Acquisition of Rifampin Resistance in Pulmonary Tuberculosis

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ABSTRACT *Mycobacterium tuberculosis* strains with spontaneous mutations conferring resistance to rifampin (RIF) are exceedingly rare, and fixed drug combinations typically prevent augmentation of resistance to single drugs. Fourteen newly diagnosed tuberculosis patients were treated with RIF alone for 14 days, and bacterial loads, including mutation frequencies, were determined. A statistical model estimated that 1% of the remaining viable mycobacteria could be RIF resistant after 30 days of monotherapy. This indicates that temporal and spatial windows of RIF monotherapy due to uneven drug distribution within lung lesions could contribute to the acquisition of resistance to RIF.

KEYWORDS tuberculosis, rifampin, treatment, resistance

Drug resistance is a peculiarity of *Mycobacterium tuberculosis* in which resistance to antibiotics is genetically encoded. Resistant *M. tuberculosis* can be transmitted from one person to another, but resistance cannot be transmitted from one bacterium to another (1). Mycobacteria with spontaneous resistance mutations are naturally present in every population of *M. tuberculosis* (2, 3). The mutation rate, i.e., the probability that a resistance-conferring mutation occurs spontaneously in a bacterium, is very low for rifampin (RIF), the cornerstone of current treatment regimens, with 3.32×10^{-9} for *M. tuberculosis* strain H37Rv (4). However, Beijing strains, which are more prevalent in the Eastern and Western Cape provinces, South Africa, were found to acquire drug resistance *in vitro* faster than other strains (5–7).

It is generally accepted that clinical drug resistance, i.e., the failure of antibiotic treatment to control tuberculosis (TB) in a patient, can emerge through augmentation of a small initial population of resistant mycobacteria that become dominant when susceptible bacteria are eliminated by antibiotic treatment (1, 8). Thus, antibiotic treatment combining different agents can prevent the possibility that a mutant which is resistant to a single antibiotic is able to become clinically relevant, and an effective combination has been shown to provide cure within 6 to 8 months, also in an outpatient setting (9–11). In the absence of a better explanation, patient noncompliance with the prescribed treatment has been blamed for drug resistance, but other lines of evidence, such as the hollow fiber model, have confirmed that noncompliance alone is unlikely to create drug resistance when a fixed-dose combination is used (12). A more convincing theory is pharmacokinetic and pharmacodynamic variability between patients and within patients, referring to differences in drug absorption and metabolism, uneven drug distribution, and bacterial subpopulations that are not equally susceptible. All these factors could conspire to create pockets or temporal windows of monotherapy within patients' lungs where resistance develops (13). Recently published work by Prideaux and coauthors appears to confirm this theory, with

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TABLE 1 CFU counts and mutation frequencies at baseline and at day 14 of treatment with RIF

Sample no.	Strain type ^a	CFU count (log ₁₀ CFU)		Mutation frequency (10 ⁻⁷)	
		Baseline	Day 14	Baseline	Day 14
1	Beijing	6.84	5.09	0.503	45.405
2	T1	NA ^b	NA	0.026	301.793
3	Beijing	5.20	2.82	0.008	64.044
4	X2	4.92	3.67	0.587	103.569
5	CAS1_KIL	5.09	3.3	0.719	97.667
6	Beijing	5.11	3.67	0.060	151.128
7	Unknown	5.06	4.16	0.555	159.136
8	Beijing	6.93	6.24	0.035	9.012
9	S	6.64	4.94	0.009	11.994
10	Beijing	4.21	4.93	0.017	6.512
11	LAM3	7.12	5.49	0.038	60.964
12	Beijing	6.00	6.31	0.442	90.420
13	LAM3	6.59	5.21	1.518	64.763
14	S	5.63	2.40	0.028	210.312
Median		5.63	4.93	0.049	77.60
Minimum		4.21	2.04	0.008	6.512
Maximum		7.12	6.31	1.518	301.80

^aT1, T1 family; X2, X2 family; CAS1_KIL, Central Asia 1 Kilimanjaro family; S, S family; LAM3, Latin American and Mediterranean 3 family.

^bNA, value not available at this time point due to contamination.

RIF being present at higher concentrations in lung lesions, including necrotic caseum mimicking RIF monotherapy (14). The pivotal question at this point is whether, and how quickly, RIF monotherapy could lead to clinically relevant RIF resistance with more than 1% RIF-resistant bacteria found in a sputum sample.

To approximate this clinical scenario, we treated 14 newly diagnosed, RIF-susceptible TB patients with RIF at the standard dose of 10 mg/kg of body weight/day for 14 days, the longest time considered safe for monotherapy, followed by a full course of standard combination treatment to ensure cure of these subjects (15). We harvested sputum before treatment and daily for the first 2 weeks to measure the total sputum bacterial load over the 14-day period as CFU counts and the mutation frequency for RIF before and at 14 days (for detailed methods, see the supplemental material). From these data, we constructed a statistical model and extrapolated, assuming linear continuation to the rates of change measured over the first 14 days, how quickly RIF resistance could become clinically relevant. Ethical approval for this study was granted by the local ethics committee and the Medicines Control Council of South Africa.

At baseline, we found a median of 5.63 (range, 4.21 to 7.12) log₁₀ CFU/ml of sputum and a mutation frequency of 4.9 (range, 0.8 to 151.8) × 10⁻⁹. Over 14 days, the CFU counts dropped by an average of 0.093 log₁₀ CFU/day, the expected effect of the antibiotic, and the mean mutation frequency increased 1,144-fold (Table 1). Reflecting the epidemiological situation in Cape Town, we found Beijing strains (43%) to be predominant (Table 1). Twenty-four out of 28 phenotypically resistant cultures recovered from the mutation frequency experiments were found resistant with the GenoType MTBDRplus line probe assay (Hain Lifescience, Nehren, Germany), which covers the region with the most common resistance-conferring mutations on the *rpoB* gene (data not shown) (16). The regions of interest of one baseline and three day 14 cultures phenotypically resistant but determined to be susceptible by the line probe assay were sequenced. They revealed one less commonly found mutation on codon 537 located outside the region of the *rpoB* gene (Table 2).

Figure 1 shows modeled (first 2 weeks) and interpolated lines for the total sputum CFU (descending black line) and mutation frequency (ascending black line) with confidence intervals. Also depicted are the predicted resistant sputum CFU (blue) as the product of mutation frequency and total CFU, and the level of clinical resistance at a

TABLE 2 Codons and changes in *rpoB* of four isolates of *M. tuberculosis* determined as susceptible to RIF by line probe assay

Isolate	Codon no.	Nucleotide sequence		Amino acid change
		Wild type	Mutation	
1	526	CAC	TAC	H→Y
2	531	TCG	TTG	S→L
3	526	CAC	TAC	H→Y
	537	GGT	AGT	G→S
4	526	CAC	GAC	H→D

1% of total CFU (red) (17). The illustration shows that, in the absence of resistance, culture-negative sputum could be expected at around 2 months of treatment with RIF alone. However, resistant CFU would become measurable at the critical proportion of 1% of the remaining CFU at about 1 month of RIF monotherapy and would subsequently dominate in sputum, preventing culture conversion to negative.

This scenario, though hypothetical and assuming linear continuation of the trends observed in the first 2 weeks, ties in very well with clinical observations made in some patients treated with RIF-based regimens. In these individuals, initial clinical improvement stagnates after a few weeks and is followed by clinical deterioration and persisting positive sputum or reversion of negative to positive sputum. Clinical guidelines that have stood the test of time recommend that patients who fail to clinically improve or convert sputum smears to negative at 2 months of treatment are at risk of having acquired resistance and should undergo sputum culture and resistance testing (9). Depending on the proportion of resistant bacteria present in these sputum samples, this could be diagnosed as heteroresistance or resistance to RIF, necessitating a switch of treatment in these patients (18).

Our findings can add to the growing evidence that pharmacodynamic and pharmacokinetic factors are potentially to blame for rising rates of clinical TB drug

FIG 1 Modeled emergence of RIF resistance in *M. tuberculosis* on monotherapy. Statistical modeling estimating the time required for the emergence of RIF resistance in the *M. tuberculosis* population when TB patients are kept on RIF monotherapy beyond 2 weeks. Two weeks of RIF monotherapy induced a significant decrease of CFU (descending black line with dashed confidence interval) ($P < 0.0001$) and a significant increase of mutation frequency (ascending black line with dashed confidence interval) ($P < 0.0001$). The blue line is the estimated count of RIF-resistant CFU and the red line is the estimated 1% of total CFU. The model estimates that the proportion of 1% CFU, considered the breakpoint to define clinical resistance, can be reached after 30 days of RIF monotherapy (blue line crosses the red) and that, after around 40 days (blue line crosses the black), all remaining CFU will be resistant.

resistance despite strong support for consequent combination treatment of TB by the public health sector. Our approach is limited by the fact that we interpolate rather than directly measure the augmentation of RIF resistance clinically, but it would be unethical to expose patients to the risk of acquisition of resistance to RIF to validate this in the field. Further research should focus on methods to measure drug concentrations, drug effects, and drug resistance at the site of disease. This will aid in developing treatment regimens that protect patients from the acquisition of drug resistance during treatment.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.02220-16>.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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REFERENCES

- Gillespie SH. 2002. Evolution of drug resistance in *Mycobacterium tuberculosis*: clinical and molecular perspective. *Antimicrob Agents Chemother* 46:267–274. <https://doi.org/10.1128/AAC.46.2.267-274.2002>.
- Colijn C, Cohen T, Ganesh A, Murray M. 2011. Spontaneous emergence of multiple drug resistance in tuberculosis before and during therapy. *PLoS One* 6:e18327. <https://doi.org/10.1371/journal.pone.0018327>.
- Davies J, Davies D. 2010. Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev* 74:417–433. <https://doi.org/10.1128/MMBR.00016-10>.
- David HL. 1970. Probability distribution of drug-resistant mutants in unselected populations of *Mycobacterium tuberculosis*. *Appl Microbiol* 20:810–814.

5. Ford CB, Shah RR, Maeda MK, Gagneux S, Murray MB, Cohen T, Johnston JC, Gardy J, Lipsitch M, Fortune SM. 2013. Mycobacterium tuberculosis mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. *Nat Genet* 45:784–790. <https://doi.org/10.1038/ng.2656>.
6. Johnson R, Warren RM, van der Spuy GD, Gey van Pittius NC, Theron D, Streicher EM, Bosman M, Coetzee GJ, van Helden PD, Victor TC. 2010. Drug-resistant tuberculosis epidemic in the Western Cape driven by a virulent Beijing genotype strain. *Int J Tuberc Lung Dis* 14:119–121.
7. Strauss OJ, Warren RM, Jordaán 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. <https://doi.org/10.1128/JCM.01938-07>.
8. Shimao T. 1987. Drug resistance in tuberculosis control. *Tubercle* 68: 5–18. [https://doi.org/10.1016/S0041-3879\(87\)80014-4](https://doi.org/10.1016/S0041-3879(87)80014-4).
9. World Health Organization. 2010. Treatment of tuberculosis: guidelines for national programmes. World Health Organization, Geneva, Switzerland. http://www.who.int/tb/features_archive/new_treatment_guidelines_may2010/en/.
10. Jindani A, Nunn AJ, Enarson DA. 2004. Two 8-month regimens of chemotherapy for treatment of newly diagnosed pulmonary tuberculosis: international multicentre randomised trial. *Lancet* 364:1244–1251. [https://doi.org/10.1016/S0140-6736\(04\)17141-9](https://doi.org/10.1016/S0140-6736(04)17141-9).
11. Miles SH, Maat RB. 1984. A successful supervised outpatient short-course tuberculosis treatment program in an open refugee camp on the Thai-Cambodian border. *Am Rev Respir Dis* 130:827–830.
12. Gumbo T, Pasipanodya JG, Romero K, Hanna D, Nuermberger E. 2015. Forecasting accuracy of the hollow fiber model of tuberculosis for clinical therapeutic outcomes. *Clin Infect Dis* 61(Suppl 1):S25–S31. <https://doi.org/10.1093/cid/civ427>.
13. Dartois V. 2014. The path of anti-tuberculosis drugs: from blood to lesions to mycobacterial cells. *Nat Rev Microbiol* 12:159–167. <https://doi.org/10.1038/nrmicro3200>.
14. Prideaux B, Via LE, Zimmerman MD, Eum S, Sarathy J, O'Brien P, Chen C, Kaya F, Weiner DM, Chen P-Y, Song T, Lee M, Shim TS, Cho JS, Kim W, Cho SN, Olivier KN, Barry CE, Dartois V. 2015. The association between sterilizing activity and drug distribution into tuberculosis lesions. *Nat Med* 21:1223–1227. <https://doi.org/10.1038/nm.3937>.
15. Heinrich N, Dawson R, du Bois J, Narunsky K, Horwith G, Phipps AJ, Nacy CA, Aarnoutse RE, Boeree MJ, Gillespie SH, Venter A, Henne S, Rachow A, Phillips PPJ, Hoelscher M, Diacon AH, Pan African Consortium for the Evaluation of Antituberculosis Antibiotics (PanACEA). 2015. Early phase evaluation of SQ109 alone and in combination with rifampicin in pulmonary TB patients. *J Antimicrob Chemother* 70:1558–1566. <https://doi.org/10.1093/jac/dku553>.
16. Hain Lifescience GmbH. 2012. GenoType MTBDRplus version 2.0. Product manual. Hain Lifescience GmbH, Nehren, Germany.
17. Canetti G, Rist N, Grosset J. 1963. Measurement of sensitivity of the tuberculous bacillus to antibacillary drugs by the method of proportions. Methodology, resistance criteria, results and interpretation. *Rev Tuberc Pneumol (Paris)* 27:217–272. (In French.)
18. Hofmann-Thiel S, van Ingen J, Feldmann K, Turaev L, Uzakova GT, Murmusaeva G, van Soolingen D, Hoffmann H. 2009. Mechanisms of heteroresistance to isoniazid and rifampin of Mycobacterium tuberculosis in Tashkent, Uzbekistan. *Eur Respir J* 33:368–374. <https://doi.org/10.1183/09031936.00089808>.