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Research Paper

Characterization of *Mycobacterium tuberculosis* var. *africanum* isolated from a patient with pulmonary tuberculosis in Brazil

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ABSTRACT

Human tuberculosis (TB) is caused by members of the *Mycobacterium tuberculosis* complex (MTBC), including *Mycobacterium tuberculosis* var. *tuberculosis* (MTB) and *Mycobacterium tuberculosis* var. *africanum* (MAF). While MTB is isolated worldwide, MAF is almost completely restricted to the African continent, and despite the historical proximity between Brazil and Africa during the slave trade, no case of TB being caused by MAF has been reported in Brazil to date. We hereby describe the first case of TB caused by MAF in Brazil comparing its genome against the published ones. A female patient who had never visited Africa presented with clinical symptoms typical of pulmonary TB. Based on 16S rRNA gene sequencing, the cultured isolate was identified as belonging to MTBC and partial sequence of the *hsp*65 gene was identical to that of MAF. This was confirmed by genotyping based on detection of Single Nucleotide Polymorphism (SNP), Region of Difference (RD) and spoligotyping. The isolate presented the Shared International Typing (SIT) 181. In the whole-genome comparison against MAF genomes available on published EMBL-EBI European Nucleotide Archive (ENA), the Brazilian genome (MAFBRA00707) was identified as belonging to Lineage 6 and clustered with isolates from The Gambia. This is

Abbreviations: AFB, (acid-fast bacilli); ATS, (American Thoracic Society); EMB, (ethambutol); CTAB, (Cetyl trimethylammonium bromide); DST, (drug susceptibility testing),INH (isoniazid); LJ, (Lowenstein-Jensen); MAF, (*Mycobacterium tuberculosis* var. *africanum*); MTB, (*Mycobacterium tuberculosis* var. *tuberculosis*); MTBC, (*Mycobacterium tuberculosis* complex); RD, (Region of Difference); RPM, (rifampicin); SIT, (Shared International Type); SNP, (Single Nucleotide Polymorphism); STR, (streptomycin); TB, (tuberculosis); WGS, (Whole Genome Sequencing)

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the first report of the isolation of MAF from a patient from Brazil, without evidence of having any contact with an African index case.

1. Introduction

Mycobacterium tuberculosis var africanum (MAF) was first isolated from a patient with pulmonary tuberculosis (TB) in 1968 in Dakar, Senegal, and described as a specie *Mycobacterium africanum* of the classic taxonomic definition, *Mycobacterium tuberculosis complex* (MTBC) (Castets et al., 1968). It presents morphologic and genetic characteristics of either or both *Mycobacterium tuberculosis var tuberculosis* (MTB) and *Mycobacterium tuberculosis* var. *bovis*. Based on these similarities and on geographical origin, MAF was divided in two main subgroups: MAF subtype I (similar to *M. tuberculosis* var. *bovis* and found mostly in West Africa) and MAF subtype II (similar to MTB and found mostly in East Africa) (DAVID et al., 1978). This division however was not entirely reliable, since studies in Guinea-Bissau identified not only these two subtypes, but a genotypic spectrum of such organisms so the MAF subtype II was reclassified as being MTB and indicated as the Uganda genotype (Brosch et al., 2002; Niemann et al., 2002; Yeboah-Manu et al., 2017).

Subtype I isolates were redefined recently into West African I (MAF1), prevalent near the Gulf of Guinea, and West African II (MAF2), prevalent in the extreme western part of Africa (Gagneux et al., 2006). Genome and SNP analysis of MTBC isolates reveals the existence of seven main lineages within the MTBC, with MAF1 belonging to lineage 5 (MAF-L5) and MAF2 belonging to lineage 6 (MAF-L6) (Comas et al., 2010).

MAF-L6 predominantly affects elderly, HIV infected and/or severely malnourished individuals (de Jong et al., 2005) and is overrepresented in extrapulmonary TB (Sharma et al., 2016). The determination of its incidence and prevalence is often complicated by the difficulty of diagnosis based on clinical symptoms and bacterial phenotyping (Prat et al., 1974). Etiologic agent of up to half of the cases of human TB in West Africa, the prevalence of MAF varies across the continent, accounting for approximately 21% of the overall TB cases but as high as 39% of TB in The Gambia (de Jong et al., 2010a, 2010b). However, outside the African continent, TB caused by this species has only sporadically been reported and if so, mostly in immigrants from West Africa (Sharma et al., 2016; Yeboah-Manu et al., 2017).

Pulmonary TB caused by MAF presents the same clinical symptoms as those caused by MTB, is quite similar in relation with the response to the tuberculin test, the incidence rate among those with BCG scar and the mortality rate during treatment among HIV negative patients. However, while transmission was reported a decade ago to be the same between both subspecies of the MTBC, progression to disease seems somewhat slower after infection with MAF (de Jong et al., 2008). In concordance, the growth rate of MAF-L6 from The Gambia is somewhat slower than that of MTB and this might be due to a frame shift mutation in genes that have been associated with growth attenuation (Gehre et al., 2013). An increased adaptation to a more persistent, anaerobic lifestyle may also explain the fact that patients infected with MAF-L6 progress to disease significantly slower when compared to MTB (de Jong et al., 2008). In addition, it seems that isolates of MAF-L6 are somewhat attenuated in their ability to cause disease in immunocompetent hosts and often correlated with HIV infection (de Jong et al., 2005). Recent data suggest that prevalence of TB caused by MAF is slowly being overwhelmed by infection with MTB (Winglee et al., 2016) and presents lower transmission rates (Asare et al., 2018).

Because the species presents a spectrum of phenotypes that range between that of MTB and *M tuberculosis* var. *bovis*, the latter showing a preference of pyruvate enriched Lowenstein-Jensen (LJ) medium, some isolates of MAF can be missed because of culture bias when compared to growth of MTB (Keating et al., 2005). This diversity of phenotypic characteristics also complicates the reliable identification, which is best obtained by genotyping, once the differentiation of the MTBC is mostly obtained by spoligotyping (de Jong et al., 2010a, 2010b), with additional genetic markers (Vasconcellos et al., 2010). We here describe the first case of TB caused by MAF in Brazil using a thorough genotypic analysis of the isolate.

2. Material and methods

2.1. Patient

A 33-year-old Brazilian woman, African-America, HIV negative, married, teacher, native and resident of Marabá, Pará, northern Brazil, was diagnosed as having pulmonary TB at the Department of Physiology of the “Hospital das Clínicas” of the Federal University of Goiás in Goiania, Brazil.

2.2. Culture and drug susceptibility testing

Specimens were decontaminated using *N*-acetyl-l-cysteine-sodium hydroxide (NALC-NaOH) and cultured in Lowenstein Jensen (LJ) medium. The isolate was sent to the National Reference Center Professor Hélio Fraga, Rio de Janeiro, Brazil for conventional identification to the MTBC level as described by the American Thoracic Society (ATS) and for evaluation of DST to STR (1.0 μg/Ml), INH (0.1 μg/mL), RPM (1.0 μg/mL) and EMB (5.0 μg/mL), was carried out on BD BACTEC™ MGIT™ 960 SIRE Kit (BD Life Sciences, Franklin Lakes, US).

2.3. Nucleic acid extraction

In October of 2014, the isolate was sent to the Laboratory of Molecular Biology Applied to Mycobacteria of the Instituto Oswaldo Cruz (IOC) in Rio de Janeiro for DNA extraction using the cetyl-trimethylammonium-bromide (CTAB) method (Van Soolingen et al., 1991).

2.4. Sanger sequencing

Sequencing of 16S was performed at the Institute of Tropical Medicine in Belgium using nested PCR with primer pairs P1 and P2, P7 and P16 (Portaels et al., 1996). Sequencing of part of *hsp*65 gene was performed at Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, Brazil, as described (Eyer-Silva et al., 2019).

2.5. Spoligotyping

Spoligotyping was performed using a nylon membrane at Institute of Tropical Medicine (Kamerbeek et al., 1997) and using microbeads in Luminex® MAGPIX® system (Thermo Fisher Scientific, Massachusetts, United States) at Fiocruz (Kiréopori Gomgnimbou et al., 2013). The patterns were entered as binary code and analysed using online database (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/) to obtain the SIT number (Couvin and Rastogi, 2014).

2.6. Whole genome sequencing

The DNA extract from the isolate was submitted to whole genome sequencing (WGS) using an Illumina NextSeq High Output chemistry with 2x150bp paired-end reads and the Nextera XT library preparation kit (Illumina, San Diego, CA) at Fundación para el Fomento de la Investigación Saniaria y Biomédica (Fisabio), Valencia, Spain.

2.7. Bioinformatic analysis

For comparison with available MAF genomes, we downloaded 221 genomes, available at the EMBL-EBI European Nucleotide Archive (ENA) originally from: Ethiopia, France, The Gambia, Sierra Leone and Ghana. We also included the MAF genome from Colombia (UT307) (Hurtado et al., 2016). Additionally, we added genomes from L1, L2, L3, L4, L5, L6 and L7 as references (Supplementary Table 1).

Reads were trimmed using Trimmomatic 0.36 (Bolger et al., 2014) and the reads were filtered against the reference genome of MTB H37Rv (RefSeq accession: NC_000962; GenBank: AL123456.3) using the Burrows Wheeler Aligner tool (BWA-MEM algorithm) (Li and Durbin, 2009). Snippy (https://github.com/tseemann/snippy) was used for variant calling from the trimmed reads. The positions associated with PE/PPE genes and the positions with a unique K-mer length below 49/50 to generate a core SNP alignment were removed. Only concordant variants were retained for downstream analysis. The pairwise SNP distance between isolates was evaluated using snp-dists (https://github.com/tseemann/snp-dists). To evaluate the model of nucleotide substitution that best fits our data we used jModelTest2. The phylogenetic tree was constructed by using the Maximum Likelihood (ML) method and General Time Reversible (GTR) model (Nei and Kumar, 2000) on MEGA X (Kumar et al., 2018). The tree with the highest log likelihood (−242,477.74) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree (s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Codon positions included were 1st + 2nd + 3rd + Noncoding. There was a total of 37,795 positions in the final dataset. The tree was annotated and rooted using the Interactive Tree of Life v5.3 (iTOL) online tool (Letunic and Bork, 2019). As an outgroup we have used the *Mycobacterium canetti* genome (SAMEA3905803).

For MAFBRA00707 we have performed a *de novo* assembly using Spades 3.12.0 assembler (Bankevich et al., 2012), annotated the genome using Prokka (Seemann, 2014) and used TB-Profiler TB-Profiler v2.8.12 to identify variations associated with drug resistance (Coll et al., 2015). To identify the genome lineage and *in silico* spoligopattern for all genomes we have used Spotyping (Xia et al., 2016), RD-analyzer (Faksri et al., 2016) and Kvarq (Steiner et al., 2014). Sequencing reads have been submitted to the ENA Sequence Read Archive (SRA) under the study accession number SRR8952882 (Bioproject PRJNA534674). All strains included and their accession numbers are listed in Supplementary Table 1.

3. Results

The patient was examined after presenting persistent cough without expectoration for over a year and with no relation to any particular period of the day. During that period, she had lost five kilograms of body weight but denied the presence of haemoptysis, fever, and dyspnea. The patient denied habits such as smoking, alcoholism and other comorbidities or previous surgeries.

She reported having contact with two individuals with pulmonary symptoms at the working environment but was not aware of their final diagnosis. She had received medication against cough and antibiotics such as amoxicillin, azithromycin, trimethoprim and sulfamethoxazole.

Upon physical examination, she presented low body weight, paleness, with blood pressure of 100/60 mmHg and a regular heart rate of 80 beats per minute. Also, she presented audible vesicular murmur in the upper half of the right hemithorax with scattered rales and 98% oxygen saturation.

We therefore examined the presence of acid-fast bacilli in the sputum and in bronchoalveolar lavage after bronchoscopy, both with negative results. Computed Tomography (CT) of the thorax revealed the presence of centrilobular micronodules with a budding tree arrangement in both upper lungs, tending to confluence. The right lobe presented a frosted glass aspect with a rounded cavity surrounded by reactional lesions (Fig. 1a).

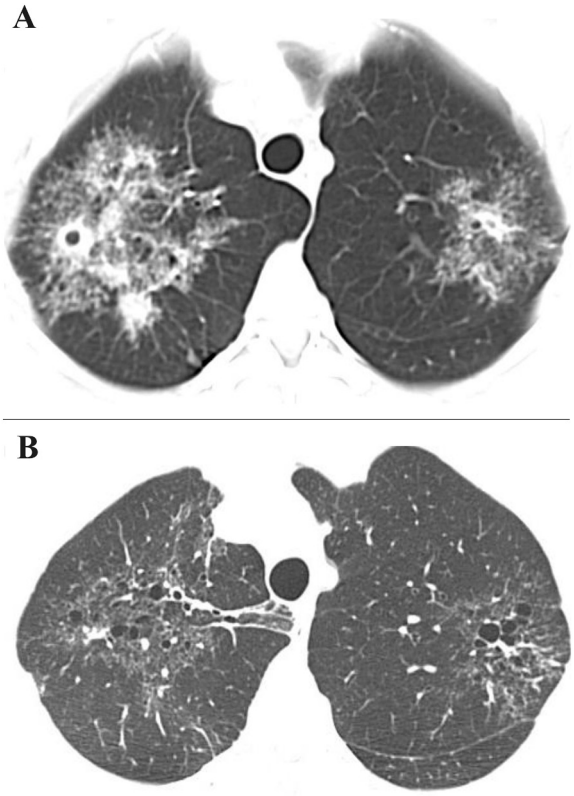

Fig. 1. A. Axial image of tomography of the chest in pulmonary window, at the level of the upper lobes, showing centrilobular micronodules with arrangement of “budding tree” (white arrow) with tendency to confluence forming larger nodules (black arrow), in addition to frosted glass of Small digging in the apical segment of the right upper lobe (arrowhead). B. Axial image of tomography of the chest in pulmonary window, at the level of upper lobes, in tomographic control after about 6 months, where residual/cicatricial alterations are observed, with traction bronchiectasis (white arrow), some cystic images (arrow Black) and parenchymal bands (arrowhead).

One year later, persistent symptoms prompted us to perform a new CT scan where residual lesions were observed, together with bronchiectasis due to scars and small cysts in the parenchymal tissue (Fig. 1b). A new bronchoscopy with bronchoalveolar lavage was performed and this time, the presence of acid-fast bacilli (AFB) was detected, and upon culturing on LJ medium bacterial growth was observed. Conventional identification and drug susceptibility testing (DST) were performed and the isolate was characterized as belonging to the MTBC because of inhibition by nitrobenzoic acid (PNB), MTB was excluded because of susceptibility to thiophene-2-carboxylic acid hydrazide (TCH) and presenting lack of niacin accumulation. The isolate tested susceptible to rifampicin (RPM), streptomycin (STR) isoniazid (INH) and ethambutol (EMB) and the patient was treated with these antibiotics as recommended for TB. After six months of treatment (RPM, INH and pyrazinamide - PZA), the patient had clinical improvement with cough reduction, increase in body weight and resumption of her usual activities. In January of 2013, a new bronchoscopy and bronchoalveolar lavage was performed and no AFB were observed, and the culture was negative, therefore considering the patient as cured.

16S rRNA gene-based identification of the bacterial culture (GO54224) confirmed that the isolate belonged to the MTBC. Because some strains of the MTBC have intermediate biochemical properties between *M. tuberculosis* var. *bovis* and MTB, partial sequencing of the

*hsp*65 gene demonstrated a SNP (G to C) at position 539 of *hsp*65, characteristic for MAF. Upon spoligotyping, the isolate demonstrated the absence of spacers 7–9 and 39, (SIT 181, octal 770,777,777,777,671) characteristic for MAF6. Additionally, the presence of some phylogenetic markers was verified, differentiating MAF from MTB, *M. tuberculosis var. bovis* and *M. bovis* BCG (TbD1 and RD4 intact while RD9 deleted) and to distinguish MAF-1 and MFA-2 (RD701 deleted and RD711 intact, confirming MAF-2 identity; RD713 failed to amplify) (Vasconcellos et al., 2010).

Paired-end NextSeq sequencing 150 bp yielded a genome (MAFBRA00707) composed of 254 contigs (longest contig composed of 224,766 bp) with N50 of 80,052 bp and average genome coverage of 160×. The total genome length was 4,307,870 bp with a GC content of 65.48% and 3973 coding sequences including 162 PE/PPE regions and containing 45 tRNAs. Compared to the Lineage 4 reference genome of MTB H37Rv, 2241 SNPs, 83 insertions and 75 deletions were observed. *In silico* analysis of the DR region confirmed the spoligotype as SIT 181as well as the MAF specific deletions of RD7, RD8, RD9, RD10 and RD702 and a microdeletion of 6-bp in pks15/1 gene (Mostowy et al., 2004). The only resistance conferring mutation identified was anonsynonymous mutation (A-T) in *emb*C (Ala307Thr).

To understand the possible route of introduction of this isolate in Brazil, we constructed a similarity matrix including genomes from 206 isolates, representing isolates of MAF from the African countries Ghana, The Gambia, Congo and Sierra Leone (Comas et al., 2013; Malm et al., 2017; Nebenzahl-Guimaraes et al., 2016; Otchere et al., 2018) (Supplementary Table 1). We also included the genome of an isolate from Colombia (SAMN04479830_UT307) (Hurtado et al., 2016). The SNP based phylogenetic tree demonstrated that the Brazilian genome (MAFBRA00707) belonged to a clade formed by isolates from The

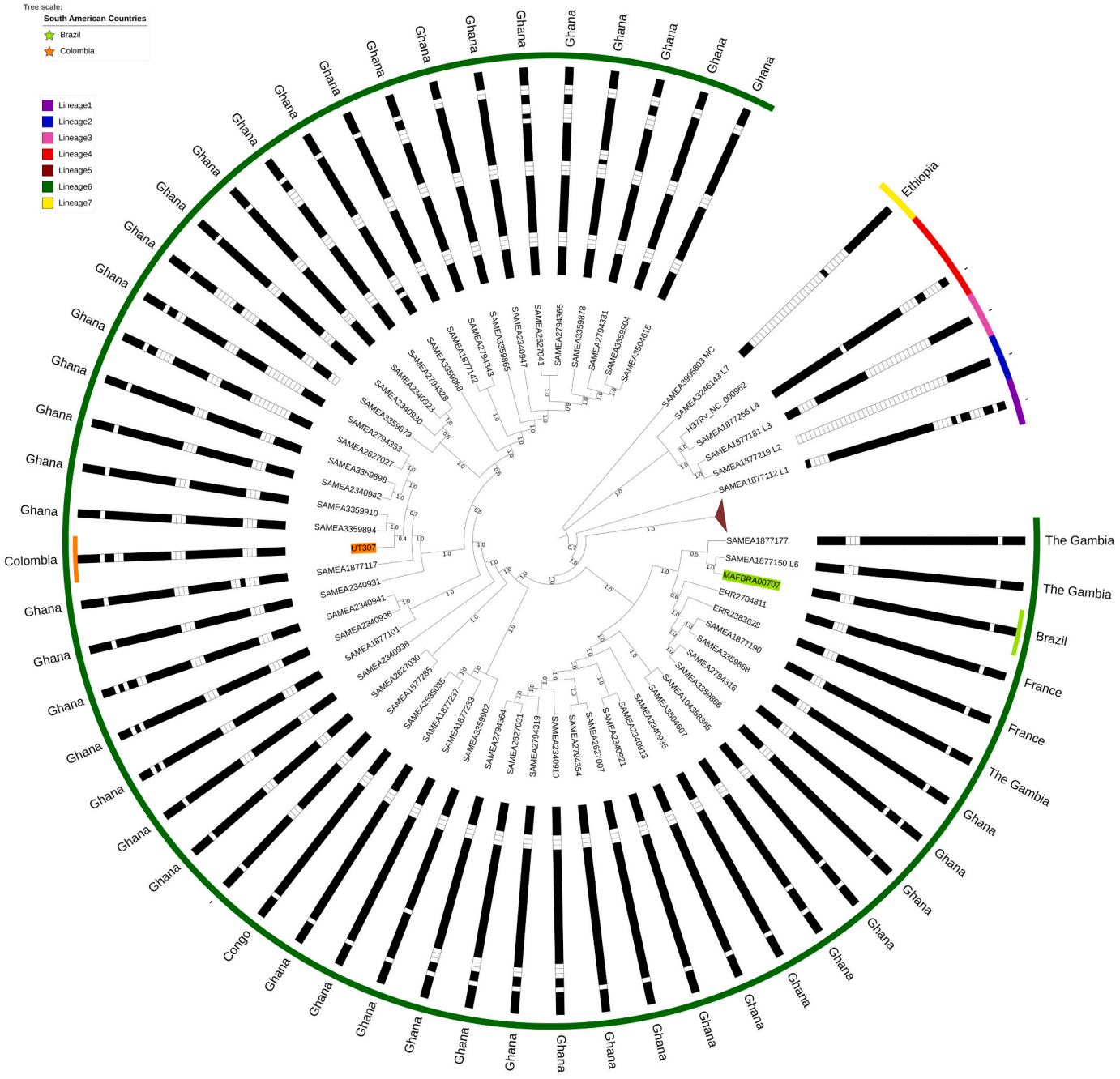

Fig. 2. Maximum Likelihood tree of the *Mycobacterium tuberculosis* var. *africanum* genomes freely available against the Brazilian MAF-L6 genome.

Gambia and closest to genome SAMEA1877150, differing by 226 SNPs (Fig. 2). The isolate from Colombia also was identified as belonging to Lineage 6 but clustered within isolates from Ghana and closest to genome SAMEA3359901, differing by 180 SNPs.

4. Discussion

Mycobacterium tuberculosis var. *africanum* is almost exclusively isolated from TB patients in the Western- and Central regions of the African continent. The restriction to Africa is surprising, given the intense slave trade out of Africa during previous centuries. Hypotheses for this remarkable geographic restriction of MAF include (i) strict adaptation of MAF to West Africans, (ii) higher virulence of MTB causing MAF to be outcompeted in other regions of the world and (iii) existence of an animal reservoir of MAF specifically in West Africa (Otchere et al., 2018).

Case reports of TB caused by MAF outside of Africa exist and refer mostly to isolated cases as observed in England, France, Spain, Germany, Italy, Denmark, California and the USA as reviewed by Yeboah-Manu et al. (Yeboah-Manu et al., 2017) and in Japan (Ueyama et al., 2014), including both pulmonary and extra-pulmonary disease. TB patients carrying MAF in these settings concerned first generation African migrants (Yeboah-Manu et al., 2017). However, in a study conducted in California, USA, two among five patients infected with MAF strains were from Vietnam and the USA respectively; neither of them had any history of travelling to Africa or African contacts with TB (Desmond et al., 2004).

This is the first report of the isolation of MAF in Brazil and the lack of overt transmission link with Africa suggests that this lineage may have been transmitted among Brazilian residents. The fact that genome analysis revealed that this MAF isolate is closely related to strains from The Gambia suggests some undescribed contact between Brazilians and an inhabitant from The Gambia. However, although the genome from the Brazilian MAF-L6 isolate belongs to the clade from The Gambia, it still differs by 226 SNPs (Supplementary Table 2) from the closest genome and this could suggest that this isolate is not part of a recent transmission chain but reflects an older event such as the slave trade from Africa to Brazil.

Special consideration should be given to the patient's residence which is the state of Pará, North Brazil: this region has been part of a particular slave trade from East Africa, as documented also by the almost exclusive presence of MTB Lineage 1 in that particular region of Brazil, which is also rare in other regions of South America (Conceição et al., 2017).

Data on genome analysis of MAF are quite scarce, including on the reference MAF-L6 strain (Bentley et al., 2012), a small number of MAF-L6 genomes (Gehre et al., 2013), on 24 isolates of MAF-L6, two MAF-L5 (Winglee et al., 2016), and on 14 MAF-L5 (Ates et al., 2018). Very recently however, Otchere et al. (Otchere et al., 2018) reported genome analysis of 253 MAF from Ghana, observing considerable genome erosion and more pseudogenes than the genomes of MTB. Besides the present data, the only other genome from an isolate of MAF from Latin America was reported in a male patient from Medellin, Colombia, (Hurtado et al., 2016).

The spoligotypes was characteristic for MAF-L6 but different from the spoligotypes of the Brazilian isolate, the Colombian strain genome clustered with those of patients from Ghana. Although there is a considerable number of SNPs that separates the genome of the Colombian strain from the genome of the closest genome from Ghana, this patient might have some relation with Africans, since Medellin is located in a route of undercover immigrants from Africa in their way to the Caribbean or Central America with North America as final destination.

The clinical symptoms caused by MAF infection are impossible to differentiate from those of lung disease caused by MTB, although the protracted symptoms of our patient may suggest an indolent infection. Together with the growth preference of MAF for pyruvate enriched medium, this may underestimate the real frequency of MAF, as supported by comparing the frequency of isolation of the species directly in sputum and after culture (Sanoussi et al., 2017). While the prevalence of MAF-L6 in Brazil is likely exceedingly low, of note, bacilloscopy analysis and GeneXpert TB do not distinguish between the MTBC members. However, a study in the city of Rio de Janeiro on human TB, that evaluated culturing on pyruvate enriched medium, did not show the presence of MAF in that area (Rocha et al., 2011; Sobral et al., 2011), nor in other regions of Brazil and Latin-America (de Kantor et al., 2008).

The response to treatment of the patient in the present study was satisfactory, and remission was achieved completely after six months of treatment, as recommended for the treatment of TB caused by MTB (Joloba et al., 2001). The SNP observed in *embC* 307 causing Ala307Thr has been previously identified but is not considered a high confidence mutation associated with resistance to ethambutol (Phelan et al., 2019).

Although MAF infections seem to present relative slowness and mildness of disease progression when compared to that of MTB, clinical findings are similar. However, contacts of patients with MAF infection had lower rate of progression to active TB in contrast to patients with MTB (de Jong et al., 2008). Also, a lower virulence of MAF was suggested when observing a slow progressing of the infection in a mouse model with mild lung pathology (Cá et al., 2019). The mildness of disease observed in the present case could however also partly be due to unreported treatment with other antibiotics or genetic background of the patient (Meyer et al., 2008).

Discrimination between infection with MTB and MAF is important because clinical recovery from disease due to infection with MAF is somewhat slower than that of MTB due to the presence of persistent bacilli (Tientcheu et al., 2016a) and patients diseased with modern MTB complex L4 respond faster to TB treatment than those with MAF L6 (Diarra et al., 2018). Also, subtle differences between MTB and MAF infected patients following anti-TB treatment have been reported in clinical features, immune response and expression of proteins associated with inflammatory processes (Tientcheu et al., 2016b).

5. Conclusions

We report the first case of TB caused by MAF-L6 in Brazil in a patient without history of travel to Africa, suggesting that transmission took place locally. The remote relatedness of this isolate to MAF-L6 isolates from Gambia does not exclude its arrival in Brazil at the time of the slave trade, nor any time since then.

Declaration of Competing Interest

The authors have no competing interests that might have influenced any part of this manuscript.

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Appendix A. The following are the supplementary data related to this article

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2020.104550.

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