

# Mycobacteria Interspersed Repetitive Units-Variable Number of Tandem Repeat, Spoligotyping and Drug Resistance of Isolates from Pulmonary Tuberculosis Patients in Kenya

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## Abstract

**Background:** Molecular typing allows a rapid and precise species differentiation and is essential in investigating the spread of specific genotypes and any relationship with drug resistance. **Methodology:** To compare the discrimination power of 24-loci Mycobacteria interspersed repetitive units-variable number of tandem repeat (MIRU-VNTR) to spoligotyping in determining the circulating genotypes of *Mycobacterium tuberculosis* in isolates from pulmonary tuberculosis patients in Kenya, a total of 204 isolates were typed. **Results:** Spoligotyping identified 22 spoligo lineages; while 36(17.6%) isolates were not determined. MIRU-VNTR typing identified 12 genotypes; Kenya H37\_Rv-like, S-like that had never been reported before and which were not identified by spoligotyping were identified. Others were Uganda I and II, LAM, Beijing, TUR, EAI, Delhi/C, S and Haarlem. Only 8 (3.9%) were not defined by MIRU-VNTR. Delhi/CAS, EAI, S, S-like, LAM and Beijing had strains that showed resistance to all the five drugs tested. Two strains of EAI and 2 of S genotypes were resistant to all the five drugs tested. Beijing genotype commonly associated with drug resistance was found to be third in drug resistance (14.7%) after Delhi/CAS (28.9%) and LAM (17.6%) with the highest resistance towards isoniazid and pyrazinamide (3.9% each). MIRU-VNTR typing was more discriminative than spoligotyping; identifying 10 unique H37\_Rv-like isolates designated KeniaH37\_Rv-like genotype and 14 S-like genotype. **Conclusion:** MIRU-VNTR typing has not been reported in any other study in Kenya and

its higher discrimination can help identify genotypes that cannot be determined by spoligotyping. Association of Beijing genotype drug resistance particularly isoniazid should be of concern since it may result in multidrug resistance in the patients.

## Keywords

Tuberculosis, MIRU-VNTR Typing, Spoligotyping

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## 1. Background

For a long time differentiation of clinical *Mycobacterium tuberculosis* (MTB) isolates was based on morphological and phenotypic characteristics such as growth morphology on Lowenstein Jensen medium (eunogic or dysnogenic) or biochemical properties such as niacin test and nitrate reduction. Since these tests need sufficient bacterial growth, are time consuming, do not allow unambiguous species identification and may not be performed by laboratories routinely, there was need for molecular methods that could allow a more rapid and precise species differentiation. Molecular typing of MTB is essential for epidemiological purposes such as investigating the spread of specific genotypes [1].

The first methods that allowed molecular fine typing of clinical isolates were IS6110 DNA fingerprinting and spoligotyping [2] [3]. These methods are used as intensive markers in epidemiological studies of MTB isolates and have revealed first insights in the population structure of clinical isolates in different geographical settings. There are several major spoligotypes of *Mycobacterium tuberculosis* namely Beijing, East African Indian (EAI) with at least five subtypes EAI1, EAI2, EAI3, EAI4, EAI5; Haarlem (H) with subtypes H1, H2, H3; Central and Middle Eastern Asia (CAS) with two subtypes CAS1 and CAS 2; European clade of IS6110 low banders X family with three subtypes X1, X2, X3; the poorly defined T family with subtypes T1, T2, T3, T4; Latino-American and Mediterranean family (LAM) with subtypes LAM1-LAM10 [4].

Spoligotyping analyzes the direct repeat (DR) locus in the genome of MTB which is composed of a cluster of 36 base pairs (bp) repeat references interspersed with unique spacers of 35 to 41 bp. By using a reverse southern blotting technique the variability in the spacer sequences can be interrogated and recorded in digital code [5]. Although this method provides a digital typing data it only measures variability in a single locus and does not generally provide sufficient discrimination for outbreak investigation [6].

The most currently used typing method is based on variable number tandem repeats (VNTR) of *Mycobacteria* interspersed repetitive units (MIRUS). The repeated units are 52 to 77 nucleotides in length and the number of repeated units can be determined by the size of the entire locus [7]. This polymerase chain reaction (PCR) based typing method was originally based on 12 human minisatellite-like regions of the MTB genome [8] [9]. A population based study in the

USA indicated that the use of the 12 locus-based MIRU-VNTR typing as a first line method, together with spoligotyping, provides adequate discrimination in most cases for large scale, prospective genotyping of MTB in the USA. IS6110 fingerprinting can subsequently be used as a secondary typing method to type the clustered isolates, when additional discrimination is needed [10].

MIRU-VNTR has the same discriminatory power in comparison to IS6110 RFLP [11]. It has also been useful in studying the population structure of MTB [12] [13] and is suitable for global epidemiological surveillance of TB due to its high resolution, simplicity, sensitivity, high reproducibility and easy inter-laboratory comparison [14] [15] [16]. MIRU-VNTR typing has become an important method as it allows high throughput, discriminatory and reproducible analysis of clinical isolates [17] [18]. First evaluations have shown that it can provide unique high-resolution insights into the population structure of the MTB and provides clear criteria for the identification of the different MTB lineages and sub-lineages [18] [19].

## **2. Methods**

### **2.1. Study Area**

A total of six TB clinics were selected for this study. These were Kangemi, Riruta and Mbagathi TB clinics, Aga Khan in Nairobi as well as Kiambu and Nazareth Hospitals in Kiambu county.

### **2.2. Study Design**

This was a hospital based prospective study.

### **2.3. Study Population**

The study population comprised 204 adult patients with pulmonary TB attending five clinics in Nairobi.

### **2.4. Sputa Collection and Processing**

An early morning and a spot sample were collected from each patient. The sputa were processed according to the protocol by Murray [20]. They were cultured in MGIT liquid medium using the Bactec 960 system and Löwenstein Jensen(LJ) solid media. All the cultures were subjected to the Capilia TB assay (TAUNS Laboratories, Inc.) to rule out Mycobacteria other than tuberculosis (MOTTs). Drug sensitivity tests were done using five first line-drugs isoniazid (INH) (0.10 µg/ml), rifampicin (RIF) (1 µg/ml), streptomycin (SM) (1 µg/ml), ethambutol (EMB) (5 µg/ml) and pyrazinamide(PZA) (100 µg/ml) using BACTEC MGIT 960 STR 4.0 and PZA kits. The procedure was performed as described by Becton, Dickinson and company 7 Loveton circle Sparks, Maryland 21152 USA. H37Rv ATCC 25618 was used for susceptibility control and was included in all test sets.

### **2.5. Deoxyribonucleic Acid (DNA) Extraction**

The isolates were grown on Löwenstein-Jensen (LJ) medium at 37°C until growth

became clearly visible. An appropriate number of bacterial cells were transferred into a microcentrifuge tube containing 400  $\mu$ l Tris-EDTA (TE) buffer. They were incubated for 20 min at 80°C in a water bath to kill the bacteria and 50  $\mu$ l of 10 mg/ml lysozyme was added and vortexed then incubated for at least 1 hour at 37°C. Then 75  $\mu$ l of 10% Sodium dodecyl sulphate (SDS)/proteinaseK mix (5  $\mu$ l Proteinase K (10 mg/ml) + 70  $\mu$ l 10% SDS) was added, vortexed shortly and incubated for 10 min at 65°C. One hundred  $\mu$ l of 5 M Sodium chloride (NaCl) was added followed by 100  $\mu$ l -N-cetyl-N, N, N-trimethyl ammonium bromide/NaCl (CTAB/NaCl) mix (prewarmed at 65°C) and vortexed until the liquid content became white and was incubated for 10 min at 65°C. Approximately 750  $\mu$ l chloroform/isoamyl alcohol mix (24:1) was added and vortexed for 10 seconds then centrifuged for 10 min at 12,000 g at room temperature. The aqueous supernatant was transferred in a new tube and 0.6 volume (450  $\mu$ l) isopropanol added, mixed carefully and incubated for 30 min at -20°C. This was then centrifuged for 15 minutes at 12,000 g. Most of supernatant was removed and 500  $\mu$ l of cold 70% ethanol was added and spinned for 5 min at 12,000 g. The supernatant was discarded and the tube spinned for 5 minutes at 12,000 g. The last  $\mu$ l of the supernatant was discarded cautiously and the pellet dried for about 10 min at room temperature. Eighty  $\mu$ l TE buffer was added and the DNA dissolved for 10 minutes at 60°C. Quality and concentration of the DNA preparation was controlled by agarose gel electrophoresis.

## 2.6. Spoligotyping and MIRU-VNTR Typing

All the samples were spoligotyped by the method described by Kremer [21]. Briefly PCR master mix was added to 5  $\mu$ l of DNA sample. This was loaded run in the PCR and run as follows: 5 minutes at 95°C for, 1 minute at 94°C, 1 minute at 58°C, 30 seconds at 72°C, 5 minutes at 72°C and  $\alpha$  at 4°C. Some 190  $\mu$ l 2XSSPE + 0.1%SDS was added to the DNA sample. Each sample was loaded on the blotter with a membrane laid on it and incubated at 65°C for 1 hour. The membrane was removed from the blotter washed in SSPE buffer on a shaker at 60°C for 15 minutes. The membrane was hybridized at 42°C for 10 minutes in the hybridization oven after which it was removed and placed on the film chamber. A film was placed on top of the membrane and detection reagent added and allowed to stand for one minute. The film was then developed in the dark room.

MIRU typing was done using the 24-loci based MIRU-VNTR typing kit (User manual [www.genoscreen.com](http://www.genoscreen.com)). Briefly, a 96-well plate, using a PCR spreadsheet was prepared. In a PCR free area, 8  $\mu$ l of triplex mixes per well were loaded in the 96-well plate according to the PCR spreadsheet. Two  $\mu$ l of DNA suspension were added, including positive and negative controls. The 96-well plate was tightly sealed with an adhesive PCR film and centrifuged at 13,000xg. This was then loaded in the PCR machine and the PCR cycle run as follows: 15 min at 95°C, 1 min at 94°C, 30 s at 59°C, 1 min 30 s at 72°C (these were repeated for 40 cycles), 10 min at 72°C, and  $\alpha$  at 4°C. For fragment analysis 9.5  $\mu$ l of HiDi for-

mamide and 0.5 µl Genescan was prepared per plate. A 96-well sequencer plate was loaded with 10 µl of the mixture. The 96-well plate containing the PCR products was centrifuged and the adhesive PCR film removed. Two µl of PCR products per well were added to the 96-well sequencer plate according to the spreadsheet. The plate was sealed with an adhesive film and centrifuged to remove bubbles. The DNA was denatured at 95°C for 5 min using PCR machine and kept on ice before loading. The plate was then loaded to the sequencer. Fragment analysis was done using ABI DNA analyzer.

## 2.7. Data Sequence Analysis

Spoligotyping data was computed using the bionumerics software (Applied maths, Sint Marten-Latem, Belgium).

A web server MIRU-VNTR plus (<http://www.miru-vntrplus.org>) created using 186 strains representing the major MTB lineage was used in interpreting the MIRU data. For each strain, species, lineage and epidemiologic information is stored together with copy numbers of 24 loci, spoligotyping patterns, regions of difference (RD) profiles and susceptibility data. Using this MIRU-VNTR service, the strains were compared with the reference strains and assigned the lineages and genotypes. The comparisons were based on MIRU-, spoligo- and susceptibility data.

## 2.8. Ethical Considerations

The study was approved by both Scientific Steering Committee (SCC) and Ethical Review Committee (ERC) (ERC NO. 1622). Permission was also granted by the administration of the respective hospitals and the Nairobi City Council Health Committee. The patients also gave informed consent.

## 3. Results

There were 22 different sub lineages of the spoligotypes isolated, while 36 were undetermined (**Table 1**). The distribution shows that the CAS1\_ KILI variety had the highest number of isolates 33 (16.1%) followed by Beijing 28 (13.7%), LAM3 and s/convergent 15 (7.3%), LAM 9 14 (6.8%) CAS1 Delhi 11 (5.3%) and S 10 (4.9%). The rest had less than ten isolates as shown in **Table 1**.

Thirty six isolates (17.6%) could not be identified by spoligotyping. Based on the 24-loci system, ten genotypes were identified by MIRU-VNTR typing; Kenia H37\_Rv\_like and S-like, which had not been determined before and which were not determined by spoligotyping were determined by MIRU-VNTR method. Others were EAI, Delhi/CAS, S, Uganda I and II, LAM, Beijing, TUR, and Haarlem. Only eight isolates could not be identified because they were not among those that were available in the database therefore could be new (**Table 2**). Delhi/CAS was the most prevalent with 59 (28.9%) followed by LAM and Beijing 36 (17.6%) and 30 (14.7%) respectively. TUR, EAI, S and KeniaH37-RV-Like were 10 (4.9%) each; the rest were less than 10.

Sensitivity patterns on each genotype showed that highest overall resistance

was by Delhi/CAS (28.9%), followed by LAM (17.6%) and Beijing (14.7%). S, EAI, Kenia H37-Rv-Like and TUR had 4.9% resistance each, while Haarlem had 4.4%. The drug which was resisted most was INH (43.1) followed by PZA (20.5%), SM (15.6%) Rif (12.2%) and finally EMB (8.8%). Five genotypes namely; EAI, Delhi/CAS, S, S-like, LAM and Beijing, had resistance to all the five drugs tested. Two strains of EAI and 2 of S genotypes were resistant to all the five drugs tested. Beijing which is commonly associated with drug resistance showed highest resistance to INH and PZA (3.9% each) and least to Rif and EMB (1.9%).

#### 4. Discussion

In our study, 22 spoligo lineages were determined by spoligotyping with 36 isolates undetermined. CAS1\_KILI had the highest prevalence with 33 (16.1%) strains. CAS1\_KILI and LAM11\_ZWE family (also called Kilimanjaro and Meru

**Table 1.** Spoligotyping lineage subtypes of the sequenced samples from patients sampled from five clinics in Nairobi.

SPOLDB4 Lineage	Strains (n)	%
CAS 1_KILI	33	16.1
BEIJING/W	28	13.7
Bovis 1_BCG	1	0.5
U	7	3.4
S	10	4.9
EAI 5 and EAI 3	8	3.9
LAM 9	14	6.8
LAM 11 ZWE	9	4.4
T1	9	4.4
LAM 3and s/convergent	15	7.3
CAS	4	1.9
CAS 1 DELHI	11	5.3
CAS 2	2	0.9
LAM 4	1	0.5
T3 ETH	3	1.4
T3	4	1.9
MANU	1	0.5
T2	3	1.4
EA11 SOM	1	0.5
EA3 IND	1	0.5
H1	1	0.5
H3	2	0.9
UNDETERMINED	36	17.6
<b>TOTAL</b>	<b>204</b>	<b>100</b>

**Table 2.** Distribution of the MIRU genotypes of the isolates taken from patients sampled from five clinics in Nairobi.

MIRU Genotype	No.	% <sup>†</sup>	Drug INH (%)	Resistance* Rif (%)	PZA (%)	EMB (%)	SM (%)
EAI	10	4.9	3(1.4)	3(1.4)	4(1.9)	2(0.9)	2(0.9)
Delhi/CAS	59	28.9	12(5.8)	7(3.4)	15(2.4)	3(1.4)	7(3.4)
Bovis	1	0.5	0(0)	0(0)	1(0.5)	0(0)	0(0)
KenyaH37_Rv_like	10	4.9	10(4.9)	0(0)	0(0)	1(0.5)	1(0.5)
S	10	4.9	5(2.4)	5(2.4)	7(3.4)	2(0.9)	5(2.4)
S-like	14	6.8	2(0.9)	1(0.5)	1(0.5)	1(0.5)	2(0.9)
Uganda I	5	2.4	1(0.5)	0(0)	0(0)	0(0)	1(0.5)
Uganda II	2	1.4	1(0.5)	1(0.5)	0(0)	0(0)	0(0)
LAM	36	17.6	6(2.9)	1(0.5)	5(2.4)	3(1.4)	10(4.9)
Beijing	30	14.7	8(3.9)	4(1.9)	8(3.9)	4(1.9)	3(1.4)
TUR	10	4.9	2(0.9)	0(0)	1(0.5)	0(0)	1(0.5)
Haarlem	9	4.4	9(4.4)	2(0.9)	0(0)	1(0.5)	0(0)
Unidentified	8	3.9	29(14.2)	1(0.5)	0(0)	1(0.5)	0(0)
Total	204	100	88(43.1)	25(12.2)	42(20.5)	18(8.8)	32(15.6)

\*First line drugs used include: INH-Isoniazid, Rif –Rifampicin, PZA-Pyrazinamid, EMB-Ethambutol, SM-Streptomycin; <sup>†</sup>% for each drug calculated over total.

families respectively) have been isolated from Tanzania where they were also predominant [22]. A study by [23] in Tanzania indicated prevalence in excess of 10% of both families. Their presence in Kenya could be as a result of cross border migration and trade with the people of Tanzania which is a neighbouring country. These may indicate introduction of new genotypes that may be due to casual contact or international travel. An earlier study in Kenya identified 47 spoligo lineages [24].

The large number of patterns shows a large diversity of spoligotypes in our study. This diversity could be attributed to human travel and migration of asylum seekers such as refugees especially because of the political instability in the neighboring Somalia and Sudan which has resulted in influx of refugees from these countries, as well as international travel.

Other studies in other regions also showed wide diversity in various countries. In Turkey by [25], 95 drug resistant isolates collected from three different centres were spoligotyped and 35 different distinct patterns were observed and 20 isolates displayed unique patterns. Five of these 20 unique patterns corresponded to orphan patterns in SITVIT2 database, while four shared types containing 8 isolates that were newly created. The most prevalent MTB lineages were Haarlem 23/95 (24.2%), T family 22/95 (23.2%), Turkey family 19/95 (20%), Beijing/W 6/95 (6.3%) and Latin American and Mediteranean (LAM) 5/95 or 5.3% followed by manu 3/95 (3.2%) and S 1/95 (1%).

In India, spoligotyping resulted in 49 patterns. The largest cluster was composed of spoligotype international T types (SITS) 26, Central Asian (CAS)1-Delhi lineage, followed by SIT 11(Eastern-African-Indian) EAI 3-Indian lineage.

A large number of isolates (75%) belonged to genotypic lineages such as CAS, EAI, and Manu with a high specificity for the Indian subcontinent [26]. A total of 273 clinical isolates showed 120 different spoligotyping patterns majority of which belonged to major clades of MTB, Haarlem 22.6%, LAM 23.3% and T 32.6% [27].

A study by [28] in Kenya spoligotyped 33 drug resistant isolates including 15 MDR and 40 susceptible isolates. The isolates were classified into 25 groups, ten of which corresponded to previously identified strains including Beijing, CAS1, S, LAM9, Haarlem, T2 and T1. One of the families, CAS1, comprised six (40%) of the 15 MDR isolates. Beijing family had 6 (8.3%) isolates of which 2 (3.3%) were MDR Beijing/W. This study was the first in Kenya to report Beijing/W type. Another study conducted in Kenya spoligotyped 536 isolates and identified 47 strain families the principal ones being CAS1-KILI (17%, T1 (12%), Beijing (12%), LAM 9 (9%), LAM3 and s/convergence (7%), LAM11\_ZWE (5%), CAS1\_DELHI (4%) and T2 (4%) [23]. New strains in this study accounted for 24/135 (17.7%) while 111 belonged to known spoligotypes.

In our study, MIRU typing identified 10 unique KeniaH37Rv\_like strains which were not identified by spoligotyping. Our study also identified 10 S genotypes two of which were resistant to all the drugs tested. Delhi/CAS genotype had the highest prevalence with 59 (28.9%) isolates. A study by [29] on MIRU types showed a predominance of the H37Rv\_like sub lineage, while strains from different sub lineages had convergent spoligotype and MIRU types. CAS1\_Delhi a subtype of CAS genotype, has been found in regions in which migration to and from India sub-continent occur such as Kenya, S. Africa, Malaysia, Myamma, Australia, USA and parts of Europe [26] because it is predominant in Northern India [30] [31] [32].

In our study, MIRU-VNTR typing showed 12 different genotypes, with only 8 unidentified strains. This shows the high discrimination capacity of MIRU-VNTR typing as opposed to spoligotyping which is the only typing method reported in Kenya, where 36 isolates could not be identified. Although there was dominance of the EAI, Beijing and East-African-Indian global lineages, MIRU-VNTR typing revealed presence of Euro-American lineage represented by Haarlem, Uganda I and II, S,TUR types all of which are dominant in the European region. This means that there has been cross transmission of TB strains from other continents.

In our study sensitivity testing showed Delhi/ CAS as having the highest resistance followed by Beijing. Beijing has been associated with resistance in different parts of the world. In 49 studies in 35 countries, it was found to be associated with drug resistance in several of those countries [33] while in China drug resistance for Beijing was 75.4% [34]. Our study also showed that Beijing had highest resistance for Isoniazid and pyrazinamide. Resistance to isoniazid is important because it has been shown to result in MDR-TB if not taken care of. In Vietnam a study showed Beijing having greatest resistance towards isoniazid and streptomycin than any other drug [35].



## 5. Conclusion

MIRU-VNTR typing identified presence of Kenia H37Rv-like and S-like genotypes that were unique as well as Euro-American lineages that could not be identified by spoligotyping. Earlier studies in Kenya have only reported on spoligotyping; no other study has reported on MIRU-VNTR typing. This means that unique or new genotypes may have been missed by earlier studies and therefore MIRU-VNTR is of importance in determining emerging genotypes in the country. Resistance of Beijing genotype to isoniazid should be of concern since it could translate to MDR-TB.

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