Seventy Years on from the Luria and Delbrück Fluctuation Analysis: A Comparison of three Methods for Estimating Mutation Rate

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Abstract
Seventy years ago, Luria and Delbrück discovered fluctuation assay for estimating mutation rates. While this method is slightly dated, it is one of the few methods for estimating mutation rates in batch culture. Mutation rates when determined expose information on cellular processes and fundamental mutagenic mechanisms. Formerly, inferences drawn from fluctuation assay were sufficient to answer a specific question inbacterial genetics. However, contemporary interpretation of results goes far beyond the motive originally intended. As the fluctuation assay has gained popularity in various scientific disciplines, analyses of results obtained are not same. This study aims to compare the estimation of mutation rates using the Poison distribution (Po) method with, the Ma-Sarka Sandri maximum likelihood estimator and the Lea-Coulson median estimator. Mycobacterium smegmatismc^{2}155was used as a model organism for *Mycobacterium tuberculosis,* and spontaneous mutations that arose in stationary phase cells exposed to antibiotic stress were investigated. Ten to twenty-four parallel cultures were tested with various anti-tuberculosis drugs; isoniazid, kanamycin, rifampicin and streptomycin. Minimum Inhibitory Concentration (MIC) of the drugs were also determinedto be; 8 µg/mL, 0.24 µg/mL, 16 µg/mL and 0.5 µg/mL for isoniazid, kanamycin, rifampicin and streptomycin respectively. The mutation rates obtained with the methods were very similar. To improve the power of deductions drawn from fluctuation assay, efforts should be made to experimentally determine the relative fitness of wild-type to mutant bacteria.This comparison is only a guide providing evidence regarding the authenticity of some of the methods currently available to researchers interested in estimating bacterial mutation rates.

Keywords: antibiotic resistance, mutation rate, fluctuation assay, fluctuation analysis calculator.

Introduction

Antibiotic resistance is a complex theme and in recent times, multi drug resistance have grabbed national headlines (McBride, 2013). Although antibiotic resistance is a serious public health problem, the emergence of multi-drug resistant tuberculosis is especially worrying as these have extended to shocking figures (Bloom and Murray, 1992; Frieden *et al*., 1993; Heym *et al*., 1994).

An organism which has the base sequence of its DNA changed is referred to as a mutant (Maloy *et al*., 1994). However for RNA-based organisms, it is a change in RNA sequence (Rosche and Foster, 2000). Furthermore, mutations occurring in the absence of exogenous agents are referred to as spontaneous mutations. This could be as a

resulting error caused by DNA during replication, repair, DNA recombination into the genome from other bacteria by transformation, spontaneous alteration of a nucleotide, movement of genetic elements, acquirement of resistance genes via plasmids or transposons or DNA damage occurring spontaneously (Maloy *et al*., 1994; Foster, 2006; Ochman *et al*, 2000; Shapiro, 1997). There is a finite likelihood that a gene will mutate per cell division in a single generation. The probability by which this happens is often estimated as the mutation rate (Maloy *et al*., 1994; Pope *et al*., 2008). Furthermore, with respect to antibiotic resistance, mutation rate is often defined as "the *in vitro* frequency at which detectable mutants arise in a bacterial population in the presence of a given antibiotic concentration" (Martinez and Bauero, 2000). Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of a drug that will inhibit the visible growth of an organism after overnight incubation (this period is extended for organisms such as anaerobes, which require prolonged incubation for growth)" (Andrews, 2001).

Drug resistance in bacteria has keenly been debated over the years in both genetics and medicine (JianLing *et al*., 2012). While some investigators believe it is drug-induced (Lewis and Taber 2008; Jin and Gao 2002), others hold that it occurs as a result of spontaneous mutations in the cells before exposure to the antibiotic (Luria and Delbruck, 1943). Nevertheless the measurement of mutation rates is invaluable in population genetics, evolution studies, microbiology, epidemiology, public health and in analysing the effect of environmental mutagens. Ultimately, calculating spontaneous mutation rates often yield valuable information on cellular processes. For instance, the manifestations of specific classes of mutations in diverse mutant backgrounds have been used to infer the significance of several DNA repair pathways (Miller, 1996; Pray, 2008).

The pioneering work of fluctuation analysis was carried out by Luria and Delbrück (L-D) for the estimation of mutation rates in a set of parallel independent cultures (Luria and Delbrück, 1943). This fluctuation analysis is still the *sine-qua-non* in estimating mutation rates, and determines how mutants are distributed in parallel independent cultures over a specific time periodto obtain the mutation rate (Rosche and Foster, 2000). L-Dtried to answer the following question- "does mutation arise due to a selective force or occur naturally irrespective of a selection force". The fluctuation test thus states the following; for every growing culture of bacteria, a cell may mutate at any time and the number of mutations occurring in any one culture is random.

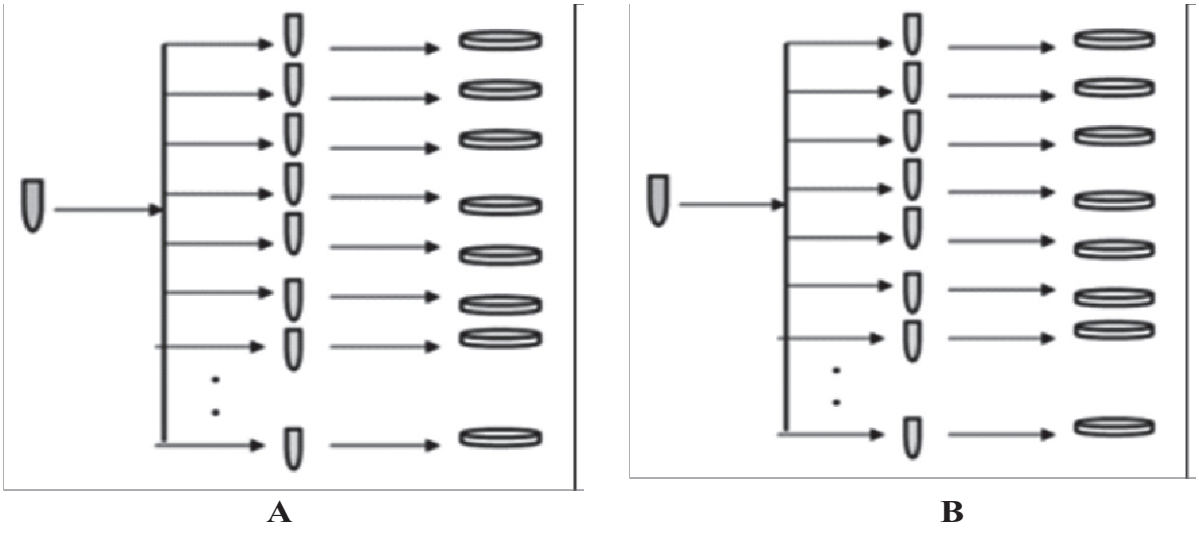

Figure 1: Schematic representation of the Luria- Delbrück fluctuation assay showing experimental set up with two replicates A and B (Adapted from JianLing et al., 2012).

This study aims to compare the estimation of *in vitro* spontaneous mutation rates using the Poisson distribution, Lea and Coulson method of the median and Ma-Sarka Sandri method all available on fluctuation analysis calculator (FALCOR). For this purpose, *Mycobacterium smegmatis* mc^2155 bacterium was studied using the antibiotics; isoniazid (INH), kanamycin (KAN), rifampicin (RIF) and streptomycin (STR).

Materials and Methods

Bacteria strain

The gram-positive *Mycobacterium smegmatis* mc^2155 was used in this experiment (Snapper *et al*., 1990).

Test drugs

INH, KAN, RIF and STR, all purchased from Sigma-Aldrich Chemical Co. Ltd. (Poole, UK) were employed in this study. Standard stock solutions of INH (50 mg mL^{-1}), KAN (10mg mL^{-1}) and STR (10mg mL^{-1}) were prepared by dissolving in sterile distilled water (SDW) and filtering using a 0.22 micrometre (µm) pore size cellulose membrane, while RIF (50mg mL^{-1}) stock solution was prepared in dimethyl sulphoxide (DMSO) (Fisher Scientific Ltd. Leicestershire, UK).Workingantibiotic solutions were prepared by diluting in SDW.

Growth medium

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

Supplements

Glycerol 0.5 % volume by volume (v/v), 0.1 % (v/v) Tween 80 was used to supplement the broth. Determination of Minimum Inhibitory Concentration (MIC)

MIC assays were determined based on log_2 serial dilution of broth using NB2 containing 0.1 % Tween 80 in 5 mL tubes using the procedure by Andrew, 2001. This was incubated for 48h at 37℃ and tubes were then observed for visible growth.

Fluctuation assay

The distribution of mutant numbers in parallel cultures was determined using Fluctuation analysis method.A small number of cells (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 appropriately by introducing 1 mL of inoculum into 1 mL sterile NB2 medium. This was to ensure the numbers of cells in all tubes were the same. About 10 to 24 parallel independent bijou bottles containing 2 mL were used in the fluctuation assay. Thereafter, 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 (1.5 mL) were then used to pellet the culture (10 000 g, 5 minutes) and made into 300 µL volume. 200 µL volume was plated on antibiotic selective plates containing > four times the MIC of the antibiotic. In order to estimate the number of viable cells, the remaining 100 µL was serially diluted and plated on non-selective NB2 agar plates. The average cell number was then calculated. This was the final number of cells, *Nt* that was used for mutation rate calculation.

Enrichment of antibiotic-resistant mutants.

To investigate which antibiotic concentrations above the MIC could select for resistant mutants, the parallel *M. smegmatis* mc^2155 cultures were plated out on NBA plates containing different antibiotic concentrations.Mutant colonies were confirmed by re-streaking on antibiotic selective plates containing the respective concentrations of antibiotics.

Mathematical and statistical analysis

The fluctuation analysis calculator also known as FALCOR is a fairly recent web based software, designed by Hall *et al*., 1999 for mutation rate determination. It is important

that experimental procedures follow the L-D fluctuation test. FALCOR was designed because the calculations of mutation rates involve complex mathematically computation and modelling which is beyond the comprehension of most bench scientists. In addition, FALCOR was aimed at standardizing the methods by which mutation rates are calculated. Currently available on FALCOR are three calculation methods namely; frequency, Lea-Coulson (L-C) Method of the median and Ma-Sandri- Sarkar Maximum Likelihood Estimator (MSS-MLE). *(Po)* (proportion of cultures without mutants) method, is the simplest method for estimating the expected number of mutations (*m*) that have occurred in each culture and is based on the fraction of cultures showing no mutants(Pope *et al*., 2008; Rosche and Foster, 2000) and was first used by L-D in their analysis. L-D assumed that the number of mutant cells obtained from replicate parallel independent cultures, followed a Poisson distribution, while the distribution of the number of mutants deviates from the Poisson distribution and is referred to as the L-D distribution (Luria and Delbrück, 1943).The zeroth term of the Poisson distribution is thus defined as; equation 1:

$$P_0 = e^{-m}; Pr = \frac{m}{r}\sum_{i=0}^{r-1}\frac{pi}{r-1+1}$$

The MSS-MLE computes the L-D distribution for any given value of m^3 from the Lea and Coulson Method (Lea and Coulson, 1949) using equation 3 above. All the terms have been explained previously except for *pi* which is probability and expresses the n^{th} derivative of a function as an integral. From equation 4, $P_0 = e^{-m}$ is used in the calculation of the proportion of cultures with zero mutants;

$$Pr = \frac{m}{r}\sum_{i=0}^{r-1}\frac{pi}{(r-i+1)}$$

is used in the calculation of all other positive values of *r*.Mutation rate is then calculated as *m/Nt.*

Data analysis

The numbers of mutant cells were analysed using Poisson distribution method and fluctuation analysis calculator (FALCOR) software. Graph was plotted using graph pad prism 6.

Results

Results for Minimum Inhibitory Concentration

In order to find out what concentration of antibiotics would result in the proliferation of resistant mutants, the minimum concentration required to prevent the visible growth was assayed, since mutants are easier to detect at concentrations greater than the MIC.

Table 1: Minimum Inhibitory Concentrations of antimycobacterial agents in Nutrient broth

Antibiotic	MIC value
INH	8 µg ml^{-1}
KAN	0.24 µg ml^{-1}
RIF	16 µg ml^{-1}
STR	0.5 µg ml^{-1}

From table 1, the minimum concentration of antibiotic required to inhibit the visible growth of *M. smegmatis* were the aminoglycosides KAN (0.24 µg ml^{-1}) and STR (0.5 µg ml^{-1}). For the antibiotics INH and RIF, MIC was 8 µg $ml^{-1}$and 16 µg ml^{-1} respectively.

Results for fluctuation assay

The numbers of selectively favourable mutants of mc^2155 that resulted in a visible antibiotic resistance phenotype were recorded.

Table 2: Resultsfor mutation rate using Poisson distribution

Mutant	Conc. µg/mL	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}

Mutant= antibiotic used in mutant selection; ^{r} = resistance; Conc. µg/ml represents the concentrations used in the selection of mutants; Mean= average of the 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 independent cultures used in the study for each antibiotic at the defined concentration; Zeros = number of cultures with zero or no mutants;Variance= the extent to which the individual numbers of mutants are offset from themean; Standard deviation (*SD*) is calculated as the square root of the variance and shows how much the number of mutants deviates from the mean; No. of bacteria (*Nt*) = final number of cells plated; P_0=Proportion of cultures without mutants (obtained by dividing the zeros by the number of cultures);Values in * are those not within the accepted values for the P_0 method ($0.7 \geq P_0 \geq 0.1$ ($0.3 \leq m \leq 2.3$);*m*= number of mutations per culture, obtained by taking the negative natural log of P_0; Mutation rate calculated is per cell per generation using $m \times In2/Nt$.

As a first step into calculating mutation rate of *M. smegmatis*, the P_0 method, the simplest and earliest method used in mutation rate calculation was used. Table 2 shows the rate of mutation for each antibiotic and respective concentration. RIF at 200 µg mL^{-1} and 500 µg mL^{-1} had the lowest mutation rates of 4.47×10^{-10} and 2.18×10^{-10}while INH at 500 µg mL^{-1} had the highest mutation rate of 1.24×10^{-7}.

Results for mutation rate using Fluctuation Analysis Calculator (FALCOR)

Table 3: Evaluation of P_0 method against two other methods available on FALCOR with experimental data

Mutant	Conc. µg/ml	Cultures	Zeros	Method	P_0	LC-MM	MSS-ML	Mutation rate
RIF^r	100	10	03	*m*	1.20	1.6821	1.57	$1.21x10^{-7a}$
				-95%CL		1.869	0.9685	$1.30x10^{-7b}$
				+95%CL		6.6176	1.2353	
RIF^r	200	24	08	*m*	1.11	1.3187	1.313	$5.29x10^{-10a}$
				-95%CL		0.0077	0.0031	$5.31x10^{-10b}$
				+95%CL		0.0079	0.0036	
RIF^r	500	24	14	*m*	0.54	UD	0.519	$2.09x10^{-10a}$
				-95%CL		0	0.0016	
				+95%CL		0.0052	0.0019	
INH^r	500	10	02	*m*	1.61	5.6511	3.484	$2.68x10^{-7a}$
				-95%CL		6.279	17.5079	$4.35x10^{-7b}$
				+95%CL		9.2067	21.1637	
INH^r	1000	20	01	*m*	2.99*	1.8709	2.048	$8.25x10^{-10a}$
				-95%CL		0.0057	0.0046	$7.54x10^{-10b}$
				+95%CL		0.0082	0.0054	
STR^r	20	10	07	*m*	0.36	UD	0.341	$2.63x10^{-8a}$
				-95%CL		0	0.2932	
				+95%CL		1.4652	0.433	
STR^r	100	10	05	*m*	0.69	0.445	0.592	$4.56x10^{-8a}$
				-95%CL		0.4945	0.4575	$3.43x10^{-8b}$
				+95%CL		0.9707	0.6344	
KAN^r	100	10	08	*m*	0.22*	UD	0.2	$1.54x10^{-8a}$
				-95%CL		0	0.1881	
				+95%CL		0.9889	0.2964	

LC-MM= Lea and Coulson method of the median; MSS -ML = *Ma-Sandri-Sarkar* Maximum Likelihood method; zeros = number of cultures with no mutants; 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* value is greater than the ideal v alue for the P_0method; UD = undefined

Table 3 shows ow calculating mutation rate using the P_0method, compares with using the L-C method of the median and the Ma Sarka Sandri method available on Fluctuation analysis calculator. This comparison was necessary because P_0 method is not applicable across all values of

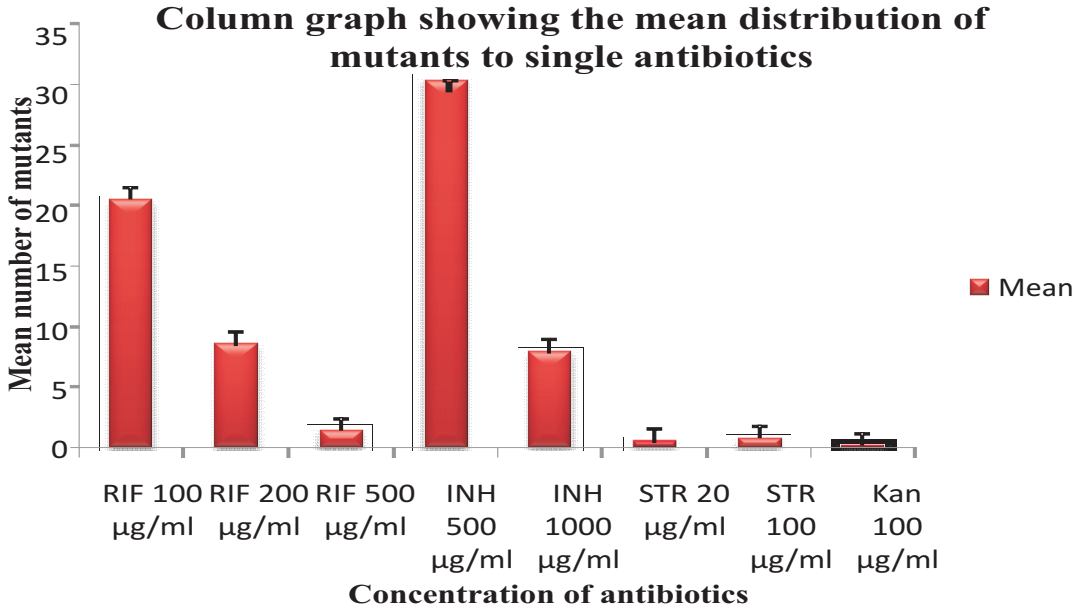

Figure 2: Column graph showing the mean distribution of mutants used in fluctuation assay (Error bars are SEM).

The number of mutant bacteria cells obtained by growing on antibiotic containing plates was divided by the total number of cultures to obtain the mean. This mean was thereafter plotted against the concentrations of antibiotics used in the study. From the column graph (Figure 2), INH 500 µg ml^{-1} had the greatest number of mutant bacteria whereas KAN 100 µg ml^{-1}had fewer numbers of mutants.

Discussion

Evaluation of minimum inhibitory concentration

Wherever microbial control is required, antimicrobial choices must be made and it is important to consider the concentrations at which these agents will be effective. MIC is considered the *sine qua non* in microbiology for assessing the susceptibility of microorganisms to antibiotics, and has been useful in comparing the performances of various susceptibility testing. In addition, they are useful in clinical diagnostic microbiology laboratories to confirm microorganisms that are unusually resistant to antibiotics (Andrews, 2001).

Although MIC obtained with INH for *M. smegmatis* in this study was found to be 8 µg mL^{-1}; studies by Pasca *et al*., 2005 found MIC to be 32 µg mL^{-1} though they used the agar dilution method for the MIC, while Teng and Dick, 2003 established 10 µg mL^{-1}as the MIC using broth. Likewise for RIF, although MIC was found to be 16 µg mL^{-1}, White, Lancini and Silvestri 1971, found the MIC to be 20 µg mL^{-1}. The result are however comparable.

Analysis of mutation rate

Information on mutation rates associated with drug resistance in *M. smegmatis* is very limited. Although some investigators have isolated antibiotic resistant mutants to *M. smegmatis*, efforts were not made to calculate a mutation rate. The hypothesis surrounding spontaneous mutation predicts a large fluctuation around the average for the count taken from the individual cultures. A mutation occurring earlier in the growth of the culture results in a higher number of mutated cells (Pope *et al*., 2008). This was observed for INH and mutation rate in this study was significantly raised (10^{-7}) for post log phase growth.

Complex networks of factors also 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 (Kohler *et al*., 1997; Hughes and Andersson, 1997). Thus it could be observed that with RIF for instance, when the concentration of RIF incorporated into the media rose from 100 to 200 and 500 µg mL^{-1}, the number of mutants selected reduced and the rate of mutations ranged from 9.24 $x10^{-8}$, 4.47 x 10^{-10}, and 2.18x10^{-10}.

In a study by Karunakaran and Davies, 2000 mutation frequencies of *M. smegmatis* $mc^{2}6$ to STR 100 µgmL^{-1} and RIF 500 µgmL^{-1} were >2 x 10^{-4} and >2.4 x10^{-5} respectively which were higher than the results obtained in the current study. This could be due to the different strain of *M. smegmatis* which was used in their study($mc^{2}6$ versus $mc^{2}155$).

It was difficult to obtain mutants after treatment with the aminoglycosides STR and KAN. Causes of resistance in STR have been extensively investigated in many bacteria and require a very specific base substitution in ribosomal genes for one of the ribosomal proteins. Mutation in the 16srRNA gene *rrs* have been found to confer mutation in STR and KAN (Suzuki *et al*.,1998), however mutation in STR is primarily caused by missense mutation in *rpsL* gene encoding the ribosomal S12 protein (Finken *et al*., 1993). Hence the mutation rates calculated using the P_o method for this study were low; 2.77 x 10^{-8} for STR at 20 µg mL^{-1} and 5.31 x 10^{-8} for STR at 100 µg mL^{-1}. These results are comparable to STR mutation rate of 10^{-8}(Sander and Böttger, 1998). For KAN, mutation rates are in the ranges of 10^{-7} to 10^{-9} (Snapper *et al*., 1988), comparable to a calculated mutation rate of 1.70 x 10^{-8} in this study. Just as it occurs in *Mtb*, RIF resistance has been found to be similar to *M. smegmatis* (White, Lancini and Silvestri, 1971). *M. smegmatis* has been found

to be naturally resistant to INH thus mutation frequency was high 10^{-6}.

Conclusions

Mutation rate was found to arise spontaneously and independent of antibiotic concentration. On comparing the P_0 method and two other methods available on FALCOR for estimating mutation rates, results obtained suggest that provided the recommendation for P_0 and m are adhered to, the methods were ideal in estimating mutation rate. Furthermore it is not recommended to use the L-C method for estimating mutation rate where over 50% of the cultures had no mutant growth as it results in an undefined m value and uncalculated mutation rate. These findings will promote a greater understanding of mutation and how the estimation of mutation rate could be of importance in the control of infectious diseases like *M. tuberculosis*.

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