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First insights into circulating *Mycobacterium tuberculosis* complex lineages and drug resistance in Guinea

Mebrat Ejo a,b,1, Florian Gehre a,c,1,⇑, Mamadou Dian Barry d, Oumou Sow d,e, Nene Mamata Bah d, Mory Camara d, Boubacar Bah e, Cecile Uwizeye a, Elie Nduwamahoro a, Kristina Fisette a, Pim De Rijk a, Corinne Merle f,g, Piero Olliaro g,h, Marcos Burgos i, Christian Lienhardt j,k, Leen Rigouts a,l, Bouke C. de Jong a,c,m

a Institute of Tropical Medicine (ITM), Antwerp, Belgium  
b University of Gondar, Gondar, Ethiopia  
c Medical Research Council (MRC), Fajara, Gambia  
d Reference Laboratory for Mycobacteria, Conakry, Guinea  
e National University Hospital IgnaceDeen, Conakry, Guinea  
f London School of Hygiene and Tropical Medicine, London, UK  
g UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR), Geneva, Switzerland  
h Centre for Tropical Medicine, Nuffield Department of Medicine, University of Oxford, Oxford, UK  
i Division of Infectious Diseases, Department of Internal Medicine, University of New Mexico, Albuquerque, United States  
j Clinical Trial Division, International Union against Tuberculosis and Lung Disease, Paris, France  
k World Health Organization, Geneva, Switzerland  
l University of Antwerp, Antwerp, Belgium  
m New York University (NYU), New York, United States

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In this study we assessed first-line anti-tuberculosis drug resistance and the genotypic distribution of *Mycobacterium tuberculosis* complex (MTBC) isolates that had been collected from consecutive new tuberculosis patients enrolled in two clinical trials conducted in Guinea between 2005 and 2010. Among the total 359 MTBC strains that were analyzed in this study, 22.8% were resistant to at least one of the first line anti-tuberculosis drugs, including 2.5% multidrug resistance and 17.5% isoniazid resistance, with or without other drugs. In addition, further characterization of isolates from a subset of the two trials (n = 184) revealed a total of 80 different spoligotype patterns, 29 “orphan” and 51 shared patterns. We identified the six major MTBC lineages of human relevance, with predominance of the Euro-American lineage. In total, 132 (71.7%) of the strains were genotypically clustered, and further analysis (using the DESTUS model) suggesting significantly faster spread of LAM10_CAM family (*p* = 0.00016). In conclusion, our findings provide a first insight into drug resistance and the population structure of the MTBC in Guinea, with relevance for public health scientists in tuberculosis control programs.

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1. **Introduction**

According to the World Health Organization (WHO), tuberculosis (TB) is among the most wide-spread global public health problems, and its implication for health and economy is increasingly recognized (Lienhardt et al., 2012; WHO, 2014a,b).

*Mycobacterium tuberculosis* and *Mycobacterium africanum* are the major pathogenic species of the *M. tuberculosis* complex (MTBC) in humans and remain one of the leading causes of adult deaths due to a bacterial infection, particularly in sub-Saharan African countries (Corbett et al., 2006; de Jong et al., 2010a,b). As the worldwide emergence of multidrug-resistant strains has become one of the greatest public health concerns (Lienhardt et al., 2012), understanding the genetic backgrounds of circulating drug-resistant strains is crucial, as observed from different studies elsewhere (Gagneux et al., 2006).

Few West African countries have conducted drug-resistance surveys and epidemiological studies on endemic *M. tuberculosis*
isolates (Godreuil et al., 2007; Affolabi et al., 2009; Yeboah-Manu et al., 2011; Traore et al., 2012), and the majority of countries in this region are still lacking such important information (WHO, 2014a,b), and the majority of countries in the study region are still lacking such important information (WHO, 2014a,b), and the majority of countries in the study region are still lacking such important information (WHO, 2014a,b), but no data on genetic diversity or drug-resistance of the prevalent MTBC strains are available up to date. Hence, we took advantage of two clinical trials recently conducted in Guinea to type the collected MTBC isolates and estimate the genetic diversity of the MTBC in the country. Obtaining a first insight into the genotypes of MTBC, we were not only able to estimate the prevalence of drug resistance among new cases in Guinea but also to describe an association of certain mycobacterial families with drug-resistance. 2. Materials and methods

2.1. Patients

This study was carried out on baseline isolates from new cases of pulmonary TB patients in Guinea recruited in 2 different clinical trials in the capital Conakry: the Oflotub Study (registration number = NCT00216385) (Merle et al., 2012, 2014) and the Study C (registration number = NCT00216333) (Lienhardt et al., 2011). A sub-set of isolates had been stored at −80 °C at the Institute of Tropical Medicine (ITM), Belgium. Both studies recruited consecutive adult smear positive patients without prior history of TB treatment (in the Oflotub study, patients with prior treatment more than 3 years ago were also eligible).

2.2. Drug-susceptibility testing (DST)

For both clinical trials, DST was conducted first in the national TB laboratory in Conakry (CHU Ignace Deen). For quality control (QC), in Study C, all isolates were sent to ITM in Antwerp, and in the Oflotub study, any rifampicin resistant isolate and every 6th rifampicin sensitive isolate were sent to Antwerp. In both CHU Ignace Deen and ITM laboratories, phenotypic DST was performed on Löwenstein–Jensen (LJ) slopes using the proportion method (Canetti et al., 1996). Specifically, isolates were inoculated on antibiotic-containing LJ slopes (rifampicin (RMP) 40 μg/ml, isoniazid (INH) 0.2 μg/ml, ethambutol (EMB) 2 μg/ml and streptomycin (SM) 4 μg/ml), incubated at 37 °C, and monitored for growth at 4 and 6 weeks. The concordance between DST results in Conakry and ITM on the subset of isolates tested in both places in the Oflotub study met international standards (Laszlo et al., 2002; WHO, 2009), with 97% each for rifampicin and isoniazid, 100% for ethambutol, and 92% for streptomycin. The complete Conakry results were used for the analysis on drug resistance, to avoid bias in the prevalence estimates.

2.3. DNA extraction and spoligotyping

Of the isolates included in the present study, we identified 184 strains from the two trials; 64 strains from Study C and 120 from Oflotub (Lienhardt et al., 2011; Merle et al., 2012, 2014), available at ITM for genotyping. DNA extraction was done by resuspending a loop-full of the stored isolate in TE buffer and boiling the mixture for 5 min. The obtained boiled lysate was subsequently spoligotyped, as described elsewhere (Kamerbeek et al., 1997). The results were recorded as the presence or absence of spacers using a binary code (Kamerbeek et al., 1997; Dale et al., 2001).

2.4. Genotype database comparison and analysis

The genotyping results were compared to the international spoligotyping (SITVIT) database of the Pasteur Institute of Guadeloupe (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo) (Brudey et al., 2006), and the newly revised publicly available international multimarker database (SITVITWEB) (Demay et al., 2012). A SIT (spoligotype international type) was assigned when two or more patient isolates in the database shared the identical spoligotype-patterns, while spoligotype-patterns that had not been described before were defined as “orphan”. To assign mycobacterial lineages and families to the genotyped isolates, spoligotypes were analyzed using TBLinage (http://tbinsight.cs.rpi.edu/about_tblindage.html) and Spotclust (http://tbinsight.cs.rpi.edu/run_spotclust.html), respectively. Lineages and families were defined according to signatures provided in SpolDB4 and SITVITWEB (Brudey et al., 2006; Demay et al., 2012) and also based on earlier reports (Gagneux and Small, 2007). To detect emerging strains of tuberculosis using Spoligotyping (DESTUS) the spoTools homepage (http://www.emi.unsw.edu.au/spoTools/) was used and manually clustered spoligotypes were analyzed with the default settings. The quantitative DESTUS model considers the mutation rate underlying each spoligotype, to detect genotypes that are spreading faster than the background population of spoligotypes (Tanaka and Francis, 2006; Tang et al., 2008).

2.5. Statistical analysis

Statistical analysis was performed using SPSS Statistic 17 (SPSS Inc., USA). Proportions of drug susceptibility test profiles for both MTBC strains were compared using Chi-Square analysis. The two sided Pearson’s Chi-Square test was used to assess associations of drug-resistance profiles with spoligotype families. Furthermore, an association of first-line anti-tuberculosis drug resistance profiles with MTBC genotypes was estimated and expressed as the odds ratio (OR) and 95% confidence interval (95% CI). A p-value of less than 0.05 was considered statistically significant.

2.6. Ethical considerations

All patients enrolled in these clinical studies had provided informed consent and studies were approved by the Guinean Ethical committee. Permission to perform this sub-study was obtained from the Institutional Research Board of the Institute of Tropical Medicine, Belgium. No patient personal identifiers were used in this study.

3. Results

3.1. Drug susceptibility profiles

A total of 359 MTBC isolates were identified, 222 isolates from Oflotub (Merle et al., 2012, 2014) collected within the period 2005–2010, and 137 from Study C (Lienhardt et al., 2011) between 2005 and 2006. Among the 222 isolates from Oflotub that had been analyzed in Guinea, 171 (77%) isolates were pan-susceptible to all first line anti-TB drugs; 51 (23%) isolates were resistant to at least one of the drugs, including 40 (18%) isolates with INH resistance, whether associated or not with resistance to other drugs, and 4 (1.8%) with resistance to both RMP and INH. Isolates from Study C included 106 (77.4%) pan-susceptible isolates and 31 (22.6%) with resistance to at least one of the drugs, including 5 (3.6%) MDR and 23 (16.8%) with any INH resistance (Table 1). Overall, among new TB patients in Guinea between 2005 and 2010, the MDR prevalence was 2.5% and 17.5% had any INH resistance.
3.2. Lineage and family assignments

Among the genotyped isolates \( n = 184 \) in this study were 50 strains from 2005, 102 from 2006, 14 from 2008 and 18 from 2010 \((\text{Lienhardt et al., 2011; Merle et al., 2012})\). The results revealed a total of 80 different patterns, including 29 ‘orphan’ spoligotypes and 51 different spoligotype patterns that had already been described before \((\text{Table S1})\). Of the total 184 isolates, 145 \((78.8\%)\) belonged to the Euro-American \((\text{Lineage 4})\), 20 \((10.9\%)\) to Lineage 6, 3 \((1.6\%)\) to the Central Asian \((\text{CAS, Lineage 3})\) lineage, 8 \((4.3\%)\) to Indo-Oceanic \((\text{Lineage 1})\), 2 \((1.1\%)\) to the East Asian \((\text{Beijing, Lineage 2})\), 6 \((3.3\%)\) to \(\text{M. africanum} \) West African 2 \((\text{MAF WA2, Lineage 6})\), 3 \((1.6\%)\) to \(\text{M. africanum} \) West African 1 \((\text{MAF WA1, Lineage 5})\), and 2 \((1.1\%)\) to the Central Asian \((\text{CAS, Lineage 3})\) lineage. The largest families were the \(\text{T} \) \((\text{including the T-sublineages: T1, T3, and T5})\) – and \(\text{Haarlem (H1 and H3)}\) – families, with 60 and 32 isolates, respectively, both members of the Euro-American lineage. Other families include \(\text{LAM, LAM10_CAM} \) \((\text{also called Cameroon family})\), X-clade and S-clade \((\text{all Euro-American})\); \(\text{Beijing (East Asian)}\); \(\text{Family34} \) and \(\text{EAI} \) \((\text{both Indo-Oceanic})\); \(\text{CAS (Central Asia)}\); and \(\text{MAF WA1&2} \) with different proportions \((\text{Table 2})\).

In total, 132 \((71.7\%)\) MTBC isolates were genotypically clustered \((28 \text{ clusters containing 2–21 isolates per cluster})\). The two largest clusters identified in the present study consisted of SIT53 isolates \((n = 21)\) of the \(\text{T-family} \) and SIT61 isolates \((n = 12)\) of the \(\text{LAM10_CAM} \) family. Other major genotypic clusters were characterized by spoligotypes SIT50 \((n = 9); \) SIT1/Beijing \((n = 8); \) SIT46 \((n = 8)\) and SIT465 \((n = 7)\). Of note, 52 \((28.3\%)\) isolates were represented by a unique spoligotype-pattern found only once in the study \((\text{Table S1})\).

3.3. Detection of emerging spoligotypes (DESTUS analysis)

Based on the spoligotype data using the DESTUS model, SIT61 of the \(\text{LAM10_CAM} \) family was detected to be spreading significantly faster \((p < 0.001)\) than the background rate of the overall spoligotype population.

3.4. Association of mycobacterial families and drug-resistance

The distribution of mycobacterial families among any resistant isolates \((43 \text{ isolates in total})\) showed a predominance of the Euro-American lineage \((\text{Tables 2 and S1})\). Of the three MDR isolates, two belonged to the \(\text{T-family} \) and one to the \(\text{LAM-family} \) \((\text{Table S2})\). In the overall statistical comparison of the drug resistance profiles, no significant associations were found \((\text{data not shown})\). However, in an exploratory analysis in which each family was compared separately against the others, Beijing strains were more likely to be poly-resistant \((\text{resistant to INH and SM but susceptible to RMP; OR = 5.61; 95\%CI, 1.23–25.56; p = 0.013})\), the \(\text{T-family} \) was associated with RMP resistance \((\text{OR = 11.18; 95\%CI, 1.28–97.98; p = 0.007})\) and \(\text{LAM10_CAM} \) isolates were correlated with mono-resistance to INH or SM \((\text{OR = 6.50; 95\%CI, 1.89–22.39; p = 0.001})\).

4. Discussion

Several studies have reported the prevalence and distribution of MTBC genotypes in the West African region \((\text{Niobe-Eyangoh et al., 2003; Godreuil et al., 2007; Affolabi et al., 2008})\). In Guinea, MTBC genotypes were predominantly the Cameroon family and \(\text{LAM10_CAM} \) family, with a high proportion of MDR isolates. However, the distribution of MTBC genotypes among new patients in Guinea is still largely unknown. The present study is the first to report the distribution of MTBC genotypes among new patients with pulmonary tuberculosis in Guinea, from 2005 to 2010. The results revealed a total of 80 different spoligotypes, including 29 ‘orphan’ spoligotypes and 51 different spoligotype patterns that had already been described before \((\text{Table S1})\). Of the total 184 isolates, 145 \((78.8\%)\) belonged to the Euro-American \((\text{Lineage 4})\), 20 \((10.9\%)\) to Lineage 6, 3 \((1.6\%)\) to the Central Asian \((\text{CAS, Lineage 3})\) lineage, 8 \((4.3\%)\) to Indo-Oceanic \((\text{Lineage 1})\), 2 \((1.1\%)\) to the East Asian \((\text{Beijing, Lineage 2})\), 6 \((3.3\%)\) to \(\text{M. africanum} \) West African 2 \((\text{MAF WA2, Lineage 6})\), 3 \((1.6\%)\) to \(\text{M. africanum} \) West African 1 \((\text{MAF WA1, Lineage 5})\), and 2 \((1.1\%)\) to the Central Asian \((\text{CAS, Lineage 3})\) lineage. The largest families were the \(\text{T} \) \((\text{including the T-sublineages: T1, T3, and T5})\) – and \(\text{Haarlem (H1 and H3)}\) – families, with 60 and 32 isolates, respectively, both members of the Euro-American lineage. Other families include \(\text{LAM, LAM10_CAM} \) \((\text{also called Cameroon family})\), X-clade and S-clade \((\text{all Euro-American})\); \(\text{Beijing (East Asian)}\); \(\text{Family34} \) and \(\text{EAI} \) \((\text{both Indo-Oceanic})\); \(\text{CAS (Central Asia)}\); and \(\text{MAF WA1&2} \) with different proportions \((\text{Table 2})\).
resistant to first line anti-tuberculosis drugs than (Traore et al., 2012) reported that conceivably that 2011; Traore et al., 2012); however, very little is known about the genotypic diversity of this pathogen in Guinea. This study reports the first use of genotyping and DST to assess the genetic diversity and resistance profiles of MTBC strains circulating in Guinea.

In the combined analysis of the baseline DST results, MDR was found in 2.5% of new pulmonary TB patients in Guinea, which is similar to estimates from neighboring countries around the same period; Guinea-Bissau (2.8%), Senegal (3.6%), Mali (2.2%), Côte d'Ivoire (2.8%) and Sierra Leone (3.1%) (Ben Amor et al., 2008; WHO, 2008); also results from other West African countries such as The Gambia (0.5%) (Adegbola et al., 2003; WHO, 2008), Benin (1%), Burkina Faso (2.9%), Ghana (2.2%) and Nigeria (2.3%) (Ben Amor et al., 2008; WHO, 2008) are in concordance with our finding. Moreover, like in other West African countries, there is considerable resistance to INH (17.5% of all strains) and/or SM (18.9% of all strains), which patients may be at increased risk of amplifying resistance. Other studies have shown various outcomes of INH monoresistant TB. In low endemic countries, such as Denmark and the US, treatment duration was typically modified when INH resistance was detected, and outcome was good (Cattamanchi et al., 2009; Bang et al., 2010). In TB endemic countries, such as South Africa, outcome of INH resistance was worse (Jacobson et al., 2011). In the context of an INH- and EMB based continuation phase in use in Vietnam (Cattamanchi et al., 2009), INH resistance was associated with an increased relapse rate. Should the high rate of INH resistance be confirmed in a drug resistance survey, the NTP could consider the addition of EMB to the INH + RMP continuation phase, as recommended by WHO, albeit based on weak evidence (WHO, 2010). We found that most MTBC lineages were identified in the present study, short of the recently identified lineage 7 (Firdessa et al., 2013) and Mycobacterium bovis or other animal strains. The majority of strains belonged to the Euro-American lineage 4, which is consistent with previous findings from neighboring countries such as Ivory Coast (Ouassa et al., 2012), Sierra Leone (Homolka et al., 2008) and Guinea-Bissau (Groenheit et al., 2011) as well as the Gambia (de Jong et al., 2009; Gehre et al., 2013). Interestingly, Sierra Leone, Guinea-Bissau and Guinea have the highest proportions (10–11%) of Indo-Oceanic strains (Lineage 1) in West Africa (Homolka et al., 2008; Groenheit et al., 2011) the reason of which is currently unknown. The wide spread of this ancestral MTBC lineage may reflect the population mobility between these neighboring countries, as currently exemplified by the Ebola epidemics. In contrast to previous reports that showed that the two M. africanum clades were among the predominant genotypes characteristic of the Western African region (de Jong et al., 2010a,b), the present findings revealed a lower than expected prevalence of 4.9% for both M. africanum clades combined in Guinea. Although surprising, this observation is in line with several reports (de Jong et al., 2010a,b) that have shown decreasing prevalence estimates of M. africanum during the last decades and at various frequencies in Cameroon (Niobe-Eyangoh et al., 2003), Burkina Faso (Godreuil et al., 2007) and in Guinea-Bissau (Groenheit et al., 2011), and also Côte d'Ivoire reporting a low prevalence (2%) of M. africanum (Ouassa et al., 2012). To date, the factors that might contribute to the decline of M. africanum strains in a particular population or region are poorly understood. It is conceivable that M. africanum is more susceptible than M. tuberculosis to TB prevention and treatment measures currently in use throughout West Africa. Previous studies from the Gambia, however, did not find greater protection from M. bovis BCG against M. africanum than M. tuberculosis (de Jong et al., 2010a,b). Publications from Ghana (Yeboah-Manu et al., 2011) and Mali (Traore et al., 2012) reported that M. africanum is less likely to be resistant to first line anti-tuberculosis drugs than M. tuberculosis clades, yet this does not explain a decrease in M. africanum prevalence, as transmission largely, if not exclusively, takes place before the initiation of TB treatment.

The most frequent mycobacterial family identified was the ill-defined T-family within Lineage 4, accounting for almost one-third of all isolates. This family was reportedly much less frequent in other West African countries such as Burkina Faso (Godreuil et al., 2007), Ghana (Yeboah-Manu et al., 2011), Sierra Leone (Homolka et al., 2008), Benin (Affolabi et al., 2009) and Nigeria (Amo et al., 2010) and therefore Guinea is the West African country with the highest prevalences of the T-clade, with SIT53 as the predominant T-family spoligotype. Even though the ill-defined T-superfamilies grouped under the modern monophyletic Lineage 4 (Coscolla and Gagneux, 2014), the sublineages of the T-family were shown to be a polyphyletic clade (Demay et al., 2012), and did not represent a family in a strict evolutionary sense (Brudey et al., 2006). Therefore future phylogenetic SNP based studies need to be conducted to elucidate the detailed population structure within this family (Stucki et al., 2012, 2014; Coscolla et al., 2015). Interestingly, spoligotype SIT61, commonly known as LAM10-CAM given its original description from Cameroon (Niobe-Eyangoh et al., 2004), is also found in Guinea, and DESTUS analysis suggests that these isolates are spreading faster than the background population of all spoligotypes in our setting. Although the exact reason for the observed emergence is elusive, a recent publication demonstrated that certain genotypes within the LAM10-CAM family were associated with HIV infections in Nigeria (Cadmus et al., 2011) and therefore HIV infection as a risk factor for increased transmission of these genotypes could be further explored in our setting in future studies, as HIV data was not consistently available within the parent studies. Moreover, our findings suggest that the various mycobacterial families may differ quantitatively and qualitatively in their individual capacity of developing drug-resistance. While Beijing strains were more frequently poly-resistant, LAM10-CAM mostly resistant to INH and SM, the T-family preferably developed RMP resistance. While an association between HIV and drug resistance has been found in some settings, yet not others (Sergeev et al., 2012), future studies should include this and other host risk factors for developing drug-resistance.

This study has limitations. We did not genotype all isolates from Guinea in these studies, which may have introduced a selection bias, although the selection was random and drug-resistance profiles of the genotyped subset were comparable to the overall bacterial population. Also, we did not correct for multiple testing, and a potential weakness is that some of the associations we identified on comparison per strain type may be spurious. Also a potential misclassification of LAM isolates as T strains, could impact on the association studies. Therefore these results should be considered exploratory, to be confirmed by larger studies in the same setting using higher resolution phylogenetic markers.

In conclusion, our findings suggest that the genotypic characteristic of the MTBC population structure analyzed in Guinea was diverse and all known MTBC lineages and families of human relevance were identified in this study. Moreover, supplementary analysis revealed the association of some of MTBC families and drug-resistance profiles. As mycobacterial population structures are often geographically restricted, genetic characterization studies to detect the presence of resistance-prone strains could be used to forecast drug-resistance profiles in a country. Therefore, our findings provide a first insight into the genotypes, population structure of MTBC, and prevalence of drug resistance in the area, contributing information for scientists as well as public health researchers in tuberculosis control programmes.
Authors’ contributions

Conceived and designed the experiments: M.E., F.G., L.R., B.D.J. Performed the research work: M.E., C.U., E.N., K.F., P.D.R. Analyzed the data: M.E., F.G., B.D.J. Supervised the experiment: F.G., B.D.J. Wrote the paper: M.E., F.G., B.D.J. Conducted the clinical studies and isolate collections: M.D.B., O.S., N.M.B., M.C., B.B., C.M., P.O., M.B., C.L., L.R. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2015.05.022.

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