RUFORUM Working Document Series (ISSN 1607-9345) No. 14 (2) : 827 - 834.
Available from *http://repository.ruforum.org*

Research Application Summary

Genetic diversity of *Mycobacterium tuberculosis*, drug resistance and atypical mycobacteria in Rwanda

Uwimana, I.,[1,2] Bwanga , F.,[1] Umubyeyi, A. N.[3] & Muvunyi, C.M.[4]
[1]Makerere University, College of Health Sciences, School of Biomedical Sciences, Department of Medical Microbiology, P.O Box 7062, Kampala, Uganda
[2]Rwanda Biomedical Centre, Biomedical Services Department, National Reference Laboratory Division, Microbiology Unit, P.O.Box 7162, Kigali, Rwanda
[3]Management Sciences for Health Laboratory Sevices, Pretoria, South Africa
[4] University of Rwanda, P.O.Box 4285, Kigali, Rwanda
Corresponding author: uwinnocent@gmail.com

Abstract

The genus *Mycobacterium* has two main groups, *Mycobacterium tuberculosis* complex (MTBC) and atypical mycobacteria. The MTBC species, the causative agents of tuberculosis (TB), has been further typed into different genotypes. Genotyping methods of *M. tuberculosis* help in identifying the most predominant circulating genotypes and their transmission dynamics and this helps in planning for a better tuberculosis drug resistance surveillance system. In Rwanda, little is known about the most predominant *M. tuberculosis* genotypes and there is limited data on drug resistance especially pyrazinamide drug resistance profile. Moreover, the distribution of atypical mycobacteria and their underlying epidemiological factors are also unknown. Our main goal is to characterize epidemiologically and genotypically *M. tuberculosis*, establish drug resistance and determine the distribution of atypical mycobacteria countrywide. Smear positives collected from suspected TB patients from Januray to July 2015 were submitted for primary culture on both solid and liquid culture media. All positive cultures on Lowenstein Jensen and BACTEC MGIT 960 liquid culture system were followed by phenotypic drug susceptibility testing (DST) to characterize resistance for first line anti-tuberculosis drugs. Isolates were tested for susceptibility to first line anti-TB drugs using Isoniazid, Rifampin, Streptomycin, and Ethambutol. Speciation of atypical mycobacteria was done using GenoType MTB DRplus Assay CM and AS. The present review presents preliminary results from primary culture, drug resistance and atypical mycobacteria. Out of 994 positive culture, MTBC accounted for 99.6% while atypical mycobacteria represented 0.4%. Of the 990 samples, 932 (94.1%) were new patients and 58 (5.9%) were patients with previous history of treatment for tuberculosis. MDR-TB was detected in 1.4% cases among new patients and 8.6% in previous treated patients. Among the mono-resistance pattern, Ethambutol represented the majority of all mono-resistance, averaging 17.4%. Non- Tuberclosis Mycobacteria (NTM) included *M. celatum* (0.1%), *M. lentiflavum* (0.1%), *M. peregrinum* (0.1%) and *M. intracellulare* (0.1%). In light of the present preliminary results, further investigations are needed to confirm the pan-resistance of Ethambutol. Molecular characterization is needed to decipher the most predominant *M. tuberculosis* genotypes, drug resistance and understand the extent of atypical mycobacteria in Rwanda. This would aid in strengthening TB treatment and control.

Key words: Atypical mycobacteria, drug resistance, genotypes, Rwanda, sequencing, tuberculosis

Résumé

Le genre Mycobacterium a deux groupes principaux, le complexe Mycobacterium tuberculosis (MTBC) et les mycobactéries atypiques. Les espèces MTBC; agents responsables de la tuberculose (TB) pulmonaire ont été davantage classées dans différents génotypes. Les méthodes de génotypage de Mycobacterium tuberculosis jouent un rôle clé pour comprendre les génotypes les plus prédominants et leur dynamique de transmission. Ceci permet de planifier et de mettre en place un meilleur système de surveillance de la résistance aux anti-tuberculeux. Au Rwanda, on en connait peu sur les génotypes de M. tuberculosis les plus prédominants et il y a peu de données sur la résistance aux médicaments en particulier le profil de résistance à la pyrazinamide. En outre, la répartition des mycobactéries atypiques et leurs facteurs épidémiologiques sous-jacents sont également inconnus. Notre objectif principal est de caractériser l'espèce de M. tuberculosis sur le plan épidémiologique et génotypique, établir la résistance aux médicaments anti-tuberculeux et déterminer la répartition des mycobactéries atypiques au niveau national. Les frottis positifs obtenus chez des patients suspects de la tuberculose à partir du mois de Janvier jusqu'au mois de Juillet 2015 ont été soumis à une culture primaire à la fois sur des milieux solides et liquides. Toutes les cultures positives sur Lowenstein Jensen et le système de culture en milieu liquide utilisant le BACTEC MGIT 960 étaient suivies par des tests phénotypiques sur la sensibilité aux médicaments pour caractériser la résistance aux médicaments antituberculeux de première ligne. La sensibilité aux anti-tuberculeux de première ligne a été testée sur toutes les souches en utilisant l'isoniazide, la rifampicine, la streptomycine et l'éthambutol. La spéciation des mycobactéries atypiques a été effectuée à l'aide du test moléculaire 'GenoType MTB DRplus Assay CM & AS'. Le présent article présente les résultats préliminaires de la culture primaire, la résistance aux anti-tuberculeux et la distribution des mycobactéries atypiques. Sur les 994 cultures positives, les MTBC représentent 99,6%, tandis que les mycobactéries atypiques représentent 0,4%. Parmi les 990 échantillons, 932 (94,1%) provenaient de nouveaux-cas tandis que 58 (5,9%) provenaient des patients ayant des cas de retraitement de la tuberculose. La tuberculose multi-résistante (TB-MR) a été détectée dans 1,4% des cas chez les nouveaux patients et 8,6% chez les patients traités précédemment. Parmi les modèles mono-résistants, éthambutol représente la majorité de toutes les mono-résistances, avec une moyenne de 17,4%. Les mycobactéries atypiques sont : M. celatum (0,1%), M. lentiflavum (0,1%), M. peregrinum (0,1%), et M. intracellulare (0,1%). A la lumière des présents résultats préliminaires, d'autres études sont nécessaires pour confirmer la pan-résistance de l'éthambutol. Les études utilisant la technologie de Biologie moléculaire sont nécessaires pour déchiffrer les génotypes des M. tuberculosis prédominants, la résistance aux médicaments et de comprendre l'ampleur des mycobactéries atypiques au Rwanda. Cela aiderait dans le renforcement du traitement de la tuberculose et de son contrôle.

Mots clés: Mycobactéries atypiques, résistance aux médicaments, génotypes, Rwanda, séquençage, Tuberculose.

Background

Globally, Tuberculosis (TB) is a major public health problem. One third of the World's population is infected with *Mycobacterium tuberculosis* complex, the causative agent of TB. Approximately 8.6 million people develop active TB disease and about 1.3 million die of TB annually, and African region carries 75% of the TB burden (WHO, 2013). In Rwanda, TB incident was 71/100.000 cases in 2014, with 25% occurring among HIV-TB co-infected people (WHO, 2015). A national drug resistance survey in Rwanda has recorded multi-drug tuberculosis (MDR-TB) cases in 3.9% and 9.4% new and retreatment patients, respectively (Umubyeyi *et al.,* 2007). The genus Mycobacteria has two main groups, atypical mycobacteria and *Mycobacterium tuberculosis* complex (MTBC). Atypical mycobacteria exist in over 20 species, among them *Mycobacterium kansasi*i, *Mycobacterium avium-intracellulare*, *Mycobacterium marinum*, *Mycobacterium ulcerans, Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacterium abscessus,* among others (Aliyu *et al.,* 2013), and they are found mainly among immune-compromised patients (Nyamogoba *et al.,* 2013).

Genotyping *M. tuberculosis* isolates has been used to demonstrate possible epidemiological links between patients, distinguish re-infection versus reactivation or to detect TB outbreak (van Embden *et al.,* 1993). Molecular genotyping helps to identify the most predominant circulating genotypes, transmission dynamics of TB, recent transmission, clustering rates, recurrent TB , single versus multiple TB infection and aids in TB drug resistance surveillance (Nagai Y I *et al.,* 2012). Different genotypic methods exist and include: Insertion sequence (IS), IS 6110- based restriction fragment length polymorphism (RFLP) (van Embden *et al.,* 1993), Spolygotyping which is labor intensive and has a low discriminatory power (Supply *et al.,* 2016), Length single polymorphism, Mycobacterial interspersed repetitive units and variable number of tandem repeats (MIRU-VNTR) and genomic DNA sequencing. The later can be either partial or whole genome sequencing that assigns lineage or different genotypes, sub-lineages, relatedness and clustering (Steiner *et al.,* 2014; Feuerriegel *et al.,* 2015). Futhermore, genome sequencing is faster and provides more information on drug resistance by detecting genes mutations confering drug resistance to the microbial agent (Sekiguchi *et al.,* 2017).

In Africa, studies have shown different genotypes of *M. tuberculosis.* These include: Beijing family, T family (T), Latino-America and Mediterranean family (LAM), Central Asia (CAS), Cameroun (CAM), Uganda I and II (Lari *et al.,* 2011, Mbug *et al.,* 2015), and others. Beijing family is the most prevalent genotype associated with drug-resistance (Marais *et al.,* 2013). In Rwanda, little is known about *M. tuberculosis* genotypes. Only two studies have so far been conducted on TB occurrence and genotyping using MIRU-VNTR (Umubyeyi *et al.,* 2017) and spoligotyping (Gafirita *et al.,* 2012). This study is aimed at molecular characterization of *M. tuberculosis* genotypes, establishing drug resistance and determining the distribution of atypical mycobacteria in Rwanda.

Study description

Sputum samples were collected from suspected TB patients from health facilities and were tested microscopically using iLED Fluorescence microscopy. Smear positives were submitted for primary culture on both solid and liquid culture media. All positive cultures on Lowenstein Jensen and BACTEC MGIT 960 liquid culture system were subjected to phenotypic drug susceptibility testing (DST) to characterize resistance for first line anti-tuberculosis drugs. Isolates were tested for susceptibility to first line anti-TB drugs using Isoniazid, Rifampin, Streptomycin, and Ethambutol. Drug susceptibility testing included testing for drug resistance pattern to Isoniazid (0.2µg/ml and 1.0 µg/ml), Rifampicin (20 µg/ml and 40 µg/ml), Ethambuthol (2.0 µg/ml) and Streptomycin (4.0 µg/ml). SD Bioline MPT64 TB Ag was done to classify mycobacteria into two groups namely: MTBC and NTM. Speciation of non-tuberculous mycobacteria (NTM) was done using GenoType MTB DRplus Assay CM and AS. The GenoType MTB DRplus Assay CM was used to identify common mycobacteria while GenoType MTB DRplus Assay AS provided probes for additional species of atypical mycobacteria.

Molecular characterization and further study. To understand molecular epidemiology of *M. tuberculosis* and drug resistance to it in Rwanda, further study will be conducted on genetic diversity of *M. tuberculosis*. Harvested pure colonies will be heat killed for genomic DNA extraction. Molecular genotyping and sequencing will be done using Mycobacterial interspersed repetitive units and variable number of tandem repeats (MIRU-VNTR) 15 loci or Single nucleotides polymorphism (SNP) and DNA sequencing to determine *M. tuberculosis* genotypes and confirm drug resistance patterns. PCR products will be analyzed using 2% agarose gel electrophoresis to determine different genotypes, and clusters. DNA sequencing will beperformed on Kat G, inhA, rpoB, pncA, rps, embB and pncA genes for first line drugs and Pyrazinamide. DNA sequencing and MIRU-VNTR will be used to determine the distribution of MTB genotypes, the most predominant MTB genotypes and sub-lineages of *M. tuberculosis*.

Results and Discussion

Mono-resistance analysis. There was a majority of monoresistant TB found in Ethambutol drug resistance (Table 1). Phenotypically, we observed predominance in Ethambutol resistance. Further analysis to sequence the highly conserved Ethambutol genes to get evidence of the pan-Ethambutol resistance in clinical isolates in Rwanda will be done since the genome sequencing provide accurate information on drug resistance and is a powerful diagnostic tool to detect antibiotic resistance. The second mono-resistance was found with Streptomycin drug resistance. Only one patient was having a mono-resistance with Rifampicin.

Table 1: Susceptibility patterns according to treatment history: new and previous treated patients

Characteristic	New n = 932	Retreatment n = 58	Total n = 990
	n (%)	n (%)	n (%)
Susceptible to all drugs	720 (77.3)	43 (87.9)	763 (77.8)
Mono resistance			
Isoniazid (INH)	6 (0.6)	1 (1.7)	7 (0.7)
Rifampicin (RIF)	1 (0.1)	0 (0.0)	1 (0.1)
Streptomycin (Strep)	15 (1.6)	0 (0.0)	15 (1.5)
Ethambutol (Eth)	164 (17.6)	8 (13.8)	172 (17.4
Poly-resistance other than MDR-TB	13 (1.4)	1 (1.7)	14 (1.4)
Multi drug resistant TB (MDR-TB)			
INH and RIF (MDR-TB)	13 (1.4)	5 (8.6)	18 (1.8)

Key: MDR-TB: Multi-drug resistant tuberculosis

Out of the 990 positive cultures, 932 (94.1%) were from new patients and 58 (5.9%) patients with previous history of treatment for tuberculosis (Table 2). Multi-drug resistant tuberculosis occurred in 1.4% cases among new patients and 8.6% in previous treated patients. Among the mono-resistance pattern, Ethambutol represented the majority (17.4%). Atypical mycobacteria represented 0.4% of all positive culture while MTBC accounted for 99.6% of the cases. The non tuberculosis mycobacteria identified included *M. celatum* (0.1%), *M. lentiflavum* (0.1%), *M. peregrinum* (0.1%) and *M. intracellulare* (0.1%).

Table 2. Distribution and speciation of a typical mycobacteria in positive culture (n=994)

Result	**Species**	**n (%)**
Positive	*M. tuberculosis complex*	990 (99.6)
	NTM	4 (0.4)
	M. celatum	1 (0,1)
NTM species	*M. lentiflavum*	1 (0.1)
	M. peregrinum	1 (0.1)
	M. intracellulare	1 (0.1)

Key: NTM: Non tuberculosis mycobacteria

Poly-resistance analysis. Poly-resistance includes either Rifampicin and Ethambutol, or Streptomycin and Ethambutol or Streptomycin and Isoniazid. The preliminary findings show that the most predominant poly-resistance was the one involving at the same time Syreptomycin and Ethambutol while Rifampicin and Ethambutol had the least occurrence in terms of poly-drug resistance. Where resistance to Rifampicin was observed it was either mono-resistant or pan-resistant to all first line drugs (SSRS or RRRR).

Multi-drug tuberculosis. Multi-drug resistant TB (MDR-TB) cases were more prevalent in retreated patients (8.6%) and low (1.4%) among new patients. This suggests that patients who have been treated for tuberculosis before are likely to get multi-drug resistant tuberculosis.

Atypical mycobacteria. In the preliminary results, atypical mycobateria represented 0.4% among all positive culture in clinical isolates. The identified and confirmed non tuberculous mycobacteria (NTM) also known as Mycobacteria other than tuberculosis (MOTT) among clinical isolates included: *M. celatum, M. lentiflavium, M. peregrinum* and *M. intracelullare.*

Gaps analysis, conclusions on preliminary and baseline results. Patients with multi-drug resistant tuberculosis are predominantly found among patients with history of previous treatment of tuberculosis. A high pan-resistance rate with Ethambuthol among all drug resistance patterns was observed. However, there is still limited data on the most predominant *Mycobacterium tuberculosis* genotypes in Rwanda. Furthermore, little is known about atypical mycobacteria in Rwanda. Moreover, its distribution and underlying epidemiological factors are also unknown. Hence, further investigations will be done to get insight on *M. tuberculosis* genotypes, drug resistance, associated risk factors and distribution of atypical mycobacteria countrywide. Further, pan-resistance in Ethambutol drug was observed among clinical isolates in Rwanda band but this will require further investigation to confirm these prevail findings for TB drug resistance control.

Genetic diversity and research application. Phenotypic drug resistance will be confirmed by genotypic drug resistance profile for better understanding of the extent of TB drug resistance. The analysis of genome sequencing will be used to determine different genotypes of *M. tuberculosis* and confirm the drug resistance patterns obtained from phenotypic drug susceptibility testing for better understanding of the extent of TB drug resistance and underlying risk factors. The prevalence of atypical mycobacteria will be defined and this will enable appropriate treatment and case management. Lastly, the findings of the present study will be used to strengthen tuberculosis surveillance system in Rwanda, the entire East African Region and hence contributing to the World Health Organization TB Stop strategy.

Acknowledgement

The authors acknowledge the financial support from RUFORUM for the dissemination of the preliminary results during the Fifth RUFORUM Biennial Conference held 17-21 October, 2016 in Cape Town, South Africa.

References

Aliyu, G., El-Kamary, S.S., Abimiku, A., Brown, C., Tracy, K. and Hungerford, L. 2013. Prevalence of non-tuberculous mycobacterial infections among tuberculosis suspects in Nigeria. *PloS one*. 8 (5):e63170.

Feuerriegel, S., Schleusener, V., Beckert, P., Kohl, T.A., Miotto, P. and Cirillo, D.M. 2015. PhyResSE: a Web Tool delineating *Mycobacterium tuberculosis* antibiotic resistance and lineage from whole-genome sequencing data. *Journal of Clinical Microbiology* 53 (6): 1908-1914.

Gafirita, J., Umubyeyi, A.N. and Asiimwe, B.B. 2012. A first insight into the genotypic diversity of *Mycobacterium tuberculosis* from Rwanda. *BMC Clinical Pathology* 12:20. DOI:10.1186/1472-6890-12-20.

Lari, N., Bimbi, N., Rindi, L., Tortoli, E. and Garzelli, C. 2011. Genetic diversity of human isolates of *Mycobacterium bovis* assessed by spoligotyping and Variable Number Tandem Repeat genotyping. Infection, genetics and evolution. *Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases* 11 (1):175-180.

Marais, B.J., Mlambo, C.K., Rastogi, N., Zozio, T., Duse, A.G. and Victor, T.C. 2013. Epidemic spread of multidrug-resistant tuberculosis in Johannesburg, South Africa. *Journal of Clinical Microbiology* 51 (6):1818-25.

Mbugi, E.V., Katale, B.Z., Siame, K.K., Keyyu, J.D., Kendall, S.L. and Dockrell, H.M. 2015. Genetic diversity of *Mycobacterium tuberculosis* isolated from tuberculosis patients in the Serengeti ecosystem in Tanzania. *Tuberculosis* 95 (2):170-178.

Nagai, Y., Hayakawa, E., Nakano, M., Sakai, T., Tanuma, M., Katayama, M., Nosaka, T. and Yamaguchi, T. 2012. Molecular genotyping of *Mycobacterium tuberculosis* in Mie Prefecture, Japan, using variable numbers of tandem repeats analysis. *Japan Jour. Infect Dis.* 65 (4): 341-344.

Nyamogoba, H.D.N., Mbuthia, G., Mining, S., Kikuvi, G., Kikuvi, R. and Mpoke, S. 2013. HIV co-infection with tuberculous and non-tuberculous mycobacteria in western Kenya: challenges in the diagnosis and management. *African Health Sciences* 12 (3): 305-11.

Sekiguchi, J., Miyoshi-Akiyama, T., Augustynowicz-Kopec, E., Zwolska, Z., Kirikae, F. and Toyota, E. 2007. Detection of multidrug resistance in *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology* 45 (1):179-192.

Steiner, A.S.D., Coscolla, M., Borrell, S. and Gagneux, S. 2014. KvarQ: targeted and direct variant calling from fastq reads of bacterial genomes. *BMC Genomics* 15 (1): 881. DOI:10.116/1471-2164-15-881.

Supply, P., Allix, C., Lesjean, S., Cardoso-Oelemann, M., Rusch-Gerdes, S. and Willery, E. 2006. l. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology* 44 (12): 4498-4510.

Umubyeyi, A.N., Rigouts, L., Dediste, A., Karita, E., Struelens, M.J. and Portaels, F. 2007. Molecular investigation of recurrent tuberculosis in patients from Rwanda. *Int J Tuberc Lung Dis.* 11 (8): 860-867.

Umubyeyi, A.N., Gasana, M., Basinga, P., Zawadi, J.P., Gatabazi, J., Pauwels, P., Nzabintwali, F., Nyiramasarabwe, L., Fissette, K., Rigouts, L., Struelens, M.J. and Portaels, F. 2007. Results of a national survey on drug resistance among pulmonary tuberculosis patients in Rwanda. *Int J Tuberc Lung Dis*. 11 (2): 189-194.

van Embden, J. D. A., Cave, M.D., Crawford, J.T., Dale, J.W., Eisenach, K.D., Gicquel,

B., Hermans, P., Martin, C., McAdam, R. and Shinnick, T.M. 1993. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J Clin Microbiol.* 31 (2): 406-409.

World Health Organization, Global tuberculosis report 2015;p.160. Available: (http://www.who.int/entity/tb/country/en/index.html). [accessed August 2015].

World Health Organization, WHO_Global tuberculosis report. 2013. Available: http://www.who.int/tb/publications/global_report/en/. [accessed August 2015].

.