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Molecular Epidemiological Interpretation of the Epidemic of Extensively Drug-Resistant Tuberculosis in South Africa

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We show that the interpretation of molecular epidemiological data for extensively drug-resistant tuberculosis (XDR-TB) is dependent on the number of different markers used to define transmission. Using spoligotyping, IS*6110* DNA fingerprinting, and DNA sequence data, we show that XDR-TB in South Africa (2006 to 2008) was predominantly driven by the acquisition of second-line drug resistance.

Molecular epidemiological studies of *Mycobacterium tuberculosis* have been instrumental in informing tuberculosis (TB) control policy in low-incidence settings (1–6). However, the accuracy of molecular epidemiological inferences depends on whether the genetic data accurately reflect the epidemiology. Within the context of the *M. tuberculosis* epidemic, clustering of identical or near-identical genotypes has been assumed to reflect transmission, while nonclustered (unique) genotypes are inferred to reflect the reactivation of a previous infection or importation of TB from another setting (7, 8). However, these assumptions do not hold true when studying drug-resistant tuberculosis, as they do not take into account the fact that drug resistance may evolve independently in strains with identical genetic backgrounds. When drug resistance patterns are ignored, the interpretation of clustered drug-resistant strains would be that they occurred by transmission, while when included in the algorithm, the interpretation could be that drug resistance was acquired, provided the mutations are different. Failure to recognize these interpretational errors might incorrectly inform policy, thereby negatively impacting TB control strategy.

In order to curb the drug resistance epidemic, it is essential to gain insight into the underlying causes of drug resistance in different geographical locations. This is particularly relevant with respect to the extensively drug-resistant TB (XDR-TB) epidemic, which is now a global phenomenon and has been identified in 100 countries (9). A recent XDR-TB review showed that spoligotyping and or mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) genotyping and IS*6110* DNA fingerprinting have been used to describe the epidemiology of XDR-TB cases in different settings (10). However, these studies have not investigated the possibility of the concurrent evolution of drug resistance within strains with identical genetic backgrounds. In this study, we aimed to investigate how the inclusion of different genetic information might influence the interpretation of the epidemiology of XDR-TB. The first available XDR-TB isolates from 118 of 127 cases (93%) diagnosed in the Western Cape Province of South Africa during the study period of November 2006 to October 2008, were included in the study. During the study period, routine drug susceptibility testing (DST) was expanded to include the second-line drugs ofloxacin and amikacin. This policy was implemented in February 2007 and applied to all existing and newly diagnosed multidrug-resistant TB (MDR-TB) patients. Thus, many of the included cases were receiving MDR-TB treatment at the time that XDR-TB was diagnosed. These isolates were genotyped using the internationally standardized methods of spoligotyping (11) and IS*6110* DNA fingerprinting (12) (see Table S1 in the supplemental material). In addition, targeted DNA sequencing was done to identify mutations in the *inhA* promoter and the *katG*, *rpoB*, *embB*, *pncA*, *gyrA*, and *rrs* genes known to confer resistance to isoniazid, rifampin, ethambutol, pyrazinamide, ofloxacin, amikacin, kanamycin, and capreomycin (13). Molecular epidemiological inferences were made using the genotyping data from either individual markers or various combinations of the different markers. A cluster was defined when isolates shared identical genotypes according to the markers included in the analysis (Table 1). When the resistance-conferring mutations were included, we assumed that the order in which resistance accumulated was isoniazid, rifampin, ethambutol, pyrazinamide, and ofloxacin, followed by aminoglycosides.

From Table 1, it is evident that spoligotyping had the lowest discriminatory index, identifying only 9 spoligotype patterns, which were grouped into 3 clusters (95% clustering) and 6 unique spoligotypes. The IS*6110* DNA fingerprinting method identified 34 strain genotypes. Of these, 11 clusters (81% clustering) and 23 unique DNA fingerprints were identified. When the spoligotype and DNA fingerprint data were combined, they did not significantly alter the proportion of clustered isolates (spoligotyping and IS*6110* DNA fingerprinting, 79%, versus IS*6110* DNA fingerprint-

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TABLE 1 Clustering of all XDR-TB strains using a combination of different genetic markers

Genetic marker(s)	Strict clustering of IS*6110*				Relaxed clustering of IS*6110*			
	No. of unique genotypes	No. of clustered genotypes	No. of clusters	% clustering	No. of unique genotypes	No. of clustered genotypes	No. of clusters	% clustering
Spoligotyping	6	112	3	94.9	6	112	3	94.9
IS*6110* DNA fingerprinting	23	95	11	80.5	11	107	4	90.7
Spoligotyping + IS*6110*	25	93	12	78.8	13	105	5	89
Spoligotyping + IS*6110* + *katG*	29	89	13	75.4	17	101	6	85.6
Spoligotyping + IS*6110* + *inhA*P	29	89	14	75.4	15	103	8	87.3
Spoligotyping + IS*6110* + *rpoB*	28	90	13	76.3	15	103	6	87.3
Spoligotyping + IS*6110* + *katG* + *inhA*P	32	86	15	72.9	18	100	9	84.7
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB*	34	84	15	71.2	20	98	9	83.1
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB* + *pncA*	46	72	14	61	32	86	11	72.9
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB* + *embB*	36	82	16	69.5	20	98	11	83.1
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB* + *pncA* + *embB*	50	68	13	57.6	33	85	13	72
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB* + *pncA* + *embB* + *gyrA*	76	42	10	35.6	62	56	13	47.5
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB* + *pncA* + *embB* + *rrs*	55	63	12	53.4	39	79	12	66.9
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB* + *pncA* + *embB* + *gyrA* + *rrs*	79	39	10	33.1	65	53	13	44.9

ing only, 81%; $P = 0.87$). These clusters remained largely intact when incorporating mutations conferring isoniazid and rifampin resistance (spoligotyping and IS*6110* DNA fingerprinting with isoniazid and rifampin resistance-conferring mutations, 71%, versus spoligotyping and IS*6110* DNA fingerprinting only, 79%; $P = 0.23$). Epidemiologic support for transmission MDR-TB was not shown, as MDR-TB cases that did not progress to XDR-TB were excluded from the study, thereby preventing the identification of contacts. The inclusion of mutations conferring pyrazinamide and ethambutol resistance reduced the proportion of clustering to 58% ($P = 0.031$). This suggests that circulating MDR-TB strains have independently acquired ethambutol and pyrazin-

TABLE 2 Clustering of atypical Beijing XDR-TB strains using a combination of different genetic markers

Genetic marker(s)	Strict clustering of IS*6110*				Relaxed clustering of IS*6110*			
	No. of unique genotypes	No. of clustered genotypes	No. of clusters	% clustering	No. of unique genotypes	No. of clustered genotypes	No. of clusters	% clustering
Spoligotyping	0	62	1	100.0	0	62	1	100.0
IS*6110* DNA fingerprinting	7	55	3	88.7	2	60	1	96.8
Spoligotyping + IS*6110*	7	55	3	88.7	2	60	1	96.8
Spoligotyping + IS*6110* + *katG*	7	55	4	88.7	2	60	2	96.8
Spoligotyping + IS*6110* + *inhA*P	9	53	4	85.5	2	60	3	96.8
Spoligotyping + IS*6110* + *rpoB*	9	53	4	85.5	3	59	2	95.2
Spoligotyping + IS*6110* + *katG* + *inhA*P	9	53	5	85.5	2	60	4	96.8
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB*	10	52	5	83.9	3	59	4	95.2
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB* + *pncA*	13	49	5	79.0	8	54	3	87.1
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB* + *embB*	10	52	6	83.9	3	59	5	95.2
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB* + *pncA* + *embB*	13	49	6	79.0	8	54	4	87.1
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB* + *pncA* + *embB* + *gyrA*	30	32	6	51.6	19	43	8	69.4
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB* + *pncA* + *embB* + *rrs*	14	48	6	77.4	9	53	4	85.5
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB* + *pncA* + *embB* + *gyrA* + *rrs*	31	31	6	50.0	20	42	8	67.7

TABLE 3 Clustering of XDR-TB strains other than atypical Beijing strains using a combination of different genetic markers

Genetic marker(s)	Strict clustering of IS*6110*				Relaxed clustering of IS*6110*			
	No. of unique genotypes	No. of clustered genotypes	No. of clusters	% clustering	No. of unique genotypes	No. of clustered genotypes	No. of clusters	% clustering
Spoligotyping	6	50	3	89.3	6	50	3	89.3
IS*6110* DNA fingerprinting	16	40	8	71.4	9	47	3	83.9
Spoligotyping + IS*6110*	18	38	9	67.9	11	45	4	80.4
Spoligotyping + IS*6110* + *katG*	22	34	9	60.7	15	41	4	73.2
Spoligotyping + IS*6110* + *inhA*P	20	36	10	64.3	13	43	5	76.8
Spoligotyping + IS*6110* + *rpoB*	19	37	9	66.1	12	44	4	78.6
Spoligotyping + IS*6110* + *katG* + *inhA*P	23	33	10	58.9	16	40	5	71.4
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB*	24	32	10	57.1	17	39	5	69.6
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB* + *pncA*	33	23	9	41.1	24	32	8	57.1
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB* + *embB*	26	30	10	53.6	17	39	6	69.6
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB* + *pncA* + *embB*	37	19	7	33.9	25	31	9	55.4
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB* + *pncA* + *embB* + *gyrA*	46	10	4	17.9	43	13	5	23.2
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB* + *pncA* + *embB* + *rrs*	41	15	6	26.8	30	26	8	46.4
Spoligotyping + IS*6110* + *katG* + *inhA*P + *rpoB* + *pncA* + *embB* + *gyrA* + *rrs*	48	8	4	14.3	45	11	5	19.6

amide resistance and were then subsequently transmitted, as demonstrated by the estimated proportion of clustering (58%). When mutations conferring second-line resistance were incorporated into the clustering algorithm, a significant decrease in the proportion of clustered isolates was observed (33% with versus 58% without; $P = 0.000235$). This suggests that resistance to fluoroquinolones and aminoglycosides was subsequently acquired in MDR-TB strains that were already resistant to ethambutol and pyrazinamide. The absence of transmission was supported by an analysis of the clinic location where each patient reported, as only 10 patients (within strictly defined clusters) or 15 patients (within relaxed clusters) were from the same community (see Table S1 in the supplemental material).

A comparison of the IS*6110* patterns with previously reported studies (13, 14) showed that 53% of the patients were infected with atypical Beijing XDR-TB strains, which were genotypically closely related to those reported in the Eastern Cape Province of South Africa. Clustering of the atypical Beijing strains was found to be significantly higher than that for the rest of the strain population (50% versus 14% without clustering; $P = 0.000036$) (Tables 2 and 3). This finding was based on the analysis of a combination of all of the markers; therefore, it is unlikely that clustering is a function of genetic stability (15) rather than transmission. These strains were genotypically identical to the atypical Beijing XDR-TB strains identified in the neighboring Eastern Cape Province (13), suggesting importation via migration (16). Analysis of the residential location of these cases showed that 10 patients were grouped within 2 suburbs, suggesting that these strains are now being transmitted within urban settings in the Western Cape Province.

We acknowledge that the proportion of clustered cases may be underestimated, as patients with XDR-TB may have died before diagnosis, patient isolates were not tested for second-line resistance, patient isolates were not available for genotyping, or diagnostic data were not available. Furthermore, our definition of an IS*6110* DNA fingerprint cluster may have been too stringent (17, 18). By relaxing the definition of a cluster to allow for 2 IS*6110* band variations, we identified 13 clusters and 65 unique cases, which increased the proportion of clustered cases to 45% (atypical Beijing, 68%, versus other, 20%). We also acknowledge that our analysis may have led to an overestimate of clustering, as the same mutation may be acquired independently in different isolates.

From the abovementioned results, it is evident that in this high-incidence setting, the estimate of the proportion of clustered cases is sensitive to the genotyping methods used. This cautions the use of a single genotyping method to describe the epidemiology of XDR-TB. Accordingly, we propose the inclusion of mutational data together with an informative genotyping method (IS*6110* DNA fingerprinting or MIRU-VNTR typing) to accurately reflect the epidemiology of XDR-TB.

Our genotyping results are in line with previous reports, which concluded that XDR-TB is acquired following the transmission of MDR-TB strains in the Western Cape Province of South Africa (19). Furthermore, we show that the XDR-TB epidemic in this region is strongly influenced by migration from the Eastern Cape (16), a region where an outbreak of an atypical Beijing XDR-TB strain has been reported (13). Given that the outcome of XDR-TB treatment is dismal in this region (20), it is essential that rapid drug susceptibility tests are implemented to guide the formulation of a strengthened MDR-TB treatment regimen to prevent the acquisition of additional resistance.

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REFERENCES

1. **Moss AR, Alland D, Telzak E, Hewlett D, Jr, Sharp V, Chiliade P, LaBombardi V, Kabus D, Hanna B, Palumbo L, Brudney K, Weltman A, Stoeckle K, Chirgwin K, Simberkoff M, Moghazeh S, Eisner W, Lutfey M, Kreiswirth B.** 1997. A city-wide outbreak of a multiple-drug-resistant strain of *Mycobacterium tuberculosis* in New York. Int J Tuberc Lung Dis **1:**115–121.
2. **Frieden TR, Munsiff SS, Ahuja SD.** 2007. Outcomes of multidrug-resistant tuberculosis treatment in HIV-positive patients in New York City, 1990–1997. Int J Tuberc Lung Dis **11:**116.
3. **Frieden TR, Sterling T, Pablos-Mendez A, Kilburn JO, Cauthen GM, Dooley SW.** 1993. The emergence of drug-resistant tuberculosis in New York City. N Engl J Med **328:**521–526. http://dx.doi.org/10.1056/NEJM199302253280801.
4. **Frieden TR, Woodley CL, Crawford JT, Lew D, Dooley SM.** 1996. The molecular epidemiology of tuberculosis in New York City: the importance of nosocomial transmission and laboratory error. Tuber Lung Dis **77:**407–413. http://dx.doi.org/10.1016/S0962-8479(96)90112-4.
5. **van Soolingen D, Borgdorff MW, de Haas PE, Sebek MM, Veen J, Dessens M, Kremer K, van Embden JD.** 1999. Molecular epidemiology of tuberculosis in the Netherlands: a nationwide study from 1993 through 1997. J Infect Dis **180:**726–736. http://dx.doi.org/10.1086/314930.
6. **Van Soolingen D.** 2001. Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. J Intern Med **249:**1–26. http://dx.doi.org/10.1046/j.1365-2796.2001.00772.x.
7. **Small PM, Hopewell PC, Singh SP, Paz A, Parsonnet J, Ruston DC, Schecter GF, Daley CL, Schoolnik GK.** 1994. The epidemiology of tuberculosis in San Francisco. A population-based study using conventional and molecular methods. N Engl J Med **330:**1703–1709.
8. **Alland D, Kalkut GE, Moss AR, McAdam RA, Hahn JA, Bosworth W, Drucker E, Bloom BR.** 1994. Transmission of tuberculosis in New York City. An analysis by DNA fingerprinting and conventional epidemiologic methods. N Engl J Med **330:**1710–1716.
9. **WHO.** 2013. Global tuberculosis report 2013. World Health Organization, Geneva, Switzerland. http://apps.who.int/iris/bitstream/10665/91355/1/9789241564656_eng.pdf.
10. **Dheda K, Gumbo T, Gandhi NR, Murray M, Theron G, Udwadia Z, Migliori GB, Warren R.** 2014. Global control of tuberculosis: from extensively drug-resistant to untreatable tuberculosis. Lancet Respir Med **2:**321–338. http://dx.doi.org/10.1016/S2213-2600(14)70031-1.
11. **Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, Bunschoten A, Molhuizen H, Shaw R, Goyal M, van Embden J.** 1997. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol **35:**907–914.
12. **Warren R, de Kock M, Engelke E, Myburgh R, Gey van Pittius N, Victor T, van Helden P.** 2006. Safe *Mycobacterium tuberculosis* DNA extraction method that does not compromise integrity. J Clin Microbiol **44:**254–256. http://dx.doi.org/10.1128/JCM.44.1.254-256.2006.
13. **Klopper M, Warren RM, Hayes C, Gey van Pittius NC, Streicher EM, Muller B, Sirgel FA, Chabula-Nxiweni M, Hoosain E, Coetzee G, David van Helden P, Victor TC, Trollip AP.** 2013. Emergence and spread of extensively and totally drug-resistant tuberculosis, South Africa. Emerg Infect Dis **19:**449–455. http://dx.doi.org/10.3201/eid1903.120246.
14. **Strauss OJ, Warren RM, Jordaan A, Streicher EM, Hanekom M, Falmer AA, Albert H, Trollip A, Hoosain E, van Helden PD, Victor TC.** 2008. Spread of a low-fitness drug-resistant *Mycobacterium tuberculosis* strain in a setting of high human immunodeficiency virus prevalence. J Clin Microbiol **46:**1514–1516. http://dx.doi.org/10.1128/JCM.01938-07.
15. **van Soolingen D, Qian L, de Haas PE, Douglas JT, Traore H, Portaels F, Qing HZ, Enkhsaikan D, Nymadawa P, van Embden JD.** 1995. Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of East Asia. J Clin Microbiol **33:**3234–3238.
16. **Müller B, Chihota VN, Pillay M, Klopper M, Streicher EM, Coetzee G, Trollip A, Hayes C, Bosman ME, Gey van Pittius NC, Victor TC, Gagneux S, van Helden PD, Warren RM.** 2013. Programmatically selected multidrug-resistant strains drive the emergence of extensively drug-resistant tuberculosis in South Africa. PLoS One **8:**e70919. http://dx.doi.org/10.1371/journal.pone.0070919.
17. **van der Spuy GD, Warren RM, Richardson M, Beyers N, Behr MA, van Helden PD.** 2003. Use of genetic distance as a measure of ongoing transmission of *Mycobacterium tuberculosis*. J Clin Microbiol **41:**5640–5644. http://dx.doi.org/10.1128/JCM.41.12.5640-5644.2003.
18. **Yeh RW, Ponce de Leon A, Agasino CB, Hahn JA, Daley CL, Hopewell PC, Small PM.** 1998. Stability of *Mycobacterium tuberculosis* DNA genotypes. J Infect Dis **177:**1107–1111. http://dx.doi.org/10.1086/517406.
19. **Ioerger TR, Feng Y, Chen X, Dobos KM, Victor TC, Streicher EM, Warren RM, Gey van Pittius NC, Van Helden PD, Sacchettini JC.** 2010. The non-clonality of drug resistance in Beijing-genotype isolates of *Mycobacterium tuberculosis* from the Western Cape of South Africa. BMC Genomics **11:**670. http://dx.doi.org/10.1186/1471-2164-11-670.
20. **Pietersen E, Ignatius E, Streicher EM, Mastrapa B, Padanilam X, Pooran A, Badri M, Lesosky M, van Helden P, Sirgel FA, Warren R, Dheda K.** 2014. Long-term outcomes of patients with extensively drug-resistant tuberculosis in South Africa: a cohort study. Lancet **383:**1230–1239. http://dx.doi.org/10.1016/S0140-6736(13)62675-6.