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Introducing the Best Six Loci in Mycobacterial Interspersed Repetitive Unit-Variable-Number Tandem Repeat (MIRU-VNTR) Typing for *Mycobacterium Tuberculosis* Genotyping

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Abstract

Background: Tuberculosis (TB) still remains endemic worldwide making epidemiological studies essential to mitigating efforts implicated in identifying its source, controlling, and preventing the spread of dangerous strains amongst humans such as *Mycobacterium tuberculosis (Mtb*).
Methods: In this study, we sought to determine the 6 Mycobacterial Interspersed Repetitive Unit-Variable-Number Tandem Repeat (MIRU-VNTR) loci with high discriminatory powers for *Mtb* genotyping as well as the loci with the highest and the lowest discriminatory powers for MIRU-VNTR. To conduct our search, we used several databases such as science direct, Embase (Elsevier), Web of Science, Scopus and Medline via PubMed. Searches were performed using key words including*: Mycobacterium tuberculosis*, MIRU–VNTR, Allele diversity, Genetic diversity and human patient. Finally, 56 articles were selected after filtering out titles, abstracts and full texts.
Results: Loci with high discriminatory powers included MIRU10 and MIRU26, while MIRU2, MIRU20, MIRU24 and ETRD had poor discriminatory powers. According to previous data in the literature, the loci MIRU10, MIRU26, MIRU40, QUB 26, QUB 11b and Mtub21 have high discriminatory powers.
Conclusions: Therefore, these loci recommended for genotyping *Mtb* to save time and cost and to ensure the production of reliable results.

Keywords: Discriminatory power, Genotyping, MIRU-VNTR, Mycobacterium tuberculosis.

Introduction

In spite of recent efforts to control and eliminate TB, this highly infectious disease still remains the second leading cause of death worldwide (1, 2). In 2018, WHO predicted that 10 million patients (ranging from 9 to 11.1 million) were stricken by TB. *Mtb* is a member of the TB complex which causes TB and can be transmitted via aerosolization of bodily fluids from coughing, sneezing or speaking (2).

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To effectively control TB, preventive policies based on transmission routes are required. Molecular epidemiology of *Mtb* involves monitoring special strains like multi-drug-resistant (MDR) *Mtb* during periods of increasing prevalence, inspecting regions of latest and potential outbreaks, locating the source and route of transmission and transmission gene sequence, discovering hidden strains and tracking circulating immigrant strains (3).

Various molecular methods are used in epidemiological studies about *Mtb* strains. Each method has a particular specificity and sensitivity. Some of these approaches include IS6110, IS6110-restriction fragment length polymorphism (RFLP), MIRU-VNTR, Spoligotyping, whole genome sequencing (WGS), Random Amplification of Polymorphic DNA PCR (RAPD_PCR), Repetitive element sequence-based PCR (rep-PCR), Pulsed-field gel electrophoresis (PFGE), Next Generation Sequencing (NGS) and finally, a combination of two or more techniques listed above will be applied. Among current typing methods, a test has to be chosen according to its feasibility, cost-benefit and discriminatory powers (4-7)

In a survey comparing PFGE, 24 locus MIRU-VNTR and IS6110-RFLP, it was revealed that the 24 locus MIRU-VNTR method was the preferred method due to a high power of discrimination and time management during epidemiological investigations (7).

This study was performed to review published applications of the Mycobacterial Interspersed Repetitive Unit-Variable-Number Tandem Repeat (MIRU-VNTR) method in *Mtb* genotyping and to introduce the best 6 loci for MIRU-VNTR in typing *Mtb* isolated from human patients along with determining the loci with highest and lowest discriminatory powers for MIRU- VNTR.

Materials and methods

To identify the best loci of the 6 loci in MIRU-VNTR method for *Mtb* genotyping, the literature search was performed using several databases including science direct, Embase (Elsevier), Web of Science, Scopus, ISC and Medline via PubMed. Chosen keywords were: *Mtb*, MIRU–VNTR, Allele diversity, genetic diversity and human patient. Inclusion and exclusion criteria were determined by the following:

1. Isolation of *Mtb* from human patients.
2. Investigating the genetic diversity among *Mtb* just based on MIRU-VNTR.
3. Excluding the studies where lineage determination was based on MIRU-VNTR where Allele diversity "*h*" was not measured for each locus.
4- Excluding the studies in which MIRU-VNTR ability in cluster analysis and Hunter-Gaston discriminatory index (HGDI) for this method was investigated and h was not estimated for each of their locus.

Several articles were excluded from our study since allele diversity (*h*) was not mentioned for each locus. Screening the articles was done in 3 steps: 1. Title screening, 2. Abstract evaluation, 3. Full text evaluation based on these criteria.

Results

A total number of 228 articles were found collectively amongst the databases. As the title screening was performed, 90 articles were removed. Abstract screening resulted in 82 more studies to be omitted during the search. Finally, after full text screening, 56 articles remained. In the remaining articles, genotyping for *Mtb* using MIRU-VNTR was investigated from 2002 to 2019. Allele diversity (*h*) was evaluated amongst the 56 articles for each locus separately (Table 1).

Mycobacterium tuberculosis genotyping was performed on 56 studies using the MIRU-VNTR technique; 39 of which were conducted in Asia, seven in America, six in Africa, three in Europe and one in a different country. The location and number of studies are shown in Figure 1. Each individual study employed a different number of loci. The results revealed that MIRU10 and MIRU26 had the highest discriminatory powers while MIRU2, MIRU20, MIRU24 and ETRD had the lowest discriminatory powers, respectively.

Table 2 shows both the number of studies in which MIRU10, MIRU26, QUB26, MIRU40, QUB11b and Mtub21 was reported to be the loci with the highest discriminatory powers ($h>0.6$), including the range of *h* for the remaining loci.

MIRU2 and MIRU20 (each in 21 studies), MIRU24 (17 studies) and ETRD (13 studies) were suggested as the loci with the lowest discriminatory powers ($h<0.3$).

Table 1. Fifty-six studies after full-text evaluation that MIRU-VNTR were used.

No.	Authors reference	year	Geographical region	Continent	Locus of MIRU-VNTR	Numbers of locus	High power	Low power
.1	Cowan et al (8)	2002	United States (Michigan)	America	ETR D,E MIRU2,10,16,20,23,24,26,27,39 ,40	12	MIRU40	MIRU2,2 0
.2	Sola et al (9)	2003	Different regions such as; USA, Thailand, Sicily, Guadeloupe and Russia		ETR A-E MIRU2,10,16,20,23,24,26,27,39 ,40	15	ETRA, MIRU40 ,26,10	MIRU2,2 0, 27
.3	Sun et al (10)	2004	Singapore	Asia	ETR D,E MIRU2,10,16,20,23,24,26,27,39 ,40	12	MIRU26,10, ETRE	MIRU2,2 0
.4	Kremer et al (11)	2005	China	Asia	ETR A-E MIRU10,16,26,39,40 QUB 11a,11b,26,1895	14	QUB 11b,11a MIRU10	MIRU16 ETRC
.5	Kovalev et al (12)	2005	Russian Federation	Asia	ETR D,E MIRU2,10,16,20,23,24,26,27,39 ,40	12	MIRU26, ETRE	MIRU24, 27
.6	Asgarzade et al (13)	2007	Iran	Asia	ETR D,E MIRU2,10,16,20,23,24,26,27,39 ,40	12	MIRU26, 40	MIRU16, 39
.7	Çavuşoğlu et al (14)	2007	Turkey	Asia	ETR D,E MIRU2,10,16,20,23,24,26,27,39 ,40	12	MIRU16,40,26	MIRU24, 27
.8	Maes et al (15)	2008	Venezuela	America	ETR A-E MIRU2,10,16,20,23,24,26,27,39 ,40 Mtub4,21,29,30,34,39 QUB 11b,26,4156	24	MIRU40,26, ETRB	MIRU20, 2, 24
.9	Alonso-Rodríguez et al (16)	2008	Spain	Europe	ETRA,C-E MIRU10,16,26,40 Mtub4,21,30,39 QUB 11b,26,4156	15	QUB, 26,11b, MIRU40, 10	ETRD
.10	Yun et al (17)	2009	Korea	Asia	ETRA-F MIRU2,10,16,20,23,24,26,27,39 ,40	6	MIRU26, ETRE,F	MIRU24, 20 ETRD
.11	Stavrum et al (18)	2009	South Africa	Africa	ETRA,C-E MIRU10,16,26,40 Mtub4,21,30,39 QUB 11b,26,4156	15	QUB 11b	ETRD
.12	Shamputa et al (19)	2010	South Korea	Asia	ETR A-E MIRU2,10,16,20,23,24,26,27,39 ,40 Mtub4,21,29,30,34,39 QUB 11b,26,4156	24	QUB 11b,26, Mtub4	MIRU2,2 4,23 ETRB,D
.13	Noguti et al (20)	2010	Brazil	America	ETR D,E MIRU2,10,16,20,23,24,26,27,39 ,40	12	MIRU40,23,10	MIRU24, 39 ETRD
.14	Jafarian et al (21)	2010	Iran	Asia	ETR D,E MIRU2,10,16,20,23,24,26,27,39 ,40	12	MIRU 26, 10,16	MIRU2, 24 ETRD
.15	Zhang et al (22)	2011	Cambodia	Asia	ETR A-E MIRU2,10,16,20,23,24,26,27,39 ,40 Mtub4,21,29,30,34,39 QUB 11b,26,4156	24	ETRD,Mtub 39, QUB26	Mtub 34, MIRU2,2 0
.16	Asgarzad et al (23)	2011	Iran	Asia	ETR A-E MIRU2,10,16,20,23,24,26,27,39 ,40	15	MIRU10,26,40	MIRU39, 2,4
.17	Bidovec-Stojkovi et al (24)	2011	Slovenia	Europe	ETR A-E MIRU2,10,16,20,23,24,26,27,39 ,40 Mtub4,21,29,30,34,39 QUB 11b,26,4156	24	QUB 26, 11b, MIRU40 ,10	MIRU24, 39
.18	Cerezo et al (25)	2012	Colombia	America	ETR D,E MIRU2,10,16,20,23,24,26,27,39 ,40	12	MIRU10, 40	MIRU24, 39
.19	Chatterjee et al (26)	2013	India	Asia	ETR D,E MIRU2,10,16,20,23,24,26,27,39 ,40	12	MIRU26, 10	MIRU2,2 0

.20	Zamani et al (27)	2013	Iran	Asia	ETRA,C-E MIRU10,16,26,40 Mtub4,21,30,39 QUB 11b,26,4156	15	MIRU16 ETRA	MIRU26, Mtub 21, 30, 39 QUB4156
.21	Joseph et al (28)	2013	India	Asia	ETRA,C-E MIRU10,16,26,40 Mtub4,21,30,39 QUB 11b,26,4156	15	ETRA,B MIRU40	MIRU2,2 0
.22	Yasmin et al (29)	2014	Pakistan	Asia	ETR A-E MIRU2,10,16,20,23,24,26,27,39 ,40 Mtub4,21,29,30,34,39 QUB 11b,26,4156	24	MIRU26 QUB26 MIRU10	MIRU2,2 0,27,24
.23	Ali et al (30)	2014	Pakistan	Asia	ETRA,C-E MIRU10,16,26,40 Mtub4,21,30,39 QUB 11b,26,4156	15	QUb26 ,MIRU10,26	ETRD
.24	Chaoui et al (31)	2014	Morocco	Africa	ETR D,E MIRU2,10,16,20,23,24,26,27,39 ,40	12	MIRU40,23,10	MIRU24, 39
.25	Zheng et al (32)	2014	China	Asia	ETR A-E MIRU2,10,16,20,23,24,26,27,39 ,40 Mtub4,21,29,30,34,39 QUB 11b,26,4156	24	QUB11b,26 Mtub 21 MIRU26	MIRU24 Mtub 34
.26	Vasconcellos et al (33)	2014	Brazil	America	ETR A-E MIRU2,10,16,20,23,24,26,27,39 ,40 Mtub4,21,29,30,34,39 QUB 11b,26,4156	24	QUB 4156, 11b MIRU10	MIRU24, 39
.27	Rindi et al (34)	2014	Italy	Europe	ETRA,D,E MIRU10,16,26,40 Mtub21 QUB 11b, 26 VNTR 42,43,47,52,53	15	QUB 26 MIRU 40 QUB 11b	MIRU 04 ETRE
.28	Bouklata et al (35)	2015	Morocco	Africa	ETR A-E MIRU2,10,16,20,23,24,26,27,39 ,40 Mtub4,21,29,30,34,39 QUB 11b,26,4156	24	QUB 26, MIRU40,26	MIRU20, 27
.29	Devi et al (36)	2015	India	Asia	ETR A-E MIRU2,10,16,20,23,24,26,27,39 ,40 Mtub4,21,29,30,34,39 QUB 11b,26,4156	24	Qub26, 11b MIRU10 QUB 4156 MIRU26 Mtub 21	MIRU2,2 0, 27
.30	Zamani et al (37)	2016	Iran	Asia	ETRA,C-E MIRU10,16,26,40 Mtub4,21,30,39 QUB 11b,26,4156	15	MIRU 40	MIRU 10 QUB 4156
.31	Hoza et al (38)	2016	Tanzania	Africa	ETRA,C-E MIRU2,10,16,20,23,24,26,27,39 ,40 Mtub4,21,29,30,39 QUB 11b,26,4156	22	MIRU26,10,16 ETRAE QUB 26, 4156	MIRU27, 2, 20
.32	Cheng et al (39)	2016	China	Asia	ETR A-E MIRU10,16,20,26,27,39,40 Mtub31 QUB 11a,11b	15	QUB11a	ETRB,C
.33	Bhembe et al (40)	2017	South Africa (Eastern Cape)	Africa	ETR D,E MIRU2,10,16,20,23,24,26,27,39 ,40	12	ETRE, MIRU27,24	MIRU40
.34	Zhang et al (41)	2017	China	Asia	ETR A-E MIRU 10,16,23,26,27,39,40 Mtub21,30,39	15	Mtub 21, MIRU26,10	ETRC,B
.35	Liu et al (42)	2017	China	Asia	ETRA-E MIRU10,16,23,26,27,39,40 Mtub21,30,39	15	MIRU26 Mtub 21	MIRU27, 23 ETRD
.36	Pasechnik et al (43)	2017	West Siberia	Asia	ETRA-D MIRU2,10,16,20,23,24,26,27,39 ,40	15	MIRU26	MIRU24 ETRB

.37	Pan et al (44)	2017	China	Asia	ETRA,D,E MIRU10,16,26,39,40 Mtub4,21,24,30,39 QUB11a,11b,18,26,3232,1895,4156 VNTR3820, 4120	22	VNTR3820 QUB3232	QUB4156 Mtub 24
.38	Khosravi et al (45)	2017	Iran	Asia	ETR D,E MIRU2,10,16,20,23,24,26,27,39,40	12	MIRU 10, 26	MIRU2,20
.39	Ravansalar et al (46)	2017	Iran	Asia	ETRA-F MIRU10,16,26,39,40 QUB 11b	12	MIRU10, 26 ETRF	ETRD
.40	Shah et al (47)	2017	Nepal	Asia	ETRA-F MIRU2,10,16,20,23,24,26,27,39,40 Mtub21,30,39 QUB 11b,11b, 26, 4156	24	QUB 26 MIRU10	ETR B,C
.41	Rasoaha et al (48)	2017	Madagascar	Africa	ETRA,C-E MIRU10,16,26,40 Mtub4,21,30,39 QUB 11b,26,4156	15	MIRU26 QUB 11b Mtub 21 QUB 26	ETRD,C Mtub4
.42	Chen et al (49)	2017	Asian countries (Cambodia, Singapore and Taiwan)	Asia	ETRA-E MIRU2,10,16,20,23,24,26,27,39,40 Mtub4,21,29,30,34,39 QUB 11b,26, 4156	24	Mtub 21 QUB 11b	MIRU2 QUB 4156
.43	Li et al (50)	2018	China	Asia	ETRA-E MIRU2,10,16,20,23,24,26,27,39,40 Mtub4,21,29,30,34,39 QUB 11b,26, 4156	24	Mtub4 MIRU40,10	Mtub21 MIRU27
.44	Xu et al (51)	2018	China	Asia	ETRA,C-E MIRU10,16,26,40 Mtub4,21,30,39 QUB 11b,26,4156	15	QUB 11b Mtub21 MIRU26	ETRC MIRU16 QUB 4156
.45	Esteves et al (52)	2018	Brazil	America	ETRA-E MIRU2,10,16,20,23,24,26,27,39,40 QUB11,26 VNTR42,1955,47,52,53,49	24	QUB26 QUB11 VNTR42	MIRU39, 24 ETRD
.46	Augusto et al (6)	2018	Brazil	America	ETRD,E MIRU2,10,16,20,23,24,26,27,39,40	12	MIRU16, 10,26	MIRU20, 24
.47	Riyahi Zaniani et al (53)	2018	Iran	Asia	ETRA-E MIRU2,10,16,20,23,24,26,27,39,40 Mtub4,21,29,30,34,39 QUB 11b,26, 4156	24	MIRU 10 QUB 26 Mtub 4,34	ETRD, MIRU 20, Mtub 29
.48	Azimi et al (54)	2018	Iran	Asia	ETRA,C-E MIRU10,16,26,40 Mtub4,21,30,39 QUB11b,26,4156	15	MIRU26, 10 Mtub 21 QUB 26	ETR D,E
.49	Li l et al (3)	2018	China	Asia	ETRA-E MIRU16,23,26,27,39,40 Mtub21,30,39	15	ETRE, MIRU10, 26,39,40 Mtub 21	ETRB, C MIRU16, 23
.50	Mansoori et al (55)	2018	Iran	Asia	ETRA-E MIRU2,10,16,20,23,24,26,27,39,40 Mtub4,21,29,30,34,39 QUB 11b,26, 4156	24	MIRU 10, 16, 26,	MIRU 2, 20 ETRD
.51	Chawla et al (56)	2018	India	Asia	ETRD,E MIRU2,10,16,20,23,24,26,27,39 40	12	MIRU 39,10,26	MIRU 2,20
.52	Shi et al (57)	2018	China	Asia	ETRA-F MIRU2,10,16,20,23,24,26,27,39,40 Mtub4,21,29,30,34,38,39 QUB 11b,26, 4156	26	QUB 11b Mtub 21 MIRU26	MIRU24, 2, 20

.53	Ei et al (58)	2018	Myanmar	Asia	ETRA-E MIRU2,10,16,20,23,24,26,27,39 ,40 Mtub4,21,29,30,34,39 QUB 11b,26, 4156	24	QUB 26 QUB 11b MIRU26	Mtu34 MIRU20
.54	Weerasekera et al (59)	2019	Sri Lanka	Asia	ETRA-E MIRU2,10,16,20,23,24,26,27,39 ,40 Mtub4,21,29,30,34,39 QUB 11b,26, 4156	24	Mtub 21,39 QUB 11b	MIRU20, 2 ,16
.55	Zarzour et al (60)	2019	Syria	Asia	ETRA-E MIRU2,10,16,20,23,24,26,27,39 ,40 Mtub4,21,29,30,34,39 QUB 11b,26, 4156	24	QUB 26 MIRU10,26	MIRU24, 20, Mtub 29
.56	Li et al (61)	2019	China	Asia	ETRA-E MIRU2,10,16,20,23,24,26,27,39 ,40 Mtub4,21,29,30,34,39 QUB 11b,26, 4156	24	QUB11b MIRU 26 Mtub21	MIRU2, 20,24

Table 2. Six MIRU-VNTR loci for *Mycobacterium tuberculosis* based on the number of studies and the reported allele diversity (h) range for each locus.

locus	Number of studies	h range
MIRU10	28	0.61 to 0.82
MIRU26	32	0.61 to 0.81
QUB26	18	0.604 to 0.89
MIRU40	17	0.604 to 0.76
QUB11b	17	0.72 to 0.84
Mtub21	12	0.64 to 0.83

Fig. 1. Location and numbers of studies was shown in the world from 2002 to 2019. Two studies were performed in different regions: Studies No. 2 and 42.

Discussion

An alarming 62% of emerging TB cases occurred in the South-East Asia and Western Pacific regions, followed by Africa, which accounts for 25% of new cases. Countries such as India, China, Indonesia, the Philippines, Pakistan, Bangladesh, Nigeria and South Africa hold a prevalence rate greater than 60% for TB. It is believed that the surge in TB research in Asia could be that TB is highly prevalent in this continent based on 2018 WHO report (2). Of the 56 studies extracted, 39 studies were conducted within Asia. More specifically, 11 were performed in China, ten in Iran, four in India, two in Pakistan, two in Korea and only one in Nepal, Singapore, Myanmar, Turkey, Cambodia, Sri Lanka, Syria, Russia, Siberia, Taiwan, and Cambodia. Among the 39 studies conducted in Asia, MIRU26 and MIRU10 was reported having high discriminatory power loci. Among the 7 studies conducted in the Western Hemisphere, four studies were carried out in Brazil, and one study was performed in Michigan, Venezuela and Colombia. Both MIRU40 and MIRU10 had high power discriminatory power loci in these ancients.

Among the six studies performed in Africa, four studies were conducted in South Africa and Morocco, respectively. One study, performed in Madagascar and Tanzania, reported that QUB26 and MIRU26 had high discriminatory power loci in this continent. Several investigations in this study used PCR-based techniques such as spacer oligonucleotide typing (spoligotyping) and mycobacterial interspersed repetitive units–variable-number of tandem repeats (MIRU-VNTR) analyses. Although spoligotyping benefits from genetic diversity, it can minimize *Mtb* clonal diversity. This method uses one direct repeat (DR) which incorporates identical alternate and variable spacers and the results are represented as a single digit pattern. The basis of MIRU-VNTR depends on the (variable) number of tandem repeat elements called mycobacterial interspersed repetitive units (MIRU). The results are represented as a code, and offers a low discriminatory power when used alone. The MIRU-VNTR method analyzes DNA segments which involves tandem repeat sequences and the copy number which differs amongst various strains. This method depends on PCR efficiency, specifically the quantity of repeats which is based on the size of the amplified product. The results are illustrated as characters that range from 15 –24 characters, in which each character represents the number of repeats at a single locus (3, 62).

These results are evaluated in comparison to a strain database on the web-based tool MIRU-VNTR plus. Therefore, MIRU-VNTR typing is considered the gold-standard for genotypic analysis of *Mtb* (7, 9). The discriminatory power of these methods was found to be different which was determined by the Hunter and Gaston Index factor .Several studies reported different Hunter-Gaston Index for MIRU-VNTR ranging from 0.951 to 0.999 (10, 13, 45, 47, 48, 53-56).

The allele diversity index (*h*) is used to describe the discriminatory power of MIRU-VNTR loci. If the index is greater than 0.6 ($h>0.6$), the discriminatory power of the locus is high. If the index lies between 0.3 and 0.6 ($0.3<h<0.6$), the locus has medium discriminatory power, however, if the index is less than 0.3 ($0.3>h$), the discriminatory power is considered weak. Our results suggest that among the 24 defined loci for MIRU-VNTR introduced by the MIRU-VNTR plus database, the MIRU26, MIRU10, MIRU40, QUB26, QUB11b and Mtub21 loci had the highest discriminatory powers, in contrast to the MIRU2, MIRU20, MIRU24 and ETRD loci which yielded low discriminatory powers.

*Mycobacterium bovis (*M. *bovis*) is the causative agent of TB in humans. When comparing the discriminatory powers of loci between *Mtb* and M. *bovis*, the loci QUB 11b and QUB 3232 have the highest discriminatory powers (for M. *bovis*), whereas ETRD (for both strains) and MIRU10 (for M. *bovis*) had the lowest discriminatory powers. Therefore, it can be concluded from our study that QUB 11b in both *Mtb* and *M. bovis* has a high discriminatory power while ETRD for both strains is considered a low power locus. However, a difference exists between the two

strains in the MIRU10 locus which has a high discriminatory power in *Mtb* but low in *M. bovis* (63).

While the 24 loci MIRU-VNTR has been introduced as the best typing method for *Mtb* based on multiple comparisons between various molecular techniques, it is, therefore, highly recommended to include the following loci, QUB26, QUB11b, MIRU10, MIRU26, MIRU40 and Mtub21, provided that MIRU-VNTR is done with less than 24 loci, in order to obtain the best results when genotyping *Mtb* isolates. In this regard, data extraction becomes inexpensive and time efficient.

Among typing methods, MIRU-VNTR is considered to be one of the best. Further, the 6 loci including MIRU10, MIRU26, MIRU40, QUB 26, QUB 11b and Mtub21, all of which have high discriminatory powers, are recommended in *Mtb* genotyping to save time and cost. Indeed, epidemiological studies are critical for disease surveillance of TB as they enable us to track circulating strains on a global scale, as well as for identifying important risk factors necessary for implementing control measures in vulnerable populations worldwide.

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