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Short Communication

# Unmasking subtle differences in the infectivity of microevolved *Mycobacterium tuberculosis* variants coinfecting the same patient

Yurena Navarro[a,b,c,d,e], Laura Pérez-Lago[a,b,e], Fernanda Sislema[a,b], Marta Herranz[a,b,e], Lucía de Juan[c,d], Emilio Bouza[a,b,e,f], Darío García-de-Viedma[a,b,d,e,*]

[a] *Servicio Microbiología Clínica y Enfermedades Infecciosas, Hospital General Universitario Gregorio Marañón, Madrid, Spain*
[b] *Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain*
[c] *Centro de Vigilancia Sanitaria Veterinaria (VISAVET), Universidad Complutense de Madrid, Avda. Puerta de Hierro s/n, 28040 Madrid, Spain*
[d] *CEI Campus Moncloa, UCM-UPM, Madrid, Spain*
[e] *CIBER Enfermedades Respiratorias, CIBERES, Spain*
[f] *Departamento de Medicina, Facultad de Medicina, Universidad Complutense de Madrid, Madrid, Spain*

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ABSTRACT

Clonal variants of *Mycobacterium tuberculosis* can emerge as a result of microevolution phenomena. The functional significance of these subtle genetic rearrangements is normally disregarded. We show that clonal variants from two patients had different infective behaviours in some *in vitro* cellular infection models but not in others. Microevolution may have a subtle impact on infectivity, but specific experimental conditions are needed to unmask it.



## Introduction

Some infections by *Mycobacterium tuberculosis* (MTB) are clonally complex; examples include reinfections, mixed infections, and compartmentalized infections (Cohen et al., 2012; Navarro et al., 2011). This complexity is not always due to different MTB strains. In some cases, a parental strain microevolves along a transmission chain or within a single episode of infection, leading to the emergence of clonal variants harbouring subtle differences with respect to the parental strain. These differences are revealed by standard genotyping techniques, namely, IS*6110*-RFLP and MIRU-VNTR (Al-Hajoj et al., 2010). Few studies have investigated the functional significance of these subtle changes or other microevolutive changes which could also be taking place in parallel in regions that are not revealed by standard genotyping techniques. Variations in the number of repetitions in specific MIRU-VNTR loci have been found to affect expression levels of neighbouring genes (Akhtar et al., 2009; Tantivitayakul et al., 2010). Strains differing according to the presence or absence of an IS*6110* copy located upstream of specific genes also show variations in the expression levels of these genes (Perez-Lago et al., 2011). However, the potential impact on the infectivity of clonal variants of the subtle rearrangements associated with microevolution phenomena has rarely been investigated. In this study, we aimed to explore potential differences between the infectivity of clonal variants coinfecting two independent cases.

## Variants involved in compartmentalized infection

Differences in infectivity have been found between MTB strains involved in compartmentalized infections (Garcia de Viedma et al., 2005), *i.e.*, cases coinfected by more than one MTB strain, each infecting a different compartment in the same patient. Therefore, if we aim to investigate differences in infectivity between clonal variants, one approach would be to select for analysis coinfecting MTB variants involved in compartmentalized infections.

Based on data from a study which systematically analyzed clonal complexity (Navarro et al., 2011), we selected a TB case (patient H) with an urotelial carcinoma and another previous TB episode, who showed at diagnosis a general health decay, fever, pain in lower limbs, lung and pleural nodules and kidney failure. In this case, an infrequently high number of coinfecting variants (A1, A3,

* Corresponding author at: Servicio de Microbiología y Enfermedades Infecciosas, Hospital General Universitario Gregorio Marañón, Instituto de Investigación Sanitaria Gregorio Marañón, C/Dr Esquerdo, 46, 28007 Madrid, Spain.
Tel.: +34 91 4265104; fax: +34 91 5044906.
*E-mail address:* dgviedma2@gmail.com (D. García-de-Viedma).

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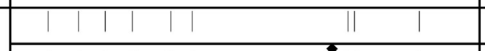
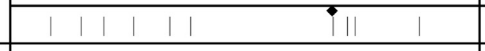
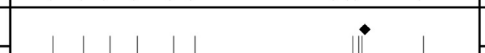
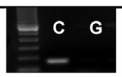
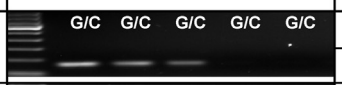
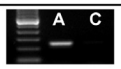
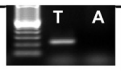
**a)**

| Clonal variant | MIRUtype | RFLPtype | Distribution | | |
|---|---|---|---|---|---|
| | | | Sputum | Blood | Urine |
| A1 | 25353323342353325**2**13423 |  | X | | X |
| A3 | |  | | X | |
| B2 | 25353323342353325**1**13423 |  | X | X | |
| C2 | 2**2**353323342353325**1**13423 | | | X | |

**b)**

| ASO-PCR | Clonal Variant | Differential alelle | Specificity | Sensitivity (2/2 0.2/2 $2x10^{-2}/2$ $2x10^{-3}/2$ $2x10^{-4}/2$) | Primers | Annealing Temp(°C) | $MgCl_2$ final concentration (µM) |
|---|---|---|---|---|---|---|---|
| 1 | A1 | G546C |   |   | F-TCAACAACAAGCGCAGCCA**C**<br>R-CTGATACAGGCCACCGATTT | 61 | 1.1 |
| 2 | B2 | C202A |   |   | F-GATCACCGCCCACACCAAC**A**<br>R-CGGCTAGGGCAGTAAATCTG | 65 | 1.0 |
| 3 | C2 | A466T |   |   | F-CGGTCAACTGCTCTGAAACA<br>R- TGGCGCTGCGCTGGATCGT**A** | 67 | 1.2 |

**c)**

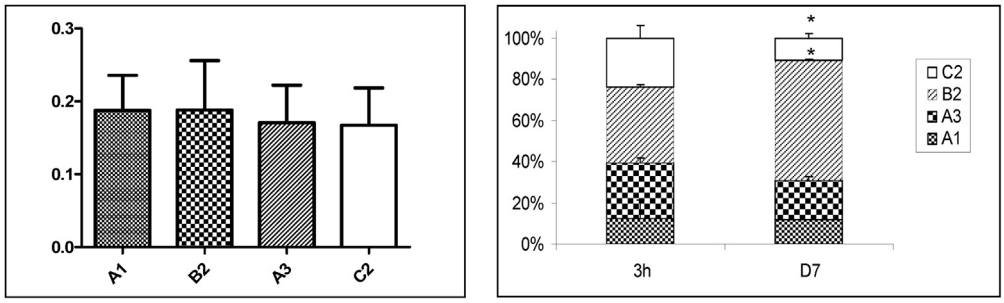



**Fig. 1.** (a) Genotypic features of the clonal variants from the patient H. The distribution of the coinfecting variants is indicated. Bold numbers and black diamonds indicate changes in the MIRU-VNTR and RFLP patterns between the variants, respectively. (b) Features of the ASO-PCRs applied. Specificity was evaluated using 5 ng of DNA representative of each of the specific or nonspecific differential alleles. The expected sizes for the specific amplicons were as follows: ASO-PCR1, 181 bp; ASO-PCR2, 239 bp; and ASO-PCR3, 227 bp. The sensitivity of each PCR for identification of the homologous variant in the presence of nonhomologous variants was evaluated using a series of templates constituted by a fixed amount of DNA representative of a nonhomologous allele, together with decreasing amounts of the DNA representative of the homologous allele (the amounts in the figure are in ng). The forward primers for ASO-PCR1 and ASO-PCR2 were complementary for the noncoding strand, whereas the primer for ASO-PCR3 was complementary for the coding strand. The 3′-end nucleotide highlighted in bold corresponds to the nucleotide involved in allelic discrimination. (c) Infection of nonactivated THP-1 cells with the clonal variants; left panel: growth of the clonal variants in the THP-1 cells infected (at a multiplicity of infection (MOI) from 1 to 8) individually (expressed as the growth rate [*y* axis], *i.e.*, the slope of the function of $\log_{10}$ CFU values during the infection period [3 h to day 7]) using ANOVA analysis of three independent experiments; CFUs were counted at 3 h and days 1, 4 and 7; right panel: percentages of representativeness of the clonal variants, after simultaneous coinfection, as determined by MIRU-VNTR analysis of 118 (3 h) and 119 (day 7) independent colonies. Two independent experiments were carried out and Bonferroni correction was used for statistical analysis. Asterisks indicate statistical significance ($p < 0.05$).

B2, and C2, all pansusceptible), revealed by standard genotyping (MIRU-VNTR and RFLP), were identified. In addition, these variants were not always isolated together from the same infected site (respiratory, blood, and urine), thus suggesting that infection was compartmentalized (Fig. 1a).

## Confirmation of compartmentalization

If compartmentalization is to be used as a criterion for suspecting that the variants involved show different phenotypes (infective behaviour), we must confirm that compartmentalization is strict and that it is not the consequence of a lack of sensitivity when identifying variants at specific infected sites. Using whole genome sequencing data for coinfecting variants (data not shown), we designed a set of allele-specific oligonucleotide (ASO) polymerase chain reactions (PCRs) in order to ensure higher sensitivity when investigating the presence of the variants at the infected sites (Fig. 1b). An ASO-PCR could not be designed for variant A3 owing to the lack of specific single-nucleotide polymorphisms for this variant. The specificity of each ASO-PCR (amplification only when the targeted variant was present), together with its analytical sensitivity, was measured using the DNAs representative of each of the alternative alleles alone or in mixtures at controlled proportions (a fixed amount of the alternative allele together with decreasing amounts of the allele homologous to the ASO-PCR) (Fig. 1b). The analytical sensitivity range was 0.02–0.002 ng (Fig. 1b).

ASO-PCRs 1, 2, and 3 were applied to investigate the presence of variants A1, B2, and C2 at the sites where they had not been identified by standard approaches (A1 in blood, B2 in urine, and C2 in sputum and urine, Fig. 1a). Variant A1 was also detected in blood, whereas variants B2 and C2 were absent from the sites where they had not been initially identified, thus confirming compartmentalization but depicting a new distribution of variants in the compartmentalized infection. These data showed the necessity of using highly sensitive tools to obtain an accurate snapshot of the true distribution of variants at the infected sites when a compartmentalized infection is detected.