

Detection of *Mycobacterium Tuberculosis* Multiple Strains in Sputum Samples from Patients with Pulmonary Tuberculosis in South Western Uganda using MIRU-VNTR

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Abstract

Infections with multiple strains of *Mycobacterium tuberculosis* are now widely recognized as a common occurrence. Identification of patients infected with multiple strains, provides both insight into the disease dynamics and the epidemiology of tuberculosis. Analysis of Mycobacterial Interspersed Repetitive Unit-Variable-Number Tandem Repeats (MIRU-VNTR) has been shown to be highly sensitive in detecting multiple *M. tuberculosis* strains even in sputum. The goal of this study was to identify cases of multiple *M. tuberculosis* strain infections among patients with pulmonary tuberculosis in south western Uganda and factors associated with multiple strain infections. Seventy-eight sputum samples were analyzed using the standard 24 loci MIRU-VNTR typing and an exact regression analysis performed using Stata version 14. Five (6.4%) of the 78 patients were infected with multiple strains of *M. tuberculosis*. All of the patients infected with multiple strains were the newly diagnosed cases while one third of them were co-infected with HIV. These findings point to a critical component of disease dynamics that is most likely being overlooked at the clinical level, emphasizing the need to further study the potential high risk of exposure to these categories of patients at the community level using a larger sample size.

Introduction

Mycobacterium tuberculosis (MTB) is one of the world's most successful human pathogen that has survived for millennia. It is associated with high morbidity and mortality worldwide, with over 1.5 million fatalities and 10 million new cases being recorded each year, making it the leading cause of death due to a single infectious agent worldwide¹⁻³. These infections are as a result of either a primary infection, an endogenous reactivation of a primary infection or exogenous re-infection with a new strain⁴. Historically, it was presumed that tuberculosis (TB) was as a result of a single strain and any recurrence was assumed to be due to reactivation of the same strain that caused the first episode⁵. Infection due to multiple strains in a patient at a single point in time was barely considered. However, in the mid-1970s using phage typing, it was discovered that different strains of MTB can infect a patient at the same time⁶ either as a result of a single transmission involving multiple distinct strains or due to multiple transmission events⁷. Moreover, distinguishing multiple strain infections from clonal diversity is essential because there is a significant difference in how these two mechanisms generate within-host diversity. Clonal diversity involves sporadic polymorphism due to sequential adaptive mutations (microevolutions)⁸⁻¹⁰, whereas multiple infections involve a host acquiring an entirely new MTB genome through successive or concurrent exposure to different strains^{11,12}. Considering that members of *Mycobacterium tuberculosis* complex (MTBC) have highly conserved genomes^{13,14}, high quality methods are required to identify small alterations within the infecting mycobacterial population. So far, various Polymerase Chain Reaction (PCR) based approaches have been utilized to demonstrate multiple strains within the same sputum sample^{10,15} or different sputum samples from the same patient^{8,16}. Mycobacterial Interspersed Repetitive Units-variable Number of Tandem Repeats (MIRU-VNTR) analysis identifies such changes in the genome by varying the copy of repeats in highly variable regions of the MTBC genome^{17,18}.

This study aimed at identifying multiple MTB strain infections among patients with pulmonary tuberculosis (PTB) in a high TB incidence area using MIRU-VNTR analysis and determining factors that could be associated with mixed infections in this area. TB incidence in south western Uganda is high at 253 cases per 100 000 people per year^{19,20}. It has been shown that multiple MTB strain infections are more common among people living in high TB burdened areas^{4,7,11,21} and accurate identification of this condition provides not only insight into the disease trends but also helps in the management and control of TB^{16,22-25}.

Results

Prevalence of multiple strains infection

MIRU-VNTR typing was performed on 78 sputum samples, each from an individual PTB patient. Majority of these samples (91%;71/78) were from newly diagnosed cases while 9% (7/78) were relapse patients. Ten (12.8%) patients were from refugees residing in the resettlement camps, 6 (7.7%) were from patients in prison. According to the HIV status records, 39.7% (31/78), 24.4% (19/78), and 35.9% (28/78) were HIV positive, negative or unknown respectively (Table 1). Five of the 78 (6.4%) patients were found to be infected with more than one strain of *M. tuberculosis* with all cases of multiple strain infections being the newly diagnosed patients. Three patients infected with multiple strains were men while the same number were HIV positive (Table 1).

One patient (#63) had strains belonging to two different lineages that also showed resistance to isoniazid while the rest belonged to the same lineage (see Table 2 and Fig. 1).

Table 1

Prevalence of *M. tuberculosis* multiple infections and comparison between patients with multiple versus single strain infections in Southwestern, Uganda

Variable	Category	Patient's characteristics n (%)	Single Strain (n = 73; 93.6%); 95% CI (0.864–0.976)	Multiple strains (n = 5; 6.4%); 95% CI (0.024–0.136)	χ^2 p-value
Age	18–24	57 (73.1)	53 (93.0)	4 (7.0)	0.799
	25–44	15 (19.2)	14 (93.3)	1 (6.7)	
	45–64	6 (7.7)	6 (100)	0 (0)	
Gender	Male	63 (80.8)	60 (95.2)	3 (4.8)	0.223
	Female	15 (19.2)	13 (86.7)	2 (13.3)	
HIV status	Positive	31 (39.7)	28 (90.3)	3 (9.7)	0.616
	Negative	19 (24.4)	18 (94.7)	1 (5.3)	
	Unknown	28 (35.9)	27 (96.4)	1 (3.6)	
Level of Income	High	7 (9.0)	7 (100)	0 (0.0)	0.468
	Low	71 (91.0)	66 (93.0)	5 (7.0)	
TB in the past	No	71 (91.0)	66 (93.0)	5 (7.0)	0.468
	Yes	7 (9.0)	7 (100)	0 (0)	
Refugee status	No	68 (87.2)	63 (92.6)	5 (7.4)	0.375
	Yes	10 (12.8)	10 (100)	0 (0.0)	
Imprisoned	No	72 (92.3)	67 (93.1)	5 (6.9)	0.505
	Yes	6 (7.7)	6 (100)	0 (0.0)	
Rif resistance	No	63 (80.8)	58 (92.1)	5 (7.9)	0.259
	Yes	15 (19.2)	15 (100)	0 (0.0)	
Inh resistance	No	63 (80.8)	67 (94.4)	4 (5.6)	0.373
	Yes	15 (19.2)	6 (85.7)	1 (14.3)	
Statistical significance considered at p-value \leq 0.05					

Table 2

Patients' in south western Uganda harboring more than one strain of *M. tuberculosis* identified using MIRU-VNTR standardized 24 loci

MIRU-VNTR loci																								
ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
63	2	2	4,5	2	3	2	2	3	1,2	4	2	4	2	2	5	1	5	3	3	4	2	4	2	2
202	2	2,3	4	2	3	3	2,3	3	1	2	4	4	2	1	5	1	4	3	3	2,3	3	7	2	2
228	2	2,3,4	4	6	3	4	2	5	2	2	7	3	2	4	5	2	2	3	3	2	2,5	6	1	3
2264	2	3	5	2	3	7	2	3	2	2	4	4	4	2	5	1	1	3	2,3,4	2,3	3	6	3	2
10546	2	3	5	2	2	3	3	3	1,2	2	4	4	2	2	5	1	5	3	2,3	3	2,5	6	2	2
The numerical figures represent the number of alleles per amplified MIRU-VNTR loci, as described by Supply et al (Supply, 2005). Where there is more than one numerical figure per locus indicates existence of more than one MTB strain in the sample, signifying an infection with multiple strains of <i>M. tuberculosis</i> . MIRU-VNTR Loci: 1 = 154, 2 = 424, 3 = 577, 4 = 580, 5 = 802, 6 = 960, 7 = 1644, 8 = 1955, 9 = 2059, 10 = 2163b, 11 = 2165, 12 = 2347, 13 = 2401, 14 = 2461, 15 = 2531, 16 = 2687, 17 = 2996, 18 = 3007, 19 = 3171, 20 = 3192, 21 = 3690, 22 = 4052, 23 = 4156, 24 = 4348. ID = patient identification number																								

Exact regression analysis of factors associated with multiple strains infection

The conditional maximum likelihood from the bivariable exact regression revealed that none of the patients' demographic variables such as age, sex was linked to multiple strain infections (Table 3). However, due to the fact that the number of individuals with multiple strain infections is so small, drawing firm inferences from these findings is difficult.

Table 3
Exact bivariate logistic regression of factors associated with multiple strains of *M. tuberculosis* infections among PTB patients in Southwestern, Uganda

Characteristics	Odds ratio	95% CI	p-value
Age group (years)			
18–24	1.780	0-16.388	1.000
25–44	0.947	0.018–10.629	1.000
45–64	1.000		
Sex			
Female	3.020	0.231–29.260	0.488
Male	1.000		
HIV status			
Positive	2.843	0.213-157.389	0.691
Negative	1.487	0.018–122.040	1.000
Unknown	1.00		
Level of income			
Low	1.483*	0-12.483	1.000
High	1.000		
Incarceration			
No	1.767*	0-15.236	1.000
Yes	1.000		
Refugees			
No	0.981*	0-7.926	1.000
Yes	1.000		
TB in the past			
No	1.483	0-12.483	1.000
Yes	1.000		
Rif			
No	0.598*	0-4.693	0.666
Yes	1.000		
Inh			
No	2.740	0.049–34.662	0.767
Yes	1.000		
*Median unbiased estimates (MUE)			

Discussion

Multiple strain infections in TB are now recognized as common occurrences and identifying patients with multiple MTB strains is critical in clinical practice, public health and molecular epidemiology. This is because not only does it provide insight into the disease patterns but also aids in the management and control of TB. This study revealed that, one out of every sixteen PTB patients (6.4%) was infected with multiple strains of MTB. This prevalence is almost similar to the 7.1% reported in Kampala, Uganda¹¹ but much lower than the 11% observed in Mubende, Uganda²¹. It is probable that the disparities in estimations are due to discrepancies in sensitivities of the genotyping techniques used to differentiate between MTB strains. While there are diverse genotyping approaches employed in the identification of mixed infections, the degree of sensitivity of each method varies. While the Mubende and Kampala investigations used 15 loci MIRU-VNTR typing, our study used 24 loci MIRU-VNTR typing with a single target conventional PCR. This method has been demonstrated to be very sensitive and discriminative, rendering it the gold standard in the diagnosis of multiple strain infections^{31,32}. Other significant discrepancies can be seen in the laboratory methods utilized. As with any other approach used to identify multiple strain infections,

detection of an underlying strain can only be established when there are sufficient DNA copies of that strain in the sample being studied. Many studies, including the Kampala and Mubende studies, use culture to increase the mycobacterial population^{11,21,33}. However, this can drastically change the clonal composition thus changing the frequency with which multiple strains are detected^{9,31,34}. In our study, we utilized DNA isolated directly from processed sputum samples. Our findings are also much higher than the 2.8%¹² reported in Malawi and 3.2% in Zambia³⁵ but lower than the 9.6%³⁶ and 10%³⁷ reported in Botswana. Differences between the study settings may partly account for this difference whereby the annual risk of TB infection in Malawi is approximately 1%^{3,38} while in Botswana its 3%³⁹.

Our study also revealed that unlike the relapse patients, who were not infected with multiple strains, all (100%) of the patients in our study with multiple strain infections were newly diagnosed cases. This is consistent with the findings of the Mubende study²¹, which observed that the majority (87.5%) of patients with multiple strains were the newly diagnosed cases. This might reflect a high level of transmission and heterogeneity of strains in this category of patients (Cohen et al., 2012). This hypothesis is supported by the proportion of newly diagnosed cases that exhibited the multiple strain infection phenomena, an attribute that is reported to indicate high transmission rates⁴⁰⁻⁴². Furthermore, a third (9.7%) of the patients with multiple strain infections were also HIV-infected. This finding is consistent with other studies, in which nearly all multiple strain TB infected people were HIV positive^{11,21}. This appears to support the notion of the link between multiple strain TB infection and HIV/TB co-infection^{16,21,36,43,44}. Given the high prevalence of HIV and HIV/TB co-infection in this region⁴⁵, it is plausible to suggest that HIV-induced immune deficiency exposes patients to the risk of concurrent infections. HIV removes the security of being reinfected as one battles an ongoing infection there by creating a scenario where one can be infected even before they clear an ongoing infection (Elizabeth Glaser Foundation, 2015).

In conclusion, patients experiencing their initial episodes of the disease and those co-infected with HIV are more susceptible to being infected with multiple strains of *M. tuberculosis*. This points to a critical component of disease dynamics that is most likely being overlooked at the clinical level, emphasizing the need to further study the potential high risk of exposure to this category of patients at the community level.

Materials And Methods

Patients, sample collection and processing

All methods of this study were carried out in accordance with the approved guidelines. Sputum samples were collected from patients seeking health care at either Nakivale HC111, Kabale Regional Referral Hospital and Mbarara Regional Referral Hospital between May 2018 and April 2019. The patients were diagnosed with PTB using either Cepheid gene X-pert or Microscopy and enrolled in an ongoing epidemiological study in south western, Uganda, from which some papers have been published²⁶. Samples were collected consecutively from patients who consented to the study upon completing an informed consent form and reported not having received treatment for TB in the preceding month. Patient demographics, sample processing and DNA extraction are described in Micheni et al (2021)²⁶ while drug susceptibility testing is described in Micheni et al (2021). All samples were confirmed as MTB by PCR-detection of a 123 bp fragment of the IS6110, which is common in the members of the MTB complex

Ethical consideration

This study was approved by the Institutional Review Board of Mbarara University of Science and Technology (MUST-IRB), the Uganda National Council for Science and Technology (with UNCST reference number HS 2379). The health facility administrators and the prime minister, granted permission to access their facilities and refugee camps, respectively. A written informed consent was obtained from each patient who participated in the study.

Typing by MIRU-VNTR PCR

The MIRU-VNTR PCRs were performed on genomic DNA extracted from the sputum samples. Using primers specific for sequences flanking the MIRU units (Table 4), the PCR was designed to amplify a standard set of 24 MIRU-VNTR loci from genomic DNA retrieved from each sample.

Table 4

PCR primer sequences and MIRU-VNTR locus designations¹ used in this study

Loci	Alias	Repeating unit length (bp)	Primer sequences (5'-3')
580	MIRU4, ETRD	77	GCGCGAGAGCCCGAACTGC GCGCAGCAGAAACGTCCAGC
2996	MIRU26	51	CCCGCCTTCGAAACGTGCT TGGACATAGGCGACCAGGCGAATA
802	MIRU40	54	GGGTTGCTGGATGACAACGTGT GGGTGATCTCGGCGAAATCAGATA
960	MIRU10	53	GTTCTTGACCAACTGCAGTCGTCC GCCACCTTGGTGATCAGCTACCT
1644	MIRU16	53	TCGGTGATCGGGTCCAGTCCAAGTA CCCGTCGTGCAGCCCTGGTAC
3192	MIRU31, ETR E	53	CTGATTGGCTTCATACGGCTTTA GTGCCGACGTGGTCTTGAT
424	Mtub04	51	GTCCAGGTTGCAAGAGATGG GGCATCCTCAAACAACGGTAG
577	ETR C	58	GACTTCAATGCGTTGTTGGA GTCTTGACCTCCACGAGTGC
2165	ETR A	75	ATTTTCGATCGGGATGTTGAT TCGGTCCCATCACCTTCTTA
2401	Mtub30	58	AGTCACCTTTCCTACCACTCGTAAC ATTAGTAGGGCACTAGCACCTCAAG
3690	Mtub39	58	AATCACGGTAACTGGGTTGTTT GATGCATGTTTCGACCCGTAG
4156	QUB-4156	59	TGACCACGGATTGCTCTAGT GCCGGCGTCCATGTT
2163b	QUB-11b	69	CGTAAGGGGGATGCGGAAATAGG CGAAGTGAATGGTGGTGGCAT
1955	Mtub21	57	AGATCCCAGTTGTCGTCGTC CAACATCGCCTGGTTCTGTA
4052	QUB-26	111	GGCCAGGTCCCTCCCGAT AACGCTCAGCTGTCGGAT
154	MIRU 2	53	TGGACTIONGAGCAATGGACCAACT TACTCGGACGCCGGCTCAAAT
2531	MIRU 23	53	CAGCGAAACGAACTGTGCTATCAC CGTGTCCGAGCAGAAAAGGGTAT
4348	MIRU 39	53	CGCATCGACAAACTGGAGCCAAAC CGGAAACGTCTACGCCCCACACAT
2059	MIRU 20	77	TCGGAGAGATGCCCTTCGAGTTAG GGAGACCGCGACCAGGTACTIONGTA
2687	MIRU 24	54	CGACCAAGATGTGCAGGAATACAT GGGCGAGTTGAGCTCACAGAA
3007	MIRU 27, QUB-5	53	TCGAAAGCCTCTGCGTGCCAGTAA GCGATGTGAGCGTGCCACTCAA

2461	ETR B, VNTR 48	57	GCGAACACCAGGACAGCATCATG GGCATGCCGGTGATCGAGTGG
2347	Mtub 29; VNTR 46	57	ATGATGGCACACCGAAGAAC AACCCATGTCAGCCAGGTTA
3171	Mtub 34; VNTR 49	54	GCAGATAACCCGCAGGAATA GGAGAGGATACGTGGATTGAG
¹ Extracted from Yasmin et al., ²⁷ . Primers were synthesized by Inqaba Biotech™ (South Africa).			

Each MIRU locus was amplified individually using a reaction mix and amplification profile described by Supply (2005)²⁸. For each reaction, DNA from *M. tuberculosis* H37Rv was used as a positive control, and sterile water was used as a negative control. Ten microliters of each PCR product were separated electrophoretically on 2% agarose gels for 3hrs, with a 100-bp DNA ladder (Solis Biodyne™, Estonia) serving as size markers. The corresponding MIRU-VNTR bands in the gel images were reported as Roman numerals representing the number of repeats per loci as described in the protocol reference table by Supply (2005)²⁸. For any sample that revealed multiple bands at any of the MIRU loci, the PCR was repeated in order to confirm the results. Multiple strains were concluded as being present if a sample had double alleles at more than one locus while those samples that had varying copy numbers at a single locus were considered as single strain evolution rather than multiple strains.

Statistical analysis

Patients' biodata and the presence or absence of multiple strain infection results were entered and validated in Microsoft Excel® 2013. The data was then exported to Stata (Stata/SE 14.2 for windows, Stata Corp, College Station, TX) for statistical analysis. Chi-square test was used to compute proportions and determine the relationship between independent factors and dependent variables (presence multiple strain infection) with statistical significance considered at 95% level of confidence. Since the feature of interest (multiple strain infections) was found in a small number of patients, an exact bivariate logistic regression analysis was performed to obtain odds ratios for factors that could be associated with the occurrence of multiple strains of *M. tuberculosis* among PTB patients in our setting. Exact logistic regression was selected because it calculates the conditional maximum chance of an event occurring within the sample population described by the model's varying factors. We did not specify a statistical significance threshold in accordance with recent statistical guidelines^{29,30}.

Declarations

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Author contributions

LNM and JB designed and funded the study; LNM and KK collected the data; LNM, KK and IN conducted data analysis and interpretation. LNM, KK, IN, HK, JB interpreted results, wrote, revised the initial and final manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figures

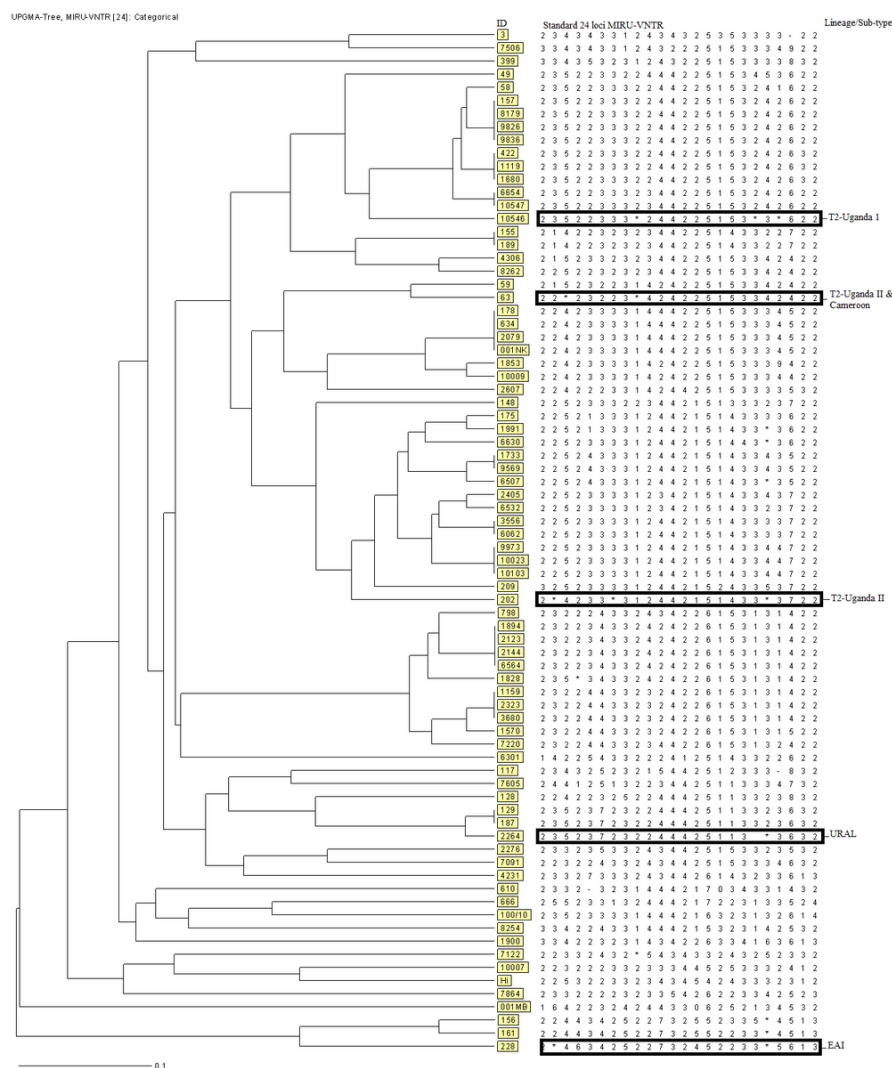


Figure 1

UPGMA tree based on the standard 24 loci MIRU-VNTR of isolates recovered from PTB patients in south western, Uganda.