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Research note

High within-host diversity found from direct genotyping on post-mortem tuberculosis specimens in a high-burden setting

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ABSTRACT

Objectives: To characterize the clonal complexity in *Mycobacterium tuberculosis* (MTB) infections considering factors that help maximize the detection of coexisting strains/variants.
Methods: Genotypic analysis by Mycobacterial Interspersed Repetitive-Unit–Variable-Number Tandem-Repeats (MIRU-VNTR) was performed directly on 70 biopsy specimens from two or more different tissues involving 28 tuberculosis cases diagnosed post-mortem in Mozambique, a country with a high tuberculosis burden.
Results: Genotypic data from isolates collected from two or more tissues were obtained for 23 of the 28 cases (82.1%), allowing the analysis of within-patient diversity. MIRU-VNTR analysis revealed clonal diversity in ten cases (35.7%). Five cases showed allelic differences in three or more loci, suggesting mixed infection with two different strains. In half of the cases showing within-host diversity, one of the specimens associated with clonal heterogeneity was brain tissue.
Conclusions: Direct MTB genotyping from post-mortem tissue samples revealed a frequent within-host *Mycobacterium tuberculosis* diversity, including mixed and polyclonal infections. Most of this diversity would have been overlooked if only standard analysis of respiratory specimens had been performed.
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Introduction

Molecular and genomic approaches have weakened the assumption that *Mycobacterium tuberculosis* (MTB) infections are clonally homogeneous. Different modalities of within-host diversity have been proposed, from mixed infections involving more than one strain [1], to polyclonal infections with related variants that emerge from a parental strain [2] with or without asymmetric distribution of the strains/variants in the infecting tissues [3]. However, in most studies cultures were analysed (which may distort specimen complexity [4,5]), extensive sampling was not assured, the studies focused on respiratory specimens, or were performed in settings with low tuberculosis (TB) incidence.

The aim of this study was to characterize the clonal complexity in TB by aiming to maximize its detection. We focused on a setting with a high burden of TB (Southern Mozambique), where the risk of overexposure is higher, and included patients with a post-mortem diagnosis of TB, candidates of prolonged disease in whom microevolution events might have occurred. An exhaustive sampling was assured and analyses were done directly on biopsies to exclude any impact of cultured samples.

Methods

Study setting

This was an ancillary study to a prospective observational post-mortem evaluation study (the CaDMIA study) conducted in Maputo, Southern Mozambique, between November 2013 and March 2015. For the 28 TB cases in the study, the clinical information is described elsewhere [6,7].

Sample processing

Tissue and blood samples were collected in buffer ATL (Qiagen) during complete diagnostic autopsies and were stored at −80°C. Samples were tested using the Xpert MTB/RIF Ultra assay (Cepheid, Sunnyvale, CA, USA) to determine MTB load and assess rifampicin resistance.

Mycobacterial Interspersed Repetitive-Unit–Variable-Number Tandem-Repeats (MIRU-VNTR) genotyping

Results of a preliminary pilot study showed that specimens with low/very low mycobacterial Xpert load values do not allow subsequent MIRU-VNTR analysis. Thus, only samples with medium and high loads were used. DNA was extracted as described elsewhere [8]. The 24-locus triplex-MIRU-VNTR method was used [9]. Simplex PCRs were carried out when the triplex format failed, to recover amplifications in certain loci. Assignation of lineages was done with TBminer (http://info-demo.lirmm.fr/tbminer/index.php).

Assignation of resistance mutations

rpoβ, katG and *mabA–inhA* regions were analysed by Sanger sequencing [10,11].

Results

Seventy clinical specimens (lung, spleen, lymph node, liver, brain biopsies, or plasma) were included—between one and five samples per patient—from 28 patients with confirmed TB by histological and microbiological (Xpert Ultra) analyses. In 25 cases (86.2%), TB was the cause of death (20 miliary TB, four pulmonary TB, and one tuberculous meningitis), whereas in the remaining three cases TB was present at death in subjects who died from other aetiologies. Thirteen cases were maternal deaths, 14 other adults, and one case was a 2-year-old child. Median age was 35 years (range 2–56 years) and 14 (50%) were female. Twenty-four cases (72.7%) were HIV-positive. TB was clinically suspected in ten patients (35.7%), but considered the main death-causing disease in six patients (21.4%).

A complete MIRU-VNTR pattern was obtained for 63 out of the 70 study samples (90%) (Table 1). No amplification in any of the loci was observed for one specimen. The six specimens with incomplete data (one locus failing in two specimens; 7–24 loci failing in five specimens) corresponded to medium viral load as per Xpert.

Most strains belonged to Lineage 4 (Euro-American) (64.3%), and four, six, and one case corresponded to Lineage 2 (Beijing), Lineage 1 (EAI1), and Lineage 3 (India and East Africa), respectively. Sixty-three different MIRU-VNTR patterns were obtained. Clusters of cases sharing the same strain were not identified.

For 23 of the 28 cases (82.1%), two or more tissues were available to assess within-patient diversity. MIRU-VNTR analysis showed some clonal diversity in ten cases (35.7%) (Table 1). In four of these cases, despite an incomplete genotype, differences were observed.

In half of the cases (5/10) with clonal heterogeneity, brain tissue was involved. Five cases showed allelic differences in three or more loci (two cases in three loci, and one case in five, six, and seven loci), suggesting mixed infection with two different strains. In the remaining five cases, lower within-host diversity was found, with allelic differences between the isolates in one or two loci. In 4/10 cases with within-host diversity, the two strains/variants were detected simultaneously in one specimen, whereas in the remaining six a compartmentalized infection was observed, with each of the strains/variants restricted to one tissue.

The ten cases with any type of clonal diversity were HIV-positive, and all except one died of miliary tuberculosis. Nine out of these ten cases had been previously diagnosed with HIV and two with TB, and were under anti-TB treatment. No significant differences were found in the previous HIV or TB history among the cases for which no clonal diversity was identified.

Rifampicin resistance was identified in four cases (Cases 1, 6, 10, and 15). Mutations could be assigned in three cases: S531L (Case 1), H526Y (Case 6), and H526D (Case 10). No changes in *katG* or *inhA* regions were identified for Case 6 and Case 15, suggesting RIF monoresistance; a −15 mutation in the *inhA* promoter region and a S315T substitution in *katG* were identified in Cases 1 and 10, respectively, indicating multidrug resistance.

Discussion

Within-host diversity has been described for TB, indicating that the idea that every TB episode involves a single strain should not be considered a generalization. However, most studies do not consider all the factors needed to assure that diversity does not remain hidden. In this study we include some of these missing factors, aiming to determine the true complexity of MTB infection from a clonal perspective.

First, we focused on a high-burden setting, where the risk of exposure to more than one infectious case is greater. Second, in our cases TB was determined post-mortem, suggesting long-term infections and, therefore, likely microevolution. Finally, exhaustive respiratory/extrapulmonary sampling and direct analysis on specimens was pursued to optimize the detection of compartmentalized infections and rule out biases derived from culturing.

Some kind of diversity was revealed in nearly half of the studied cases. Most likely, several were mixed infections, even involving

Table 1
MIRU-VNTR types for the specimens analyzed.

Patient	Specimen	MIRU-VNTR locus																								Lineage	GeneXpert load	
		2	20	23	24	27	39	4	26	40	10	16	31	42	43	ETRA	47	52	53	Qub11B	1955	Qub26	46	48	49			
1	SPLEEN	2	2	5	1	3	1	2	6	1	3	3	4	3	5	4	4	3	3	5	4	5	4	2	3	L2_Beijing	medium	MDR
	LYMPH NODE	2	2	5	1	3	3	2	6	1	3	3	5	3	5	4	4	3	3	5	4	9	4	2	3	L2_Beijing	high	
2	CSF	2	1	6	2	3	3	5	2	3	4	3	5	2	4	9	2	5	3	11	6	7	3	3	3	L1_EAI	medium	
	BRAIN	2	1	6	2	3	3	5	2	3	4	3	5	2	4	9	2	5	3	11	6	7	3	3	3	L1_EAI	medium	
3	LUNG	2	2	6	1	3	2	2	6	4	4	2	3	4	2	2	2	1	2	4	3	2	4	2	1	L4_LAM	medium	
	BRAIN	2	2	6	1	3	2	2	6	-	4	2	6	4	2	2	2	1	2	4	3	2	4	2	1	L3_CAS	medium	
4	PLASMA	2	2	6	1	2	1 | 2	2	5	1	3	1	3	1	4	2	1	2	2	4	3	7	4	1	5	L4_LAM	medium	
	LUNG	2	2	6	1	2	2	2	5	1	3	1	3	4	4	2	1	2	2	4	3	7	4	1	5	L4_LAM	medium	
	BRAIN	2	2	6	1	2	1	2	5	1	3	1	3	4	4	2	1	2	2	4	3	7	4	1	5	L4_LAM	medium	
5	LUNG	2	2	6	1	2	2	2	6	1	4	2	3	1	4	2	1	2	2	4	3	8	4	2	3	L4_LAM	high	
	CSF	2	2	6	1	2	2	2	6	1	4	2	3	1	4	2	1	2	2	4	3	-	4	2	3	L4_LAM	medium	
	BRAIN	2	2	6	1	2	2	2	6	1	4	2	3	1	4	2	1	2	2	4	3	8	4	2	3	L4_LAM	medium	
6	BRAIN	3	2	5	1	3	2	3	4	5	3	3	3	3	4	4	2	3	2	3	1	9	4	2	2	L4_S	medium	Rif mono-resistance
	LUNG	3	2	5	1	3	2	3	4	5	3	3	3	3	4	4	2	3	2	3	1	9	4	2	2	L4_S	medium	
	PLASMA	3	2	5	1	-	1 | 2	6 | 2	6	5	-	-	-	-	2	4	1	3	1	7	1	-	4	2	-	L4	medium	
7	LUNG	2	2	6	1	3	2	2	5	3	3	3	3	4	4	2	2	1	2	3	7	7	4	2	1	L4_LAM	medium	
	PLASMA	2	2	6	1	3	2	2	5	3	3	3	3	4	4	2	2	1	2	3	7	7	4	2	1	L4_LAM	high	
8	LUNG	2	2	8	1	3	2	2	5	3	6	3	3	2	3	3	4	3	3	3	3	4	4	2	3	L4_H1-2	medium	
	SPLEEN	2	2	8	1	3	2	2	5	3	6	3	3	2	3	3	4	3	3	3	3	4	4	2	3	L4_H1-2	medium	
9	LUNG	2	2	6	1	3	2	2	5	1	3	2	3	1	4	2	1	2	2	2	3	4	5	2	3	L4_LAM	medium	
	BRAIN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		medium	
10	BRAIN	2	2	5	1	3	2	2	5	3	4	3	3	2	4	3	4	4	3	3	4	9	4	2	3	L4_X	high	MDR
	SPLEEN	2	2	5	1	3	2	2	5	3	4	3	3	2	4	3	4	4	3	3	4	9	4	2	3	L4_X	medium	
	LIVER	2	2	5	1	3	2	2	5	3	4	3	3	2	4	3	4	4	3	3	4	9	4	2	3	L4_X	medium	
	LUNG	2	2	5	1	3	2	2	5	3	4	3	3	2	4	3	4	4	3	3	4	9	4	2	3	L4_X	medium	
	CSF	2	2	5	1	3	2	2	5	3	4	3	3	2	4	3	4	4	3	3	4	9	4	2	3	L4_X	high	
11	CSF	2	2	5	1	-	1	-	6	5	3	-	-	-	2	4	-	2	-	4	-	-	4	2	-	L4	medium	
	BRAIN	2	2	5	1	3	2	3	5	4	3	3	3	3	4	4	2	3	2	4	1	8	4	2	2	L4_S	medium	
	LUNG	2	2	5	1	3	2	3	5	4	3	3	3	3	4	4	2	3	2	4	1	8	4	2	2	L4_S	medium	
	PLASMA	2	2	5	1	3	2	3	5	4	3	3	3	3	4	4	2	3	2	4	1	8	4	2	2	L4_S	medium	
12	LUNG	2	2	6	1	2	2	2	2	1	4	1	3	3	4	1	1	2	2	3	3	8	4	1	5	L4_LAM	medium	
	PLASMA	2	2	6	1	2	2	2	2	1	4	1	3	3	4	1	1	2	2	3	3	8	4	1	5	L4_LAM	medium	
13	BRAIN	2	2	6	2	3	1	5	2	3	4	3	5	2	2	9	2	10	1	3	11	5	3	4	3	L1_EAI1	medium	
	LUNG	2	2	6	2	3	1	5	2	3	4	3	5	2	2	9	2	10	1	3	11	5	3	4	3	L1_EAI1	high	
	PLASMA	2	2	6	2	3	1	5	2	3	4	3	5	2	2	9	2	10	1	3	11	5	3	4	3	L1_EAI1	high	
14	BRAIN	2	2	4	1	3	2	2	2	1	-	-	-	-	4	-	-	2	-	3	3	-	-	-	-	L4	medium	
	LUNG	2	2	6	1	2	2	2	6	1	4	2	3	1	4	2	1	2	2	3	3	8	4	2	2	L4_LAM	medium	
	PLASMA	2	2	6	1	2	2	2	6	1	4	2	3	1	4	2	1	2	2	3	3	8	4	2	2	L4_LAM	medium	
15	LUNG	2	2	5	1	3	1	3	2	3	3	3	3	2	2	4	2	4	2	4	1	3	4	2	2	L4	medium	Rif mono-resistance
	BRAIN	2	2	5	1	3	1	3	2	3	3	3	3	2 | 3	2	4	2	4	2	4	1	3	4	2	2	L4	medium	
16	BRAIN	2	1	6	2	3	3	5	2	2	4	3	5	2	4	9	2	6	1	10	6	7	3	5	3	L1_EAI	medium	
	LUNG	2	1	6	2	3	3	5	2	2	4	3	5	2	4	9	2	6	1	10	6	7	3	5	3	L1_EAI	high	
	PLASMA	2	1	6	2	3	3	5	2	2	4	3	5	2	4	9	2	6	1	10	6	7	3	5	3	L1_EAI	high	
17	BRAIN	2	1	6	1	3	2	2	2	2	4	2	3	2 | 5	4	2 | 9	1 | 2	2	2	4	3	8	4	1	5	L4	medium	

	LUNG	2	2	6	1	3	2	2	2	1 | 2	4	2	3	5	4	2	1 | 2	2	2	4	3	8	4	1	5	L4	medium
	PLASMA	2	2	6	1	3	2	2	5	1	4	2	3	5	4	2	1	2	2	4	3	8	4	1	5	L4_LAM	high
18	BRAIN	2	2	5	1	4	3	2	7	3	3	3	5	4	4	4	4	3	2	5	5	8	4	2	3	L2_Beijing	medium
	LUNG	2	2	5	1	4	3	2	7	3	3	3	5	4	4	4	4	3	2	5	5	8	4	2	3	L2_Beijing	high
19	SPLEEN	2	2	5	1	4	1	2	7	3	3	3	6	4	2	4	4	3	3	5	5	8	4	2	3	L2_Beijing	medium
	LUNG	2	2	5	1	4	1	2	7	3	3	3	6	4	2	4	4	3	3	5	5	8	4	2	3	L2_Beijing	medium
20	PLASMA	2	2	6	1	2	2	2	5	1	4	1	3	4	4	2	1	2	2	1	3	6	4	1	4	L4_LAM	high
	BRAIN	2	2	6	1	2	2	2	5	1	4	1	3	2	4	2	1	2	2	1	3	6	4	1	4	L4_LAM	medium
	CSF	2	2	6	1	2	2	2	5	1	4	1	3	4	4	2	1	2	2	1	3	6	4	1	4	L4_LAM	medium
	LUNG	2	2	6	1	2	2	2	5	1	4	1	3	4	4	2	1	2	2	1	3	6	4	1	4	L4_LAM	high
21	BRAIN	2	2	6	1	3	2	2	4	3	3	3	3	2	4	3	2	3	2	3	2	5	4	2	1	L4	medium
	LUNG	2	2	6	1	3	2	2	4	3	3	3	3	2	4	3	2	3	2	3	2	5	4	2	1	L4	medium
22	SPLEEN	2	2	6	1	3	2	2	5	3	4	3	3	2	4	1	2	1	2	4	4	5	4	2	1	L4_LAM	medium
	LIVER	2	2	6	1	3	2	2	5	3	4	3	3	2	4	1	2	1	2	4	4	5	4	2	1	L4_LAM	medium
	LUNG	2	2	6	1	3	2	2	5	3	4	3	3	2	4	1	2	1	2	4	4	5	4	2	1	L4_LAM	medium
	PLASMA	2	2	6	1	3	2	2	5	3	4	3	3	2	4	1	2	1	2	4	4	5	4	2	1	L4_LAM	medium
23	BRAIN	2	1	6	2	4	3	5	2	3	3	3	5	2	4	10	2	6	1	4	6	7	3	5	3	L1_EAI	medium
	CSF	2	1	6	2	3	3	5	2	3	3	3	5	2	4	10	2	6	1	5	6	7	3	5	3	L1_EAI	medium
	LUNG	2	1	6	2	3	3	5	2	3	3	3	5	2	4	10	2	6	1	5	6	7	3	5	3	L1_EAI	high
	SPLEEN	2	1	6	2	3	3	5	2	3	3	3	5	2	4	10	2	6	1	5	6	7	3	5	3	L1_EAI	high
	LIVER	2	1	6	2	3	3	5	2	3	3	3	5	2	4	10	2	6	1	5	6	7	3	5	3	L1_EAI	medium
24	LUNG	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1	4	3	-	3	-	5	3	3	5	L4	medium
25	LUNG	2	1	5	1	3	1	2	6	1	4	1	2	3	2	3	4	2	4	3	2	5	4	3	2	L4_H3	medium
26	SPLEEN	2	2	6	2	3	1	5	2	3	4	3	5	2	2	1	2	1	1	3	11	7	3	3	3	L1_EAI1	medium
27	LUNG	2	2	5	1	3	3	2	7	3	3	2	5	4	5	4	4	4	2	5	5	8	4	2	3	L2_Beijing	medium
28	LUNG	2	2	4	2	3	1	5	2	3	4	3	5	2	2	9	2	2	1	3	3	4	3	4	4	L1_EAI1	medium

Grey boxes: Locus with allelic differences.

strains from different lineages, and a high proportion of cases showed clonal diversity.

Resistance to anti-TB drugs was found in around 10% of the cases, which is in agreement with previous studies [12,13]. Among the four cases with RIF resistance, two were multidrug resistant (MDR) while the remaining two were most likely RIF monoresistant (RMR). Overinterpretation of RMR as MDR based on Xpert results has been reported elsewhere [14], which is worrying when RMR rates are increasing [15].

In summary, MTB genotyping from post-mortem tissue samples by MIRU-VNTR is successful with moderate/high bacterial loads, which may facilitate MTB surveillance and epidemiological studies in low-income countries in which performing cultures may be challenging. Our analysis of within-host diversity yielded a high percentage of cases with non-homogeneous infections, including mixed infections and polyclonal infections. In this study the combination of a high-TB-burden setting, long-term infections, exhaustive respiratory and extra-respiratory sampling, and direct pre-culture genotypic analysis of specimens most likely maximized the detection of diversity. Most within-host diversity in our study would have been overlooked if only standard analysis of respiratory specimens had been performed.

Ethical approval

The study received the approval of the Clinical Research Ethics Committee of the Hospital Clinic of Barcelona (File 2013/8677) and the National Bioethics Committee of Mozambique (Mozambique; approved, Ref. 342/CNBS/13).

Author contributions

Conceptualization: DGV and MJM. Methodology: DGV, MJM and CRG. Formal analysis: DGV, MJM and CRG. Investigation: CRG, JCH, SRM, IC, PC, MN, NR, AGB, CC, FF, LL, DJ, MRI, CL, AC, IM, QB, CM and JO. Resources: DGV, MJM and PM. Writing original draft: DGV and CRG. Writing—review and edit: DGV, CRG, MJM and PM. Supervision: DGV and MJM.

Transparency declaration

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