1 Spontaneous mutations conferring antibiotic resistance to antitubercular drugs at a range of concentrations in

2 *Mycobacterium smegmatis*

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9 Abstract

10 Mycobacteria population can undergo mutations in their DNA sequence during replication, which if not repaired, would be
 11 transferred to future generations. In this study, *in vitro* spontaneous mutations in *Mycobacterium smegmatis* mc²155 (*Msm*)
 12 conferring resistance to isoniazid (INH^r), rifampicin (RIF^r), kanamycin (KAN^r) and streptomycin (STR^r) were determined at several
 13 concentrations in a fluctuation assay. Mutation rate was estimated using the P_0 method, and estimates were then compared with
 14 the Lea-Coulson method of the median and Ma-Sandri- Sarkar Maximum Likelihood Estimator (MSS-MLE) method available on the
 15 Fluctuation analysis calculator (FALCOR). The mutation rates of RIF^r ranged from 9.24×10^{-8} - 2.18×10^{-10} , INH^r 1.2×10^{-7} -

16 1.20×10^{-9} , STR^r 2.77×10^{-8} - 5.31×10^{-8} and KAN^r 1.7×10^{-8} mutations per cell division. This study provides mutation rate estimates
17 to key antitubercular drugs at a range of concentrations.

18

19 Keywords: *Mycobacterium smegmatis*, Mutation rate, Fluctuation Analysis Calculator, Antitubercular drugs

20

21 **Introduction**

22 Antibiotic resistance allows bacteria to withstand the inhibitory effects of high concentrations of antibiotics, typically beyond the
23 minimum concentration required to inhibit the growth of the bacterium. In mycobacteria, drug resistance occurs exclusively as a
24 result of spontaneous mutations of genes encoding drug target (1), through a natural process of DNA replication. As such, resistant
25 mutants can then be selected for in the presence of an antibiotic (2). *Mycobacterium tuberculosis* (*Mtb*) immune to several
26 antitubercular drugs is referred to as multi-drug resistant and accounted for 490,000 cases of MDR-TB infections in 2017 and
27 caused 190,000 deaths globally (3). The antibiotics used in this study are shown (Table 1, Figure 1).

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30 Kanamycin (KAN) is an aminoglycoside antibiotic (Fig. 1a), belonging to the same oligosaccharide group of water-soluble antibiotic,
 31 as streptomycin (STR). Administered as a second-line anti TB drug, especially against STR-resistant *Mtb* (4), KAN is bactericidal
 32 and acts, by binding irreversibly to the 16s rRNA of the 30S ribosomal subunit thereby inhibiting the synthesis of protein (6). *In vitro*
 33 KAN-resistant *Mycobacterium smegmatis* (*Msm*) have been shown to possess altered ribosomes (9), which appear to be conferred
 34 by mutation in the *rrs* gene (6). STR (Fig. 1b) is an aminocyclitol aminoglycoside that inhibits translation during protein synthesis
 35 (6). Mutations in the *rpsL* gene and *rrs* genes result to STR-resistance (2, 10). RIF (Fig. 1c) binds to the β subunit of RNA
 36 polymerase, the enzyme responsible for transcription of RNA inhibiting transcription (11). RIF-resistance is due to altered β sub-unit
 37 of the polymerase at one of the three loci in *rpoB* gene (7). Together with RIF, INH (Fig. 1d) forms the core of TB therapy. Catalase-
 38 peroxidase enzyme (*katG*) activates INH into its active form and exerts its effect by inhibiting mycolic acid synthesis, which makes
 39 up a large amount of mycobacterial cell envelope (4). Generally, resistance to INH is often linked to a range of mutations affecting
 40 one or more genes e.g. mutations in the catalase-peroxidase gene, *KatG*, *inhA*-encoded long chain enoyl-ACP reductase, which is
 41 involved with the biosynthesis of mycolic acid (12). Recently also alkyl-hydroperoxide reductase (*aphC*) that is involved in the
 42 cellular response of oxidative stress (13, 5) has been reported to cause resistance in INH.

43 In this study, we isolated *Msm* mutants at a variety of concentrations and determined mutation rates using the P_0 method (15), and
 44 estimates were then compared with the Lea-Coulson method of the median (LC-MM) and the Ma-Sandri- Sarkar Maximum
 45 Likelihood Estimator method (MSS-MLE), available on the fluctuation analysis calculator (16).

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47 **Results**

48 **Results for Minimum Inhibitory Concentration**

49 Mutants are easier to detect at concentrations greater than the MIC, hence to find out what antibiotic concentration will result in the
 50 proliferation of resistant mutants, the minimum concentration, required to inhibit the visible growth of *Msm* was assayed. The results
 51 are shown (Table 2).

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53 **Results for fluctuation assay**

54 Table 3 shows the results for mutation rate calculated using the P_0 method.

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56 **Results for mutation rate using Fluctuation Analysis Calculator (FALCOR)**

Due to the fact that the P_o method is not applicable across all values of m i.e. the number of mutations per culture (15), the LC-MM and MSS-MLE, available on the Fluctuation analysis calculator (16) were also employed in estimating mutation rate of Msm to the test antibiotics (Table 4) and then and compared with the P_o method.

Discussion

We have isolated mutants to Msm at a range of antitubercular drug concentrations. RIF was tested at 100, 200 and 500 $\mu\text{g ml}^{-1}$, INH at 500 and 1000 $\mu\text{g ml}^{-1}$, STR at 20 and 100 $\mu\text{g ml}^{-1}$ and KAN at 100 $\mu\text{g ml}^{-1}$ using the fluctuation assay. We next estimated mutation rates using the P_o method, and estimates were then compared with LC-MM and MSS-MLE methods obtainable on the fluctuation analysis calculator. In our study, the mutation rates estimated using the three methods were comparable. For instance at 100 $\mu\text{g ml}^{-1}$ KAN, the mutation rate was 1.70×10^{-8} mutations per cell division with the P_o method and 1.54×10^{-8} with the LC-MM.

The hypothesis surrounding spontaneous mutation predicts a large fluctuation around the average for the count taken from the individual cultures. As such, a mutation occurring earlier in the growth of the culture results in a higher number of mutated cells (17). This was observed for 500 $\mu\text{g ml}^{-1}$ INH and mutation rate in our study was significantly raised (10^{-7}) for post log phase growth. Complex networks of factors influence the rate and type of mutants that can be selected with a given antibiotic. One of such factors that play a significant part in the mutation rate is the concentration of the antibiotic (18, 19). Thus it could be observed that, when

72 the concentration of RIF increased from 100 to 200 and 500 $\mu\text{g ml}^{-1}$ on agar plates, the number of mutants selected reduced and
73 the rate of mutations ranged from 9.24×10^{-8} , 4.47×10^{-10} , and 2.18×10^{-10} . Respectively for estimation using the P_o method, LC-
74 MM and MSS-MLE.

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76 In a study by (20), mutation frequencies of *Msm* mc²6, rather than mutation rate was estimated, to 100 $\mu\text{g ml}^{-1}$ STR and 500 $\mu\text{g ml}^{-1}$
77 RIF and were $>2 \times 10^{-4}$ and $>2.4 \times 10^{-5}$ respectively which were higher than the results obtained in the current study. However, the
78 *Msm* strains employed in both studies were different (mc²6 versus mc²155). It was difficult to obtain STR^r and KAN^r mutants.
79 Causes of resistance in streptomycin have been extensively investigated in many bacteria and require a very specific base
80 substitution in ribosomal genes for one of the ribosomal proteins. Mutations in the 16srRNA gene *rrs* have been found to confer
81 mutation in streptomycin and kanamycin (6). Hence the mutation rates calculated using the P_o method for this study were low i.e.
82 2.77×10^{-8} for streptomycin at 20 $\mu\text{g ml}^{-1}$ and 5.31×10^{-8} for streptomycin at 100 $\mu\text{g ml}^{-1}$. These results are comparable to
83 streptomycin mutation rate of 2.29×10^{-8} in *Mtb* (2). Mutation rate of *Msm* to 100 $\mu\text{g ml}^{-1}$ KAN was 1.70×10^{-8} in this study. Just as it
84 occurs in *Mtb*, rifampicin resistance mutation rate of 3.32×10^{-9} (2) has been found to be similar to *Msm* mutation rate of 10^{-8} - 10^{-10}
85 as we have found in our study.

86 **Conclusions**

87 It was not surprising that *M. smegmatis* been naturally resistant to isoniazid had the lowest mutation rate by comparison to
 88 kanamycin, streptomycin and rifampicin. Furthermore, we found that since the L-C method estimates mutation rate based on the
 89 median value, it was not ideal for estimating mutation rate where greater than 50 per cent of cultures had no growth (15). This
 90 resulted in both an undefined m value and uncalculated mutation rate. All the three methods were ideal in estimating mutation rate
 91 provided the recommendations for P_0 and m values are adhered to. These findings will promote a greater understanding of the
 92 phenomenon of mutation and how the estimation of mutation rate could be of importance in the control of multi-drug resistant *M.*
 93 *tuberculosis* infection.

94 **Materials and Methods**

95 **Bacteria strain**

96 *Mycobacterium smegmatis* mc²155 was employed in our experiments (21).

97 **Test drugs**

98 Isoniazid, kanamycin, Rifampicin and Streptomycin all purchased from Sigma-Aldrich Chemical Co. Ltd. (Poole, UK) were
 99 employed in this study. Standard stock solutions of isoniazid (50 mg/mL), Kanamycin (10 mg/mL) and Streptomycin (10mg/mL)
 100 were prepared by dissolving in sterile distilled water (SDW) and filtering using a 0.22 micrometre (μ m) pore size cellulose

101 membrane, while RIF (50 mg/mL) stock solution was prepared in DMSO (Fisher Scientific Ltd. Leicestershire, UK). Working
102 solutions were prepared by diluting in SDW.

103 **Growth medium**

104 Nutrient broth No. 2 (NB2; Lab lemco powder 10, Oxoid Ltd, Basingstoke, England), composed of: peptone 10g, sodium chloride
105 5g, beef extract 5g and reverse osmosis water, to make 1000 mL. Agar Technical No. 3 (Oxoid Ltd, Basingstoke, England) at 1.5 %
106 was used. Sterilisation was by autoclaving at 121°C, 15 pound per square inch (psi) for 20 minutes. Antibiotics were added to
107 media after cooling to 55°C.

108 **Supplements**

109 Glycerol 0.5 % volume by volume (v/v), 0.1 % (v/v) Tween 80 was used to supplement the broth. DMSO was also used in rifampicin
110 stock solution preparation (Fisher Scientific Ltd, Leicestershire, UK).

111 **Determination of Minimum Inhibitory Concentration (MIC)**

112 MIC assays were determined based on log₂ serial dilution of broth using NB2 containing 0.1 % Tween 80 in 5 mL tubes using the
113 procedure by (22). This was incubated for 48 h at 37°C and tubes were then observed for visible growth.

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115 **Fluctuation assay**

116 The distribution of mutant numbers in parallel cultures was determined using Fluctuation analysis method. A small number of cells
 117 (OD, 0.002) were grown under non-selective conditions in a 15 mL centrifuge tube. After about 36 h, a 1:1 serial dilution was done
 118 appropriately by introducing 1 mL of inoculum into 1 mL sterile NB2 medium. This was to ensure the numbers of cells in all tubes
 119 were the same. About 10 to 24 parallel independent bijou bottles containing 2 mL were used in the fluctuation assay. Thereafter,
 120 the cells were grown to saturation (after 4 to 7 days), resulting in 10^6 to 10^9 cells, and selected for mutant growth. Microfuge tubes
 121 (1.5 mL) were then used to pellet the culture (10 000 g, 5 minutes) and made into 300 L volume. 200 μ L volume was plated on
 122 antibiotic selective plates at the indicated concentrations. In order to estimate the number of viable cells, the remaining 100 μ L was
 123 serially diluted and plated on non-selective NB2 agar plates. The average cell number was then calculated. This was the final
 124 number of cells, Nt that was used for mutation rate calculation.

125 **Isolation of antibiotic-resistant mutants**

126 *Msm mc²155* cultures were spread out on NBA plates at the desired antibiotic concentrations and incubated for 4-6 days at 37 °C.
 127 Mutant colonies were confirmed by re-streaking on antibiotic selective plates containing the antibiotics at their respective
 128 concentrations.

129 **Data analysis**

130 The numbers of mutant cells were analysed using P_0 method and fluctuation analysis calculator (FALCOR) available online.

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133 **Conflicts of interest**

134 None declared.

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Figures

182
183 **Figure 1:** Structures of the anti-tubercular drugs used in this study- isoniazid (a) Kanamycin (b) Streptomycin (c) Rifampicin (d)
184 Isoniazid (14).

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187 **Tables**

188 Table 1: Modes of action of anti-TB drugs employed in this study.

Antibacterial agent (Year of discovery)	First/Second Line drug	MIC $\mu\text{g ml}^{-1}$ ¹ for <i>M. tb</i>	Resistance genes	Function of gene	Mode of action	References
Isoniazid (1952)	First-line	0.02-0.2	<i>KatG</i> , <i>inhA</i> , <i>aphC</i>	Catalase- peroxidase Enoyl ACP reductase	Inhibits mycolic acid synthesis	4, 5
Kanamycin (1957)	Second-line	≤ 3	(<i>rrs</i>)	16S rRNA	Inhibition of protein synthesis	6

Rifampicin (1966)	First-line	0.05-1	<i>rpoB</i>	β -subunit of RNA polymerase	Inhibition of RNA synthesis	
Streptomycin (1944)	Second-line	2-8	<i>rpSL</i>	S12 ribosomal	Alters protein	7
			<i>rrs</i>	protein 16S rRNA	synthesis	

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189 Table 2: Antitubercular drugs, targets and Minimum Inhibitory Concentrations results obtained in our study

Drug	Symbol	Mechanism	MIC value
Isoniazid	INH	Mycolic Acid synthesis inhibitors	8 $\mu\text{g ml}^{-1}$
Kanamycin	KAN	Protein synthesis inhibitor	0.24 $\mu\text{g ml}^{-1}$
Rifampicin	RIF	DNA-dependent RNA synthesis inhibitor	16 $\mu\text{g ml}^{-1}$
Streptomycin	STR	Protein synthesis inhibitor	0.5 $\mu\text{g ml}^{-1}$

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197 Table 3: Results for mutation rate using the P_o method

Mutant	Conc. $\mu\text{g ml}^{-1}$	Mean	Cultures	Zeros	Variance	SD	No. of viable bacteria (Nt)	P_0	m	Mutation rate
RIF ^r	100	20.5	10	03	2323.39	48.20	9.00E+06	0.3	1.20	9.24×10^{-8}
RIF ^r	200	8.5	24	08	247.22	15.72	1.72E+09	0.33	1.11	4.47×10^{-10}
RIF ^r	500	1.42	24	14	8.60	2.93	1.72E+09	0.58	0.54	2.18×10^{-10}
INH ^r	500	30.4	10	02	1901.6	43.61	9.00E+06	0.2	1.61	1.24×10^{-7}
INH ^r	1000	7.9	20	01	94.2	9.71	1.72E+09	0.05*	2.99	1.20×10^{-9}
STR ^r	20	0.5	10	07	0.72	0.85	9.00E+06	0.7	0.36	2.77×10^{-8}
STR ^r	100	0.8	10	05	0.84	0.92	9.00E+06	0.5	0.69	5.31×10^{-8}
KAN ^r	100	0.2	10	08	0.18	0.42	9.00E+06	0.8*	0.22	1.70×10^{-8}

198 Mutant = antibiotic used in mutant selection; ^r = resistance; Conc. $\mu\text{g/ml}$ represents the concentrations used in the selection of mutants; Mean= average of the
199 number of mutants in the cultures. The mean was calculated by dividing the total number of colonies by the number of cultures; Cultures = number of parallel
200 independent cultures used in the study for each antibiotic at the defined concentration; Zeros = number of cultures with zero or no mutants; Variance = the
201 extent to which the individual numbers of mutants are offset from the mean; Standard deviation (SD) is calculated as the square root of the variance and

202 shows how much the number of mutants deviates from the mean; No. of bacteria (N_t) = final number of cells plated; P_o = Proportion of cultures without
 203 mutants (obtained by dividing the zeros by the number of cultures); Values in * are those not within the accepted values for the P_o method ($0.7 \geq P_o \geq 0.1$ (0.3
 204 $\leq m \leq 2.3$); m = number of mutations per culture, obtained by taking the negative natural log of P_o ; Mutation rate calculated is per cell per generation using m
 205 $\times \ln 2 / N_t$.

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217 Table 4: Evaluation of P_o method against two other methods available on the Fluctuation analysis calculator with experimental

218 data

Mutant	Conc. $\mu\text{g ml}^{-1}$	Cultures	Zeros	Method	P_0	LC-MM	MSS-ML	Mutation rate
RIF ^r	100	10	03	m	1.20	1.6821	1.57	1.21×10^{-7a}
				-95%CL		1.869	0.9685	1.30×10^{-7b}
				+95%CL		6.6176	1.2353	
RIF ^r	200	24	08	m	1.11	1.3187	1.313	5.29×10^{-10a}
				-95%CL		0.0077	0.0031	5.31×10^{-10b}
				+95%CL		0.0079	0.0036	
RIF ^r	500	24	14	m	0.54	UD	0.519	2.09×10^{-10a}
				-95%CL		0	0.0016	
				+95%CL		0.0052	0.0019	

INH ^r	500	10	02	<i>m</i>	1.61	5.6511	3.484	2.68×10 ^{-7a}
				-95%CL		6.279	17.5079	4.35×10 ^{-7b}
				+95%CL		9.2067	21.1637	
INH ^r	1000	20	01	<i>m</i>	2.99*	1.8709	2.048	8.25×10 ^{-10a}
				-95%CL		0.0057	0.0046	7.54×10 ^{-10b}
				+95%CL		0.0082	0.0054	
STR ^r	20	10	07	<i>m</i>	0.36	UD	0.341	2.63×10 ^{-8a}
				-95%CL		0	0.2932	
				+95%CL		1.4652	0.433	
STR ^r	100	10	05	<i>m</i>	0.69	0.445	0.592	4.56×10 ^{-8a}
				-95%CL		0.4945	0.4575	3.43×10 ^{-8b}
				+95%CL		0.9707	0.6344	
KAN ^r	100	10	08	<i>m</i>	0.22*	UD	0.2	1.54×10 ^{-8a}
				-95%CL		0	0.1881	
				+95%CL		0.9889	0.2964	

219 LC-MM= Lea and Coulson method of the median; MSS-ML = *Ma-Sandri-Sarkar* Maximum Likelihood method; zeros = number of cultures with no mutants;
 220 P_0 = proportion of cultures with no mutants; a = mutation rate calculated with m value from MSS-ML; b = rate calculated using m value from LC-MM; * = m
 221 value is greater than the ideal value for the P_0 method; UD = undefined
 222