pietermaritzburg, South Africa[3]; National Health Laboratory Service, Inkosi Albert Luthuli Central Hospital, Mayville, South Africa[4]; and Infectious Disease Unit, Massachusetts General Hospital, Boston, Massachusetts[5]

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We performed spoligotyping and 24-locus mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) typing on *M. tuberculosis* culture-positive biopsy specimens collected from adults dying in a hospital in KwaZulu-Natal. Of 56 culture-positive samples genotyped, we detected mixed strains in five (9%) and clonal heterogeneity in an additional four (7%).

The application of molecular approaches for detecting variation among *Mycobacterium tuberculosis* isolates has generated new appreciation for the diversity present within this relatively genetically conserved bacterial species (4, 17). Genotyping methods, such as insertion sequence typing (IS*6110*), spacer oligonucleotide typing (spoligotyping), and mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) typing, have been used to identify transmission chains (1, 10, 18, 26), to classify strains into families and lineages (2, 5, 9, 18, 27, 29), to identify episodes of exogenous reinfection (3, 20, 25, 31), and, most recently, to detect the presence of within-host genetic heterogeneity (11, 19, 24, 33).

Genetic heterogeneity of *M. tuberculosis* within a host may arise by one of two mechanisms: (i) within-host diversification following a single infection event or (ii) reinfection resulting in a mixed infection with more than one strain (12, 16). The clinical consequences of within-host diversity are most obvious when manifesting as subpopulations of bacteria with resistance to tuberculosis (TB) antibiotics, either reflecting acquired drug resistance (mechanism 1) or transmitted drug resistance (mechanism 2). Furthermore, because individuals can be simultaneously infected by strains with different phenotypic characteristics (e.g., growth rates, drug resistance), the within-host competition between strains may influence the clinical outcomes for coinfected patients (30) and may affect the population dynamics of the pathogen in the community (6, 8).

While within-host *M. tuberculosis* genetic diversity has been documented in many settings, systematic efforts to measure the prevalence of these complex infections have rarely been attempted. In this study, we report the results of a genotyping analysis on isolates collected from young adults dying in a hospital in KwaZulu-Natal, South Africa.

We conducted limited autopsies on adults aged 20 to 45 years who died after admission to Edendale Hospital in KwaZulu-Natal, South Africa, between October 2008 and August 2009. The incidence of tuberculosis in KwaZulu-Natal is 1,094 cases/100,000 persons per year, and the HIV prevalence among women in antenatal clinical settings is 39% (14, 21). Our primary aim was to investigate the burden of tuberculosis as a contributing cause of death in this highly vulnerable community. Of the 240 decedents enrolled in the study, 94% were HIV seropositive. Fifty-eight percent of those on treatment for tuberculosis at the time of death were still infected with viable *M. tuberculosis*, while 42% of those not receiving treatment for TB also had positive *M. tuberculosis* cultures at the time of death. These results suggest that delayed and missing diagnoses of tuberculosis and the emergence of drug resistance contribute to the large burden of tuberculosis-related mortality in this setting. A more detailed account of the design and findings of this study is available in a related report (7).

Here we present a genotyping analysis of *M. tuberculosis*-positive specimens collected from a subset of *M. tuberculosis* culture-positive decedents included in the larger postmortem study; Table 1 displays the characteristics of decedents with samples included in this analysis. Briefly, for each decedent, we conducted limited autopsies to retrieve respiratory tract secretions as well as needle core biopsy specimens from lung, liver, and spleen. These specimens were pooled and grown in liquid culture medium (BACTEC MGIT 960; Becton-Dickinson, NJ). Positive cultures were identified as *M. tuberculosis* by the niacin-nitrate test. Mycobacterial DNA was then extracted from the liquid medium and shipped to a commercial laboratory (Genoscreen, Campus de l'Institut Pasteur de Lille, France) for spoligotyping and 24-locus MIRU-VNTR analysis (23). Of the 58 samples sent to the laboratory, 2 were of insufficient quality to perform genetic analysis.

Consistent with existing definitions (16, 24, 28), we categorized infections as mixed-strain infections when we identified more than one allele at more than one MIRU-VNTR locus and as clonal population infections when there was more than one allele at a single locus. By this classification approach, we found that five decedents (9%) had infection with more than

* Corresponding author. Mailing address: Division of Global Health Equity, Brigham and Women's Hospital, Boston, MA 02115. Phone: (617) 432-6783. Fax: (617) 432-2565. E-mail: tcohen@hsph.harvard.edu.

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TABLE 1. Characteristics of individuals with genotyped samples

Characteristic	Result
Total no. of samples genotyped	56
No. (%) of male subjects	36 (64)
Median age in yr (range) at time of death	34 (21–45)
No. (%) of HIV-infected subjects	54 (96)
No. (%) on ARVs[a]	8 (15)
No. (%) with MDRTB	12 (21)
No. (%) on TB treatment at time of death	34 (60)
No. of days (range) of final hospitalization	2 (0–40)

[a] Number and percentage of HIV-infected subjects on antiretroviral drugs (ARVs).

one strain and four (7%) had evidence of clonal heterogeneity (Table 2). Previous examination of worldwide samples of *M. tuberculosis* isolates found that certain MIRU-VNTR loci were more variable than others (28). In our sample, heterogeneity in the four polyclonal populations we detected was due to variation at four different loci: 4052 (Qub26), 2996 (MIRU 26), 960 (MIRU 10), and 802 (MIRU 40). These loci were (respectively) the first, fourth, sixth, and seventh (out of 24) most variable loci in the international sample. Each individual with a complex infection was HIV coinfected.

After grouping mixed-strain infections and clonal heterogeneity into a single category of complex infection, we investigated if any measured clinical and demographic variables were associated with this outcome. Univariate logistic regression did not identify any host or bacterial variables that were statistically significantly associated with this outcome, and neither did male gender (odds ratio of complex infection [OR], 2.41; 95% confidence interval [CI], 0.45 to 12.84; *P*, 0.30), increases in age (OR, 0.98 per year; 95% CI, 0.87 to 1.10; *P*, 0.75), antiretroviral use (OR, 1.95; 95% CI, 0.33 to 11.69; *P*, 0.46), or multidrug-resistant (MDR) TB (OR, 0.47; 95% CI, 0.05 to 4.33; *P*, 0.51). While it did not reach the level of statistical significance, there is some suggestion that being on treatment for TB at the time of death reduced the probability of complex infection (OR, 0.27; 95% CI, 0.06 to 1.20; *P*, 0.08). This may reflect the selective pressure of standard drug treatments, which we expect should rapidly eliminate drug-sensitive bacteria; this hypothesis is supported by the strong positive association we observed between being on treatment at the time of death and the detection of MDRTB among these individuals (OR, 9.0; 95% CI, 1.06 to 76.48; *P*, 0.04). This finding is important because it indicates that in areas where complex infections occur, individuals who appear to have acquired drug resistance while on therapy (i.e., individuals whose drug sensitivity tests indicated sensitivity prior to treatment and resistance after treatment has begun) may instead have experienced transmitted drug resistance which was subsequently "unmasked" by treatment (30). Such unmasking may, in turn, contribute to ongoing transmission of drug-resistant tuberculosis during first-line treatment.

The diversity of strain lineages present in our sample is qualitatively similar to the diversity of other strains that have been genotyped in Africa and submitted to international collections (5) (Table 3). The most common lineages and sublineages in our sample included LAM (16 isolates, 33% of all classified strains), T (11 isolates, 23%), Beijing (8 isolates,

TABLE 2. Samples categorized as complex infections

Sample population type and study ID no.	Allele(s) at MIRU-VNTR locus:																					
	580	2996	802	960	1644	3192	424	577	2165	2401	3690	4156	2163b	1955	4052	154	2531	4348	2059	2687	3007	2347	2461	3171
Mixed																								
BN093	2	4	3 + 4[b]	—[a]	—	2	2	4	3	2	3	—	2	2	3	2	5 + 6[b]	2 + 3[b]	2	1	2	5	2	3
BN099	2	6	1	—	—	2 + 3[b]	2 + 3[b]	3 + 4[b]	3	4	2	—	4	2	—	2	3 + 5[b]	2	1	1	2 + 3[b]	2 + 4[b]	2 + 3[b]	2
BN112	2	4 + 5[b]	1 + 4 + 5[b]	3 + 4[b]	1	3	2	3 + 4[b]	2 + 3[b]	2 + 4[b]	3	—	2	2 + 4[b]	4	2	5	2	2	1	3	4	1 + 2[b]	3
BN123	2	7	3	2	3	5	4	3 + 4[b]	3	4	4	2	2	5	6	2	5	4	2	1	3	4	2	2 + 3[b]
BN154	3	4	4	3	3	3	3	4	1 + 4[b]	2	1 + 3[b]	2	3	1	—	3	5	2	2	1	3	4	2	2
Clonal																								
BN090	2	5	3	1 + 4[b]	—	3	4	4	2	2	1	2	2	4	—	2	6	2	2	1	3	4	2	1
BN160	2	5	3	5	3	3	2	3	1	3	3	3	4	3	6 + 7[b]	2	3	2	1	1	3	2	2	3
BN177	4	5 + 6[b]	3	4	3	3	4	4	2	2	1	2	3	4	—	2	6	2	2	1	3	4	2	1
BN220	2	5	3 + 4[b]	4	3	3	3	4	2	1	2	3	2	3	—	1	6	2	2	1	3	4	2	3

[a] —, nonamplified markers after two rounds of independent and monoplex PCR.
[b] Double alleles that were confirmed by two independent rounds of PCR.

TABLE 3. Spoligotypes of samples and association with complex infections

SIT[a]	Lineage/sublineage	Frequency	No. of samples showing complex infection
1	Beijing	8	0
33	LAM3	8[c]	1 (clonal heterogeneity)
34	S[b]	5[c]	1 (mixed infection)
37	T3	1	0
39	T4-CEU1	2	0
51	T1	1	0
52	T2	1[c]	1 (mixed infection)
54	MANU2	1[c]	1 (mixed infection)
60	LAM4	5	1 (clonal heterogeneity)
62	H1	1	1 (clonal heterogeneity)
71	S[b]	1	0
73	T2-T3	2	0
88	S	1	0
92	X3	1	0
207	H3	1	0
336	X1	1	0
719	T1	4	1 (clonal heterogeneity)
815	LAM11_ZWE	3	0
1196	Undesignated	1[c]	1 (mixed infection)
1915	S	1	0
No SIT type		7[c]	1 (mixed infection)

[a] SIT, shared international type (according to SpolDB4 [5]).
[b] These spoligotypes are the same as those for the F28 family (32).
[c] Frequency reported includes one sample in which mixed strains were detected by MIRU-VNTR; thus the SIT assignments for these particular samples may not be accurate.

17%), and S (8 isolates, 17%). Six of the eight strains in the S lineage had spoligotypes matching the F28 family identified by Warren et al. in Cape Town, South Africa (32). In Table 3, while we have provided the shared international type (SIT) grouping assigned to those samples in which we detected mixed infections, we note that these assignments may be misleading. In a mixed infection, the spoligotype pattern may in some cases reflect the cumulative presence of spacers in all strains present or in other cases may accurately report the spoligotype from the dominant strain. Accordingly, in areas where mixed infections are common, the usefulness of spoligotyping may be compromised.

We note several limitations to this study. First, nearly half of the *M. tuberculosis* culture-positive samples (47%) from the postmortem exams were not stored at the laboratory and thus were not available for genotyping. Samples collected from males (OR, 2.43; 95% CI, 1.13 to 5.25; P value = 0.02) and samples that were MDR (OR, 3.74; 95% CI, 1.13 to 12.39; P value = 0.03) were more likely to be saved and thus are overrepresented in this analysis. Although this limits the generalizability of our results, the finding that a substantial fraction of these individuals had active infection with more than one strain is noteworthy. While MIRU-VNTR appears to be a specific test for mixed infections, its sensitivity is likely to be quite limited, since detection of a mixed infection requires that clinical samples collected from the patient actually contain some of each strain present, that the culture procedures are permissive enough to allow growth of minority strains (15), and that the DNA isolation procedures from these cultures capture material from all strains present. While our strategy for pooling all autopsy material from a single individual before culturing may have increased the sensitivity for detecting mixed infections (especially if different strains were present in different organs [13]), we were not able to identify the anatomic distribution of these strains or strain variants.

In conclusion, we found mixed-strain infections and clonal heterogeneity in a substantial fraction of young adults dying in a hospital in KwaZulu-Natal, South Africa. Because of the limited sensitivity of our testing procedures (22), the frequency of complex infections is probably higher than we found in this setting. The high frequency of HIV coinfection in our study population and the absence of timely TB treatment may contribute to the risk of both mixed *M. tuberculosis* infection and clonal heterogeneity in these patients; however, the effect of HIV on the risk of complex infection cannot be teased out directly from our data, since nearly everyone in our sample had HIV infection. Future studies that compare the risk of mixed infection among those with and without severe immunosuppression within the same community will help clarify the impact of HIV on the within-host diversity of TB infection.

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REFERENCES

1. **Alland, D., G. E. Kalkut, A. R. Moss, R. A. McAdam, J. A. Hahn, W. Bosworth, E. Drucker, and B. R. Bloom.** 1994. Transmission of tuberculosis in New York City. An analysis by DNA fingerprinting and conventional epidemiologic methods. N. Engl. J. Med. **330:**1710–1716.
2. **Allix-Beguec, C., D. Harmsen, T. Weniger, P. Supply, and S. Niemann.** 2008. Evaluation and strategy for use of MIRU-VNTRplus, a multifunctional database for online analysis of genotyping data and phylogenetic identification of Mycobacterium tuberculosis complex isolates. J. Clin. Microbiol. **46:**2692–2699.
3. **Andrews, J. R., N. R. Gandhi, P. Moodley, N. S. Shah, L. Bohlken, A. P. Moll, M. Pillay, G. Friedland, and A. W. Sturm.** 2008. Exogenous reinfection as a cause of multidrug-resistant and extensively drug-resistant tuberculosis in rural South Africa. J. Infect. Dis. **198:**1582–1589.
4. **Brown, T., V. Nikolayevskyy, P. Velji, and F. Drobniewski.** 2010. Associations between Mycobacterium tuberculosis strains and phenotypes. Emerg. Infect. Dis. **16:**272–280.
5. **Brudey, K., J. R. Driscoll, L. Rigouts, W. M. Prodinger, A. Gori, S. A. Al-Hajoj, C. Allix, L. Aristimuno, J. Arora, V. Baumanis, L. Binder, P. Cafrune, A. Cataldi, S. Cheong, R. Diel, C. Ellermeier, J. T. Evans, M. Fauville-Dufaux, S. Ferdinand, D. Garcia de Viedma, C. Garzelli, L. Gazzola, H. M. Gomes, M. C. Guttierez, P. M. Hawkey, P. D. van Helden, G. V. Kadival, B. N. Kreiswirth, K. Kremer, M. Kubin, S. P. Kulkarni, B. Liens, T. Lillebaek, M. L. Ho, C. Martin, I. Mokrousov, O. Narvskaia, Y. F. Ngeow, L. Naumann, S. Niemann, I. Parwati, Z. Rahim, V. Rasolofo-Razanamparany, T. Rasolonavalona, M. L. Rossetti, S. Rusch-Gerdes, A. Sajduda, S. Samper, I. G. Shemyakin, U. B. Singh, A. Somoskovi, R. A. Skuce, D. van Soolingen, E. M. Streicher, P. N. Suffys, E. Tortoli, T. Tracevska, V. Vincent, T. C. Victor, R. M. Warren, S. F. Yap, K. Zaman, F. Portaels, N. Rastogi, and C. Sola.** 2006. Mycobacterium tuberculosis complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol. **6:**23.
6. **Cohen, T., C. Colijn, and M. Murray.** 2008. Modeling the effects of strain diversity and mechanisms of strain competition on the potential performance of new tuberculosis vaccines. Proc. Natl. Acad. Sci. U. S. A. **105:**16302–16307.
7. **Cohen, T., M. Murray, K. Wallengren, G. G. Alvarez, E. Y. Samuel, and D. Wilson.** 2010. The prevalence and drug sensitivity of tuberculosis among

patients dying in hospital in KwaZulu-Natal, South Africa: a postmortem study. PLoS Med. **7:**e1000296.
8. **Colijn, C., T. Cohen, and M. Murray.** 2009. Latent coinfection and the maintenance of strain diversity. Bull. Math. Biol. **71:**247–263.
9. **Comas, I., S. Homolka, S. Niemann, and S. Gagneux.** 2009. Genotyping of genetically monomorphic bacteria: DNA sequencing in Mycobacterium tuberculosis highlights the limitations of current methodologies. PLoS One **4:**e7815.
10. **Daley, C. L., P. M. Small, G. F. Schecter, G. K. Schoolnik, R. A. McAdam, W. R. Jacobs, Jr., and P. C. Hopewell.** 1992. An outbreak of tuberculosis with accelerated progression among persons infected with the human immunodeficiency virus. An analysis using restriction-fragment-length polymorphisms. N. Engl. J. Med. **326:**231–235.
11. **Garcia de Viedma, D., N. Alonso Rodriguez, S. Andres, M. J. Ruiz Serrano, and E. Bouza.** 2005. Characterization of clonal complexity in tuberculosis by mycobacterial interspersed repetitive unit–variable-number tandem repeat typing. J. Clin. Microbiol. **43:**5660–5664.
12. **Garcia de Viedma, D., M. Marin, M. J. Ruiz, and E. Bouza.** 2004. Analysis of clonal composition of Mycobacterium tuberculosis isolates in primary infections in children. J. Clin. Microbiol. **42:**3415–3418.
13. **Garcia de Viedma, D., M. Marin, M. J. Ruiz Serrano, L. Alcala, and E. Bouza.** 2003. Polyclonal and compartmentalized infection by Mycobacterium tuberculosis in patients with both respiratory and extrarespiratory involvement. J. Infect. Dis. **187:**695–699.
14. **The Italian Cooperation in South Africa.** 2009. 1 March 2010, accession date. Demographic and health indicators, KwaZulu-Natal. The Italian Cooperation in South Africa, Pretoria, South Africa. http://www.italcoop.co.za/PublicDocuments/Demographic_Health_Statisticaldata_KW_EC_SA%20_EN.pdf.
15. **Martin, A., M. Herranz, M. J. Ruiz Serrano, E. Bouza, and D. Garcia de Viedma.** 2010. The clonal composition of Mycobacterium tuberculosis in clinical specimens could be modified by culture. Tuberculosis (Edinb.) **90:**201–207.
16. **Martin, A., M. Herranz, M. J. Serrano, E. Bouza, and D. Garcia de Viedma.** 2007. Rapid clonal analysis of recurrent tuberculosis by direct MIRU-VNTR typing on stored isolates. BMC Microbiol. **7:**73.
17. **Mathema, B., N. E. Kurepina, P. J. Bifani, and B. N. Kreiswirth.** 2006. Molecular epidemiology of tuberculosis: current insights. Clin. Microbiol. Rev. **19:**658–685.
18. **Mazars, E., S. Lesjean, A. L. Banuls, M. Gilbert, V. Vincent, B. Gicquel, M. Tibayrenc, C. Locht, and P. Supply.** 2001. High-resolution minisatellite-based typing as a portable approach to global analysis of Mycobacterium tuberculosis molecular epidemiology. Proc. Natl. Acad. Sci. U. S. A. **98:**1901–1906.
19. **Mokrousov, I., V. Valcheva, N. Sovhozova, A. Aldashev, N. Rastogi, and J. Isakova.** 2009. Penitentiary population of Mycobacterium tuberculosis in Kyrgyzstan: exceptionally high prevalence of the Beijing genotype and its Russia-specific subtype. Infect. Genet. Evol. **9:**1400–1405.
20. **Narayanan, S., S. Swaminathan, P. Supply, S. Shanmugam, G. Narendran, L. Hari, R. Ramachandran, C. Locht, M. S. Jawahar, and P. R. Narayanan.** 2010. Impact of HIV infection on the recurrence of tuberculosis in South India. J. Infect. Dis. **201:**691–703.
21. **National Department of Health, S. A.** 2007. 1 March 2010, accession date. The national HIV and syphilis prevalence survey, South Africa, 2006. Department of Health South Africa, Pretoria, South Africa. http://www.doh.gov.za/docs/reports/2007/hiv/partl.pdf.
22. **Niemann, S., C. U. Koser, S. Gagneux, C. Plinke, S. Homolka, H. Bignell, R. J. Carter, R. K. Cheetham, A. Cox, N. A. Gormley, P. Kokko-Gonzales, L. J. Murray, R. Rigatti, V. P. Smith, F. P. Arends, H. S. Cox, G. Smith, and J. A. Archer.** 2009. Genomic diversity among drug sensitive and multidrug resistant isolates of Mycobacterium tuberculosis with identical DNA fingerprints. PLoS One **4:**e7407.
23. **Oelemann, M. C., R. Diel, V. Vatin, W. Haas, S. Rusch-Gerdes, C. Locht, S. Niemann, and P. Supply.** 2007. 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. **45:**691–697.
24. **Shamputa, I. C., L. Jugheli, N. Sadradze, E. Willery, F. Portaels, P. Supply, and L. Rigouts.** 2006. Mixed infection and clonal representativeness of a single sputum sample in tuberculosis patients from a penitentiary hospital in Georgia. Respir. Res. **7:**99.
25. **Shen, G., Z. Xue, X. Shen, B. Sun, X. Gui, M. Shen, J. Mei, and Q. Gao.** 2006. The study recurrent tuberculosis and exogenous reinfection, Shanghai, China. Emerg. Infect. Dis. **12:**1776–1778.
26. **Small, P. M., P. C. Hopewell, S. P. Singh, A. Paz, J. Parsonnet, D. C. Ruston, G. F. Schecter, C. L. Daley, and G. K. Schoolnik.** 1994. The epidemiology of tuberculosis in San Francisco. A population-based study using conventional and molecular methods. N. Engl. J. Med. **330:**1703–1709.
27. **Sola, C., I. Filliol, E. Legrand, S. Lesjean, C. Locht, P. Supply, and N. Rastogi.** 2003. Genotyping of the Mycobacterium tuberculosis complex using MIRUs: association with VNTR and spoligotyping for molecular epidemiology and evolutionary genetics. Infect. Genet. Evol. **3:**125–133.
28. **Supply, P., C. Allix, S. Lesjean, M. Cardoso-Oelemann, S. Rusch-Gerdes, E. Willery, E. Savine, P. de Haas, H. van Deutekom, S. Roring, P. Bifani, N. Kurepina, B. Kreiswirth, C. Sola, N. Rastogi, V. Vatin, M. C. Gutierrez, M. Fauville, S. Niemann, R. Skuce, K. Kremer, C. Locht, and D. van Soolingen.** 2006. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of Mycobacterium tuberculosis. J. Clin. Microbiol. **44:**4498–4510.
29. **Supply, P., S. Lesjean, E. Savine, K. Kremer, D. van Soolingen, and C. Locht.** 2001. Automated high-throughput genotyping for study of global epidemiology of Mycobacterium tuberculosis based on mycobacterial interspersed repetitive units. J. Clin. Microbiol. **39:**3563–3571.
30. **van Rie, A., T. C. Victor, M. Richardson, R. Johnson, G. D. van der Spuy, E. J. Murray, N. Beyers, N. C. Gey van Pittius, P. D. van Helden, and R. M. Warren.** 2005. Reinfection and mixed infection cause changing Mycobacterium tuberculosis drug-resistance patterns. Am. J. Respir. Crit. Care Med. **172:**636–642.
31. **van Rie, A., R. Warren, M. Richardson, T. C. Victor, R. P. Gie, D. A. Enarson, N. Beyers, and P. D. van Helden.** 1999. Exogenous reinfection as a cause of recurrent tuberculosis after curative treatment. N. Engl. J. Med. **341:**1174–1179.
32. **Warren, R. M., E. M. Streicher, S. L. Sampson, G. D. van der Spuy, M. Richardson, D. Nguyen, M. A. Behr, T. C. Victor, and P. D. van Helden.** 2002. Microevolution of the direct repeat region of Mycobacterium tuberculosis: implications for interpretation of spoligotyping data. J. Clin. Microbiol. **40:**4457–4465.
33. **Warren, R. M., T. C. Victor, E. M. Streicher, M. Richardson, N. Beyers, N. C. Gey van Pittius, and P. D. van Helden.** 2004. Patients with active tuberculosis often have different strains in the same sputum specimen. Am. J. Respir. Crit. Care Med. **169:**610–614.