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Physiological fitness of drug-resistant *Mycobacterium tuberculosis* isolates in Hong Kong

Key Messages

1. Drug target gene mutations in *Mycobacterium tuberculosis* conferred drug resistance at the expense of physiological fitness of the pathogen, rendering it less capable of multiplying even in a nutrient-rich environment.
2. Isolates containing mutations in *gyrA* experienced the strongest growth inhibition.
3. The growth fitness cost of resistance-gene mutations may only slow down mycobacterial growth at the initial phase (immediately after inoculation), possibly with little negative effect on the overall ability of this pathogen to cause disease in humans.
4. In addition to the mutation-induced effects, growth and survival fitness of multi-drug-resistant tuberculosis (MDR-TB) is also highly dependent on overall genetic constraints, which are unique to each organism.
5. Such MDR-TB mutants are fully capable of residing in human host-reservoirs and are as effective as their non-resistant counterparts in causing infections in the community.

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Introduction

Multi-drug-resistant tuberculosis (MDR-TB) strains are less contagious than their drug-sensitive counterparts,[1,2] based on the perception that mutations conferring drug resistance deleteriously affect the normal physiological functions of the pathogen. Drug-resistant mutants are expected to grow more slowly and/or exhibit milder virulence. This is known as the 'fitness cost' of drug resistance.[3] This may confine the frequency and seriousness of infections caused by drug-resistant pathogens. However, mutations in *Mycobacterium tuberculosis* (MTB) do not always result in loss of fitness.[3] Reduction in fitness of the pathogen can be restored by compensatory mutations in the genome,[4] hence MDR-TB strains can be as infectious and invasive as the drug-sensitive, non-mutated form.

This study aimed to determine the physiological fitness of MDR-TB isolates in Hong Kong by evaluating their infectivity and virulence. First, whether the target gene mutations suppressed the growth fitness of the organisms was determined. Second, the relative virulence of selected MTB isolates was assessed in terms of their ability to survive and replicate within macrophages. Third, the proteomic profiles of selected MDR-TB isolates were studied to determine whether drug-resistant organisms undergo significant physiological changes to compensate for the effects of resistance gene mutations.

Methods

This study was conducted from January 2007 to December 2008. The growth rate of 88 selected MDR-TB strains was measured using the BBL Mycobacteria Growth Indicator Tube (MGIT) and the BACTEC MGIT 960 system. A fluorescent sensor sensitive to quenching by dissolved oxygen was used. Bacterial growth consumes oxygen and therefore enables the sensor to fluoresce, producing growth signals. Equal-sized populations of drug-resistant and drug-susceptible strains were inoculated in parallel into separate tubes and then incubated in BACTEC MGIT 960 for up to 2 weeks. Fluorescence changes during this period were recorded and the growth rate of each test strain was measured by recording the time required to produce an arbitrary breakpoint value in growth signal.

The human macrophage-like cells were derived from phorbol-ester-activated THP1 cells. Eight selected MTB strains of different resistance phenotypes were mixed with human macrophages at a ratio of one to ten bacterial cells to one macrophage in a well of a 24-well plate. After overnight incubation, the non-phagocytosed MTB cells were removed by filtration. The macrophages were lysed at day 4 and 7 to determine the number of viable MTB organisms recoverable to determine their ability to survive or replicate inside the macrophages. The data were compared to those of drug-sensitive organisms.

Total proteins were prepared for eight selected MTB strains that exhibited specific drug-resistance phenotypic patterns, followed by simultaneous analysis by two-dimensional gel electrophoresis alongside with drug-sensitive strains. Comparison of the proteomic profiles pinpointed proteins that were expressed specifically in either drug-resistant or -sensitive strains. Technically, total protein prepared from each strain was resolved on Bio-Rad Proteome

Works two-dimensional gel electrophoresis system. After electrophoresis, the gel was stained and scanned to produce a proteomic profile, which was analysed using the software provided by the manufacturer.

Results

Analysis of mycobacterial growth

In a preliminary attempt to assess the fitness cost of resistance gene mutations in MDR-TB isolates, the in vitro growth rate of 59 selected isolates with known drug susceptibility and genetic profiles were measured by recording the mean time required to reach a breakpoint value of 200 signal units in the MGIT system. To examine the correlation between growth rate and the number of mutations in the resistance genes, mutation profiles of the fastest and slowest growing strains were compared. The eight fastest-growing strains took a mean of 3.06 days to reach 200 signal units, and harboured two or fewer mutations. In contrast, the eight slowest-growing strains took a mean of 5.41 days to produce 200 signal units, and harboured at least three mutations (Table 1). Seven of these strains contained *gyrA* mutations, and five contained *pncA* mutations. Regarding the effect of mutations in specific resistance genes, wild-type strains required a mean of 3.58 days to produce 200 signal units, whereas the mean time taken by isolates containing mutations was much longer, ranging from 4.52 days for strains containing *katG* mutations to 4.91 days for strains containing *gyrA* mutations (Table 2). Strains carrying mutations in *gyrA* and *pncA*, with or without mutations in other genes, were the slowest-growing organisms, taking 4.91 and 4.83 days, respectively, to produce the reporter signal. The effects of mutations in specific genes were depicted by the growth rate in single mutants (Table 2). The mean time for strains harbouring a single mutation in *rpoB*, *embB*, and *katG* was 3.75, 4, and 4.36 days, respectively. Single mutants of the *gyrA* and *pncA* genes were not available for analysis.

To test whether mutation-induced fitness cost can attenuate the ability of MDR-TB to spread between human hosts, the growth rate of organisms that had been isolated from unrelated individuals and yet displayed almost identical genetic and drug-resistance profiles were measured (Table 3). The recovery of genetically identical organisms from up to 10 unrelated patients provided evidence that they possessed the ability to survive in human hosts and spread in the community. As these organisms also exhibited resistance to multiple antibiotics, we speculated that their survival fitness and hence infectivity might also be constrained by mutation-induced fitness cost to some extent. Measurement of the in vitro growth capacity of these organisms was therefore expected to reveal the actual impact of such fitness cost on the infectivity of MDR-TB organisms. Most of the 29 cluster isolates exhibited a significantly slower growth rate than did the drug-sensitive controls and other genetically diverse MDR-TB strains. Nevertheless, upon prolonged incubation, significant growth was recorded for these cluster strains that eventually produce a population size comparable to that of other MDR-TB organisms.

Mycobacterial survival fitness in macrophages

An intracellular survival assay was used to assess the in vivo fitness and virulence of eight selected drug-resistant MTB strains. The number of organisms taken up by macrophages was the difference between the viable cell counts before and after ingestion by macrophages. The numbers of viable organisms collected from washing the macrophage pellet as well as from lysis of the macrophage population at different time points after the ingestion step were recorded. A formula was derived to calculate the survival rate of MTB in macrophages (Table 4).

Drug-resistant MTB isolates generally exhibited reduced survival fitness when compared to drug-sensitive strains, which did not harbour mutations; such reduction was more apparent in the long term (day 7). Table 4 shows the relative survival rate of a wild-type strain (25117), a drug-sensitive clinical isolate (B8), and a representative multi-drug-resistant strain which contained mutations in five resistance

Table 1. The mean initial growth rate of the fastest and slowest growing *Mycobacterium tuberculosis* isolates

Strain no.	Resistance gene mutations involved	Mean initial growth rate (days to reach 200 signal units)
Fastest-growing strains		
M12	*embB, katG*	3
M14	*rpoB, katG*	3
M16	*rpoB*	3
M48	*rpoB*	3.5
M58	*rpoB, embB*	3
188a	-	3
417a	-	3
B7	-	3
Slowest-growing strains		
M1	*rpoB, embB, katG*	5.5
M15	*rpoB, embB, pncA*	5.3
M29	*gyrA, rpoB, embB, katG*	5.35
M96	*gyrA, rpoB, embB, katG, pncA*	5.43
484	*gyrA, rpoB, embB, pncA*	5.32
G53211	*gyrA, rpoB, embB, pncA*	5.6
Sc1	*gyrA, rpoB, embB, pncA*	5.32
Y777	*gyrA, rpoB, embB, pncA*	5.43

Table 2. Mean initial growth rate of isolates harbouring mutations in specific genes, with or without further mutations in other genes

Resistance gene	Mean initial growth rate (days to reach 200 signal units)
No mutation (n=6)	3.58
rpoB (single mutant, n=4)	3.75
katG (single mutant, n=7)	4.36
embB (single mutant, n=1)	4.0
rpoB (multiple mutant, n=50)	5.59
katG (multiple mutant, n=29)	4.52
embB (multiple mutant, n=39)	4.59
gyrA (multiple mutant, n=24)	4.91
pncA (multiple mutant, n=26)	4.83

Table 3. Mean initial growth rate and drug susceptibility profiles of 29 genetically related multi-drug-resistant *Mycobacterium tuberculosis* isolates

Cluster/strain no.	Minimal inhibitory concentration of					Mean initial growth rate (days to reach 200 signal units)
	Ofloxacin	Rifampicin	Ethambutol	Isoniazid	Pyrazinamide	
C9-1	0.6/S	>64/R	2/S	1/R	50/S	9.4
C9-2	1.2/S	>64/R	2.8/S	1/R	50/S	11.5
C9-3	1.2/S	>64/R	2.8/S	1/R	50/S	9.0
C9-4	0.6/S	>64/R	2/S	1/R	50/S	9.4
C9-5	0.6/S	>64/R	2/S	1/R	50/S	8.3
C9-6	0.6/S	>64/R	2/S	1/R	50/S	9.0
C17-1	1.2/S	>64/R	2/S	>1/R	-	9.0
C17-2	1.2/S	>64/R	2/S	1/R	-	9.4
C17-3	1.2/S	64/R	2/S	>1/R	-	9.4
C17-4	0.6/S	>64/R	2/S	>1/R	50/S	9.6
C17-5	1.2/S	64/R	2/S	>1/R	50/S	8.7
C17-6	1.2/S	64/R	2/S	>1/R	50/S	8.8
C22-1	1.2/S	>64/R	2.8/S	1/R	50/S	9.8
C22-2	2.4/S	>64/R	>4/R	>1/R	>50/R	9.0
C22-3	>4.8/R	>64/R	4/R	1/R	>50/R	8.6
C22-4	>4.8/R	>64/R	4/R	1/R	-	5.2
C33-1	1.2/S	>64/R	2.8/S	>1/R	50/S	9.5
C33-2	>4.8/R	>64/R	>4/R	>1/R	>50/R	8.8
C33-3	>4.8/R	>64/R	>4/R	1/R	>50/R	8.9
C33-4	>4.8/R	>64/R	>4/R	1/R	>50/R	9.0
C33-5	>4.8/R	>64/R	>4/R	1/R	>50/R	9.1
C33-6	>4.8/R	>64/R	>4/R	1/R	>50/R	4.5
C39-1	2.4/S	>64/R	4/R	1/R	50/S	9.3
C39-2	>4.8/R	>64/R	>4/R	>1/R	-	9.5
C39-3	4.8/R	>64/R	>4/R	1/R	-	12.8
C39-4	2.4/S	>64/R	>4/R	1/R	>50/R	9.4
C39-5	4.8/R	>64/R	4/R	1/R	>50/R	10.7
C39-6	2.4/S	>64/R	>4/R	1/R	>50/R	5.4
C39-7	4.8/R	>64/R	4/R	1/R	>50/R	5.7
188a*	1.2/S	16/S	2/S	0.2/S	-	3.0
417a*	1.2/S	16/S	2/S	1/S	-	3.0

* Drug-sensitive strain as controls

Table 4. Intracellular survival rate of *Mycobacterium tuberculosis* (MTB) in macrophages

Calculation of intracellular survival rate*	25117 (wild-type strain)		B8 (drug-sensitive strain)		M96 (multi-drug-resistant strain)	
	Day 4	Day 7	Day 4	Day 7	Day 4	Day 7
VCLP	$4x10^4$	$3x10^4$	$1.2x10^2$	$1.8x10^2$	$4x10^4$	$3x10^4$
VCULP	$2x10^4$	$1.3x10^3$	50	20	$3x10^4$	$4x10^3$
VCI	$2.5x10^5$	$1.6x10^6$	$3x10^4$	$3x10^5$	3x106	$2x10^7$
VCUT	$1x10^4$	$6x10^4$	30	500	$3x10^4$	$6x10^4$
VCLP-VCULP	$2x10^4$	$2.87x10^4$	70	$3.6x10^3$	$1x10^4$	$2.6x10^4$
VCI-VCUT	$2.4x10^5$	$1x10^5$	$3x10^4$	$3x10^5$	$3x10^6$	$2x10^7$
(VCLP-VCULP)/(VCI-VCUT)	8.3%	28.7%	0.2%	1.2%	0.3%	0.13%

* VCLP denotes viable count obtained after plating out a lysed macrophage pellet without washing, VCULP viable count obtained after plating out wash fluid collected from washing a macrophage pellet, VCI the initial viable count prior to macrophage uptake, and VCUT viable count of remaining free MTB after the macrophage uptake step. Hence, (VCLP-VCULP) depicts the number of organisms that survived intracellular stress, and (VCI-VCUT) depicts the number of organisms taken up by macrophages. Noted that the macrophage pellet might contain extracellular MTB which were neither ingested by macrophages nor eradicated by antibiotic or filtration

genes (M96) in macrophages 4 and 7 days after inoculation and uptake by macrophages. The survival rate of the wild-type strain in macrophages was much higher than that of the clinical isolates, regardless of whether they harboured mutations. At day 4, both clinical isolates tested exhibited a survival rate of less than 1%, whereas that of the wild-type strain (strain 25117) was 8%. At day 7, strains 25117 and B8, both harbouring wild-type nucleotide sequences in the resistance genes, displayed a higher cell count than that of day 4. This suggests that intracellular organisms regained growth fitness during the intervening period. In contrast, the viability count of strain M96, which harboured multiple mutations in five resistance genes (*rpoB*, *katG*, *embB*, *gyrA*, and *pncA*) declined during this period. This indicates that mutation bearing organisms were less fit to survive macrophage-induced intracellular stress. Nevertheless, a small number of viable cells were still recoverable.

Proteomic analysis of drug-resistant strains

To determine whether drug-resistant isolates undergo significant physiological changes to accommodate the structural changes imposed by drug-resistance gene mutations, preliminary proteomic analyses on MDR-TB strains that harboured mutations in up to five drug-

resistance genes were performed. The MDR-TB isolates exhibited a small but discernable difference in proteomic profiles when compared to the drug-sensitive strains. Further experiments are necessary to confirm whether drug-resistance development involves physiological changes other than those concerning the drug target functions.

Discussion

Drug target gene mutations in MTB conferred drug resistance at the expense of physiological fitness of the pathogen, rendering them less capable of multiplying even in a nutrient-rich environment. Isolates containing mutations in *gyrA* experienced the strongest growth inhibition. However, since these isolates also harboured mutations in other resistance genes, we could only indirectly assess the relative impact of *gyrA* mutations on growth inhibition by comparing the effects of single mutations in other resistance genes. Mutations in *rpoB* or *katG*, the two genes involved in resistance to the first-line drugs rifampicin and isoniazid, exhibited weaker growth retardation than multiple mutations. The significant growth inhibition phenomenon observed in multiple mutants was attributed to additional *gyrA* mutations. Structural alteration of gyrase is expected to affect the efficiency of DNA and cellular replication, leading to a slower growth rate. The fact that isolates that harboured a single mutation in the *rpoB* gene exhibited a growth rate similar to that of wild-type strains indicated that RNA polymerase, the *rpoB* gene product, was more flexible in accommodating mutation-directed structural changes. In contrast, isolates carrying mutations in the *katG* gene grew much slower. This suggested that the *katG* gene product played an important role in defending against oxidative stress during the growth process when reactive oxygen species were being produced.

Despite a reduced initial growth rate, all tested mutants eventually grew to a population size comparable to that of the wild type. This suggested that the growth fitness cost of resistance-gene mutations only slowed down mycobacterial growth at the initial phase immediately after inoculation, possibly with little negative effect on the overall ability to cause disease in human hosts. The growth fitness data on clonal MDR-TB strains also suggested that a severe growth fitness cost did not necessarily hamper the ability of the organism to infect humans and survive. In addition to the mutation-induced effects, growth and survival fitness of MDR-TB was also highly dependent on the overall genetic constraints, which are unique to each organism. The nature of constraint factors other than those in drug-resistance genes has yet to be determined. This finding is alarming as it indicates that MDR-TB mutants are fully capable of residing in human host-reservoirs and are as effective as their non-resistant counterparts in causing infections in the community.

In addition, MDR-TB strains were also found to exhibit reduced survival fitness, especially in the long term, after they had been engulfed in macrophages. Reduced survival of resistant MTB strains was likely to be due to their impaired ability to produce stress responses as a result of genetic mutations in resistance genes. *katG* is likely to be a major candidate gene which contributes to intracellular survival by producing catalase-mediated antioxidant responses against the detrimental effects of reactive oxygen species produced by macrophages. Other drug-resistance genes may also affect survival fitness indirectly. For example, mutations in the *embB* gene affect cell wall integrity, leading to a weaker structure that is less resistant to oxidative stress in the intracellular compartment of macrophages.

Resistance-gene mutations could cause detectable changes in proteomic profiles, indicating that they elicited secondary or compensatory changes in the physiology of the resistant strains. Our study revealed the effects of the fitness costs of resistance gene mutations on the infectivity and virulence of MDR-TB organisms. Such data should facilitate development of novel strategies for the control of this important pathogen.

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