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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