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Original Article

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

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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 predominace (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.

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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 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 chemiluminiscence 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 µL). 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 µL) and proteinase K (50 µL) were added, mixed and incubated at 60 °C for 1 hour. Then 5 M NaCl (100 µL) 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 µL) was added followed by centrifugation (12,000 rpm, 10 minutes). The supernatant was then aspirated and transferred into a new tube. Cold isopropanol (700 µL) 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 µL).

DNA quantification and spoligotyping

All samples were subjected to quantification of genomic DNA and evaluation of protein and RNA impurities using a µLITE instrument (Biodrop, UK) following the manufacturer's instructions. PCR reaction mixtures (25 µL) 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 spogliotyping 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 dendogram 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

Table 2. Spoligopatterns for *M. tuberculosis* isolates from Balochistan, Pakistan.

S. No	Binary form	Octal form	Similarity with family	ST No.	No. of Isolates
1	■■■□□□□■■■■■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■	703777740003771	CAS1 Delhi	26	75
2	□□■□□□□■■□■■■□□■■■■■■■□□□□□□□□□□□□■■■■□■■□■	703347740003661	CAS 1 Delhi	26	3
3	□□■□□□□■■□■■■□□■■■■■■■□□□□□□□□□□□□■■■■□■■■■	103347740003671	CAS 1 Delhi	26	2
4	■■■□□□□■■□■■■□■■■■■■■■□□□□□□□□□□□□■■■■■■■■■	703357740003771	CAS 1 Delhi	26	1
5	□□■□□□□■■■■■■■□■■■■■■■□□□□□□□□□□□□■■■■■■■■■	103767740003771	CAS 1 Delhi	26	3
6	□□■□□□□■■□■■■□□■■■■■■■□□□□□□□□□□□□■■■■□■■□■	103347740003661	CAS	26	1
7	■■■□□□□■■■■■■■□■■■■■■■□□□□□□□□□□□□■■■■□■■■■	703767740003671	CAS1 Delhi	26	4
8	■■■■■■■□□□□■■■■■■■■□□□□□■■■■■■■■■□□■■■■■■■■	774177407771771	LAM3	33	3
9	■■■■■■■■■□■■■■■■■■■■■■□■■■■■■■■■■□□□□■■■■■■■	777377757760771	T1	53	1
10	■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■	777377777577771	EAI 5	236	4
11	■□□■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■	777777777413771	EAI 5	236	3
	Total isolates				**100**

■ = presence of spacer (1); □ = absence of spacer (0); ST. No = spoligotype number; Key for octal formation: 000 = 0, 001 = 1,010 = 2, 011 = 3, 100 = 4, 101 = 5, 110 = 6, 111 = 7, 0 = 0, 1 = 1.

isolates with six unique spoligopatterns (octal format 703347740003661, 103347740003671, 703357740003771, 103767740003771, 103347740003661, 703767740003671) were also found that matched best with the ST 26 strain genotype according to addition or deletion of spacers (Table 2 and Figure 1).

East African Indian (EAI) was the second most predominant clade with octal formats 777377777577771 (n = 4) and 777777777413771 (n = 3), accounting for seven isolates with the ST 236 genotype. Three isolates were best matched with a Latin American Mediterranean strain LAM3 with octal format 774177407771771 (ST 33). A single isolate was best matched with the T1 family having octal format 777377757760771 (ST 53) (Table 2 and Figure 1).

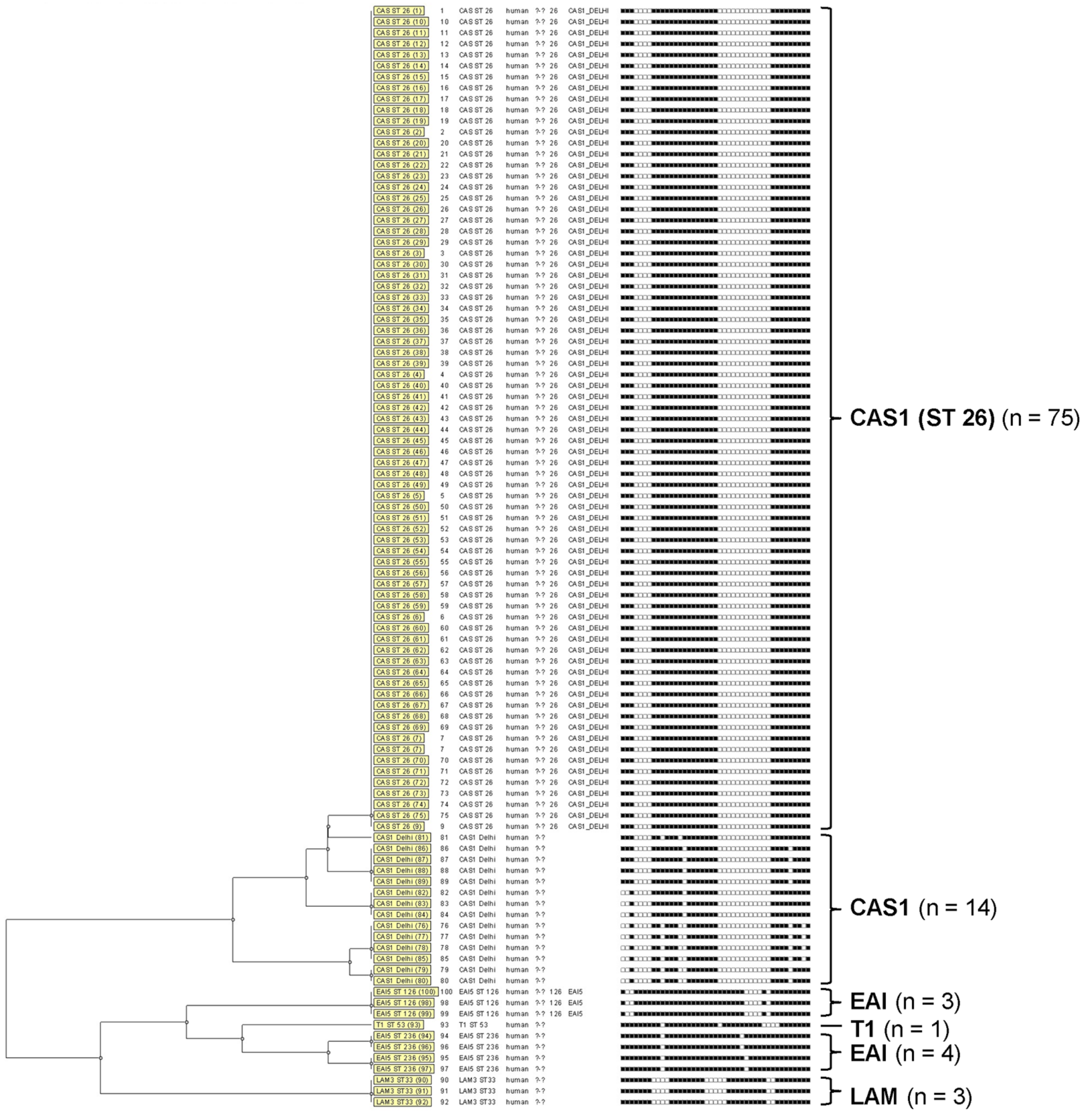
Discussion

Tuberculosis is a major infectious disease that leads to serious health effects and is prevalent in many parts of the world. It is the ninth leading cause of death worldwide and the leading cause from a single infectious agent, now ranking above HIV/AIDS. In this respect, Pakistan is included in the top five list of high burden countries [13]. The various techniques used for genotyping of Mycobacterium strains include spoligotyping, for which different spoligopatterns have been reported in different countries [21]. Seven distinct lineages of *M. tuberculosis* strains predominate in various parts of the world. The lineages are Indo-Oceanic, East Asian (including "Beijing"), Central Asian (CAS)/Delhi, Euro-American (including Latin American-Mediterranean, Haarlem, X type and T families), West African 1, West African 2, respectively, and lineage 7 in Ethiopia [28,29].

Our study evaluated *M. tuberculosis* genotypes in 100 clinical isolates from the Balochistan region of Pakistan using reverse line dot blot spoligotyping. The main genotypes identified were CAS, EAI, LAM and T1, for which the CAS family accounted for 89% of spoligopatterns, followed by EAI (n = 7), LAM (n = 3) and T1 (n = 1) (Table 2). In a previous study from Karachi, Pakistan 445 isolates were reported as CAS1 (ST 126) [19]. Indeed, our findings corroborate a number of previous studies from Karachi, which identified CAS1 in 41% [18], 44% [19] and 66% [30] and of isolates. In our study, a spoligopattern for CAS1 (ST 26) characterized by deletion of spacers 4-7 and 23-34 was predominant (n = 75). This spoligopattern was found in other studies from Karachi, which reported prevalences of 39% [18] and 40% [30]. A predominance of CAS1 has also been reported from Punjab [19,21,31], which is the largest province in Pakistan and shares a border with India. CAS1 does circulate in India as reported in Delhi [32] and Mumbai [33,34], so there is likely to be transmission of CAS1 across the border. It is evident from our study and from other studies that the CAS family is the most common circulating genotype of *M. tuberculosis* in the Balochistan region and in Pakistan as a whole.

The Balochistan region of Pakistan shares borders with Afghanistan and with Iran and there is easy and frequent movement of people across the country borders. The high circulation of CAS1 in Balochistan might be due to the fact that Afghanistan and Iran both have high levels in their populations. Frequent influx from Afghanistan and Iran may contribute to transmission and distribution of the CAS1 genotype in the Balochistan region [35]. It is noteworthy that the CAS1 genotype is predominantly limited to Middle Asian countries [36].

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.

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