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DRUG DISCOVERY AND RESISTANCE

Fitness of drug resistant *Mycobacterium tuberculosis* and the impact on the transmission among household contacts☆

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SUMMARY

There has been an on-going debate on whether the development of drug resistance in *Mycobacterium tuberculosis* reduces its relative fitness and its ability to cause disease. The aim of this study was to explore this relationship. For this purpose, we evaluated the in vitro growth of clinical isolates and the transmission of the strains within the patients' households. Clinical and epidemiological data from patients in households, drug-susceptibility and genetic patterns of the isolates were collected. BACTEC MGIT 960™ system with the Epicenter™ software was used to perform fitness experiments and calculate the relative fitness (RF) comparing with the H73Rv reference strain. From 39 households, 124 patients and 388 contacts were included. Concerning transmission, 20 Multi drug-resistant (MDR) and 16 drug sensitive (DS) index cases generated 23 and 28 secondary cases, respectively. An average RF drop of 16.7% was found for MDR strains, but only mutations in rpoB codons 531 were associated with reduced fitness. When the strains were transmitted, their RF tended to decrease, and strains with low RF were less frequently transmitted. Within the limitations of this study, the results showed that the decrease in RF was associated to a limited transmission among the households' contacts.

1. Introduction

Mycobacterium tuberculosis complex bacteria can infect many mammalian hosts, as they are able to adapt and alter gene expression and metabolism in response to the conditions of intracellular growth, acidity, starvation, and the innate immune response [1]. Some strains are also capable of severe outbreaks, despite the presence of resistance mutations in several essential genes that are the targets of different antibiotics. Fitness has been defined as the ability of a microorganism to survive, reproduce and be transmitted [2]. When mycobacteria are exposed to a specific antibiotic, those bacteria with preexisting mutations conferring resistance to the drug have an obvious survival advantage over susceptible cells that will be inhibited or killed. The antibiotic pressure selects for the resistant bacteria, thereby producing a quantitative and qualitative change in the whole bacterial population [3,4].

MDR *M. tuberculosis* strains have mutations that confer resistance to at least isoniazid and rifampicin, and are associated with treatment failure [5,6]. However, there has been a polemical debate about whether these mutations may concomitantly decrease the virulence and transmissibility of the mycobacteria [4,7–11]. While bacteria with resistance mutations associated with a major decrease in fitness are unlikely to thrive, mutations that only modestly reduce fitness would be difficult to detect because of the strong positive selection for drug resistance. The primacy of drug resistance over putative associated minor decreases in fitness is illustrated by the worsening patient prognosis as an MDR strain acquires resistance to additional drugs. For example, this is the case of the extensively drug-resistant strains (XDR), MDR strains that are also resistant to an injectable agent and a fluoroquinolone [12].

However, not all MDR/XDR strains seem to have similar fitness; for instance, some are repeatedly isolated from many individuals,

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while most are isolated only once or twice [13]. Several possible reasons could explain why some strains have only limited transmission, such as a mutation that confers a moderate level of resistance but also decreases the bacteria's "fitness" or ability to cause disease. Another possible explanation is that the presence of several mutations in different essential genes, each conferring resistance to an antibiotic but also slightly reducing the "fitness", may cumulatively result in a bacterium whose "fitness" or viability has been sufficiently diminished so that it is unable to cause illness. From the point of view of the host, individuals with an intact immune system may be able to control an infection with a reduced-fitness strain and avoid progression to disease. These strains may, however, still be capable of causing a fatal illness in patients with a compromised immune system.

The determination of *M. tuberculosis* isolate fitness might help understand why some MDR and XDR strains are fully capable of disseminating and causing fatal outbreaks within a community [14]. The growth rate of a bacteria is often used as in vitro means of estimating its fitness under different environmental conditions, such as oxygen deprivation [4] or the presence of antimycobacterial drugs in the culture medium. The infection of an animal model with an engineered bacterial mutant library [15] might help identifying those bacteria with resistance mutations that result in a critical loss in viability or in the capacity to cause disease. Obviously, this strategy cannot be performed in human patients. We reasoned, however, that less severe defects in relative fitness might be finding by examining differences in the ability of clinical isolates to be transmitted and cause secondary cases. With this in mind, we looked for the effect of drug-resistance mutations on the in vitro fitness of TB strains and then attempted to correlate this with transmission as detected by secondary cases arising within household contacts.

2. Material and methods

2.1. Patients and clinical isolates

From 2001 to 2009, 2736 TB cases were reported and analyzed. Thirty nine families comprising 124 patients in total were selected for this study. The selection criteria included:

2.2. Epidemiological data

Identification of the household, residence, household contacts, other type of contact, age and gender.

2.3. Clinical data

Localization of the disease, HIV status, co-morbidities, alcoholism, previous anti-TB treatment background.

2.4. Bacteriological data

Direct smear examination and culture results, identification of the microorganism as belonging to the *M. tuberculosis* Complex, drug-susceptibility testing to first and second-line drug results.

2.5. Criteria for households inclusion

Identification of at least one household contact infected by a MDR-TB strain, household with more than three members with fully drug-susceptible and no MDR strains, families with more than three members with drug-resistant (DR) and MDR strains. The medical records and the social query included in the NTBCP guidelines were utilized to collect epidemiological and clinical information as well as information about contacts [16].

2.6. Reference strain

M. tuberculosis H37Rv ATCC 27294 was used as reference for drug susceptibility testing (DST), fitness and molecular experiments.

2.7. Ethical approval

This work has only used *M. tuberculosis* isolates from a collection of Dr. Cetrangolo Hospital. No patients were directly involved in the study nor the treatments were changed due to its results. The study protocol has obtained the approval from the Dr. Cetrangolo Hospital Research and Teaching Committee. Patients gave their consent for their information to be stored in the hospital database and used for research.

2.8. Definition of terms

The index case or primary case was considered the initial patient in the population under an epidemiological investigation [13,17].

Lag phase (t0): time from the start of cultivation to the beginning of detectable growth in MGIT [18].

Growth units (GU): data given by the EpiCenter® and related to the colony forming unit concept (CFU).

Generation number (GN): for this work purposes, GN is measured in hours to reach 200 GU.

Growth rate (GR): based on Toungoussova OS et al. [19], the mean time was calculated considering the time from the beginning of growth up to reaching 200 units.

Fitness: Ability of an organism to survive, adapt and replicate into its biological nitche [2].

Relative fitness (RF): the RF was calculated as the relationship expressed by this formula: GN MX/GN H37Rv, where GN MX is the generation number of the strain under analysis in relation with that from the reference strain *M. tuberculosis* H37Rv (GN H37Rv). The GN and GR for the reference strains was measured in every experiment. GR ranges from 16 to 18 h for *M. tuberculosis* H37Rv.

Secondarily, and in order to propose a potential chain of transmission among members of each one of the families, the year of disease diagnosis, t0, GU, the obtained RF and the drug-resistant profiles were considered.

2.9. Laboratory methods

To search for acid-fast bacilli (AFB), direct smear microscopy were performed by Ziehl–Neelsen stain after decontamination – and concentration of natural contaminated specimens such as sputa, bronchial washings, brochioalveolar washings, gastric aspirates, feces [20,21]. Specimens such as pleural, cerebral–spinal and ascetic fluid were aseptically obtained and concentrated by centrifugation before smears preparation.

Each sample was inoculated in MGIT 960, as well as in a tube containing either Lowenstein–Jensen medium. MGIT 960 incubation and positive detection were automatically done by the device. The solid media were incubated at 37 °C for 60 days [22].

First-line DSTs were performed using the proportion method on Lowenstein–Jensen and/or the MGIT 960 system following the manufacturer's instructions [23,24]. All the drug-susceptibility testing were performed by MGIT when the patient had a previous anti-TB treatment history. In case of detecting any drug-resistance, this was confirmed by the proportion method on Lowenstein–Jensen. When the patient did not have a previous contact

with the specific therapy, its isolated was tested by the proportion method on solid medium. Any drug-resistance detected was later confirmed by the MGIT SIRE kit. In both cases resistance to isoniazid and/or rifampicin were confirmed by GenoType MTBDRPlus.

Species identification was performed by means of biochemical tests and/or spoligotyping [25]. Genotyping analysis was based on IS6110-RFLP typing and spoligotyping carried out as described previously [26] All the isolates belonging to the same household showing the same spoligotyping pattern were analyzed by IS6110 RFLP in order to confirm or discard the unique isolated transferred from one patient to another. RFLP was also performed to isolates from the same household members with different drug-resistance patterns.

Mutations in *rpoB*, *katG* and *inhA* of *M. tuberculosis* were analyzed by a multiplex allele specific polymerase chain reaction (MAS-PCR) and also by sequencing [27].

2.10. Fitness experiments

M. tuberculosis Complex strains were sub-cultured on Lowenstein–Jensen or Stonebrink solid media during 20 days. After this time, bacterial suspensions were prepared in 2.0 mL of distilled sterile water From this original suspension (A), another one was prepared showing turbidity comparable with that of 1 McFarland standard and containing approximately 10^8 bacilli/mL (CFU/mL) (B). This B bacterial suspension was passed by a needle (21GX1); thus this suspension was diluted 1:500 in distilled sterile water obtaining the third suspension (C). A total of 500.0 μL of C (with approximately 10^5 CFU/mL) was finally inoculated in MGIT 960 in duplicate. The tubes were placed in the MGIT 960 apparatus. For a further counting of colonies, 200.0 μL of suspension C was also inoculated onto solid media also in duplicate. These tubes were incubated as above mentioned [18,19,28].

2.11. Statistical analysis

Epidemiological, clinical and microbiological data were analyzed by MedCalc software (MedCalc Software, Mariakerke, Belgium). Descriptive statistics and analysis of the differences were analyzed by ANOVA or Fischer Exact test and multivariate analysis were performed on these data.

Molecular epidemiology analysis of the genetic patterns found by IS6110 RFLP and spoligotyping were performed using the BioNumerics Software (Applied Maths NV, Sint-Martens-Latem, Belgium).

For fitness analysis, growth records were obtained from the EpiCenterTM software of the MGIT 960 system. The lag phase (t0, hours), time (hours) to reach 200 GU, GR and RF of each one of the isolates included in the study were calculated.

3. Results

The 124 TB cases included in this study belong to 39 households. The mean age of the TB patients was 30.7 years (range <1.0–66.0). Patients, isolates and the households herein studied are detailed in Table 1. Molecular epidemiology showed that all the multi-drug resistant (MDR) isolates belong to spoligotype defined families [29].

Compared to fully-DS strains, the RF of the MDR and XDR isolates showed an average decrease of 13.2% (0.98 vs. 0.85, range: 11.0–17.0%) and 24.4% (0.98 vs. 0.74, range: 22.3–29.4%), respectively (Table 2) and these differences were significant ($p < 0.0001$). The most large variations (showed as standard deviation) were found in RFs of the resistant isolates The standard deviation for the RF of MDR and XDR strains showed increases of 45.0% (p: 0.001) and 64.2% (p: 0.003) respectively, compared to that of the DS isolates, (Table 2). These higher standard deviation values in MDR and XDR may be explained because various mutations may cause these phenotypes, while the genotypes of the DS strains are fairly constant.

Table 1
Epidemiological, clinical and microbiological characteristics of the patients whose isolates were included in the study.

Study compounds details	N° (%)	Patients/household N° (%)
Patients	124 (100.0)	124/39 (3.2) (range: 16–2)
Households	39 (100.0)	range: 16–2
Household contacts	388 (100.0) (average: 3.2)	388/39 (9.9) (range: 11–2)
HIV co-infection	23 (18.5)	23/39 (0.6)
Women	58 (46,8)	58/39 (1.5)
Pulmonary TB	120 (96.8)	120/39 (3.1)
Previous treatment history	39 (31,5)	39/39 (1.0)
MDR TB isolates XDR TB	47 (38.0) [4 (3.2)]	51/24 (2.1) 4/39 (0.1)
DS isolates	51 (41.1)	51/24 (2.1)
DR isolates	18 (14.5)	18/39 (0.5)

DR, MDR and XDR: drug-resistant, multidrug-resistant and extensively drug-resistant *M. tuberculosis* isolates respectively; DS: fully drug-susceptible *M. tuberculosis* Complex.

The lag phase (t_0) of the isolates was also determined. The mean t_0 values for M/XDR isolates were increased 49% and 42%, respectively compared to that for DS controls ($p < 0.0001$), while the difference between the t_0 means for DR and fully-DS isolates was not significant ($p < 0.9798$). The standard deviation obtained for the M/XDR t_0 distribution also showed a significant increase of 41.6% compared to the t_0 standard deviation for fully-DS strains ($p < 0.012$).

The growth rate (GR) to reach 200 growth units (GU) was also calculated and compared according with the DST patterns of the isolates. The average GRs were (in hours) as follows: XDR, 32.3; MDR, 29.0; DR, 24.6; DS controls, 21.9. The mean GR for the M/XDR strains were 32.3% ($p < 0.0001$) and 17.9% ($p < 0.0001$) greater, respectively, than those for fully-DS and DR strains (Table 2).

Table 3 shows the tuberculosis cases found in each of the households included in the study, along with the drug sensitivity pattern of the presumed index case and secondary cases. Households 2, 4, 7, 11, 16, 17, 21, 27, 28, 32, 33, 34, 36, 37, and 39 showed only different drug-resistance pattern from that of the index case. Three households (# 5, 6, 10) were composed by mixed bacterial populations, since the secondary cases showed drug-sensitivity and genetic patterns distinct from those of their respective index cases.

Household 5 with 1 MDR case and 4 contacts affected by fully-DS TB illustrates the particular case of an acquired MDR developed in the family father after several years of non-adherence to the treatment. This patient presumably was the infectious source for the children (10, 7 and 1 years old), from whom the fully-DS bacteria with the same spoligotype were isolated. The RF of the MDR isolate was 0.53 while that of the fully-DS was 0.98 (data not shown). The mother was the fifth member of the family who was diagnosed and successfully treated soon before the rest of her family. The genetic patterns of the mother's isolate were different from those of the husband and children, who were diagnosed afterwards.

The secondary cases were nomined after assigning the index case of the household. Its assumption was made based on the bacteriological diagnostic dates and the growth characteristics represented by the RF, t_0 and GR obtained values (Figure 1). According to an *a priori* possibility, we assumed that the strain whose RF, t_0 and GR were similar to the parental strain could have the

Table 2
Relative fitness and lag phase distributions obtained from clinical isolates according to their drug-resistant profile.

DRP	Relative fitness		Lag phase (hours)		Growth rates	
	Mean ± SD (Range)	95% CI	Mean ± SD (Range)	95% CI	Mean ± SD (Range)	CI 95%
DS	0.98 ± 0.058 (0.87–1.17)	0.94–1.00	116.7 ± 32.9 (64.0–198.0)	105.4–127.8	21.5 ± 2.65 17.0–22.9	17.0–25.9
DR	1.04 ± 0.073 (0.93–1.14)	0.98–1.09	117.7 ± 39.7 (64.0–197.0)	95.6–139.7	24.5 ± 2.77 20.2–24.7	13.8–27.2
MDR	0.85 ± 0.106 (0.44–0.96)	0.84–0.86	173.4 ± 54.6 (97.0–283.0)	155.5–191.4	29.0 ± 6.79 23.5–53.1	26.8–32.0
XDR	0.74 ± 0.162 (0.63–0.86)	0.58–0.88	166.7 ± 56.3 (153.0–194.0)	107.8–225.5	32.3 ± 7.07 27.3–37.3	25.8–33.5

P values in the text.
DRP: drug-resistance pattern; DS: fully drug-susceptible *M. tuberculosis* Complex; DR, MDR and XDR: drug-resistant, multidrug-resistant and extensively drug-resistant *M. tuberculosis* isolates respectively; 95% CI: confidence interval of 95%; SD: standard deviation. Significance level: 0.05.

opportunity to be transmitted to other household member. In this assumption, we also took into account the order in which the cases appeared or were diagnosed.

A total of 20 households had a MDR TB index case related to 33 secondary cases (1.65/index case); 23 (1.15/index case) of which were MDR secondary cases (Figure 1). Furthermore, 16 index cases with a fully-DS TB produced 43 secondary cases (2.7/index case) and 3 DR index case gave 3 secondary cases (1/index case) (Figure 1). Differences in secondary cases generation from MDR and fully-DS index cases were detected (χ^2: 0.043).

MDR isolates carrying the *rpoB 531* mutations showed an average RF drop of 14.6% compared to MDR cases with other *rpoB*

Table 3
Households *M. tuberculosis* strains distribution and generation of secondary cases from presumable index cases.

Household N°	Total presumible index cases			Total 2ary cases			No household transmission	Total cases
	MDR	**DS**	**DR**	**M/XDR**	**DS**	**DR**		
1	1	0	0	3	0	0	1	5
2†	1	0	0	0	0	1	0	2
3	1	0	0	2	0	0	0	3
4†	1	0	0	0	0	1	0	2
5*	1	0	0	0	4	0	1	6
6*	0	1	0	0	1	3	0	5
7†	1	0	0	0	1	0	0	2
8	1	0	0	1	0	0	0	2
9	0	1	0	0	2	0	0	3
10*	0	1	0	0	2	1	0	4
11†	1	0	0	0	0	0	1	2
12	1	0	0	4§	0	0	0	5
13	1	0	0	1	0	0	0	2
14	0	0	1	0	0	1	0	2
15	0	1	0	0	2	0	0	3
16†	1	0	0	0	0	0	1	2
17†	0	1	0	0	11	3	0	15
18	0	1	0	0	1	0	0	2
19	0	1	0	0	3	0	0	4
20	1	0	0	2	0	0	1	4
21†	0	1	0	1	0	1	0	2
22	0	1	0	0	1	0	0	2
23	1	0	0	1	0	0	0	2
24	1	0	0	2	0	0	0	3
25	1	0	0	1**	0	0	0	2
26	0	0	1	0	0	1	0	2
27†	1	0	0	0	1	0	0	2
28†	0	1	0	0	0	2	0	3
29	0	1	0	0	2	0	0	3
30	0	1	0	0	2	0	0	3
31	0	1	0	0	1	0	0	2
32†	0	1	0	2	0	0	0	3
33†	0	0	1	1	0	0	1	3
34†	0	1	0	1	0	0	0	2
35	1	0	0	1	0	0	0	2
36†	1	0	0	1	0	1	0	3
37†	0	1	0	1	0	0	0	2
38	1	0	0	1	0	0	0	2
39†	1	0	0	3	0	1	0	5
Total	20	16	3	29	34	16	6	124

* Households containing strains with different genetic and drug-susceptibility patterns.
† households containing strains with different drug-resistance pattern but identical genetic patterns.
§ 3XDR secondary cases.
** 1 XDR secondary case.

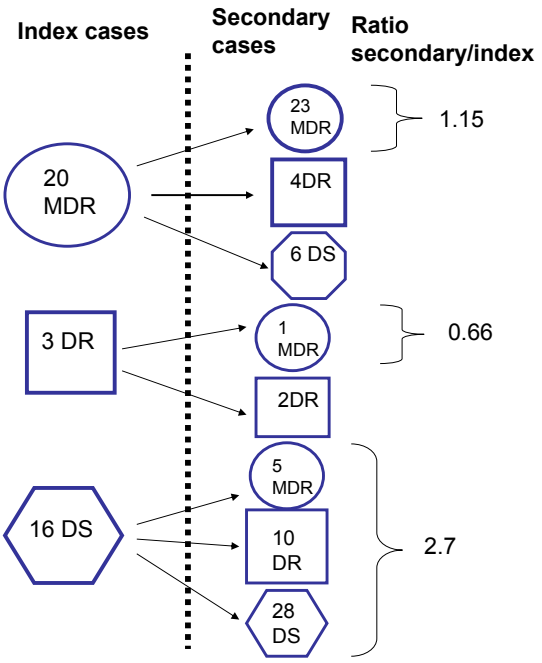

Figure 1. Generation of secondary cases from the putative index cases according to their respective drug-resistance profile.

mutations, DR and fully-DS strains. The strains with *rpoB* substitutions in nucleotides at the 526 or 531 positions generally had high MIC's for rifampicin. The multivariate analysis of RF, the t0 duration, and MIC values for RIF resistant strains with mutations in the rpoB gene at positions 526 and 531, showed a correlation coefficient of 0.88. It was observed that only mutations in rpoB 531 ($p < 0.0222$) were associated with a decrease in RF. Mutations conferring isoniazid resistance, strains with a substitution in 315 of *katG* showed high MIC's for isoniazid [34]. On the other hand, strains with mutations in the *inhA* promoter region (-15) displayed low MIC's. However, no differences were found in transmission of strains mutated in *katG* or inhA *genes* (p: 1.000).

4. Discussion

This study assesses *M. tuberculosis* strain transmission among members of the same household and to observe the growth behavior during host transmission.

In general, decreasing RF values were observed while the isolates were transmitted among households. In average, MDR and XDR isolates suffer a drop of 13.2% and 24.4% and this decline is apparently enough to cause a decrease in multiplication and transmission among humans. The lag phase and growth rate also decrease in MDR strains; which suggests that the last and less fit mycobacterium was not further transmitted to another household member. This assumption would explain why several household members became infected by an external infectious source or remained healthy after two years follow-up, such as the XDR strains that belonged to a MDR outbreak involving healthcare workers and their household contacts (household 12, Table 3). The first XDR case (a middle age no compliant man) transmitted the disease to a young child whose XDR strain – with a low RF – was not further found in either any other household member or close contact studied. The observation that MDR/XDR strain are less transmitted to other member of the household may be attributable to transmission of MDR bacteria from a non detected mixed infection in the index case to contacts and not to an infection from other source than the index case, as we postulated here. In any case the secondary cases did not acquire MDR isolates. Albeit most of the isolates had genetic patterns grouped in clusters, households composed mostly by MDR cases had at least one member infected by a DS organism presumably form a different community source.

The lower transmission of MDR and XDR isolates is also reflected by the presumptive generation of secondary cases from the assigned index cases being this generation index lower in MDR and XDR strains. One may suppose that a strain with lower fitness may not overwhelm the immune response.

The multivariate analysis showed that only the mutations in the *rpoB* at position gene 531 were associated to a RF decrease, in agreement with observations of Spies et al. [30]. This finding differs from a previous publication [4] but in the core of each family group. Thus, these RIF-resistant strains harboring these common *rpoB* mutations would become disadvantaged against a DS strain from the community and the final competition effect seems to be the strain self limitation. Most of M/XDR strains harbored mutations in *katG* 315 genes, but the drop in RF was not associated to these mutations. This is consistent with the role of catalase-peroxidase (KatG) in the pathogenicity of *M. tuberculosis*, which is a known virulence factor [31,32]. A strain with *katG* S315T mutation produces a KatG enzyme with a substitution resulting in a functional enzyme that retains its catalase–peroxidase activity but with a highly diminished capacity to activate INH. This fact is in concordance which those reported by Gagneux et al. regarding transmission of isoniazid-resistant strains in San Francisco [33].

The drug-resistance level (expressed by MIC values) was neither related to a decreased RF nor the strain transmission among the household contacts studied.

A decrease in the RF was found in MDR isolates compared to fully-DS and DR isolates. The adaptive cost may explain why MDR/XDR isolates are successfully transmitted among households' members until a moment in which the strain is no longer isolated. However we cannot assume that the observed alterations of in vitro growth parameters may affect the growth during infection. The detected and described mutations as well as the addition of different changes in the same microorganism might explain the sudden disappearance of the mycobacteria in some of the studied human group.

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NSM: conceived and designed the experiments.

NSM, BRI, ABD and MJZ performed the experiments.

NSM BRI AAC, HT: analyzed the data.

NSM ABD AAC: contributed with reagents, materials and analysis tools.

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