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Predominance of a single genotype of *Mycobacterium tuberculosis* in regions of Southern Africa

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SUMMARY

SETTING: Zimbabwe and Zambia.

OBJECTIVE: To determine the genetic diversity of *Mycobacterium tuberculosis* strains isolated from tuberculosis (TB) patients in Zimbabwe and Zambia.

DESIGN: *M. tuberculosis* isolates cultured from TB patients presenting at referral hospitals in Zimbabwe and health care clinics in Zambia were characterised by IS*6110* genotyping and/or spoligotyping using internationally standardised methods. Genotypic data were compared to those from Cape Town and the SpolDB3.0 database.

RESULTS: A predominant group of strains could be identified among 116/246 (47.2%) Zimbabwean isolates by their characteristic IS*6110*-banding pattern and unique spoligotype signature, where spacers 21–24, 27–30 and 33–36 were deleted. Comparison with strains from Cape Town showed that they were closely related to a family of strains present in 2.3% of Cape Town patients. Comparison of the spoligotypes with those obtained from 114 isolates from Zambia showed that 74 (65%) of these isolates had the same spoligotype signature. Spoligotypes in the SpolDB3.0 database showed that this group of strains was rarely isolated in other parts of the world, but was commonly isolated in Southern Africa.

CONCLUSION: A predominant group of strains infecting approximately half of the patients in the study are major contributors to the TB epidemic in this region. We have designated this group of strains the Southern Africa 1 (SAF1) family.

KEY WORDS: tuberculosis; *Mycobacterium tuberculosis*; molecular epidemiology; IS*6110* restriction fragment length polymorphism; spoligotyping

IN TERMS of its historical and current disease burden, *Mycobacterium tuberculosis*, the aetiological agent for tuberculosis (TB), is undeniably the most successful human pathogen. Approximately one third of the world's population is infected by *M. tuberculosis*; however, fewer than 10% of these develop active TB during their lifetime. Sub-Saharan Africa has had the highest annual TB incidence rates since the emergence of the human immunodeficiency virus (HIV) epidemic, and most countries in this region belong to the group of 22 high-burden countries that collectively account for 80% of cases worldwide.[1]

In Zimbabwe, the incidence of TB is estimated at 604 new cases per 100 000 population.[2] The situation is exacerbated by HIV co-infection, with approximately 60% of cases presenting with TB also co-infected with HIV.[2] Sputum smear microscopy is the mainstay for the diagnosis of pulmonary tuberculosis (PTB) where culture is not available. However, it is well established that smear-negative cases may be disproportionately higher among HIV-positive than in HIV-negative individuals,[3,4] and the true denominator of total cases is therefore largely unknown. Although countries in Southern Africa have by far the largest TB disease burden, very little is known about the disease dynamics and characteristics of the bacterial populations circulating in these communities.

Approaches to understanding the disease dynamics of TB have included the use of molecular methods together with conventional TB epidemiology.[5–7] Molecular epidemiology techniques have enabled the differentiation of clinical isolates of *M. tuberculosis* into distinct genotypes, which may be important in understanding bacterial pathogenesis. The most commonly used method is insertion sequence (IS) IS*6110* genotyping.[8] The use of additional markers such as spoligo-

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typing[9] and mycobacterial interspersed repetitive units (MIRUs)[10,11] has greatly enhanced the accuracy in differentiating *M. tuberculosis* strains.

The lack of comprehensive molecular epidemiological data from most countries in Africa, such as Zimbabwe and Zambia, has limited the understanding of TB disease dynamics in these areas. A molecular epidemiology study conducted in Harare reported a high level of strain clustering.[12] However, no comprehensive studies have been conducted in Zimbabwe to describe the degree of genetic heterogeneity of *M. tuberculosis* isolates circulating in this region. Earlier attempts to characterise *M. tuberculosis* strains from Zimbabwe using spoligotyping were limited by small sample size, use of one marker and study period.[13] In the absence of such information it is possible that the molecular epidemiology data may have been misinterpreted.

In this study we have used the internationally standardised IS*6110* genotyping method together with spoligotyping to determine the genetic diversity of *M. tuberculosis* strains isolated from TB patients in Harare and Gweru, Zimbabwe. Analysis of this data identified a predominant strain family contributing to the high TB incidence rates. To gain an insight into the global significance of this strain family, genotypic data from Zimbabwe were compared to those from other countries in the Southern Africa region, thereby allowing an assessment of how molecular epidemiology could be used to determine the dynamics of the current TB epidemic, such as transmission of the disease between communities, regions and countries.

MATERIALS AND METHODS

Patient recruitment

The *M. tuberculosis* strains were cultured from patients presenting with PTB in two cities in Zimbabwe. During the period October to December 2001, sputum samples were collected from a total of 120 consecutive smear-positive PTB patients presenting at the Beatrice Road Infectious Diseases Hospital (BRIDH), the main referral centre for infectious diseases in Harare, Zimbabwe. During the period September 2000 to September 2001, sputum samples were collected from 300 consecutive smear-positive or smear-negative PTB patients presenting at the Gweru Provincial Hospital (GPH).

The Zambian isolates were collected as part of a national drug resistance surveillance study and were obtained from new smear-positive patients presenting at various medical centres in different provinces of Zambia.

This study was approved by the Medical Research Council of Zimbabwe* and the Stellenbosch University Faculty of Health Sciences.†

Bacterial strains

The sputum samples from BRIDH were sent to the Biomedical Research and Training Institute (BRTI) in Harare, Zimbabwe, for sputum microscopy and subsequent culture on Löwenstein-Jensen (LJ) slants using conventional methods. Sputum samples from GPH were also sent to BRTI for sputum microscopy and culture on Mycobacteria Growth Indicator Tube (MGIT).[14]

All positive cultures were sub-cultured onto LJ slants and allowed to grow at 37°C until confluent growth was observed, to ensure adequate DNA for subsequent IS*6110* genotyping. Only one culture from each patient was used for IS*6110* genotyping and interpretation of the data.

The samples from Zambia were cultured on LJ slants at the Chest Diseases Laboratory (CDL) in Lusaka, Zambia, using conventional culture methods. All culture-positive isolates confirmed as *M. tuberculosis* were sent to the Tropical Disease Research Centre (TDRC) for DNA extraction. A total of 114 isolates had DNA that could be used for genotyping.

*IS*6110 *genotyping*

DNA was extracted from *M. tuberculosis* cultures from Zimbabwe and Cape Town, as previously described.[15] IS*6110*-genotyping was performed using the international standard typing method for *M. tuberculosis*.[8] Briefly, genomic DNA was digested with the restriction endonuclease *Pvu*II and subjected to electrophoresis in 0.8% agarose. Each lane on the gel included an internal marker (Marker X; Boehringer Mannheim, Mannheim, Germany) to enable normalisation between lanes, while each gel included two external marker lanes (MTB 14323) to ensure inter-gel comparisons. The DNA fragments on the gel were Southern-blotted to Hybond N+(Amersham Pharmacia-Biotech, Amersham, Bucks, UK) and IS*6110*-containing fragments were visualised after hybridisation with enhanced chemiluminescence (ECL) labelled probes IS*6110*-3′ and Marker X DNA. Each probe was stripped from the membrane by denaturation before hybridisation with the subsequent probe. Each DNA genotype was normalised and analysed using Gelcompar (Version 4.0, Applied Maths, BVBA, Kortrijk, Belgium) and entered into a database. The IS*6110* genotype patterns were aligned using the GelCompar programme, with tolerance parameters allowing a 6% shift in each pattern as a whole and a 0.4% variance in individual band positions. Cluster analysis was done using the unweighted pair group method with arithmetic mean (UPGMA), based on Dice coefficient. The isolates were classified into strain families according to the similarity of the IS*6110* genotype patterns. Strain families were defined according to a similarity index of $\geqslant$70%.[16] Isolates within strain families were classified into clusters of identical strains (strains sharing an identical IS*6110* genotype pattern) or uniques (strains with a unique IS*6110* genotype pattern). The IS*6110* genotype

* MRCZ/A/895, MRCZ/A/967.
† 2003/022/N.

database for Zimbabwe was compared to the IS*6110* genotype database of isolates from the epidemiological study site in Cape Town (maintained at Stellenbosch University),[15,17,18] to identify similarities in IS*6110* genotype patterns.

As there was insufficient DNA, IS*6110* genotyping could not be performed for the isolates from Zambia.

Spoligotyping

Isolates from Zimbabwe, Cape Town and Zambia were spoligotyped according to a standardised protocol[9] to detect DNA polymorphism in the direct repeat (DR) region. Spoligotyping was done at the Stellenbosch University Department of Medical Biochemistry; resultant autoradiographs were analysed manually and information entered into a Microsoft Excel database (Microsoft Corporation, Redmond, WA, USA) to allow comparisons between isolates from the different regions.

To determine the global distribution of the spoligotypes identified in this study, the spoligotypes were compared with the spoligotypes deposited in the SpolDB-3.0 worldwide database.[19]

RESULTS

*IS*6110 *genotype patterns of* M. tuberculosis *strains isolated in Zimbabwe and Cape Town*

Sputum cultures were available for genotyping from 246 different patients resident in Harare ($n = 62$) and Gweru ($n = 184$). Among these isolates, 220 (89.4%) harboured more than six IS*6110* copies (high copy number strains), 24 (9.8%) had six or fewer IS*6110* copies (low copy number strains) and the remaining two isolates had poor IS*6110* genotype patterns due to poor quality DNA. Strains lacking IS*6110* were not identified in either of the study sites.

Cluster analysis based on the UPGMA and Dice coefficient grouped the isolates into a number of defined strain families (according to a similarity index of >70%). A total of 15 strain families were identified among the Zimbabwean isolates (Figure 1). However, 116 of the 246 isolates (47.2%) belonged to a group of strains sharing a similarity of >80%. These 116 isolates were characterised by 8–17 IS*6110* hybridising fragments (Figure 2). The majority of these isolates shared six IS*6110-Pvu*II fragments of 0.9, 1.67, 2.43, 2.60, 2.7, 4.16 and 4.6 kb (Figure 2). The IS*6110* genotyping analysis revealed 73 different patterns among these 116 isolates. Sixty-three isolates belonged to 20 clusters, each containing between two and eight isolates. The remaining 53 IS*6110* genotype patterns were unique. Smear microscopy data were available for 75 isolates from this strain family; 51 of these (68%) were smear-positive and 24 (32%) were smear-negative.

Analysis of M. tuberculosis *strains with spoligotyping*

To confirm that the isolates belonged to a closely related group, representing clonal expansion, the 116 isolates were further characterised by analysis of the DR region using spoligotyping. Of the isolates, 115 shared a characteristic spoligotype signature that lacked spacers 21–24, 27–30 and 33–36 (Figure 2). One isolate only lacked spacers 21–24 and 33–36, but had the characteristic IS*6110* genotype pattern, possibly representing a more ancestral strain. A total of 27 distinct spoligotypes were identified among these 116 isolates. Twelve of the isolates each had a unique spoligotype pattern, and the remaining 104 (89.7%) belonged to 15 clusters (Figure 3).

Comparison of isolates belonging to the major family grouping with other databases

Comparison of the IS*6110* genotype patterns from the Zimbabwean isolates with those from the Cape Town IS*6110* genotype database showed >70% similarity to 19 Cape Town isolates, representing 2.3% of TB patients in that study setting.[16] Four different IS*6110* genotype patterns were shared between isolates from Cape Town and from Zimbabwe. Eighteen of the Cape Town isolates had the characteristic spoligotype signature, lacking spacers 21–24, 27–30 and 33–36, and one isolate had the ancestral spoligotype (Figure 3). Three spoligotype patterns (811, 815 and 59) were shared among isolates from Zimbabwe and Cape Town (Figure 3).

We compared the characteristic spoligotype patterns with spoligotype patterns from 114 Zambian isolates. Of these isolates, 72 (63.2%) showed the characteristic spoligotype pattern, while a further two isolates showed the ancestral spoligotype (Figure 3). Restricted strain diversity was observed among the Zambian isolates, with a total of 17 spoligotype patterns being identified among the 74 isolates. Of the 17 different patterns, four were shared among 61 isolates and 13 were unique (Figure 3). Six spoligotype patterns were shared among isolates from Zimbabwe and Zambia, while four spoligotypes were shared between the Zambian and Cape Town isolates. Spoligotype pattern 59 formed the largest clusters in Zambia (44 isolates), Zimbabwe (36 isolates) and Cape Town (6 isolates).

Dissemination of M. tuberculosis *isolates of the identified major strain family grouping in other countries*

The updated SpolDB 3.0 database[19] was searched for isolates with a spoligotype signature identical to those found in Zimbabwe, Zambia and Cape Town. Strains with similar spoligotype signatures have only rarely been isolated from other regions such as the Americas, Europe and Madagascar.[19] With the exception of the isolates with spoligotype pattern 20, 42, 59, 412 and 753, this family of strains appears to be predominant in central regions of Southern Africa (Zimbabwe and Zambia), but not in Cape Town. Based on these findings we have now called this strain family Southern Africa Family 1 (SAF1).

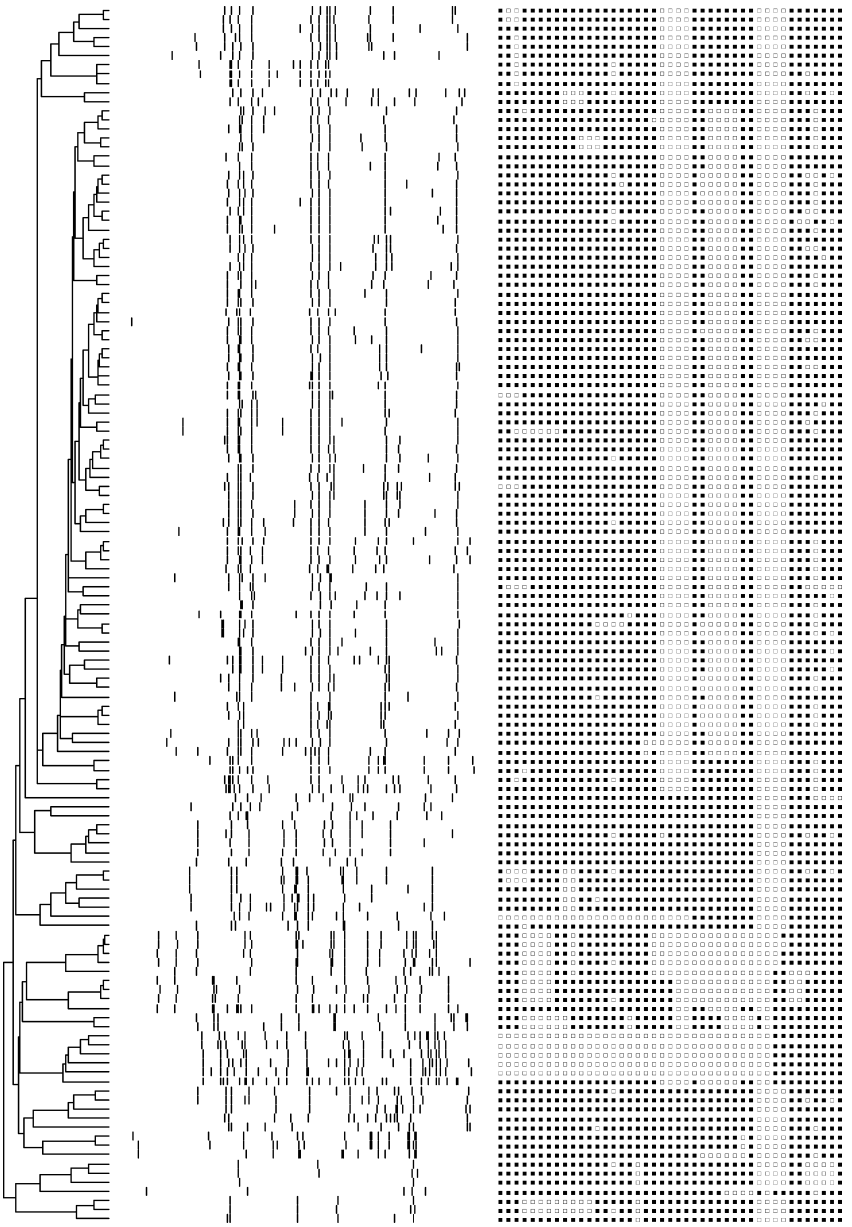


Figure 1 IS*6110* genotype and spoligotype patterns of the *M. tuberculosis* isolates from Zimbabwe. Cluster analysis of *M. tuberculosis* isolates from Harare and Gweru, Zimbabwe. The analysis was based on UPGMA and Dice coefficient. The resulting dendrogram shows the IS*6110* similarity index on the left and the IS*6110* genotype banding patterns in the middle. Strain families were assigned according to an IS*6110* similarity index of >70%.[16] On the extreme right are the corresponding DNA polymorphisms in the DR region (spoligotype patterns). IS = insertion sequence; UPGMA = unweighted pair group method with arithmetic mean; DR = direct repeat.

DISCUSSION

This study represents the first genetic study on *M. tuberculosis* using IS*6110* genotyping in combination with spoligotyping in Zimbabwe. This investigation has led to the identification of a predominant group of strains infecting approximately half of the patients in the study, and which are thereby major contributors to the TB epidemic in this region. The strains are characterised by a distinct IS*6110* genotype pattern and a characteristic spoligotype signature suggesting clonal expansion from a common progenitor. This characteristic spoligotype signature was found not only among isolates from Zimbabwe but also predominantly from isolates from Zambia and in a minority of isolates from Cape Town, South Africa. We have named this strain family SAF1. Only rarely were SAF1 spoligotypes identified in other regions of the world.[19]

Previous comparative genomic studies have shown that the SAF1 genotype is a member of the Latin-American-Mediterranean (LAM) family,[20] which includes F11, F13, F14, F15 and F26 from Cape Town.[21] IS*6110* insertion site mapping suggests that these strain families form part of a group of strains that have

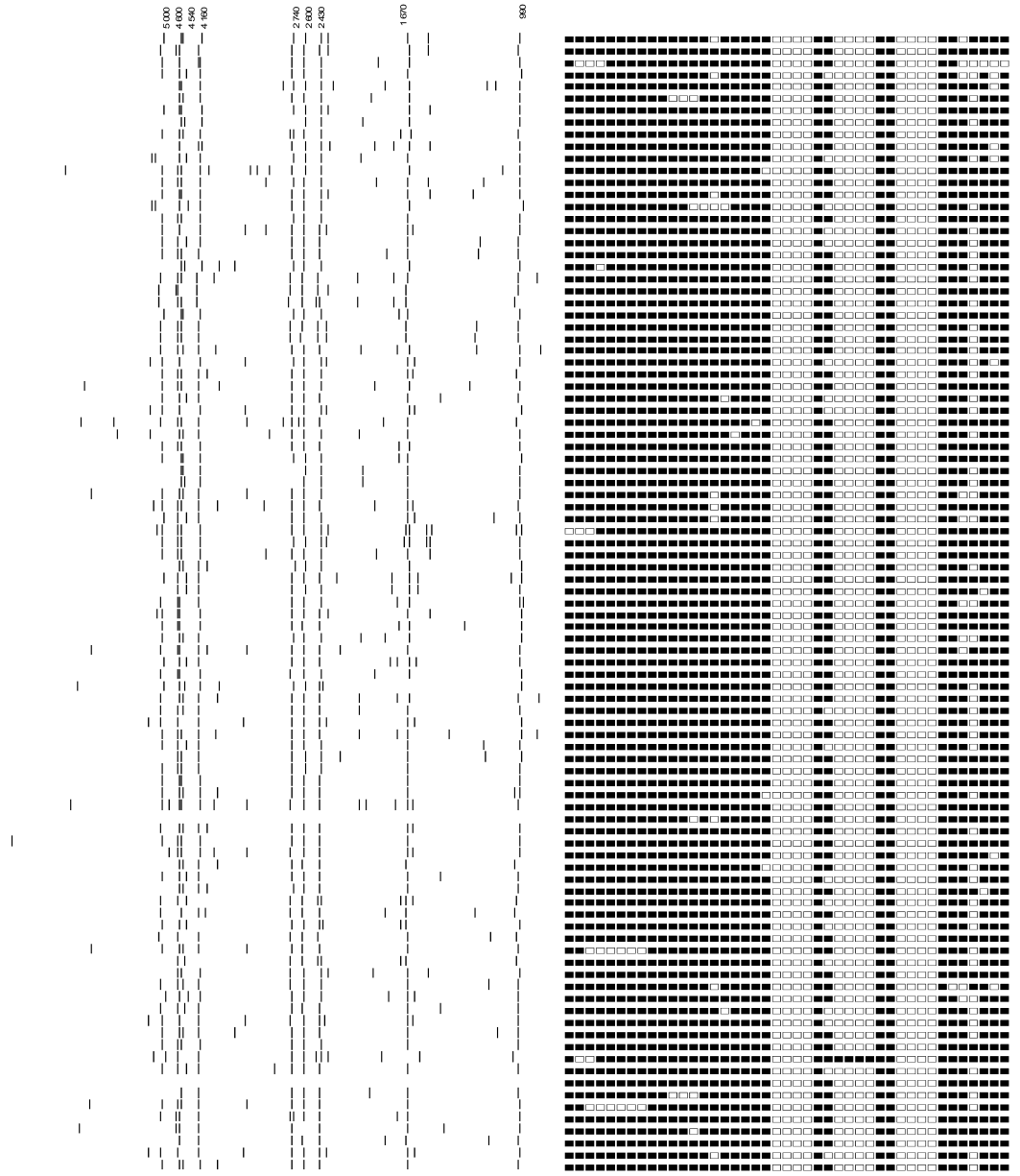
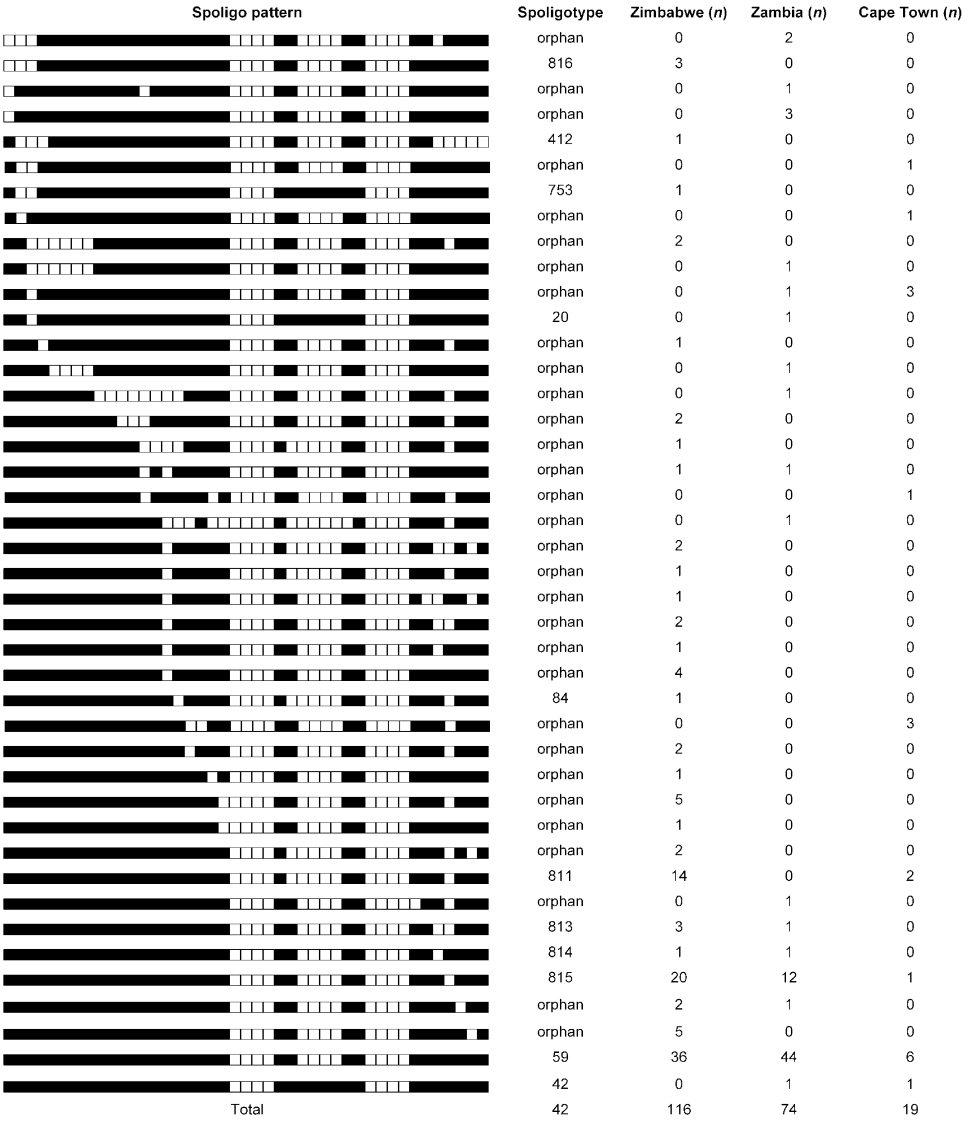


Figure 2 IS*6110* genotype and spoligotype patterns of the 'SAF1' family isolates from Zimbabwe. IS*6110* genotype banding patterns of isolates from Zimbabwe showed a similarity of >70%. All isolates shared a common banding pattern of six IS*6110-PvuII* fragments, 0.9, 1.67, 2.43, 2.60, 2.7, 4.16 and 4.6 kb, respectively. In addition, a spoligotype 'signature' lacking spacers 21–24, 27–30 and 33–36 was observed among the isolates. The two isolates with no IS*6110*-RFLP due to poor DNA had this characteristic spoligotype pattern. IS = insertion sequence; SAF1 = Southern Africa Family 1; RFLP = restriction fragment length polymorphism.

originated from a common progenitor.[21] This would therefore imply that the SAF1 family is a branch within this superfamily of genotypes, where different branches appear to have different frequencies in different settings. The strain family F11 occurs as the highest frequency strain family in Cape Town, South Africa, being isolated in 21.4% of the patients.[22]

We acknowledge that this study has certain limitations. First, the isolates were collected from different settings and the epidemiological relationships according to clustering were therefore not appropriate to define transmission events. Our data set could only be applied to demonstrate the broad distribution of closely related genotypes. To gain further insight into the disease dynamics in Southern Africa, future studies will need to comprehensively analyse isolates of *M. tuberculosis* over an extended period. Such studies must be able to establish whether the *M. tuberculosis* population structure is influenced by HIV co-infection or whether there is an association between clinical presentation and strain genotype. Second, the restricted time interval over which the isolates were collected has limited the application of this study to determining only the population structure of *M. tuberculosis*. However, we do not believe that this has introduced a significant bias, given that specimens were cultured from patients from



Spoligotype	Zimbabwe (*n*)	Zambia (*n*)	Cape Town (*n*)
orphan	0	2	0
816	3	0	0
orphan	0	1	0
orphan	0	3	0
412	1	0	0
orphan	0	0	1
753	1	0	0
orphan	0	0	1
orphan	2	0	0
orphan	0	1	0
orphan	0	1	3
20	0	1	0
orphan	1	0	0
orphan	0	1	0
orphan	0	1	0
orphan	2	0	0
orphan	1	0	0
orphan	1	1	0
orphan	0	0	1
orphan	0	1	0
orphan	2	0	0
orphan	1	0	0
orphan	1	0	0
orphan	2	0	0
orphan	1	0	0
orphan	4	0	0
84	1	0	0
orphan	0	0	3
orphan	2	0	0
orphan	1	0	0
orphan	5	0	0
orphan	1	0	0
orphan	2	0	0
811	14	0	2
orphan	0	1	0
813	3	1	0
814	1	1	0
815	20	12	1
orphan	2	1	0
orphan	5	0	0
59	36	44	6
42	0	1	1
Total	116	74	19

Figure 3 Schematic representation of spoligotype patterns obtained from Cape Town, Zambia and Zimbabwe. The spoligotype nomenclature is according to the SpolDB3.0 database.[19] Orphan patterns did not have matches in the SpolDB3.0 database. Spoligotypes 811, 813, 814 and 815 have previously been reported only from Zimbabwe.[12,19]

diverse geographical settings. Last, it is possible that the *M. tuberculosis* strain population structure may differ between smear-positive and smear-negative cases.

Despite these limitations, our results provide evidence that the burden of TB disease in the central Southern African regions is largely due to a relatively small subset of actively circulating strains. The success of particular predominant clones related to high incidence of TB was also reported in earlier studies.[18,23–25] The identification of a predominant strain genotype in central parts of Southern Africa may reflect that this strain has either gained a higher degree of virulence, or that the strains in these high-incidence countries may have originated from a few clones that expanded rapidly in the recent past. The reasons for the selection and dissemination of these strains are unknown. A recent study on the global genetic population structure for *M. tuberculosis* has suggested that the organism has adapted to specific human populations in different geographic settings.[26]

As postulated for other predominant strain families, the success of these strains may be a result of increased transmissibility or from expression of an as yet unidentified virulence factor.[27] The reason for the high frequency of the Beijing family has been related to virulence and mutator phenotypes, and this may explain the better adaptability to stress conditions and hostile intracellular conditions.[28]

This study may have important implications for the interpretation of molecular epidemiology data. In molecular epidemiological analysis, the predominance of closely related strains implies restricted genetic diversity, which may be interpreted as transmission. This may hinder the quantification of the contribution of

reactivation and recent transmission to the epidemic. Furthermore, it may be difficult to define the mechanism leading to recurrent TB. However, the interpretation of genetic diversity will generally depend on the method of genotyping used, underscoring the need to develop molecular epidemiological tools appropriate to settings with low *M. tuberculosis* IS*6110* genotypic diversity.

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RÉSUMÉ

CONTEXTE : Zimbabwe et Zambie.
OBJECTIF : Déterminer la diversité génétique des souches de *Mycobacterium tuberculosis* isolées chez les patients tuberculeux au Zimbabwe et en Zambie.
SCHÉMA : Les isolats de *M. tuberculosis* cultivés à partir de patients consultant dans les hôpitaux de référence du Zimbabwe et dans les dispensaires de soins de santé de Zambie ont été caractérisés par un génotypage IS*6110* et/ou par spoligotypage en utilisant des méthodes standardisées internationales. Les données génotypiques ont été comparées avec celles de Cape Town et de la base de données SpolDB3.0.
RÉSULTATS : Un groupe prédominant de souches a pu être identifié chez 116 des 246 (47,2%) isolats du Zimbabwe par leur type de caractéristiques de bande IS*6110* et une signature spoligotypique avec délétion des espaceurs 21–24, 27–30 et 33–36. La comparaison de ces souches avec celles provenant de Cape Town a montré qu'elles étaient étroitement en relation avec une famille de souches présente chez 2,3% des patients de Cape Town. La comparaison des spoligotypes avec ceux obtenus à partir des 114 isolats de Zambie a montré que 74 (65%) de ces isolats avaient la même signature spoligotypique. Les spoligotypes dans la base de données SpolBB3.0 ont montré que ce groupe de souches n'était que rarement isolé dans d'autres parties du monde, mais fréquemment en Afrique du Sud.
CONCLUSION : Un groupe prédominant de souches infectant près de la moitié des patients de l'étude est un élément contributif majeur de l'épidémie de TB dans cette région. Nous avons dénommé ce groupe de souches la famille Southern Africa 1 (SAF1).

RESUMEN

MARCO DE REFERENCIA : Zimbabwe y Zambia.
OBJETIVO : Determinar la diversidad genética de las cepas de *Mycobacterium tuberculosis* aisladas de pacientes tuberculosos en Zimbabwe y Zambia.
MÉTODOS : Se realizó la caracterización genética de los aislados de *M. tuberculosis* provenientes de pacientes con tuberculosis (TB) que acudieron a los hospitales de referencia en Zimbabwe y a los centros de salud en Zambia por genotipificación con la secuencia de inserción IS*6110* y una espoligotipificación (*spoligotyping*), utilizando métodos normalizados a escala internacional. Los datos así obtenidos se compararon con las bases de datos de Ciudad del Cabo y la SpolDB3.0.
RESULTADOS : En los 246 aislados de Zimbabwe se definió un grupo predominante de 116 cepas (47,2%) por su perfil de bandas de IS*6110* y un aspecto distintivo único de espoligotipificación, con deleción de los espaciadores 21 a 24, 27 a 30 y 33 a 36. La comparación con las cepas de Ciudad del Cabo puso en evidencia su relación cercana con una familia de cepas presentes en el 2,3% de los pacientes de Ciudad del Cabo. La comparación de los espoligotipos con aquellos obtenidos de los 114 aislados de Zambia demostró que 74 de ellos (65%) presentaban el mismo espoligotipo distintivo. La comparación con los espoligotipos de la base de datos SpolDB3.0 indicó que este grupo de cepas se ha aislado raramente en otras regiones del mundo, pero se observa con frecuencia en el sur de África.
CONCLUSIÓN : Un grupo predominante de cepas infecta a cerca de la mitad de los pacientes en esta región y constituye la principal causa de la epidemia de TB. Este grupo de cepas se ha denominado la familia del sur de África 1 (SAF1).