The Most Predominant Spoligopatterns of Mycobacterium tuberculosis Isolates among Iranian, Afghan-Immigrant, Pakistani and Turkish Tuberculosis Patients: A Comparative Analysis

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**Key Words**
Ancient/modern tuberculosis \cdot Iran, tuberculosis \cdot Mycobacterium tuberculosis \cdot Spoligopattern

**Abstract**

**Background:** The aim of this study was to identify prevalent spoligopatterns of Mycobacterium tuberculosis (MTB) strains within Iran and its neighboring countries. **Methods:** The spacer oligonucleotide typing (spoligotyping) was performed on 1,742 MTB strains isolated from Iranian and Afghan patients. Subsequently, these results were compared with published results of Pakistan and Turkey. **Results:** Based on the international spoligotyping database T, Central Asian Strain (CAS), East African-Indian and Latin American and Mediterranean families were prevalent in the region. T was dominant among Iranian (32.3%) and Turkish (36.5%), whereas CAS was more prevalent among Pakistani (61.3%) and Afghan-immigrant (27.4%) TB patients. **Conclusion:** The majority of strains isolated from Pakistan and Afghanistan belonged to ancient MTB genotypes (principal genetic group, PGG, I). In contrast, in Turkey there was prevalence of modern TB (PGG II and III), whereas in Iran, the prevalence of ancient and modern TB was almost equal. Therefore, Iran may be considered as the connecting geographical location between ancient and modern TB.

**Introduction**

Tuberculosis (TB) is a major global public health problem, particularly in developing countries like Afghanistan, Pakistan, Iran and Turkey. The total number of TB cases is still increasing worldwide, particularly in countries where HIV infection is epidemic. The recently published WHO report stated that there were 9.27 million new cases of TB in 2007, 1.8 million deaths from TB and 13.7 million prevalent cases [1].

Recently, Mycobacterium tuberculosis (MTB) has been intensely studied because of its resurgence and the emergence of drug-resistant strains [2–4]. Multidrug-resistant TB (MDR-TB), that is MTB strains resistant to at least isoniazid and rifampicin, is becoming extensively widespread today, with 0.5 million estimated new cases in 2007 [1]. Hence, epidemiological studies of this disease...
are of high importance to define its origin and propagation in communities, and for effective control and prevention [5]. Nowadays, molecular methods are used to assess the transmission of TB in high-incidence areas, where infection and disease patterns are highly heterogeneous [6]. Spoligotyping is a polymerase chain reaction (PCR)-based method of a highly polymorphic direct repeat (DR) locus in the MTB genome. It provides epidemiological information on strain identities, and is useful in the surveillance of TB transmission and in intervention to prevent further spread of the disease [7]. Although spoligotyping is cheap, rapid and highly reproducible, it remains less discriminatory than the standard IS6110 restriction fragment length polymorphism (RFLP) method for isolates with a high copy number of IS6110, but more discriminatory for isolates containing few IS6110 [8]. The recently introduced mycobacterial interspersed repetitive unit typing is based on polymorphisms of variable-number tandem repeats at multiple loci, and has a higher discriminatory power than spoligotyping and slightly less than RFLP [9, 10].

By spoligotyping, seven potential families of MTB have been identified, namely East African-Indian (EAI), Beijing, Haarlem, Latin American and Mediterranean (LAM), Central Asian Strain (CAS), X and T [8]. These families possess major differences in the spoligotyping pattern and geographical distribution. The characterization of each lineage is as follows: the EAI family has a low copy number of IS6110 (absence of spacers 29–32; presence of spacer 33, and absence of spacer 34), the Beijing genotype has an inverted IS6110 copy within the DR region (absence of spacer 1–33 and presence of spacer 34–43), the CAS shows absence of spacers 4–27 and 23–34, the Haarlem family reveals absence of spacers 29–31 and 33–36 [11], the LAM family is defined by the finding of linkage disequilibrium between the absence of spacers 21–24 and the presence of an ETR-A allele equal to 2 [12], the X family has two concomitant characteristics, a low copy number of IS6110 and absence of spacer 18 [13], and the T family is characterized by the absence of spacers 33–36 [14]. The geographical prevalence of the spoligotypes in SpolDB4 [8] is as follows: EAI (South-East Asia, South India and East Africa; may have originated in Asia), Beijing genotype (Far-East Asia, but also in Middle-East-Central Asia and Oceania), CAS lineage is split into CAS1-Delhi type (ST26; India and in the Indian subcontinent) and CAS1-Kilimanjaro (ST21; Tanzania), Haarlem family (Armenia, Austria, Finland, Georgia, Iran and Russia), LAM family (Mediterranean countries, Venezuela and the Caribbean region), X family (North America and Central American regions) and T family (all continents).

The transmission and circulation of these spoligotypes are yet not well characterized within communities in our region. Although the prevailing spoligopatterns have been reported in Iran, Afghan-immigrant, Pakistani and Turkish TB patients [15–17], the relationships among circulating strains in the region have not been fully elucidated. In regard to the incidence rates of TB in 2007, Iran and Turkey have nearly similar incidence rates of TB, 22 and 30 per 100,000, respectively, whereas Pakistan and Afghanistan are among the countries with high TB burden, with estimated incidence rates of 168 and 181 per 100,000, respectively [1].

The aim of this project was to study prevalent spoligopatterns of MTB strains within Iran and its neighboring countries. To achieve this, we identified the spoligopatterns among Iranian and Afghan-immigrant TB patients and compared the results to the reported spoligotypes of Pakistan and Turkey [16, 17].

**Patients and Methods**

**Study Population**

The study included patients with confirmed TB culture. A total of 3,812 patients with suspected TB were referred to the National Research Institute of Tuberculosis and Lung Diseases for diagnosis and treatment between December 2000 and June 2005. The National Research Institute of Tuberculosis and Lung Diseases is the only center for treatment of drug-resistant TB in Iran.

Of 3,812 patients, 1,583 (41.5%) were excluded because they were either culture negative (916, 24%) or had smear examination only (667, 17.5%). In addition, another 394 patients (10.3%) were excluded due to culture contamination. Further, 93 patients (2.4%) were infected with non-TB mycobacteria. Overall, 2,070 patients were excluded from the study: 1,180 (57%) were Iranian and 890 (43%) were Afghan cases. The study, therefore, consisted of 1,074 (28.2%) Iranian and 668 (17.5%) Afghan patients. Regarding gender, of the Iranian patients 579 (53.9%) were male and 495 (46.1%) were female, whereas 356 Afghan cases (53.3%) were male and 312 (46.7%) were female. The mean age of Iranian cases was 46.1 ± 17.6 years (SD) and of Afghani it was 39.8 ± 16.3 years (SD). The MTB isolates studied included pulmonary and extra-pulmonary specimens. There were 981 and 634 pulmonary and 93 and 34 extrapulmonary specimens in Iranian and Afghan patients, respectively.

**Bacterial Strains**

Primary isolation and culturing of mycobacterial isolates were performed in accordance with a previously described method [18]. All isolates were identified as MTB complex using biochemical tests, including production of niacin, catalase activity, nitrate reduction, pigment production and growth rate. Drug susceptibility testing against isoniazid, rifampicin, streptomycin and eth-

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**Spoligopatterns of Mycobacterium tuberculosis Isolates**

Chemotherapy 2010;56:248–257

249
ambutol was performed at concentrations of 0.2, 40, 4.0 and 2.0 μg/ml, respectively, by the proportional method on Lowenstein-Jensen media. Susceptibility to pyrazinamide was tested using a two-phase medium and two different concentrations of the inoculums.

**Spoligotyping**

Spoligotyping was performed on genomic DNA extracted from clinical specimens by standard phenol-chloroform methods, as described by Kamerbeek et al. [19], with a commercially available kit, according to the manufacturer’s instructions (Isogen Bioscience, The Netherlands). In brief, the DR region was amplified by PCR using primers [DrA (5’-CCG AGA GGG GAG GGA AAC-3’) and DrB (5’-GTT TTT GGG TCT GAC GAC-3’)] derived from the DR sequence. The amplification was carried out in a MasterCycler 5330 (Eppendorf, Germany) using 53 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C and extension for 1–2 min to ECL-Hyper film (Amersham) detection kit (Amersham, UK) and by exposing the membrane for 30 s at 72°C. The first denaturation and final extension steps were performed for 10 min. The amplified DNA was hybridized to a set of 43 immobilized oligonucleotides derived from the spacer sequences of MTB H37Rv and Mycobacterium bovis BCG P3 by reverse line blotting. Hybridized DNA was revealed with the ECL detection kit (Amersham, UK) and by exposing the membrane for 1–2 min to ECL-Hyper film (Amersham) [19].

**Definitions**

MDR-TB was defined as MTB strains that were resistant to at least isoniazid and rifampicin [1]. A cluster (shared types, STs) was defined as two or more isolates obtained from different patients having identical spoligotype patterns, whereas ‘orphan’ designates patterns reported for a single isolate [20].

**Statistical Analysis**

The results obtained were analyzed by entering the data in a binary format as Excel spreadsheet (Microsoft) and compared with published data [20–23]. Categorical data in each group (Iranian and Afghan TB cases) were compared by χ² test or Fisher’s exact test. Subsequently, the results of Iranian and Afghan-immigrant TB patients were compared with published results of Pakistani and Turkish countries [16, 17]. Based on Sreevatsan et al. [24], MTB genotypes are classified into three principal genetic groups (PGG). PGG I: EAI, Beijing, CAS and Bovis families; PGG II: Haarlem, X and LAM families, and PGG III: T family. The same classification has been used in our analysis.

### Results

**Drug Susceptibility**

Overall, antimicrobial susceptibility testing was performed for 1,074 Iranian and 668 Afghan cases. A total of 712 (66.3%) and 274 (41%) MTB strains were sensitive to all drugs tested in Iranian and Afghan immigrants, respectively (Table 1). The prevalence of MDR-TB was higher in previously treated cases in both Iranian (64, 20.8%) and Afghan cases (127, 42.9%). The rate of MDR-TB among Afghan immigrants (175, 26.2%) was higher than in Iranian (88, 8.2%) TB cases (Table 1).

**Analysis of Spoligotyping Data**

A total of 133 distinct spoligopatterns were identified, including 63 unique patterns (orphan) and 1,679 isolates (contained ≥2 isolates per cluster) within 71 clusters. The diversity of clusters identified was 0.076 (diversity = the number of STs divided by the total number of isolates found, i.e. 133 of 1,742; 0.076). All spoligotype patterns were definable. Among clustered isolates, two broad categories of clusters (STs) were identified. First, the clusters that contained more than 10 isolates, i.e. 12 clusters containing 1,385 isolates. Second, the clusters that contained less than 10 isolates, i.e. 59 clusters containing 294 isolates (12 clusters contained 2 isolates, 9 clusters contained 3 isolates, 10 clusters contained 4 isolates, 8 clusters contained 5 isolates, 7 clusters contained 7 isolates, 3 clusters contained 8 isolates and 10 clusters contained 9 isolates).

**Epidemiological Analysis of Patients in Clusters**

Afghan-Immigrant Cases in Clusters. Of 668 isolates, 640 (95.8%) were clustered in 32 clusters. Nine clusters contained 556 isolates (>10 isolates per cluster) and the remaining 84 isolates fell under 23 clusters (<10 isolates per cluster; fig. 1). An epidemiological link was expected

### Table 1. Rates of drug resistance in Iranian and Afghan-immigrant TB cases

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Spoligopatterns of Mycobacterium tuberculosis Isolates

for 252 Afghan patients (39.3%) in different clusters; 240 were family members. Among the clusters identified, the Beijing family and ST 253 had a unique appearance. In the Beijing family, 55/80 (68.7%) of patients were below the age of 30 years, 50/80 (62.5%) were family or close contacts, and 47/80 (58.8%) were MDR-TB. On the other hand, 65.4% of patients in the cluster ST 253 were older than 45 years and 74.5% had a previous history of TB. The age differences between the Beijing family and ST 253 were statistically significant.

Iranian Cases in Clusters. Out of 1,074 isolates, 1,039 (96.7%) were clustered in 69 clusters. Twelve clusters contained 829 isolates (>10 isolates per cluster) and the remaining 210 fell under 57 clusters (<10 isolates per cluster; fig. 1). An epidemiological link was expected for 212 patients (20.3%) in different clusters; 162 (15.5%) were family members or close contacts. In the Beijing family, 6/12 (50%) were MDR-TB.

Pulmonary versus Extrapulmonary Isolates

The MTB families consisted of 981 Iranian and 634 Afghan pulmonary specimens, whereas 93 Iranian and 34 Afghan cases were extrapulmonary. The distribution of spoligotypes (T, CAS, EAI, Haarlem, X, Beijing, Orphan and infrequent ST) between Iranian and Afghan immigrants according to the site of recovery is shown in table 2. Six (6.5%) and 5 (14.7%) MTB orphan types were recovered from extrapulmonary specimens among Iranian and Afghan cases, respectively.

Description of Spoligotypes and Its Shared Types between Iranian, Afghan-Immigrant, Pakistani [16] and Turkish [17] TB Cases

Four families, i.e. T, CAS, EAI and LAM, were prevalent in the region. T was dominant among Iranian (32.3%) and Turkish (36.5%) [17], whereas CAS was more prevalent among Pakistani (61.3%) [16] and Afghan cases (27.4%; table 3). EAI and CAS genotypes could not be detected in the studied Turkish population, instead the LAM family (18.4%) was reported as the second most prevailing family in Turkey [17].

The distribution of shared types, their respective sizes and their relative distribution in different locations are summarized in figure 2. The 14 most frequent shared
types totaled 2,084 (68.6%) isolates (fig. 2a); 7 types were highly frequent, representing 1,748 (57.3%) isolates. The CAS, T, and EAI families were most frequent in the region and represented 30.6, 19.0 and 14.7%, respectively (table 3).

Regarding the relative size of 104 STs, 18 STs (17.3%) contained only 2 isolates. In contrast, 8 STs (7.7%) contained >80 isolates [total 1,846 (60.5%) isolates (fig. 2b)]. Figure 2c shows the distribution of STs reported in one location versus that found in two or more locations; 68 (65.4%) STs were reported from a single location, 22 (21.2%) were from two locations, 11 (10.6%) were from three locations, and 3 (2.9%) were from four locations. Most MTB STs containing a low number of isolates were confined to a single location, whereas a minority containing a high number of isolates was from all locations. The most ubiquitous STs in decreasing order of distribution were ST53, ST1 and ST50. The percentage of unique patterns was almost similar among Pakistani (22.2%) and Turkish (22.8%) TB patients [16, 17], but the rate was lower in Iranian (3.3%) and Afghan (4.2%) cases. In accordance with Sreevatsan et al. [24], the number and percentage of the studied population PGG (PGG I, ancient TB, and PGG II/III, modern TB) are shown in table 4 and figure 3.

### Discussion

Geographically, Iran is located in South-West Asia with total land boundaries of 5,440 km. Iran’s western border is with Turkey (499 km) in the north; to the east there is Afghanistan (936 km) on the north and Pakistan (909 km) on the south (fig. 3). These countries have many
Fig. 2. Histograms summarizing the distribution of shared types (a), their respective sizes (b) and their relative distribution in different locations (c).

Fig. 3. Geographic comparison of old and ancient TB in Iran, Afghanistan (Afghan immigrants in Iran), Pakistan and Turkey. Iran is the geographical link between ancient and modern TB from South Asia to Western Asia.
social, cultural and economical relationships. Here, we studied the prevalent spoligopattern within Iran and compared it with those of its neighboring countries. Although Iran also shares its northern border with Armenia, Azerbaijan and Turkmenistan, and its western border with Iraq in the south, we did not include these countries in our comparison because of either lack of reports on the prevailing spoligopattern or lack of a sufficient number of immigrants in Iran.

Recently, investigators showed intra-community transmission among circulating strains of MTB in different countries [16, 25]. Generally, spoligotyping demonstrates the predominance of certain families of MTB in specific locations. In the present study, the T family of MTB was dominant among Iranian patients. The same family was reported to be common in Turkey [17], whereas in Pakistan and among Afghan cases the percentage was lower than 3%. The high prevalence of the T family among Iranian and Turkish TB cases (32.3 and 36.5%, respectively) might be of great interest for further studies and phylogeographical interpretation. In Pakistan, the CAS family of MTB was identified as dominant genotype (61.3%) [16]. Its prevalence was highest in the Punjab province, which might be attributed to the shared border and homogeneity of the population with North India. Similarly, the same family was the most prevalent genotype (27.4%) among Afghan cases, whereas its prevalence in Iran was 16.9%. The second frequent spoligopattern in Afghan cases was EAI (26.8%); this may partly be explained by the fact that Afghanistan borders the North West Frontier Province of Pakistan, which has the highest prevalence of EAI strains. In this study, we have shown that the EAI family represented 21.1% of all cluster isolates among Iranian patients. We could not find data about the prevalence of CAS and EAI lineages in Turkey, but based on Gencer and Shinnick’s report [17], these two families were considered less frequent circulating strains. This may be due to differences in civilization and agri-

culture histories between Turkey and other populations studied. Indeed, from a geographical, anthropological aspect, Turkey is a link between Europe and Asia, a region of early human settlement, located at the crossroads of Eurasia, to its western part [26]. Therefore, CAS and EAI genotypes are considered prevalent in Pakistan, Afghanistan and Iran, whereas the T family is prevalent in Iran and Turkey.

In this study, Beijing was the other detected family, which was originally described by van Soolingen [27] in China. The percentage of Beijing strains was 1.1, 12, 2.7 and 1.8% in Iranian, Afghan, Pakistani and Turkish cases, respectively (table 3). Further, Beijing isolates were more frequently found at younger age (<30 years) both among Iranian and Afghan cases. In agreement with this, Tanveer et al. [16] documented a significant association of Beijing strains with young age. This may suggest active transmission of Beijing strains in young-age groups, as demonstrated by other investigators [28]. ST 253 had an appearance unique to Beijing genotype in Afghan cases in clusters and it has been found to be potentially specific to Russia [29]. Thus, it may be transmitted to Afghan cases as a result of population movements from the former Soviet Union through Afghanistan. Similar to Beijing strains, some MTB genotypes have demonstrated a great ability to be transmitted and distributed throughout the world, such as the Haarlem family [30]. This family was more frequent among Turkish (10.2%) patients than Iranian (4.1%), Afghan (4.9%) and Pakistani (1.6%) cases. Generally speaking, the finding of identical STs in different countries in our study may be explained either by recent or past transmission events (fig. 2).

In this study, we found that the Beijing strains were associated with MDR-TB in 50% of Iranian and 58.8% of Afghan cases. Furthermore, the prevalence of MDR-TB was higher in previously treated cases in both Iranian (20.8%) and Afghan cases (42.9%). In support of our finding, other Iranian researchers demonstrated an association of MDR strains with the Beijing genotype [15]. Further studies documented an association between Beijing and Haarlem families and drug resistance [30–32]. In one report from Iran, 40% of the isolated Beijing strains occurred among Afghan-immigrant TB cases [33]. The high rate of MDR Beijing strains among Afghan immigrants in Iran may play an important role in the dissemination of this genotype to Iranians. Overall, the finding of a high rate of drug resistance among Afghan patients as well as a >8-fold increase in the incidence rate in Iranian patients should guide policy mak-

Table 4. Spoligopattern distribution according to PGG in Iranian, Afghan-immigrant, Pakistani and Turkish TB cases

<table>
<thead>
<tr>
<th>TB patients</th>
<th>PGG I</th>
<th>PGG II</th>
<th>PGG III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iranian</td>
<td>420 (39.1%)</td>
<td>62 (5.8%)</td>
<td>347 (32.3%)</td>
</tr>
<tr>
<td>Afghan immigrant</td>
<td>442 (66.2%)</td>
<td>44 (6.5%)</td>
<td>70 (10.5%)</td>
</tr>
<tr>
<td>Pakistani</td>
<td>635 (68.5%)</td>
<td>17 (1.8%)</td>
<td>23 (2.5%)</td>
</tr>
<tr>
<td>Turkish</td>
<td>7 (1.8%)</td>
<td>109 (28.6%)</td>
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ers to consider TB screening for migrants originating from high-burden settings. In Pakistan, 43% of the studied TB cases were MDR, and MDR was correlated with a high relative risk to Beijing strains, reflecting the association with previous anti-TB treatment [16]. A similar finding was documented from another study on Pakistan [34]. Although the rate of drug resistance is alarming in Pakistan, the percentage of the Beijing genotype (2.7%) reported by Pakistani researchers was lower than expected [16]. There is no adequate explanation for this finding. The high rate of MDR-TB in Pakistan demands serious public health attention as Pakistan is ranked 8th among 22 high-burden countries [1]. In contrast, Turkey had reported about 4% of MDR-TB, but MDR-TB was more closely associated with previous treatment [17]. Therefore, since the Beijing strains are more closely associated with young age and MDR, urgent prevention strategies should be applied to tackle the transmission of this strain. It has been shown that a few families have a particular tendency to be transmitted and causing disease because of certain criteria like antigen and higher virulence factors. The most documented example is the Beijing strain family [32, 35].

In the present study, a low percentage of the X family was noted among Iranian and Afghan cases (1.7 and 1.6%, respectively). This family was not reported by Pakistani and Turkish investigators [16, 17]. Hence, we believe that the X family may be imported to Iran as it has not yet been reported in neighboring countries; accordingly, we suggest further detailed investigation to know its exact phylogeographical origin.

In Turkey, the second frequent spoligotype was the LAM family (18.4%) [17], which is in concordance with the finding of Rahim et al. [4], who documented that this family is the second most prevalent clade in modern TB. LAM prevalence in Pakistan was negligible [16] and was not classified in our spoligopattern neither in Iranian nor in Afghan cases. The percentage of unique patterns was almost similar among Pakistani (22.2%) and Turkish (22.8%) TB patients [16, 17]. On the contrary, the rate was lower in Iranian (3.3%) and Afghan-immigrant (4.2%) patients, because the vast majority of spoligotypes were recognized in our study. In a recent study from Iran [36], a higher prevalence rate of orphan spoligotypes (10.4%) among relapse cases from Iranian and Afghan immigrants was reported. Therefore, a study in a larger cohort should be performed to understand the real picture of orphan spoligotypes in Iranian and Afghan cases.

According to Sreevatsan et al. [24], >66% of MTB isolates from Pakistan and Afghanistan belonged to ancient MTB genotypes (PGG I). Group I is evolutionarily older and has further evolved into PGG II and III organisms [21]. In contrast, in Turkey the prevalence of modern TB (PGG II/III) was 65.1%, whereas in Iran about half of MTB strains belonged to ancient TB and the rest to modern TB (table 4, fig. 3). Hence, Iran may be considered as the connecting geographical location between ancient and modern TB from South Asia to Western Asia. Therefore, the genetic diversity of MTB genotypes and their population structure is strongly linked to geography at a specific location. Thus, strengthening the localized effort to control TB is very important.

This study has several limitations. Different sample sizes and different time periods of the study reduce the validity of the comparative finding. The predominant patterns of MTB spoligotypes detected among Afghan-immigrant cases may not represent the real circulating strains in Afghanistan. Other genotyping techniques like IS6110 and mycobacterial interspersed repetitive units-variable number of tandem repeat were not included to show the exact transmission rate within these countries.

**Conclusion**

The T family was the most frequent genotype among Iranian and Turkish TB cases, which might be of great interest for further studies and phylogeographical interpretation. The CAS family (ancient origin) was most prevalent among Pakistani and Afghan cases. The Beijing strains were more closely associated with young age and MDR-TB. Therefore, urgent prevention strategies should be applied to tackle the transmission of these strains. The high rate of drug resistance among Afghan-immigrant cases in Iran as well as an 8-fold increase in the incidence rate in Iranian patients should guide policymakers to consider TB screening for migrants originating from high-burden settings. The X family was found among Iranian and Afghan cases only. Accordingly, further detailed investigation is required to elucidate its exact phylogeographical origin, since data in neighboring countries have not been published. The majority of MTB isolates from Pakistan and Afghanistan belonged to ancient MTB genotypes. In contrast, in Turkey the prevalence of modern TB was high, whereas in Iran about half of MTB strains belonged to ancient and the rest to modern TB. Hence, Iran may be considered as the geograph-
ical link between ancient and modern TB from South Asia to Western Asia. Therefore the genetic diversity of MTB genotypes and their population structure is strongly linked to geography at a specific location. Thus, strengthening the localized effort to control TB is very important.

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