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Analysis of potential household transmission events of tuberculosis in the city of Belem, Brazil

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ABSTRACT

Tuberculosis (TB) is an infectious disease with a higher risk for infection and disease among household contacts (HHC). Here, we report a molecular epidemiology-based approach to study disease transmission and the genetic characteristics of *Mycobacterium tuberculosis* (Mtb) strains among HHC in the city of Belem, the capital of the state of Para in north Brazil. The study included 63 TB patients belonging to 26 HHC groups (HHC1 to HHC26). Spoligotyping and 24-loci Mycobacterial Interspersed Repetitive Unit - Variable Number of Tandem Repeat (MIRU-VNTR) revealed indistinguishable bacterial genotypes among 26 patients in 14 (53.8%) HHC groups. Drug susceptibility testing (DST) revealed that 45 (71.4%) of the Mtb isolates were multidrug resistant. The major cluster composed of isolates from five HHCs and on three of these, whole genome sequencing (WGS) was performed confirming their high genetic similarity. These results pinpoint the need for improved vigilance for TB control in households in the city of Belém. When comparing WGS *versus* phenotypic resistance detection methods as DST and Minimum Inhibitory Concentration (MIC) our data suggest that depending on the colonies selection, results may present variation.

Tuberculosis (TB) transmission among household contacts (HHC) is well documented and related with increased risk of infection due to higher proximity and exposure duration to a source of infection [1]. However, in areas where TB is endemic, like in Brazil, the overall contribution of disease transmission to HHC is not well documented. Besides that, a small percentage of families with microepidemics generates a large number of secondary cases who are diagnosed during the screening of TB contacts [2].

Thus, studies about microepidemics of TB are significant particularly involving drug resistant (DR) and multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* (Mtb). It has been postulated that such DR strains suffer from a fitness cost and are therefore less

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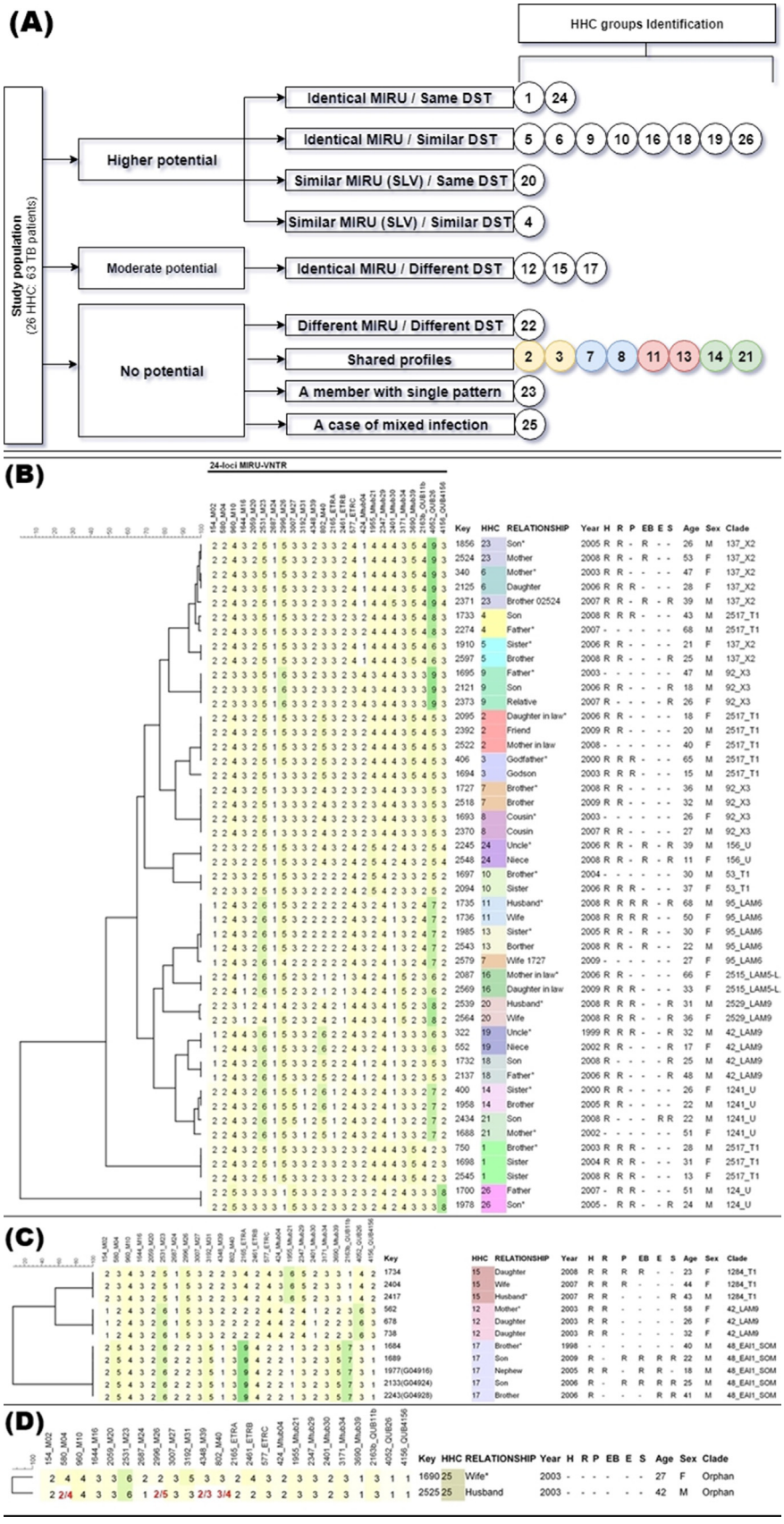

Fig. 1. Potential transmission of household contacts based on MIRU-VNTR clustering associated to drug susceptibility test and and epidemiological information. A) Tree mean classification including the 26 household contacts (HHC). The highlighted HCCs (2 and 3, 7 and 8, 11 and 13) are paired by MIRU-VNTR. B) Higher Potential and No Potential clustering groups. C) Moderate Potential group. D) Mixed infection case.

transmissible than drug susceptible strains. However recent evidence shows that at least some MDR strains are fully transmissible, which is partly due to the presence of compensatory mutations that restore the virulence of DR strains when compared to their susceptible ancestors [3].

DR and MDR TB complicates disease control because these patients do not respond to conventional treatment schemes and therefore continue transmitting Mtb for longer periods and to a larger number of contacts.

Molecular typing of Mtb isolates has added to the understanding of the epidemiology of TB and opened new directions for studying transmission dynamics in conjunction with classical epidemiological approaches. Currently, 24 loci Mycobacterial Interspersed Repetitive Unit - Variable Number of Tandem Repeat (MIRU-VNTR) typing is the reference technique for Mtb genotyping. In addition, Whole Genome Sequencing (WGS) is becoming financially more affordable [4] and in countries where the TB rates are lower than that of Brazil (41.5/100.000) such as the United Kingdom (6.6/100.000) and Italy (3.5/100.000) [5] and shown to be more accurate to study TB transmission and implemented for routine diagnosis [6,7].

To better characterize the transmission dynamics among HHC resident of the state of Pará, Brazil, we performed a molecular epidemiological investigation among 63 HHC representing 6.4% of a collection of 980 Mt b isolates, of which the population structure has recently been described by spoligotyping at Instituto Evandro Chagas [8]. For this, we performed 24-loci MIRU-VNTR typing of the Mtb isolates. We identified 26 HHC groups (HHC1 to HHC26) defined by a specific index case, by convention assumed to be the first TB case diagnosed and HHC being an individual that resided in the household for at least three months prior to the diagnosis of TB in the index case. Culturing, drug susceptibility testing (DST) and spoligotyping were performed as described earlier [8] and 24-loci MIRU-VNTR was performed as described by Supply et al. [9]. To determine genotyping lineage, it was used the SITVIT2 and MIRU-VNTRplus databases. In addition, we performed WGS of three samples (1977, 2133 and 2243) the biggest cluster by preparing sequence libraries using the Nextera XT DNA Library Preparation Kit (Illumina, California, USA) using an Illumina Hiseq2500 sequencer. The mean read depth was $> 20\times$. Read trimming, H37Rv (NC000962_3) reference-based assembly, Single Nucleotide Polymorphism (SNP) variant calling and clustering analysis were performed using the software Bionumerics 7.0 (Applied Maths, Sint-Martens-Latem, Belgium). For detection BioProject of mutations related to DR we used TB-Profiler, as well an active search in all genes related to compensatory mutations. Sequencing reads have been submitted to the National Center of Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the study accession number (BioProject) PRJNA494931.

Among the 63 patients in our study, 34 (54%) were males, with a median age of 30.0 years and ranging from 11 to 68 years (IQR 17.0). DST revealed that 45 isolates (71.4%) were MDR, eight (12.6%) exhibited other patterns of resistance and 10 (15.8%) were fully susceptible to the drugs tested, including isoniazid (INH), rifampicin (RIF), ethambutol (EMB), streptomycin (STM) and ethionamide (ETH). The Evandro Chagas Institute is a reference in northern Brazil for TB diagnostic by performing smear microscopy, culture and DST supporting the Laboratório Central (LACEN) of Pará and the reference hospital for MDR-TB Hospital Universitário João de Barros Barreto (HUJBB). We observed an unexpected high proportion of DR and MDR among HHC contacts, however, this is probably related to the fact that these TB cases were from HUJBB, patients suspected of treatment failure, disease relapse or TB contact. For contacts without a previous history of TB prophylaxis is a treatment with INH for at least six months.

Based on 24- loci MIRU-VNTR and DST, we observed tree scenarios among the 26 HHC groups regarding the potential household TB transmission: 14 (53.8%) HHC with a Higher potential (26 patients), tree (11.5%) as a Moderate Potential and nine (34.6%) with no potential (Fig. 1-A). We assumed as Single-locus variations (SLV) clusters harboring until two loci variation. Important details (Fig. 1-B) about the 26 HHCs are: (i) 12 with identical patterns are not shared in others (HHC1, HHC5, HHC6, HHC9, HHC10, HHC14, HHC16, HHC17, HHC18, HHC19, HHC24 and HCC26); (ii) four with identical patterns shared in others (HHC7/HHC8; HHC11/HHC13); and (iii) a member of HHC7 and HHC24 do not cluster with any other group which means community TB acquisition; (iv) four with SLV shared in others (HHC2/HHC3 and HHC6/HHC23) (Fig. 1-B); (v) one with different MIRU and DST suggesting different strains therefore no TB household transmission (HHC22); (vi) tree with similar MIRU and similar DST (HHC4, HHC20 and HHC21); (vii) tree with identical pattern and many differences among DST profile (HHC12, HHC15 and HHC17). For those that we have observed identical genotypes between Mtb isolated from patients belonging to different HHC we could not confirm TB transmission through but this is highly analysis of the epidemiologic data, but this is highly suggestive for transmission of DR and MDR strains between different HHC groups. This is strengthened by identical SLV on MIRU-VNTR by spoligotypes-based genotypes and coherent DST results. Moreover, those Mtb strains with SLV are associated to microevolution and it is highly unlikely that they are independent infections involving highly similar strains. A slightly different DST heterogenicity observed within the Higher Potential group is coherent to time and the concept of drug-evolution in each patient once the therapeutic history may not be the same for all of them or because from infection, the evolution of the disease may be different among individuals (developing the disease as soon as get infected or later when there is a deficiency in the immune system).

Patients and HHC with Mtb isolates showing identical MIRU patterns ,we tried to interview for better understanding of the reason of their link but without success because the hospitals and neighborhood were different. A recent study conducted from an outbreak of 25 TB cases in United Kingdom showed that although MIRU-VNTR was vital in identifying linked cases, a deep epidemiological investigation was essential for understanding disease transmission among TB contacts. Among the shared places were barbers' shop and football club, for example. The study result was informed to the public health action and has facilitated screening to prevent ongoing transmission [10].

The group Moderate Potential harbors three HHCs, including the bigger cluster HHC17, shows different DST results among strains (Fig. 1-C). It is because each HCC group has more member within their household with TB who were not captured in the present study. The TB treatment is not mandatory by law and the its adherence is not well adopted.

In the group of "No Potential" includes those isolates that are part of clusters that include isolates from patients from the general population. Among those is a sample from the single HIV positive patient (02525) belonging to HHC25 that carried a drug susceptible isolate and presented an orphan spoligotype pattern; however, the MIRU-VNTR pattern presented alleles differences in copy number among four of the VNTRs, characteristic for a mixed infection with at least two different Mtb strains (Fig. 1-D). This HHC includes a woman diagnosed earlier and her husband who was diagnosed during the TB contact screening. Interestingly the media of C content was identical in and an orphan pattern by spoligotyping with some similarity by MIRU-VNTR. A study based on WGS suggested that complex sub-populations of Mtb might coexist within patient and contribute with disparate responses to antibiotic treatment [11]. To clarify this better, we pretend to isolate separate colonies from these isolates and perform DST, MIRU-VNTR and WGS.

According to the SITVIT2 and the MIRU-VNTR plus database, spoligotyping profiles belong to five known lineages: Latin-American-Mediterranean (LAM); T; X; East-African-Indian (EAI); Haarlem, four to the undesignated group (“Unknown” - U) and two showing “orphan” patterns.

As this biggest cluster deserves a more specific analysis, so far three

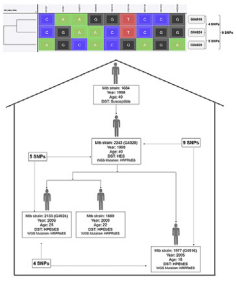

Fig. 2. Single Nucleotide Polymorphism network acquisition among three members of household contact (HCC) 17. Among the five members of HHC17, whole genome sequencing was performed for three of them.

of these isolates: 1977 (G04916), 2133 (G04924) and 2243 (G04928), belonging to rare a lineage in South-America (EAI), were submitted to WGS, which contributed to evaluate the accuracy of genotyping to study transmission among HHC in the present setting.

When performing wgSNP analysis, variants selected (manually inspected) had a minimum read depth of 70. The genome coverage was 172, 137 and 203, respectively; in addition, GC content was 62.56%. The maximum number of SNPs was nine, observed between G04916 and G04928 (Fig. 2). Literature data on the use of WGS for epidemiological studies indicate that most patients in direct transmission chain have Mtb isolates presenting five or less SNPs, mostly of them making part of an TB outbreak [10,12–15], which is not the case of this present study and confirming that each setting might be different. Other recent studies on use of WGS for evaluating TB transmission considered isolates with as much as 12 or 14 SNPs difference (13) as belonging to a recent transmission chain also in non-endemic areas. Our study adds to the available data to evaluate the cut-off of SNP difference for definition of recent transmission and confirms that 24-MIRU-VNTR typing can be used in the present endemic setting.

Regarding to relation between conventional DR results and those of WGS, identical patterns were observed for all genomes indicating an INH, RIF, PZA, EMB, ETH resistance-mutation. Unlike the others, the isolate G04916 did not present a mutation associated to STM, that should be there according to DST result. For these tree cases we observed that WGS agreed more with MIRU-VNTR and SNPs than the DST results for genomes G04924 (RIF) and G04928 (RIF and PZA) (Table 1). For this we performed the Minimum Inhibitory Concentration (MIC) assay using Resazurin Microtiter Assay method (REMA) recorded as the lowest antibiotic concentration that reduced visible growth [16,17]. We observed that the Mtb strains were strongly resistant (MIC $\geq 2\,\mu g/mL$) to INH in agreement with both DST and WGS; regarding the strains G04924 (2133) and G04928 (2243) that were susceptible to RIF by DST but presenting a rpoB mutation, they were considered resistant (MIC $\geq 2.5\,\mu g/mL$). However, the strain G04916 (1977) that was resistant to RIF, both by WGS and DST (even with a low percentage of 5% compared to others with 100%), was considered susceptible by MIC ($> 0.25\,\mu g/mL$). It seems that there is a certain percentage of resistant colonies and depending on the selection, as well as the phenotypic DST test, results may present variation, meaning that high discrepancy rate of one's chosen method will have direct clinical consequences [17,18]. The number of acid-fast bacilli (AFB) observed at 1000× magnification was 3+ (> 10 bacilli/20 fields). Unfortunately, it was not possible to perform MIC to PZA. The network of the chronology of acquisition of SNPsis presented in Fig. 2. The ideal scenario would be to have all Mtb genomes of the present study sequenced, being therefore a limitation. In addition, there is a very strong sampling the bias about all isolates are from patients that have been attended at the MDR hospital reference. Nevertheless, based on these results we hope to be able to propose additional studies on a long term focusing on WGS also including an external control group of non-household contacts known.

As yet, the only study in southeast of Brazil focusing on microepidemics of TB is based on IS*6110*-RFLP based genotyping and demonstrated that no particular genotype of Mtb was associated with disease transmission to HHC [19]. Therefore, our findings could be representative for similar situations in other major cities in Brazil and pinpoint the need of a stronger vigilance among HHC on a country level. This study suggests that the detection of already known mutations should be investigated to improve the diagnosis together with DST.

Table 1
Description of drug susceptibility test result and resistance mutation detection based on whole genome sequencing for tree isolates.

		Genomes Identification		
		G04916 (1977)	G04924 (2133)	G04928 (2243)
INH[a]	DST[g]	R (100%)[i]	R (100%)[i]	R (100%)[i]
	WGS[h]	inhA (I194T)/katG (S315T)	inhA (I194T)/katG (S315T)	inhA (I194T)/katG (S315T)
RIF[b]	DST	R (5%)[j]	S[i]	S[i]
	WGS	rpoB (L430P/ G490H*/L511P*)	rpoB (L430P/ G490H*/L511P*)	rpoB (L430P/ G490H*/L511P*)
PZA[c]	DST	S	R (100%)	S
	WGS	pncA (P54Q)[k]	pncA (P54Q) [k]	pncA (P54Q)[k]
EMB[d]	DST	R (100%)	R (100%)	S
	WGS	embB (M306V)	embB (M306V)	embB (M306V)
ETH[e]	DST	R (100%)	R (100%)	R (50%)
	WGS	embB (M306V)	embB (M306V)	embB (M306V)
STM[f]	DST	S	R (100%)	R (50%)
	WGS	S	rpsL (K43R)	rpsL (K43R)
YEAR		2005	2006	2006

[a] INH (isoniazid).
[b] RIF (rifampicin).
[c] PZA (pyrazinamide).
[d] EMB (ethambutol).
[e] ETH (ethionamide).
[f] STM (streptomycin).
[g] DST (drug susceptibility test based on Proportion Method).
[h] WGS (Whole Genome Sequencing); R (resistant); S (susceptible).
[i] Resistance detected by MIC $\geq 2\,\mu g/mL$.
[j] According to MIC it was susceptible ($> 0.25\,\mu g/mL$).
[k] Mutations not detected by TB-Profiler.

Authors' contribution

Conceived and designed the experiments: ECC, KVBL, HMG and PNS. Performed the experiments: ECC, MLP, AESG and YCR. Provided additional epidemiological information: NCC, WAB. Provided the reagents and performed the experiment for whole genome sequencing: LKR, SG. Analyzed the data: ECC, KVBL, IPF, PNS and SG. Genomes analysis: ECC, MLC, SG, AESG, AS and PNS. MIC assay: KVBL, LNGCL and CF. Wrote the paper: ECC, PNS, KVBL, RSD and SG. Potential conflicts of interest: none reported.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2018.09.011.

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