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Original Article

Occurrence of RD149 and RD152 deletions in *Mycobacterium tuberculosis* strains from Pakistan

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Abstract

Introduction: Central Asian Strain 1 (CAS1) is the predominant *Mycobacterium tuberculosis* genotype in Pakistan. The occurrence of deletions in regions of differences (RDs) among CAS1 and other predominant genogroups in the country were investigated.

Methodology: Using stratified random sampling, 235 *M. tuberculosis* (185 pulmonary; 50 extrapulmonary) strains were selected from 926 previously spoligotyped isolates, including 171 CAS strains (133 CAS1 (ST26), 38 CAS subfamily), 8 Beijing isolates, 47 isolates belonging to other previously defined ("Other") clusters, and 9 previously undefined Unique isolates. Commonly reported RD deletions, RD1, RD750, RD207, RD149, RD152, RD105, RD150, RD142 and RD181, were investigated using a PCR-based method.

Results: Deletions in RDs 750, 149 and 152 were identified among CAS strains, and in RDs 207, 149, 152, 105, 150, 142 and 181 in Beijing isolates. CAS1 strains showed more frequent RD149 deletions compared with CAS subfamily strains ($p=0.036$), and more frequent RD152 deletions compared with "Other" clusters ($p=0.003$). RD149 and RD152 deletions were more frequent in Beijing isolates compared with CAS1 strains ($p < 0.001$). Concurrent RD149 and RD152 deletions were more frequent in CAS1 compared with "Other" clusters ($p < 0.001$) and in Beijing strains compared with CAS1 ($p < 0.001$). No significant difference was detected in RD deletion patterns between pulmonary and extra pulmonary isolates.

Conclusion: Higher frequencies of RD149 and RD152 deletions and of concurrent RD149 and RD152 deletions were found in CAS1 and Beijing strains compared with CAS subfamilies, "Other" clusters and Unique strains. No association between these deletions and disease presentation, pulmonary or extrapulmonary tuberculosis, was observed.

Key words: *Mycobacterium tuberculosis*; region of differences; Central Asian Strain 1

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Introduction

Pakistan ranks eighth on the list of high disease burden countries and accounts for 44% of all tuberculosis (TB) cases in the Eastern Mediterranean regions [1]. Molecular epidemiological studies have identified the predominance of Central Asian Strain 1 (CAS1) (39%) in this population, with Beijing strains constituting 3% of the *Mycobacterium tuberculosis* isolates from the country [2].

It is recognized that variation in clinical *M. tuberculosis* strains are a result of repeated insertion or deletion events in specific genomic regions known as regions of differences (RDs) [3]. Long sequence polymorphisms (LSPs) such as RDs have been used to dissect strain diversity and differentiate various species of the *M. tuberculosis* complex (MTC) on the basis of genomic deletions. [4]. Sixty-eight RDs have been identified thus far [5].

A number of studies have linked deletions of certain RDs with changes in mycobacterial epidemic potential and virulence. Deletion of RD1 has been documented in avirulent Bacille Calmette-Guérin (BCG) strains as compared to wild types and *M. tuberculosis* H37Rv [6,7]. RD1 deletion, however, has also been reported in *M. tuberculosis* strains from patients with active tuberculosis (TB) [8] and thus the significance of this deletion is yet to be established.

A population-based study has shown an association between extrapulmonary tuberculosis and concurrent deletions of RD105, RD181 and RD142 in Beijing strains [9]. Deletion of the RD207 region is reported to be a marker for Beijing strains. Beijing strains have also been shown to harbour deletions in RD149 and RD152 [10]. Although the impact of these deletions on disease outcome and epidemiology is not well defined, genes within RD152 and RD207 have been shown to code for putative transposases

and are thought to be required for transposition of the insertion element IS6110 [10]. The Rv1759c gene (*wag22*) within RD152 of these strains encodes a protein with fibronectin binding activity which may be involved in mediating bacterial attachment to host cells during infection [11].

CAS strains have been shown to harbor deletions in the RD750 region [12]. These deletions affect the Rv1519 and Rv1520 genes which have been shown to up-regulate the anti-inflammatory cytokine IL-10 in the human monocyte derived macrophages; thus the deletions appear to favor the intracellular survival of the pathogen [13].

The aim of this study was to determine the frequency of RD deletions in clinical *M. tuberculosis* strains prevalent in Pakistan. Given that very little information is available relating to RD deletions among the prevalent CAS1 genotype, the presence of commonly reported RD deletions, RD1, RD750, RD207, RD149, RD152, RD105, RD150, RD142 and RD181, were investigated.

Methodology

Strain selection

A strain bank of *M. tuberculosis* clinical isolates (n = 926) collected at the Aga Khan University Hospital Laboratory between 2003 and 2005 and characterized using spoligotyping [2] was used. This included 850 pulmonary and 76 extrapulmonary isolates. A total of 185 pulmonary and 50 extrapulmonary strains were selected for study purposes using stratified random sampling.

Spoligotypes of these strains had been determined by comparison with the International Spoligotyping Database SpolDB4 [14]. Strains with 90% homology to Central Asian Strain 1 (CAS1) were identified as belonging to the CAS subfamily [2].

The diversity of spoligotypes included in the study is shown in Table 1. *M. tuberculosis* isolates from pulmonary sources were grouped into 17 different clusters, while isolates from extrapulmonary sites were grouped into 16 clusters.

M. tuberculosis *growth and DNA extraction*

Mycobacteria were cultured on Middlebrook 7H10 agar (Becton Dickinson (BD), New Jersey USA). The extraction of genomic DNA from *M. tuberculosis* clinical isolates was performed by the cetyltrimethylammonium bromide (CTAB) method [15].

Determination of RD deletions

A PCR based RD deletion detection method was used to determine the presence or absence of RD1, RD750, RD207, RD149, RD152, RD105, RD150, RD142 and RD181 regions using specific primers [12,16-20] as indicated in Table 2. All PCRs were performed in a volume of 25 ul using 50 ng of DNA template. The *Tfl* (Epicenter, Madison, Wisconsin, USA) DNA polymerase enzyme was used to amplify fragments (> 1kb) for RD105, RD142, RD150, RD181, 149, RD152, RD207 and RD750. The Red Hot DNA polymerase enzyme (ABgene, Rockford, Illinois, USA) were used to amplify fragments (< 1kb) for 16S rRNA gene and the RD1 region for CAS1 and CAS subfamilies, "Other" clusters, and Beijing *M. tuberculosis* strains [17].

A 16S rRNA gene PCR was performed and used as internal control to determine the quality of genomic DNA [20] for all *M. tuberculosis* strains studied. The *M. tuberculosis* H37Rv, Beijing and *M. bovis BCG* (Montreal) vaccine strains were used as positive controls. To determine the quantity of DNA, 16S rRNA gene PCR was performed with 1X Taq PCR buffer (Epicenter, Madison,Wisconsin, USA) 2 mM $MgCl_2$, 0.2 mM dNTP, 0.2 pmole of each primer, and 0.25 U of Red Hot *Taq* DNA polymerase (ABgene, Rockford, Illinois, USA). Amplification was performed at 40 cycles, 95°C for 1 minute, 55°^{0}C for 1 minute and 72°C for 1 minute with a final extension at 72°C for 10 minutes.

The PCR for RD1 deletion was performed with 1X PCR buffer, 2 mM $MgCl_2$, DMSO, 0.2 mM dNTP, 1 pmole of each primer, and 1.25 U of Red Hot *Taq* DNA polymerase (ABgene, Rockford, Illinois, USA). Amplification was performed at 30 cycles, 94°C for 30 seconds, 65°C for 30 seconds and 72°C for 30 seconds with a final extension at 72°C for 10 minutes.

The PCR for RD181 deletion was performed with 1X PCR buffer, 2 mM $MgCl_2$, 1X Enhancer (Betaine) (Epicenter, Madison, Wisconsin, USA), 0.2 mM dNTP, 1 pmole of each primer, and 1.25U of *Tfl* DNA polymerase (Epicenter, Madison, Wisconsin, USA). Amplification was performed at 35cycles, 94°C for 1 minute, 60°C for 1 minute and 72°C for 2 minutes with a final extension at 72°C for 10 minutes.

The PCR for the RD149 (Rv1585c-1587c) deletion was performed with 1X PCR buffer, 2.5 mM $MgCl_2$, 1X Enhancer (Betaine), 0.25 mM dNTP, 1 pmole of each primer, and 1.25U of *Tfl* DNA polymerase. Amplification was performed at 35

Table 1. Spoligotypes and distribution of RD deletions amongst *M. tuberculosis* strains

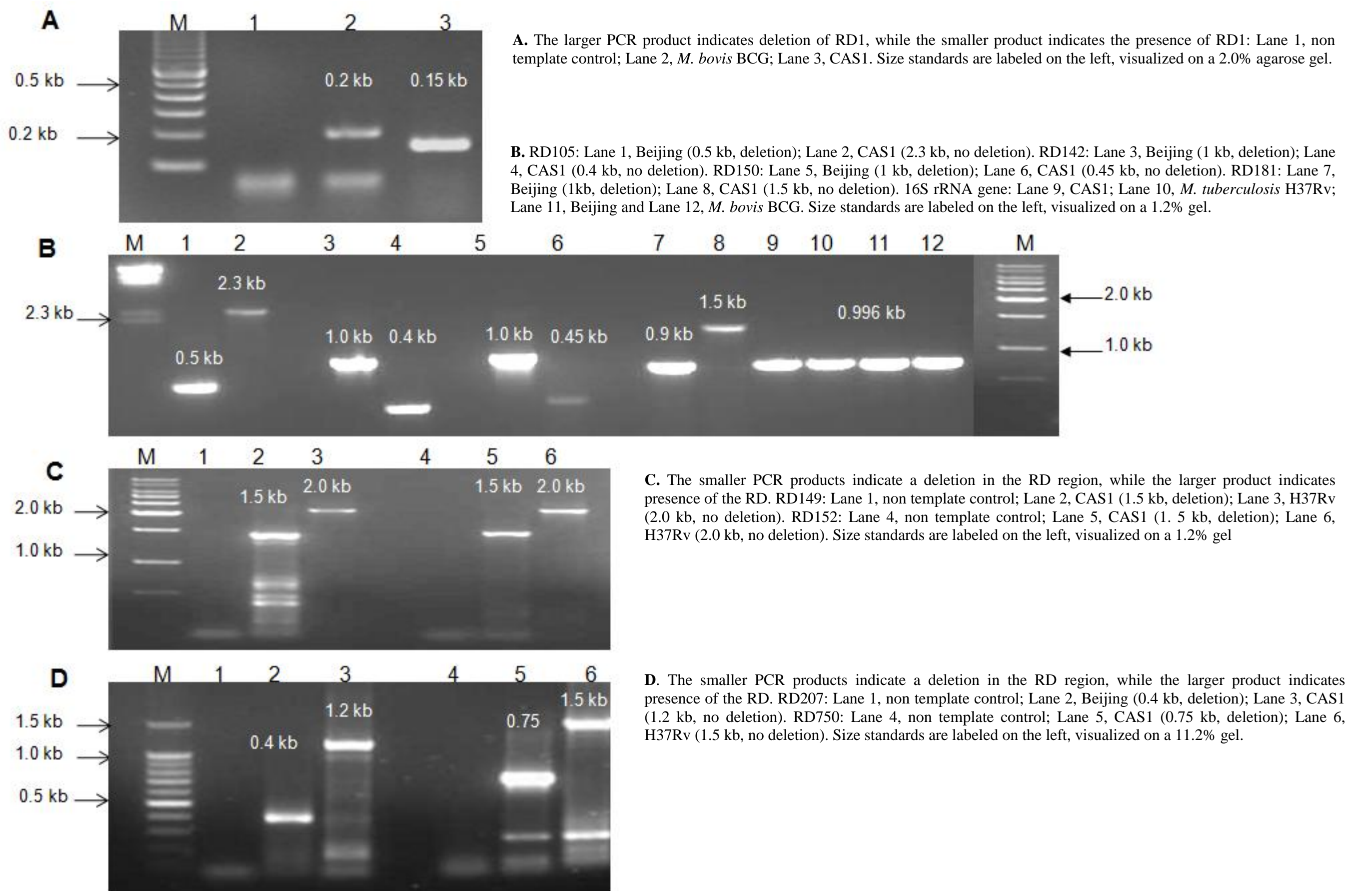
Spoligotype groups	Total isolates	Pulmonary isolates n (%)[a]	Extrapulmonary Isolates n (%)[a]	RD1	RD750	RD207	RD149	RD152	RD105	RD150	RD142	RD181
CAS1(ST26)	**133**	**122 (52)**	**11 (4.7)**	-	133	-	53	29	-	-	-	-
CAS subfamily	**38**	**27 (11.5)**	**11 (4.7)**		38	-	8[b]	8	-	-	-	-
ST357	8	7 (3)	1 (0.42)	-	8	-	-	4	-	-	-	-
ST794	6	5 (2.13)	1 (0.42)	-	6	-	-	1	-	-	-	-
ST25	6	3 (1.3)	3 (1.3)	-	6	-	3	-	-	-	-	-
ST1264	4	3 (1.3)	1 (0.42)	-	4	-	1	2	-	-	-	-
ST486	3	2 (0.85)	1 (0.42)	-	3	-	1	-	-	-	-	-
ST203	2	2 (0.85)	-	-	2	-	1	-	-	-	-	-
ST288	2	2 (0.85)	-	-	2	-	-	-	-	-	-	-
ST142	2	-	2 (0.85)	-	2	-	-	-	-	-	-	-
ST1327	1	1 (0.42)	-	-	1	-	-	1	-	-	-	-
ST754	1	1 (0.42)	-	-	1	-	-	-	-	-	-	-
ST1343	1	-	1 (0.42)	-	1	-	-	-	-	-	-	-
ST1093	1	-	1 (0.42)	-	1	-	1	-	-	-	-	-
ST54	1	1 (0.42)	-	-	1	-	1	-	-	-	-	-
Beijing (ST1)	**8**	**5 (2.13)**	**3 (1.3)**	-	-	8	8[c]	8[c]	8	8	5	7
Other clusters	**47**	**31 (13.2)**	**16 (6.8)**		-		14	1[b]				
T1 (ST53)	15	13 (5.5)	2 (0.85)				9	-	-	-	-	-
EAI3 (ST11)	12	7 (3)	5 (2.1)	-	-	-	2	-	-	-	-	-
H4 (ST127)	10	7 (3)	3 (1.3)	-	-	-	1	-	-	-	-	-
EAI5 (ST126)	3	3 (1.3)	-	-	-	-	1	-	-	-	-	-
LAM6 (ST64)	1	1 (0.42)	-	-	-	-	-	1	-	-	-	-
T1 (ST628)	1	-	1 (0.42)	-	-	-	1	-	-	-	-	-
T1 (ST1877)	2	-	2 (0.85)	-	-	-	-	-	-	-	-	-
T2 (ST52)	2	-	2 (0.85)	-	-	-	-	-	-	-	-	-
PAK28	1	-	1 (0.42)	-	-	-	-	-	-	-	-	-
Unique	**9**	**-**	**9 (3.83)**	-	-	-	-	-	-	-	-	-
Total Strains (%)	**235**	**185 (78.7)**	**50 (21.3)**	-	**171**	**8**	**83**	**46**	**8**	**8**	**5**	**7**

CAS1: Central Asian Strain1, EAI: East African Indian, H: Haarlem, T1: Undefined group LAM: Latin American Mediterranean. ST (shared type) as identified by SpolDB4.0.

[a] Percentage (%) values: number of specific clusters expressed as a percentage of total isolates in the study (N=235) [b]Significantly fewer deletions compared with CAS1 ($p<0.05$)

[c]Significantly higher number of deletions compared with CAS1 ($p<0.05$)

Figure 1. Results of PCR for RD1, RD105, RD142, RD150, RD181, 16S rRNA gene, RD149, RD152, RD207 and RD750



A. The larger PCR product indicates deletion of RD1, while the smaller product indicates the presence of RD1: Lane 1, non template control; Lane 2, *M. bovis* BCG; Lane 3, CAS1. Size standards are labeled on the left, visualized on a 2.0% agarose gel.

B. RD105: Lane 1, Beijing (0.5 kb, deletion); Lane 2, CAS1 (2.3 kb, no deletion). RD142: Lane 3, Beijing (1 kb, deletion); Lane 4, CAS1 (0.4 kb, no deletion). RD150: Lane 5, Beijing (1 kb, deletion); Lane 6, CAS1 (0.45 kb, no deletion). RD181: Lane 7, Beijing (1kb, deletion); Lane 8, CAS1 (1.5 kb, no deletion). 16S rRNA gene: Lane 9, CAS1; Lane 10, *M. tuberculosis* H37Rv; Lane 11, Beijing and Lane 12, *M. bovis* BCG. Size standards are labeled on the left, visualized on a 1.2% gel.

C. The smaller PCR products indicate a deletion in the RD region, while the larger product indicates presence of the RD. RD149: Lane 1, non template control; Lane 2, CAS1 (1.5 kb, deletion); Lane 3, H37Rv (2.0 kb, no deletion). RD152: Lane 4, non template control; Lane 5, CAS1 (1. 5 kb, deletion); Lane 6, H37Rv (2.0 kb, no deletion). Size standards are labeled on the left, visualized on a 1.2% gel

D. The smaller PCR products indicate a deletion in the RD region, while the larger product indicates presence of the RD. RD207: Lane 1, non template control; Lane 2, Beijing (0.4 kb, deletion); Lane 3, CAS1 (1.2 kb, no deletion). RD750: Lane 4, non template control; Lane 5, CAS1 (0.75 kb, deletion); Lane 6, H37Rv (1.5 kb, no deletion). Size standards are labeled on the left, visualized on a 11.2% gel.

cycles, 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes 30 seconds with a final extension at 72°C for 10 minutes.

The PCR for RD152 (MT1798/Rv1755c) deletion was performed with 1X PCR buffer, 2.5 mM $MgCl_2$, 1X Enhancer (Betaine), 0.25mM dNTP, 1pmole of each primer, and 1.25 U of *Tfl* DNA polymerase. Amplification was performed at 35 cycles, 94°C for 1 minute, 57°C for 1 minute and 72°C for 2 minutes 30 seconds with a final extension at 72°C for 10 minutes.

The PCR for RD207 deletion was performed with 1X PCR buffer, 2.0 mM $MgCl_2$, 1X Enhancer (Betaine), 0.25 mM dNTP, 1 pmole of each primer, and 1.25 U of *Tfl* DNA polymerase. Amplification was performed at 35cycles, 94°C for 1 minute, 58°C for 1 minute and 72°C for 2 minutes with a final extension at 72°C for 10 minutes.

The PCR for RD105, RD142 and RD150 deletions was performed with 1X PCR buffer, 2.0 mM $MgCl_2$, 1X Enhancer (Betaine), 0.2 mM dNTP, 0.8 pmole of each primer, and 1.25U of *Tfl* DNA polymerase. Amplification was performed at 35 cycles, 94°C for 1 minute, 63°C for 1 minute and 72°C for 2 minutes 30 seconds with a final extension for 72°C for 10 minutes.

The PCR for RD750 deletion was performed with 1X PCR buffer, 2.0 mM $MgCl_2$, 1X Enhancer (Betaine), 0.2 mM dNTP, 0.8 pmole of each primer, and 1.25 U of *Tfl* DNA polymerase. Amplification was performed at 35cycles, 94°C for 1 minute, 60°C for 1 minute and 72°C for 2 minutes with a final extension for 72°C for 10 minutes.

PCR results are illustrated in Figure 1 to depict a reference PCR for each RD PCR performed. All PCR products were visualized on a 1.2% agarose gel except for the RD1 deletion, where a 2% gel was employed.

Data analysis

SPSS for Windows version 16.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The variables for RD deletion analysis were compared using Fisher exact test of significance.

Table 2. Primer Sequences for the detection of RD1, RD105, RD142, RD150, RD181, RD207, RD149, RD152 and RD750 deletions

Primer	Sequence	PCR product (kb)	Reference
16S rRNA	F: 5'CCA GCA GCC GCG GTA ATA CG 3' R: 5'ATC GGC TAC CTT GTT ACG ACT TC 3'	0.996	Lu,JJ *et al.* 2000
RD1	ET1 :.5'AAG CGG TTG CCG CCG ACC GAC C 3' ET2: 5'CTG GCT ATA TTC CTG GGC CCG G 3' ET3: 5'GAG GCG ATC TGG CGG TTT GGG G 3'	0.2 (deletion) 0.15 (no deletion)	Parsons, LM et al. 2002
RD105	F ACA GCG CGG GTC ATA TCA C I GCA ACA CCC GCT TGT CTT TG R AAC CAG CTC CTC GAC GCT ATC	0.5 (deletion) 2.3 (no deletion)	Hanekom, M *et al*. 2007
RD142	F: CCG GTG GTA CGG GTA TTT CC I: GCT CGA GCA TGA TCA GCA AAG R: TAG CAC CAG TAC CGG ATG TCC	1 (deletion) 0.4 (no deletion)	Hanekom, M *et al*. 2007
RD150	F: AGT GCT GGC AAT AGC GGT TG I: CAC CGG CAC TTA CCA TCT CG R: CCA GCA CTT GTT GCA ACT TCG	1 (deletion) 0.4 (no deletion)	Hanekom, M *et al*. 2007
RD 181:	F: 5' CGC AAC GGC CGC GGT GAA CTC T 3' R: 5' CGG GCG GCT GCG GGA ACC TT 3'	1 (deletion) 1.5 (no deletion)	Hanekom, M *et al*. 2007
RD207	F:GATCGCTTGTTCTCAGTGCAG 3' R: CGAAGGAGTACCACGTGGAG 3'	0.4 (deletion) 1.2 (no deletion)	Warren, RM et al. 2004
RD149 (Rv1585c-1587c)	F: 5' GACGATCACGATGTTGTGGTGC 3' R: 5' CACCCTGACCGACCTGCAAAC 3'	1.5 (deletion) 2.0 (no deletion)	Stavrum *et al.* 2008
RD152 (MT1798/ Rv1755c)	F: 5' CGTCAGCTGGAAGGTGTATCGCA 3' R:5' GTCACCGGATGTCACATGAACTC 3'	1.5 (deletion) 2.0 (no deletion)	Stavrum *et al.* 2008
RD750 (Rv1519-Rv1520)	F: 5'GTCGGCGGTCTGCTTCGTTCC 3' R: 5' CCTGTCGGCCGGGTGTCTTTC 3'	0.75 (deletion) 1.5 (no deletion)	Gagneux *et al.* 2006

A 95% level of significance was used for the test. A *P*-value of ≤ 0.05 was considered significant.

Results

Occurrence of RD deletions in different M. tuberculosis *spoligotypes*

The spoligotypes and distribution of RD deletions in the RD1, RD105, RD142, RD149, RD150, RD152, RD181 and RD750 regions were determined for all strains studied (Table 1).

All the 133 CAS1 isolates studied had RD750 deletions. RD149 and RD152 deletions were observed in 39.8% (n = 53) and 21.8 % (n = 29) of CAS1 strains respectively. All the 38 CAS subfamily isolates were characterized by a RD750 deletion; 21.05% (n = 8) had RD149 deletions and 21.05% (n = 8) had RD152 deletions. Of the "Other" cluster strains, 29.8% (n = 14) had RD149 deletions, followed by 2.13% (n = 1) with RD152 deletions. No deletions were observed in the Unique strains (n = 9). Overall, the frequency of strains with RD149 deletion was significantly greater in CAS1 compared with the CAS subfamily (p = 0.036). The number of CAS1 strains with RD152 deletions was also significantly higher compared with "Other" cluster strains (p = 0.0003). The frequency of Beijing strains with

Table 3. Comparison of RD149, RD152 and concurrent RD149 & RD152 deletions in Pulmonary and Extrapulmonary isolates

Spoligotypes	**Pulmonary isolates (n=185)**				**Extrapulmonary isolates (n=50)**			
	Total strains	**RD149 n (%)[a]**	**RD152 n (%)[a]**	**RD149 & RD152 n (%)[a]**	**Total strains**	**RD149 n (%)[a]**	**RD152 n (%)[a]**	**RD149 & RD152 n (%)[a]**
CAS1 (ST26)	**122**	51 (41.8)	26 (21.3)	21 (17.2)	**11**	2 (18.2)	3 (27.3)	4 (36.4)
CAS subfamily	**27**	8 (29.6)	8 (29.6)	2 (7.4)	**11**	1 (9.1)	0	0
Beijing	**5**	5 (100)[c]	5 (100)[c]	5 (100)[c]	**3**	3 (100)[c]	3 (100)[c]	3 (100)[c]
Other clusters	**31**	13 (42)	1 (3.2)[b]	0[b]	**25**	1 (4)	0[b]	0[b]

CAS1: Central Asian Strain1, ST26 (shared type 26) as identified by SpolDB4.0.
[a]Percentage (%) values: number of strains with deletions as a percentage of the total strains within the specific cluster.
[b]Significantly fewer deletions compared with CAS1 ($p<0.05$)
[c]Significantly higher number of deletions compared with CAS1 ($p<0.05$)

RD149 deletions was significantly higher compared with CAS1 strains ($p < 0.001$). Similarly, RD152 deletions within Beijing strains were also significantly higher compared with CAS1 strains ($p < 0.001$).

Deletions in RD149, RD152 as well as concurrent RD149 and RD152 deletions in pulmonary and extrapulmonary isolates were compared (Table 3). CAS1 strains with RD152 deletions were significantly more frequent compared with “Other” clusters in both pulmonary ($p = 0.017$) and extrapulmonary ($p = 0.023$) isolates. RD149 and RD152 deletions were further identified in all Beijing strains studied, significantly more frequently compared with CAS1 strains ($p < 0.001$). In the case of RD149 deletions, these observations held true for both pulmonary ($p = 0.015$) as well as extrapulmonary ($p = 0.027$) strains, and in the case of RD152, for pulmonary isolates ($p = 0.001$) as analyzed by Fisher’s exact test. Statistical analysis showed concurrent RD149 and RD152 deletions were more frequent in CAS1 compared with “Other” clusters in both pulmonary ($p = 0.007$) as well as extrapulmonary ($p = 0.006$) isolates analyzed by Fisher’s exact test (Table 3). Concurrent deletions, on the other hand, were highest among the Beijing isolates, significantly more frequently as compared with CAS1 (pulmonary isolates ($p < 0.001$) analyzed by Fisher’s exact test. The difference between Beijing and CAS1 for extrapulmonary isolates, however, did not achieve statistical significance, most probably due to a small sample size.

Discussion

The Central Asian strains which include the CAS1 and CAS subfamilies have been shown to be the predominant *M. tuberculosis* spoligotype in southern Asian countries including Pakistan, India, and Bangladesh. Little is known about any mechanisms which may be responsible for the persistence of this genogroup in the region.

This study reports the epidemiological data on the presence of regions of differences (RDs) among CAS1 and CAS subfamilies which belong to the East African Indian lineage otherwise known as lineage 3 [12]. Developments in comparative genomics have revealed differences in the presence or absence of regions of differences (RDs) between the *M. tuberculosis* H37Rv and the *M. bovis* BCG. Presence or absence of these RD regions, thought to harbor several important genes and virulence factors, could help identify lineages of different isolates in a particular geographical region [21].

A previous study has suggested that some RD deletions are likely to offer advantages, including escape from the host immune system, antibiotic resistance, curtailing latency and promoting transmission [5]. Deletion of RD1 from *M.*

tuberculosis, resulting in an attenuation strikingly like that of BCG, suggested the use of RD1 mutant strains for improvement of the tuberculosis (TB) vaccine [22]. Consistent with this hypothesis, RD1 deletion was not detected in any of the clinical strains in our study.

RD105, RD142, RD150, RD181 and RD207 deletions have been demonstrated in the Beijing strains [10]. RD207 (*Rv2815c-Rv2820c*), a Beijing-defining deletion [19], along with RD105, RD142, RD150 and RD181 deletions, were also observed in our Beijing isolates but not in CAS1, CAS subfamilies and "Other" cluster strains. The contribution of this difference to strain properties and disease pathogenesis is difficult to explain at this stage and needs to be explored further.

Deletions in the CAS1 isolates were limited to RD149 and RD152 in addition to RD750, a defining marker for CAS (also known as the East African-Indian lineage, Delhi, or South Asian lineage) [12]. It has been shown that strains with the RD750 deletion are a prominent cause of tuberculosis among Asians in the United Kingdom and in the Indian subcontinent. The RD750 deletion appears to have increased, rather than decreased, the capacity of this lineage of MTB to cause immune deviation and contribute to its persistence and outbreak potential in human populations [13]. It has been suggested that deletions in the RD149 and RD152 regions may be due to transposition and homologous recombination events between adjacent IS6110 fragments. These deletions have previously been reported in the Beijing strains [10]. RD149 (also known as RD3) has been reported to be important as an evolutionary marker to differentiate *M. tuberculosis* strains independently of their geographical origin [23]. The RD149 deletion is reported in all Beijing strains, while RD152 was found to be variably deleted in Beijing strains [19]. In our samples, however, both RD149 and RD152 deletions were identified in all Beijing strains studied.

Deletions in RD149 and RD152 have also been reported in non-Beijing shared types (STs) (ST42, ST48 and ST947) and (ST42, ST89 and TB21) respectively [19]. These deletions, however, have not been studied in CAS1 (ST26) strains. Our study points to a higher frequency of RD149 deletion in CAS1 strains compared with the CAS subfamily, and of the RD152 deletion compared with "Other" clusters ($p < 0.05$, Fisher's exact test). The advantage of these deletions to CAS1 strains was not studied; however, the RD149 and RD152 deletions in this population did not appear to be associated with extrapulmonary disease. Our data is consistent with earlier reports wherein RD149 and RD152 deletions have so far not been shown to be associated with extrapulmonary disease [10,19].

Frequency of concurrent RD149 and RD152 deletions in CAS1 strains was significantly higher compared with "Other" clusters ($p < 0.05$, Fisher's exact test) and also higher compared with the CAS subfamily, although the latter did not achieve statistical significance. Surprisingly, concurrent RD149 and RD152 deletions were not noted in strains belonging to the "Other" clusters. Concurrent deletions of RD105, RD181 and RD150 and RD105, RD181 and RD142 in Beijing strains have been shown to be significantly associated with extrathoracic TB [9]. We did not observe any association between concurrent RD149 and RD152 deletions and extrapulmonary TB. However, functional studies of the genes involved in RD149 and RD152 deletions would be important to enhance our understanding epidemiological and clinical relevance of these RDs in the pathogenesis of *M. tuberculosis* and of CAS strains in particular.

Conclusions

Deletions in RD149 and RD152 were noted among the prevalent CAS1 and CAS subfamilies and Beijing strains prevalent in Pakistan. RD149 and RD152 concurrent deletions were also found to be present in some of these strains; however, no association between these deletions and disease presentation, Pulmonary or extrapulmonary tuberculosis was observed.

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