

Original Article

## Predominance of Central Asian strain (ST 26) in *Mycobacterium tuberculosis* isolates from Balochistan by spoligotyping

Muhammad Shafee<sup>1</sup>, Ferhat Abbas<sup>1</sup>, Zunera Tanveer<sup>2</sup>, Andrew Whitelaw<sup>3</sup>, Lemese Ah Tow<sup>4</sup>, Muhammad Ashraf<sup>5</sup>, Irshad Ahmad<sup>6,7</sup>, Simon G Patching<sup>6</sup>, Abdul Jabbar<sup>8</sup>, Ali Akbar<sup>9</sup>, Mohammad Zahid Mustafa<sup>1</sup>

<sup>1</sup> Centre for Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan, Quetta, Pakistan

<sup>2</sup> Department of Physiology, Bloan University of Medical and Health Sciences, Quetta, Pakistan

<sup>3</sup> Division of Medical Microbiology, University of Stellenbosch, Cape Town, South Africa

<sup>4</sup> Division of Medical Microbiology, Department of Pathology, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa

<sup>5</sup> Fatima Jinnah Chest and General Hospital, Quetta, Pakistan

<sup>6</sup> School of Biomedical Sciences and Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, United Kingdom

<sup>7</sup> Institute of Basic Medical Sciences, Khyber Medical University, Peshawar, Pakistan

<sup>8</sup> Department of Medical Lab Technology, University of Haripur, Haripur, Pakistan

<sup>9</sup> Department of Microbiology, Faculty of Life Sciences, University of Balochistan, Quetta, Pakistan

### Abstract

**Introduction:** Tuberculosis is a chronic debilitating infectious disease causing a severe challenge to public health, especially in developing countries. The aim of this study was to examine genetic diversity in *Mycobacterium tuberculosis* strains circulating in the Balochistan region of Pakistan.

**Methodology:** One hundred isolates collected from patients visiting the Fatima Jinnah TB Hospital in Quetta were subjected to genotype analysis by spoligotyping.

**Results:** Three main genotypes were identified: Central Asian Strain 1 (CAS1) (n = 89), East African Indian (EAI) strain (n = 7) and Latin American Mediterranean (LAM) strain (n = 3). The CAS1 clade (ST 26) had high genetic diversity represented by seven different spoligopatterns, of which one had major predominance (n = 75).

**Conclusions:** This is the first insight into the genotype of *M. tuberculosis* strains in the Balochistan region that might serve as a base line study for control of tuberculosis in the community.

**Key words:** Genotyping; spoligotyping; *Mycobacterium tuberculosis*; Central Asian strain; Pakistan.

*J Infect Dev Ctries* 2019; 13(7):619-625. doi:10.3855/jidc.10803

(Received 24 August 2018 – Accepted 06 June 2019)

Copyright © 2019 Shafee *et al.* This is an open-access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

### Introduction

Tuberculosis remains a major health concern throughout the world, especially in developing countries. This is despite effective treatments, diagnostic and preventive measures that have become available in the last few decades. Molecular based typing techniques for the causative *Mycobacterium tuberculosis* (*M. tuberculosis*) complex have emerged as effective tools based on variation in insertion sequences and repetitive genomic sequences [1-3]. *M. tuberculosis* has a highly conserved genome containing useful markers that can be exploited in different typing techniques, such as those using Insertion Sequence

IS6110 [4], Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats (MIRU-VNTR) [5], Large Sequence Polymorphism (LSP) [6], Restriction Fragment Length Polymorphism (RFLP) [7] and reverse line dot blot spoligotyping [8,9].

Based on sequencing of *M. tuberculosis* strain H37Rv, the complete genome contains 4,411,539 base pairs with densely packed coding regions. It comprises approximately 4,000 protein coding regions and has a very high guanine and cytosine content of 65.6%. Comparative genome sequencing of *M. tuberculosis* strains revealed that they have an overall genomic similarity of 99.9% [10]. The genome is rich in

repetitive DNA with two types of repeat sequences, interspersed repeats and tandem repeats. Interspersed repeats include insertion sequences (IS), which are small mobile genetic elements of less than 2.5 kb in size, and direct repeats (DRs), which are 36-bp sequences in the chromosomal region interspersed by unique 35 to 41 bp spacer DNA sequences. DRs are members of the universal Clustered Regularly Interspersed Palindromic Repeats (CRISPR) DNA sequence family also present in the *M. tuberculosis* complex with poorly known physiological activity [11]. Tandem repeats are arrays of consecutive base pair repeats in the noncoding region of *M. tuberculosis* DNA.

Spoligotyping (or spacer oligonucleotide typing) is a PCR-based reverse hybridization technique widely used for strain typing of *M. tuberculosis* [8,9]. It is based on polymorphism in the DR region of *M. tuberculosis* strains, where it identifies the presence or absence of 43 spacer DNA sequences between the variable DRs. PCR amplification of the DR locus uses primers, one of which is marked with biotin. PCR products are hybridized perpendicularly to a membrane containing 43 oligonucleotides of known sequence. The membrane is incubated with streptavidine-peroxydase conjugate, which links to biotin on the PCR products, then hybridization signals are detected by a chemiluminescence system. The order of spaces in the DR regions of different strains and isolates can then be compared. Spoligotyping is a robust, rapid, highly sensitive, specific and cost-effective technique alternative to traditional IS6110 genotyping. It is a valuable tool for epidemiological studies and for investigating genetic diversity of *M. tuberculosis* isolates.

Tuberculosis cases have highest prevalence in developing countries, especially those in Asia. Indeed, the countries of China, India, Bangladesh, Indonesia and Pakistan collectively contribute 50% of the global burden [12], with Pakistan standing at fifth position [13]. The population of Pakistan, which is approximately 193 million people, has a tuberculosis incidence rate of 268 per 100,000 with an estimated 510,000 new cases emerging each year (World Health Organization, 2017) [13]. The emergence and spread of multidrug-resistant tuberculosis in Pakistan, approximately 15,000 cases per year, presents a further critical challenge [14-17]. There are limited studies on the molecular epidemiology of tuberculosis in Pakistan. Using spoligotyping to test isolates from all over the country, Hasan *et al.* [18] reported 22 different types of genogroups with 39% Central Asian Strains (CAS) and

6% Beijing strains. A study of 926 *M. tuberculosis* isolates from all over the country identified the predominant genotypes as Central Asian Strains (including CAS1, CAS sub-families and Orphan Pak clusters), East African-Indian (EAI) strains (4%), Beijing strains (3%), poorly defined TB strains (2%), 2% Haarlem and LAM (2%) [19]. Interestingly, the prevalence of CAS1 was significantly higher in Punjab than in Sindh, North West Frontier Province and the Balochistan province. Also, multidrug resistance was significantly associated with the Beijing strains and not with the CAS strains [19]. A study of 1,004 pulmonary tuberculosis patients in Karachi identified the most prevalent genotype as CAS (55.6%), followed by East African Indian (EAI) (9.6%), T clade (4.9%), Haarlem (3.1%), Beijing (2.7%), U clade (2.5%), MANU (0.2%) and Lam American Mediterranean (LAM) (0.4%). Multidrug resistance was significantly associated with the Haarlem genotype [20]. MIRU-VNTR typing of *M. tuberculosis* isolates from Pakistan revealed that they were comprised of 113 CAS lineages and 87 non-CAS lineages [21]. A recent study of multidrug-resistant *M. tuberculosis* isolates (n = 127) from the Punjab region identified three main clades of CAS1\_DELHI (n = 53, 41.7%), T1 (n = 14, 11.0%) and Beijing (n = 10, 7.8%) strains [22].

Despite global and regional efforts to combat the disease, tuberculosis still poses a great threat at the international, national and regional level. Balochistan is the largest province of Pakistan comprising of 44% of the land with a scattered population. The large majority of people in Balochistan live in rural areas that have poor access to health care facilities. A large proportion of the provincial population is also poorly educated and poverty prevails throughout the province. This study was designed to explore genetic diversity in *M. tuberculosis* among clinical isolates from Balochistan.

## Methodology

### Study area

This study was conducted at the Fatima Jinnah TB Hospital in the city of Quetta (Balochistan, Pakistan). Sputum samples were routinely collected from patients visiting the hospital from all remote areas of the province. The study was approved by the Ethics Committee of the Fatima Jinnah TB Hospital and all patients provided written informed consent in accordance with the Declaration of Helsinki.

### Sample collection and preparation

At the Fatima Jinnah TB Hospital, sputum samples were routinely tested for presence of the tuberculosis

bacterium by smear microscopy with Ziehl-Neelsen staining [23,24]. A total of 100 tuberculosis-infected sputum samples were randomly selected from the hospital laboratory. Epidemiological information for the 100 patients from which clinical samples were used is given in Table 1. Cultures were isolated from the samples by growth on Lowenstein-Jensen media in order to have colonies for strain typing. All of the isolates were aseptically collected and mixed with distilled water (700  $\mu$ L). The isolates were then heat-killed at 80 °C for 60 minutes and shipped to the Division of Medical Microbiology at the University of Cape Town, South Africa following International Air Transport Association (IATA) shipment rules.

#### Genomic DNA extraction

DNA was extracted from heat-killed bacterial cultures using a previously established cetyl trimethylammonium bromide (CTAB) method [25]. Briefly, after thawing the samples at 60 °C in a thermo mixer, 10% SDS (70  $\mu$ L) and proteinase K (50  $\mu$ L) were added, mixed and incubated at 60 °C for 1 hour. Then 5 M NaCl (100  $\mu$ L) and CTAB (10% final concentration) were added to each tube followed by incubation at 60 °C for 15 minutes. The tubes were held at -70 °C for 15 minutes followed by thawing at 60 °C for 15 minutes. After cooling, 24:1 chloroform/isoamyl alcohol (700  $\mu$ L) was added followed by centrifugation (12,000 rpm, 10 minutes). The supernatant was then aspirated and transferred into a new tube. Cold isopropanol (700  $\mu$ L) was added to the tube and incubated at -20 °C for 30 minutes followed by centrifugation (12,000 rpm, 10 minutes). The supernatant was then discarded and the DNA pellet was dried with 70% ethanol and resuspended in molecular grade water (100  $\mu$ L).

#### DNA quantification and spoligotyping

All samples were subjected to quantification of genomic DNA and evaluation of protein and RNA impurities using a  $\mu$ LITE instrument (Biodrop, UK) following the manufacturer's instructions. PCR

reaction mixtures (25  $\mu$ L) were prepared using specific biotinylated primer pairs (DRa-F 5'-GGTTTTGGGTCTGACGAC-3' and DRb-R 5'-CCGAGAGGGGACGGAAAC-3) as described previously [25]. PCR amplification was performed over 35 cycles with initial denaturation at 96 °C for 3 minutes, denaturation at 96 °C for 60 seconds, annealing at 55 °C for 60 seconds and extension at 72 °C for 30 seconds. The final extension was carried out at 72 °C for 5 minutes and the banding pattern was confirmed by running the product on a 2% agarose gel.

For hybridization of the membrane, four different solutions were prepared from the stock solution: 250 mL of 2X SSPE/0.1% SDS and 250 mL of 2X SSPE kept at 60 °C; 250 mL of 2X SSPE/0.5% SDS and 250 mL of 2X SSPE kept at 42 °C. Spoligotyping based on the presence or absence of any of 43 spacers in the direct repeat region in the *M. tuberculosis* genome was carried out using a spoligotyping kit from Ocimum Biosolutions (India) following the manufacturer's instructions. The membrane was then placed in developer for 2 minutes and in fixer for 1 minute followed by rinsing with water. The black spots/blocks identified the presence or absence of any spacer.

The spoligotyping banding pattern was converted to the octal format using a two-stage process. First, the 43-digit binary codes were divided into 14 sets of three digits plus the last additional remaining digit. Secondly, each three-digit binary set was converted to its octal equivalent code. Genotypes were assigned using the MIRU-VNTRplus web tool (<https://www.miru-vntrplus.org/MIRU/index.faces>) [26,27]. A dendrogram was produced by unweighted pair group analysis using average linkage.

## Results

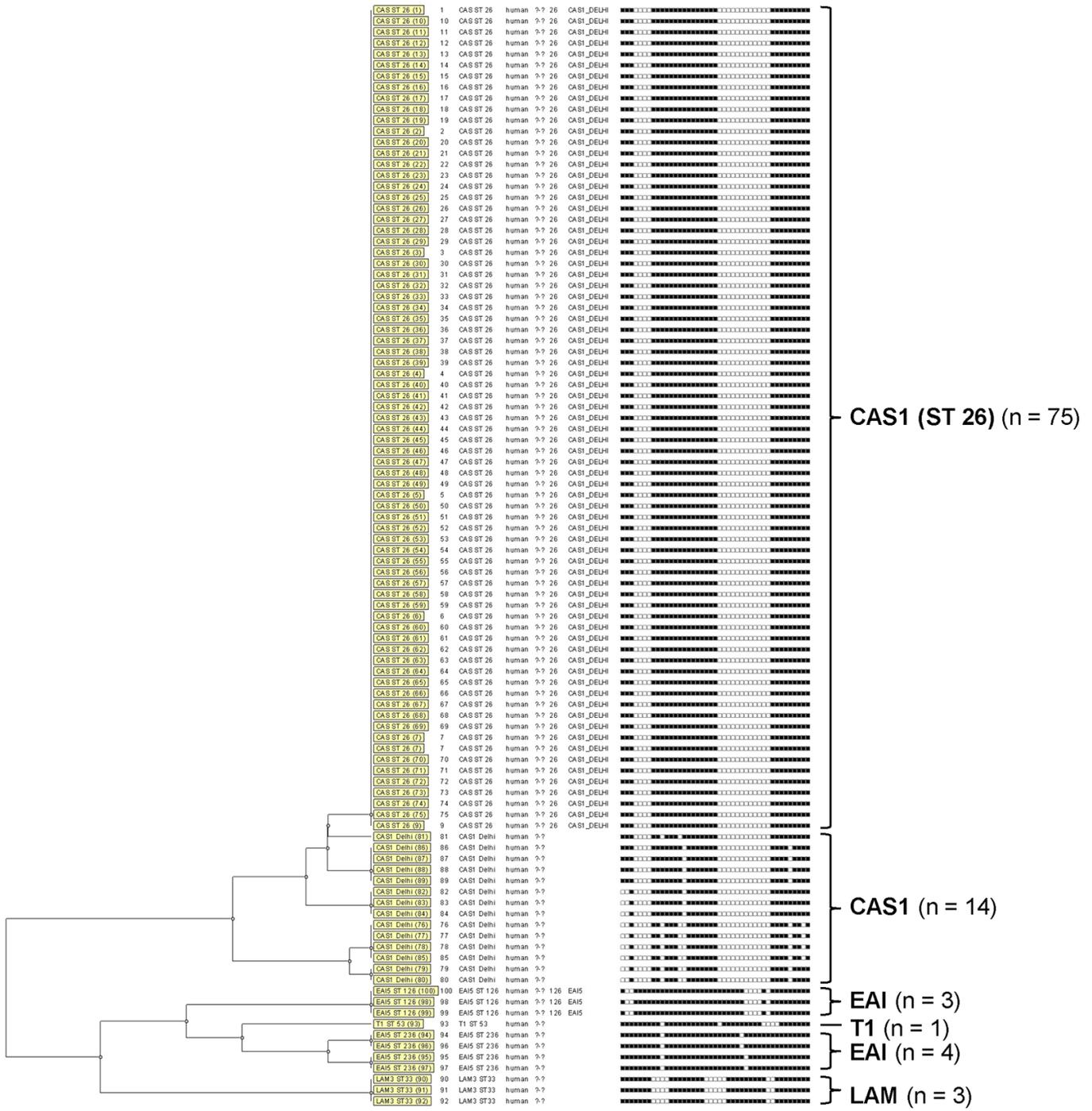
Out of 100 isolates, CAS was the predominant clade (n = 89), represented by a CAS1 Delhi (ST 26) spoligopattern (octal format 703777740003771) characterized by deletion of spacers 4-7 and 23-34 in the large majority of cases (n = 75). Fourteen other

**Table 1.** Epidemiological information for patients from which clinical samples were used.

Patient characteristic		Number (out of 100)
Gender	Male	40
	Female	60
Age groups	20-40 years	30
	41-60 years	55
	>60 years	15
Location	Urban	20
	Rural	80



Figure 1. Dendrogram of *M. tuberculosis* isolates from Balochistan, Pakistan.



A dendrogram was constructed based on the Jacquard index for pairwise analysis of strains. The clustering pattern of 100 isolates is illustrated. The four most predominant shared spoligotypes, CAS (n = 89), EAI (n = 7), LAM (n = 3) and T1 (n = 1) were recorded.

EAI was the second most prevalent genotype in our study (7%), which has a spoligopattern characterized by presence of spacer 33 and absence of spacer 34. The EAI genotype has been isolated from all provinces of Pakistan [18,19,30] and has also been documented in the neighboring countries of Afghanistan, India, Bangladesh and Iran. For example, EAI was documented in India with prevalences of 8% from Delhi [32] and 17% from Mumbai [37]. A much higher prevalence of 80% has been recorded from southern India. Ancient EAI strains have also been documented from Bangladesh, Sri Lanka, Europe, Far East countries and the Indo-Pak subcontinent [38].

The LAM genotype (3%) was found in our study with a unique spoligopattern (ST 33). LAM was also isolated from water samples in Iran [39], suggesting that it might be transmitted across the border between the two countries. The LAM genotype is relatively rare in Pakistan, but a few cases have been reported from the Sindh province [19], which shares a border with Balochistan. A single isolate was identified as belonging to the T1 family (ST 53).

## Conclusion

The results of our study reflect high prevalence and high genetic diversity of the *M. tuberculosis* CAS family (ST 26) in the Balochistan (city of Quetta) region of Pakistan, followed by EAI, LAM and T1 genotypes. This provides baseline information on the genetic diversity of *M. tuberculosis* in the region. Further research encompassing a greater population throughout the province should be planned for a more detailed study of the genotypes in a diversified population of Balochistan.

## Acknowledgements

The authors are thankful to Prof. Mark Nicol, Widad Zemanay, Rajesh Sarkar, Layla Adonis Hendrick, Clinton Moodley, Felix Dube and Mrs Shima at the Division of Medical Microbiology, University of Cape Town, South Africa for their full support and technical guidance.

## Financial support

The authors thank the Higher Education Commission (HEC) of Pakistan International Research Support Initiative Program (IRSIP) for financial support to conduct the experiments abroad.

## References

- van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, Hermans P, Martin C, McAdam R, Shinnick TM (1993) Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J Clin Microbiol* 31: 406-409.
- Ei PW, Aung WW, Lee JS, Choi GE, Chang CL (2016) Molecular strain typing of *Mycobacterium tuberculosis*: a review of frequently used methods. *J Korean Med Sci* 31: 1673-1683.
- Ravansalar H, Tadayon K, Ghazvini K (2016) Molecular typing methods used in studies of *Mycobacterium tuberculosis* in Iran: a systematic review. *Iran J Microbiol* 8: 338-346.
- Roychowdhury T, Mandal S, Bhattacharya A (2015) Analysis of IS6110 insertion sites provide a glimpse into genome evolution of *Mycobacterium tuberculosis*. *Sci Rep* 5: 12567.
- Iakhiaeva E, McNulty S, Brown Elliott BA, Falkinham JO 3rd, Williams MD, Vasireddy R, Wilson RW, Turenne C, Wallace RJ Jr (2013) Mycobacterial interspersed repetitive-unit-variable-number tandem-repeat (MIRU-VNTR) genotyping of *mycobacterium intracellulare* for strain comparison with establishment of a PCR-based database. *J Clin Microbiol* 51: 409-416.
- Alland D, Lacher DW, Hazbón MH, Motiwala AS, Qi W, Fleischmann RD, Whittam TS (2007) Role of large sequence polymorphisms (LSPs) in generating genomic diversity among clinical isolates of *Mycobacterium tuberculosis* and the utility of LSPs in phylogenetic analysis. *J Clin Microbiol* 45: 39-46.
- Williams RC (1989) Restriction fragment length polymorphism (RFLP). *Am J Phys Anthropol* 32 Suppl 10: 159-184.
- Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, Bunschoten A, Molhuizen H, Shaw R, Goyal M, van Embden J (1997) Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J Clin Microbiol* 35: 907-914.
- Driscoll JR (2009) Spoligotyping for molecular epidemiology of the *Mycobacterium tuberculosis* complex. *Methods Mol Biol* 551: 117-128.
- Orduz ML, Ribón W (2015) Molecular epidemiology of tuberculosis. In Ribón W, editor. *Tuberculosis - expanding knowledge*. London: IntechOpen. 43-64.
- Mojica FJ, Diez-Villasenor C, Garcia-Martinez J, Soria E (2005) Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J Mol Evol* 60: 174-182.
- Mathema B, Kurepina NE, Bifani PJ, Kreiswirth BN (2006) Molecular epidemiology of tuberculosis: current insights. *Clin Microbiol Rev* 19: 658-685.
- World Health Organisation (2017) Global Tuberculosis Report Available: [http://www.who.int/tb/publications/global\\_report/en/](http://www.who.int/tb/publications/global_report/en/). Accessed: 20 March 2019.
- Hasan R, Jabeen K, Ali A, Rafiq Y, Laiq R, Malik B, Tanveer M, Groenheit R, Ghebremichael S, Hoffner S, Hasan Z (2010) Extensively drug-resistant tuberculosis, Pakistan. *Emerg Infect Dis* 16: 1473-1475.
- Akhtar AM, Arif MA, Kanwal S, Majeed S (2016) Prevalence and drug resistance pattern of MDR TB in retreatment cases of Punjab, Pakistan. *J Pak Med Assoc* 66: 989-993.
- Javaid A (2017) Multi-drug-resistant tuberculosis: Current situation in Pakistan. *Pak J Chest Med* 23: 28-30.

17. Sheikh AS, Aziz M, Ayaz M (2018) Rising trends of multidrug resistant (MDR) tuberculosis in Pakistan. *Biomed J Sci Tech Res* 12: 9245-9248.
18. Hasan Z, Tanveer M, Kanji A, Hasan Q, Ghebremichael S, Hasan R (2006) Spoligotyping of *Mycobacterium tuberculosis* isolates from Pakistan reveals predominance of Central Asian Strain 1 and Beijing isolates. *J Clin Microbiol* 44: 1763-1768.
19. Tanveer M, Hasan Z, Siddiqui AR, Ali A, Kanji A, Ghebremichael S, Hasan R (2008) Genotyping and drug resistance patterns of *M. tuberculosis* strains in Pakistan. *BMC Infect Dis* 8: 171.
20. Ayaz A, Hasan Z, Jafri S, Inayat R, Mangi R, Channa AA, Malik FR, Ali A, Rafiq Y, Hasan R (2012) Characterizing *Mycobacterium tuberculosis* isolates from Karachi, Pakistan: drug resistance and genotypes. *Int J Infect Dis* 16: e303-309.
21. Ali A, Hasan Z, Jafri S, Inayat R, Hasan R (2014) *Mycobacterium tuberculosis* Central Asian Strain (CAS) lineage strains in Pakistan reveal lower diversity of MIRU loci than other strains. *Int J Mycobacteriol* 3: 108-116.
22. Bakula Z, Javed H, Pleń M, Jamil N, Tahir Z, Jagielski T (2019) Genetic diversity of multidrug-resistant *Mycobacterium tuberculosis* isolates in Punjab, Pakistan. *Infect Genet Evol* 72: 16-24.
23. Bishop PJ, Neumann G (1970) The history of the Ziehl-Neelsen stain. *Tubercle* 51: 196-206.
24. Singhal R, Myneedu VP (2015) Microscopy as a diagnostic tool in pulmonary tuberculosis. *Int J Mycobacteriol* 4: 1-6.
25. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Sturhl K (1995) Current protocols in molecular biology, 3rd edition. New York: John Wiley and Sons. 4648 p.
26. Allix-Béguec C, Harmsen D, Weniger T, Supply P, Niemann S (2008) Evaluation and user-strategy of MIRU-VNTRplus, a multifunctional database for online analysis of genotyping data and phylogenetic identification of *Mycobacterium tuberculosis* complex isolates. *J Clin Microbiol* 46: 2692-2699.
27. Weniger T, Krawczyk J, Supply P, Niemann S, Harmsen, D (2010) MIRU-VNTRplus: a web tool for polyphasic genotyping of *Mycobacterium tuberculosis* complex bacteria. *Nucleic Acids Res* 38: W326-W331.
28. Gagneux S, DeRiemer K, Van T, Kato-Maeda M, de Jong BC, Narayanan S, Nicol M, Niemann S, Kremer K, Gutierrez MC, Hilty M, Hopewell PC, Small PM (2006) Variable host-pathogen compatibility in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* 103: 2869-2873.
29. Yimer SA, Norheim G, Namouchi A, Zegeye ED, Kinander W, Tønjum T, Bekele S, Mannsäker T, Bjune G, Aseffa A, Holm-Hansen C (2015) *Mycobacterium tuberculosis* lineage 7 strains are associated with prolonged patient delay in seeking treatment for pulmonary tuberculosis in Amhara Region, Ethiopia. *J Clin Microbiol* 53: 1301-1309.
30. Shakoor S, Tanveer M, Rafiq Y, Hasan Z, Javed A, Rizvi N, Rehman N, Hasan R (2009) Prevalence of ST26 among untreated smear-positive tuberculosis patients from Karachi indicating ongoing transmission. *Scand J Infect Dis* 41: 714-719.
31. Kanji A, Hasan Z, Tanveer M, Laiq R, Hasan R (2011) Occurrence of RD149 and RD152 deletions in *Mycobacterium tuberculosis* strains from Pakistan. *J Infect Dev Ctries* 5: 106-113. doi: 10.3855/jidc.1112
32. Singh UB, Suresh N, Bhanu NV, Arora J, Pant H, Sinha S, Aggarwal RC, Singh S, Pande JN, Sola C, Rastogi N, Seth P (2004) Predominant tuberculosis spoligotypes, Delhi, India. *Emerg Infect Dis* 10: 1138-1142.
33. Arora VK, Chopra KK (2007) Extra pulmonary tuberculosis. *Indian J Tuberc* 54: 165-167.
34. Vadwai V, Shetty A, Supply P, Rodrigues C (2012) Evaluation of 24-locus MIRU-VNTR in extrapulmonary specimens: study from a tertiary centre in Mumbai. *Tuberculosis* 92: 264-272.
35. Mozafari M, Farnia P, Afraei M, Derakhshani-Nezhad Z, Masjedi MR, Velayati AA (2013) Molecular diversity of *Mycobacterium tuberculosis* strains in different provinces of Iran. *Iran J Microbiol* 5: 366-373.
36. Merza MA, Farnia P, Salih AM, Masjedi MR, Velayati AA (2011) First insight into the drug resistance pattern of *Mycobacterium tuberculosis* in Dohuk, Iraq: using spoligotyping and MIRU-VNTR to characterize multidrug resistant strains. *J Infect Public Health* 4: 41-47.
37. Kulkarni S, Sola C, Filliol I, Rastogi N, Kadival G (2005) Spoligotyping of *Mycobacterium tuberculosis* isolates from patients with pulmonary tuberculosis in Mumbai, India. *Res Microbiol* 156: 588-596.
38. Khorshidi A, Rohani M, Moniri R, Torfeh M (2009) Comparison of culture and microscopic methods by PCR for detection of *Mycobacterium tuberculosis* in sputum. *Iran J Clin Infect Dis* 4: 228-232.
39. Velayati AA, Farnia P, Mozafari M, Malekshahian D, Farahbod AM, Seif S, Rahideh S, Mirsaeidi M (2015) Identification and genotyping of *Mycobacterium tuberculosis* isolated from water and soil samples of a metropolitan city. *Chest* 147: 1094-1102.

### Corresponding author

Muhammad Shafee, DVM, MPhil, PhD  
 Centre for Advanced Studies in Vaccinology and Biotechnology,  
 University of Balochistan, Brewery Road, Quetta, 87300, Pakistan  
 Tel: 0092-81-2853843  
 Cell: 0092-333-7837828  
 Fax: 0092-819213134  
 E-mail: shafeedr73@gmail.com, muhammad.shafee@mail.com

**Conflict of interests:** No conflict of interests is declared.