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Predominance of a Single Genotype of *Mycobacterium tuberculosis* in Countries of East Asia

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Analysis of the population structure of *Mycobacterium tuberculosis* strains from the People's Republic of China showed that the vast majority belong to a genetically closely related group. These strains shared the majority of their IS*6110* DNA-containing restriction fragments, and also, the DNA polymorphism associated with other repetitive DNA elements, like the polymorphic GC-rich sequence and the direct repeat, was very limited. Because the majority of these strains originated from the province of Beijing, we designated this grouping the "Beijing family" of *M. tuberculosis* strains. Strains of this family were also found to dominate in neighboring countries such as Mongolia, South Korea, and Thailand, whereas a low prevalence of such strains was observed in countries on other continents. These data indicate that strains of the Beijing family recently expanded from a single ancestor which had a selective advantage. It is speculated that long-term *Mycobacterium bovis* BCG vaccination may be one of the selective forces implicated in the successful spread of the Beijing genotype.

Recent studies on repetitive DNA elements in *Mycobacterium tuberculosis* have shown that these elements are associated with DNA polymorphism in the chromosome. This polymorphism has enabled investigators to distinguish different strains of *M. tuberculosis*. Therefore, these elements have been found to be extremely useful for epidemiological studies of tuberculosis, such as outbreak investigations, transmission in the community, and the dissemination of multidrug-resistant clones (1–5, 9, 11–13, 17). Two classes of repetitive DNA elements have been used for the molecular epidemiology of tuberculosis: (i) insertion sequence (IS) elements, which have the capacity to move within the genome and which have a size of about 1,300 bp, and (ii) small repetitive DNAs, varying in size from 3 to 36 bp (14). The DNA polymorphism driven by insertion elements is due to their inherent capacity to move within the genome, with little target specificity. The nature of the genetic rearrangements driven by the small repetitive elements has been investigated only for the direct repeat (DR) region. Homologous recombination between DRs was found to be the predominant kind of rearrangement (6). It is likely that the same mechanism contributes to the DNA polymorphism associated with other small repetitive sequences, for example, the polymorphic GC-rich repeat sequence (PGRS), the major polymorphic tandem repeat, and the GTG repeat (9, 12, 19).

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The insertion element IS*6110* is the most widely used marker for epidemiological studies because of the high degree of discrimination obtained with this element. Furthermore, international consensus exists on a standardized method of IS*6110* fingerprinting, thus enabling the comparison of DNA types from different laboratories (15).

Most studies on DNA polymorphism in *M. tuberculosis* have been used to answer short-term epidemiological questions. Identical fingerprints from different isolates are usually evidence of recent person-to-person transmission or endogenous reactivation (1–5, 9, 11–13, 17). However, little is known about the degree of IS*6110*-associated DNA polymorphism of *M. tuberculosis* isolates from different human populations. Because the transposition of IS*6110* is a time-dependent process, the degree of IS*6110* polymorphism among the descendents of a particular clone in a population is a reflection of the time that has elapsed since their divergence. Thus, analysis of the *M. tuberculosis* population structure by IS*6110*-associated restriction fragment length polymorphism (RFLP) may provide information about the evolutionary history and the dissemination of particular clones in a given geographic region.

Van Soolingen et al. (17) observed that the degree of IS*6110*-associated RFLP is less among *M. tuberculosis* strains isolated from Central Africa compared with that among strains originating from The Netherlands. A recent study on the population structure of *M. tuberculosis* strains in Ethiopia, Tunisia, and The Netherlands suggested that there are a small number of predominant families of genetically related strains in Ethiopia and Tunisia, whereas no such distinct groupings predominate in The Netherlands (7). The latter study and additional

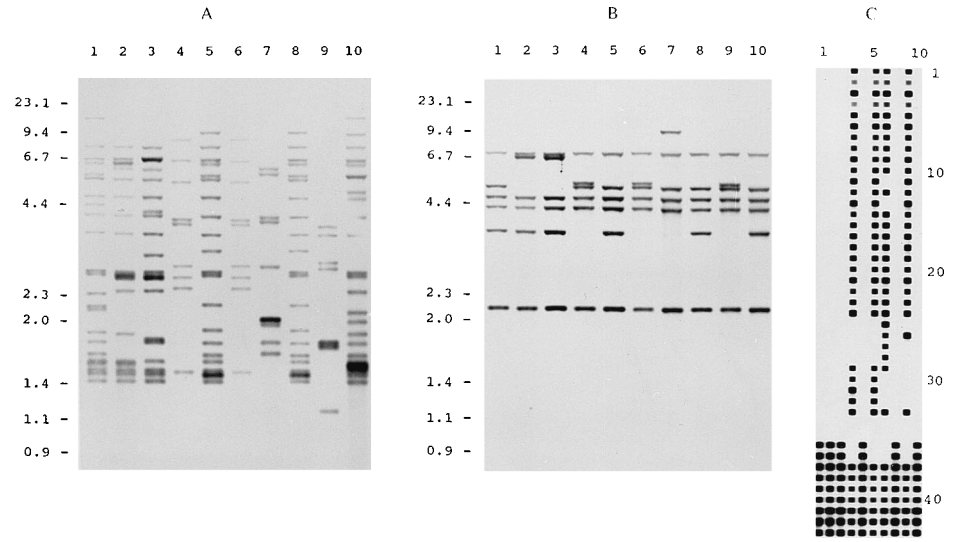

FIG. 1. DNA typing of 10 *M. tuberculosis* strains originating from Mongolia with IS*6110* (A) and IS*1081* (B) and by hybridization with 43 oligonucleotides derived from spacer sequences in the DR region (C). Lanes 1 to 3, 5, 8, and 10, DNAs from strains displaying IS*6110* DNA fragment patterns of the Beijing family. The sizes of the restriction fragments in panels A and B are given on the left (in kilobases). The 43 spacer oligonucleotides in panel C are numbered as described by Kamerbeek et al. (9b).

data (15b) have reinforced the idea that the success of particular clones in predominating is related to a high incidence of tuberculosis.

Preliminary investigations on the population structure of *M. tuberculosis* strains from the People's Republic of China and Mongolia suggested that isolates from these regions belong to a remarkably homogeneous family of strains, sharing the majority of their IS*6110*-containing restriction fragments. In the present study the population structure of this family of strains is described by using IS*6110* and other genetic markers. We show that strains of this family evolved from recent clonal expansion. Furthermore, the dissemination of strains of this family to other countries is described. The possible reasons for the expansion of this exceptionally successful family are discussed.

MATERIALS AND METHODS

Bacterial strains. Nineteen *M. tuberculosis* isolates obtained from Wang Guozi and Wang Zeng, Beijing, People's Republic of China, were tested. These strains were isolated from patients who were hospitalized for tuberculosis in the National Institute for Preventive Medicine during 1992 to 1993. Another group of strains from the People's Republic of China, comprising 30 isolates, was obtained from Z. Q. Huang of the National Tuberculosis Control Centre in Beijing. These strains were isolated from patients with tuberculosis hospitalized in the hospital of the National Tuberculosis Control Centre during 1993 and 1994. All except 2 of these 30 patients originated from the Beijing Province. Twenty strains isolated in Mongolia in 1992 and 1993 were obtained from G. Tsogi, National Tuberculosis Center, Ministry of Health, Ulan Bator, Mongolia. These strains were isolated from hospitalized tuberculosis patients and nonhospitalized patients from various parts of Mongolia, with the majority being from the city of Ulan Bator. All other *M. tuberculosis* strains were selected from a collection of strains from various parts of the world. This collection contained 2,594 strains isolated in The Netherlands and 934 strains from 16 other countries. All strains in this collection were isolated from 1992 to 1994 and have been typed by IS*6110* fingerprinting at the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands.

DNA fingerprinting. Extraction of DNA from *M. tuberculosis* strains and Southern blotting with labeled IS*6110* DNA as a probe were done by the standardized method of fingerprinting (15, 16). Each Southern blot contained DNA from *M. tuberculosis* Mt14323 as an external standard (15). IS*1081*, DR, and PGRS fingerprinting was done as described previously (16). The isolates from the National Institute of Tuberculosis Control, Beijing, were typed in Antwerp, Belgium. All other strains were typed in Bilthoven.

Spoligotyping. Spacer oligonucleotide typing (spoligotyping) is a novel method of differentiating *M. tuberculosis* complex strains. The method relies on the in vitro amplification of the DNA of the unique, highly polymorphic DR locus in the *M. tuberculosis* chromosome containing multiple short DRs. Each DR is interspersed by a nonrepetitive spacer sequence of 35 to 41 bp (8). The amplified DNA was hybridized to a set of spacer oligonucleotides derived from spacer sequences of the laboratory strain *M. tuberculosis* H37Rv (9a) and *Mycobacterium bovis* BCG (8). The resulting hybridization patterns are strain specific because of the strain-dependent presence or absence of spacer sequences in different isolates (6). We used 37 spacers derived from strain H37Rv and 6 spacers derived from *M. bovis* BCG. The DR region was amplified by PCR with oligonucleotide primers of 18 residues derived from the DR sequence which drive an outward polymerization of DR DNA. Hybridization was done by annealing the amplified DNA to multiple synthetic spacer oligonucleotides covalently bound to a membrane in parallel lines, a procedure described by Kaufhold et al. (10). Details of the entire method are described elsewhere (9b).

Computer analysis. The computer-assisted analysis of the IS*6110* fingerprints and spoligotypes was done with the Windows version of Gelcompar (version 3.10; Applies Maths, Kortrijk, Belgium) as described previously (7). Imaging of the autoradiograms was done with a scanner at 190 dpi (HP Scanjet IIcx/T; Hewlett-Packard, Camas, Wash.). The mobilities of the IS*6110*-containing restriction fragments were compared with those of a set of internal molecular weight markers by superimposing the autoradiographs containing the IS*6110* DNA fingerprints and the autoradiographs of the internal markers of known molecular size (15). This procedure enables one to normalize the position of each IS-containing fragment, irrespective of the autoradiogram, gel position, and gel distortions. The accuracy of the procedure was evaluated by comparing the IS banding patterns of *M. tuberculosis* Mt14323, which was present as an external marker in a single lane on each autoradiogram (15). All corresponding bands of strain Mt14323 on different images matched 100%, irrespective of the autoradiogram or the laboratory that made the fingerprint. Comparisons of patterns were done by the umweighted pair group method using arithmetic averages (UPGMA) clustering method by using the Dice coefficient according to the instructions of the manufacturer of Gelcompar. The analysis of the fingerprints from the set of 2,594 strains from Dutch patients and the set of 934 strains from other countries was done with a novel, experimental version of Gelcompar (18a).

RESULTS

IS*6110* restriction fragment patterns of *M. tuberculosis* strains isolated in the People's Republic of China and Mongolia. Forty-nine *M. tuberculosis* strains isolated from patients in the People's Republic of China and 20 strains from patients in Mongolia were fingerprinted by using IS*6110*. Figure 1A shows the IS*6110* fingerprints of *Pvu*II-restricted chromosomal DNAs of 10 isolates from Mongolia. The number of IS*6110* DNA-containing *Pvu*II fragments in most strains was found to be high, varying between 15 and 20, indicating that these strains contain 15 to 20 copies of the IS*6110* element. Six of the

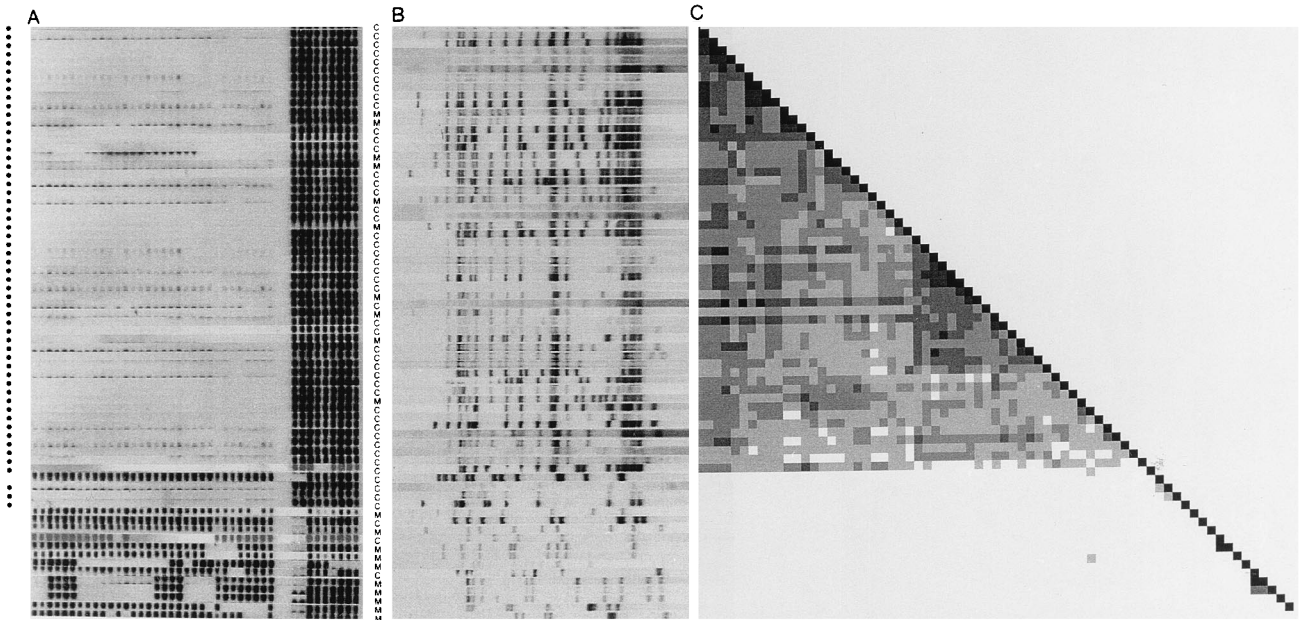

FIG. 2. Spoligotypes (A), IS*6110* banding patterns (B), and similarity matrices (C) of 69 strains isolated in the People's Republic of China (C) and Mongolia (M). The IS*6110* banding patterns were ordered by similarity. Note that the position of the IS*6110*-containing restriction fragments in each lane is corrected (normalized) in such a way that the band positions of all strains are mutually comparable. The value of the similarity coefficient between values of 65 and 100% is depicted by the five different grey tones in the matrices. The diagonal is formed by the 100% similarity coefficient values of the corresponding strains. Note that all strains having the characteristic IS*6110* pattern of the Beijing family are identical by spoligotyping (marked with a dot at the left).

fingerprints shown in Fig. 1 share the majority of their IS*6110*-containing restriction fragments and thus seem to belong to a group of closely related strains. To analyze the relatedness of all 69 strains originating from the People's Republic of China and Mongolia, the IS*6110* banding patterns of these isolates were ordered by similarity as calculated by the UPGMA method, and the result is shown in Fig. 2B. Fifty-two of the 69 fingerprints shared at least two-thirds of the IS-containing *Pvu*II fragments, indicating that the strains with those fingerprints belong to a closely related group. For convenience we refer to this grouping as the "Beijing family," because the vast majority of the Chinese strains originated from patients in Beijing. Only 17 strains, 7 from the People's Republic of China and 10 from Mongolia, did not belong to this family. Two of the seven non-Beijing family Chinese strains were known to have been isolated from patients in provinces other than Beijing. The remaining five isolates are assumed to have originated from Beijing. Thus, 89% (42 of 47) of the strains originating from patients in Beijing were closely related, and 50% (10 of 20) of the strains from Mongolia shared the same IS*6110* family pattern. To visualize the relatedness between the banding patterns of all *M. tuberculosis* isolates more objectively, a similarity matrix was generated. In this matrix the degree of relatedness of each IS banding pattern with any other IS banding pattern in the collection is shown. The degree of similarity of each pair of strains is visualized by a square box with various shades of grey, with the more similar strains being darker. The result (Fig. 2C) shows that 52 strains of the Beijing type are highly related, whereas the remaining 17 strains are quite different from this Beijing family and share little relatedness among themselves as well.

Analysis of *M. tuberculosis* strains belonging to the Beijing family with other genetic markers. To confirm that *M. tuberculosis* isolates of the Beijing family belong to a genetically closely related group that clonally expanded, we analyzed the DNA polymorphisms of strains from the People's Republic of China and Mongolia using the three repetitive DNA elements DR, PGRS, and IS*1081*, which drive DNA polymorphism independently of the insertion element IS*6110*. The polymorphism in the DR region was determined by spoligotyping, and the spoligotypes of the 69 strains from the People's Republic of China and Mongolia are depicted in Fig. 2. All strains of the Beijing family as defined by IS*6110* had identical spoligotypes. These strains contained only 9 of the 43 spacer sequences tested. In contrast, the spoligotypes of 14 of the 17 strains not belonging to the Beijing family were very different. These strains generally harbored many more spacers than isolates of the Beijing family type (Fig. 2). Furthermore, the spoligotypes of the non-Beijing family strains were highly diverse, consistent with the high degree of IS*6110*-associated RFLP observed among these strains.

To further substantiate the clonality of the strains of the Beijing family, we subjected 13 strains of the Beijing family type and 5 non-Beijing family strains to Southern blot analysis using PGRS DNA and IS*1081* DNA as a probe, respectively (16, 18). All PGRS DNA-containing restriction fragment patterns of the Beijing family were highly similar, whereas those of the other five strains greatly differed from those of the Beijing family (data not shown). The IS*1081* fingerprints of the strains belonging to the Beijing family differed from those of other strains in that they contained a characteristic *Pvu*II fragment of 3.6 kb which was absent from the non-Beijing family strains. We conclude that *M. tuberculosis* isolates grouped into the Beijing family by their IS*6110* fingerprints also share a similar grouping pattern when other genetic markers are used. These characteristics are not generally shared with other strains outside the Beijing family. These observations suggest that strains of the Beijing family constitute a genetically closely related group of bacteria.

Dissemination of *M. tuberculosis* isolates of the Beijing family among other countries. The database of IS*6110* fingerprints in Bilthoven contains the patterns of thousands of *M. tubercu-*

TABLE 1. Prevalence of strains of various geographic origins displaying IS*6110* banding patterns characteristic of the Beijing family

Origin	No. of strains investigated	No. of strains of the Beijing type	% of strains of the Beijing type
People's Republic of China	49	42	86
Mongolia	20	10	50
Thailand	19	7	37
South Korea	14	6	43
India	63	3	5
Iran	28	1	4
Russia (Moscow)	5	1	20
The Netherlands	2,594	82	3
Greenland	21	0	0
Spain	76	0	0
South Africa	46	7	15
Tanzania	88	4	4.5
Ethiopia	159	1	0.7
Tunisia	243	1	0.5
Zambia	36	0	0
Bolivia	41	1	2.5
Honduras	11	0	0
Brazil	81	0	0
Chile	3	0	0

losis strains originating from The Netherlands and many other countries. Recently, the Gelcompar software has been updated, permitting the comparison of tens of thousands of fingerprints (18b). This novel software was used to search the IS*6110* fingerprint database in Bilthoven for the presence of strains having characteristics of the banding pattern of the Beijing family of strains. For this purpose we selected strains from the IS*6110* library whose fingerprints matched any of the 52 fingerprints of the Beijing family obtained from the strains isolated in the People's Republic of China and Mongolia by 80% or more. The results are presented in Table 1. At least 40% of the *M. tuberculosis* strains originating from the countries bordering China, i.e., Thailand and South Korea, were of the Beijing family. In contrast, the prevalence of the Beijing type among strains from parts of the world more distant from the People's Republic of China except for strains originating from South Africa was less than 5%. Fifteen percent of the isolates from South Africa were of the Beijing type.

It should be noted that the fingerprints in the database are not necessarily a representative reflection of the *M. tuberculosis* population in a given country, because the numbers of strains from the various countries are very limited and regional differences may exist within a given country. However, the strains originating from The Netherlands do not share this limitation, since virtually all *M. tuberculosis* isolates obtained from patients in The Netherlands in 1993 and 1994 have been typed. Eighty-two of the 2,594 Dutch isolates grouped in the Beijing family. Twenty of the strains with Beijing family type patterns occurred in five clusters with two or more identical IS*6110* patterns, suggesting an epidemic spread of these strains (14, 15). The largest cluster of identical strains consisted of eight strains, indicating a microepidemic. In order to confirm that these strains were indeed related to the Beijing family of strains, we investigated the spoligotypes of eight strains from four different countries (Thailand, India, Russia, and The Netherlands). All of these strains were found to have spoligotypes characteristic of those of the Beijing family (data not shown).

DISCUSSION

Our investigations on the IS*6110*-associated RFLPs among *M. tuberculosis* isolates from the People's Republic of China and Mongolia led to the identification of a distinct group of strains which share more than two-thirds of their IS*6110*-containing *Pvu*II restriction fragments. Furthermore, isolates belonging to this group share few of their IS*6110*-containing restriction fragments with other isolates, suggesting that strains of this family (which we designated the Beijing family) constitute a distinct group. This grouping was supported by our observation of a low degree of DNA polymorphism with other repetitive genetic elements which drive DNA polymorphism in *M. tuberculosis* isolates independently of the insertion sequence IS*6110*. All of the 52 strains from the People's Republic of China and Mongolia that grouped in the Beijing family were found to be identical by spoligotyping, which is based on the DNA polymorphism in the DR region. Strains of the Beijing family contained only 9 of the 43 spacer sequences tested (Fig. 2). These spacer sequences correspond to nine contiguous spacers located near the 3′ end of the DR region of strain H37Rv (9b). This characteristic presence of the nine spacers in strains belonging to the Beijing family was found not only among strains from the People's Republic of China and Mongolia but also among a minority of strains from other countries. The characteristic spoligotype of the Beijing family of strains is very uncommon among strains isolated in The Netherlands for which extensive spoligotyping has been performed. All Dutch strains with this spoligotype exhibited the characteristic IS*6110* fingerprint pattern of the Beijing family, confirming its uniqueness (15a).

Another characteristic property shared by all *M. tuberculosis* strains belonging to the Beijing family is the presence of a 3.5-kb *Pvu*II fragment carrying IS*1081*. The presence of this fragment is associated with the modification of one of the chromosomal *Pvu*II recognition sites in the mycobacterial genome, resulting in a partial protection of cleavage by *Pvu*II (15c).

The present study shows the existence of a family of related strains sharing several independent genetic properties. The shared genetic properties were found not only among strains from the People's Republic of China and Mongolia but also among a minority of strains isolated from patients on other continents. Therefore, these strains belong to a genetically closely related group which may have diverged from a common ancestor in the recent past. Unfortunately, it is impossible to calculate the time that has elapsed since the divergence from this putative common ancestor. To determine the time that has elapsed, one would need to know the pace of the molecular clocks associated with the different polymorphic genetic markers. However, on the basis of observations of the IS*6110*-associated RFLPs among *M. tuberculosis* strains which have been circulating in the community (in the United States [10a] and in The Netherlands [15b]) for several years, we estimate that less than a century has elapsed since the clonal dissemination of the common ancestor of the Beijing family of strains. These strains apparently have had some selective advantage in the Beijing area. As a result, it may be possible that the Beijing family is an aggressively expanding clone that is spreading to neighboring countries and other continents. In the neighboring countries in Asia rates of infection with the Beijing family strains are higher than those in the more distant countries, suggesting that the Beijing family may have radiated from the Beijing area to other

regions. The nature of the force(s) that contributed to the selection and dissemination of strains of the Beijing family is unknown. A factor common to all countries in southeast Asia is BCG vaccination, which has been used for the past two to six decades. A recent study on the population structure of *M. tuberculosis* in Ethiopia, Tunisia, and The Netherlands (7) suggested more DNA polymorphism in Ethiopia than in Tunisia, although the incidence of tuberculosis in Ethiopia is about five times that in Tunisia. Because BCG vaccination in Ethiopia is not a common practice, in contrast to Tunisia, where it is common practice, it has been suggested that vaccination in Tunisia may have favored the selection of *M. tuberculosis* strains that resist BCG-induced immunity (7). A similar mechanism may have operated in southeast Asia, favoring the dissemination of strains of the Beijing family. If so, one would expect that *M. tuberculosis* strains of the Beijing type are more prevalent among BCG-vaccinated individuals than among nonvaccinated individuals in a population in which a significant fraction has not been covered by BCG vaccination. Such studies are in progress.

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